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MYELOID NEOPLASIA

The use of targeted sequencing and flow cytometry to identify patients with a clinically significant monocytosis

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KEY POINTS

- **Somatic mutations are detected at high frequency in patients with a monocytosis and are associated with significantly reduced survival.**
- **In those without a WHO-defined diagnosis, patients with a mutation have laboratory and clinical features indistinguishable from CMML.**

The diagnosis of chronic myelomonocytic leukemia (CMML) remains centered on morphology, meaning that the distinction from a reactive monocytosis is challenging. Mutational analysis and immunophenotyping have been proposed as potential tools for diagnosis; however, they have not been formally assessed in combination. We aimed to investigate the clinical utility of these technologies by performing targeted sequencing, in parallel with current gold standard techniques, on consecutive samples referred for investigation of monocytosis over a 2-year period (N = 283). Results were correlated with the morphological diagnosis and objective outcome measures, including overall survival (OS) and longitudinal blood counts. Somatic mutations were detected in 79% of patients, being invariably identified in those with a confirmed diagnosis (99%) but also in 57% of patients with nondiagnostic bone marrow features. The OS in nondiagnostic mutated patients was indistinguishable from those with CMML (*P* = .118) and significantly worse than in unmutated patients (*P* = .0002). On multivariate analysis, age, ASXL1, CBL, DNMT3A, NRAS, and RUNX1 mutations retained significance. Furthermore, the presence of a mutation was associated with a progressive decrease in hemoglobin/platelet levels and increasing monocyte counts compared with mutation-negative patients. Of note, the immunophenotypic features of nondiagnostic mutated patients were comparable to CMML patients, and the presence of aberrant CD56 was highly specific for detecting a mutation. Overall, somatic mutations are detected at high frequency in patients referred with a monocytosis, irrespective of diagnosis. In those without a World Health Organization–defined diagnosis, the mutation spectrum, immunophenotypic features, and OS are indistinguishable from CMML patients, and these patients should be managed as such. (*Blood*. 2019;133(12):1325-1334)

Introduction

Distinguishing a reactive monocytosis from chronic myelomonocytic leukemia (CMML) is challenging for the hematopathologist. Using current World Health Organization (WHO) diagnostic criteria, a persistent monocytosis is the hallmark of disease, and demonstrating clonality is not a definitive requirement.[1] This leads to a greater risk for misdiagnoses or misclassification, particularly in patients with prolonged reactive changes.

More recently, alternative techniques, in particular flow cytometry, have provided a potential objective tool to identify patients with disease. Skewing of the distribution of monocyte subsets in the peripheral blood (PB; >94% M1 monocytes) has been reported to be sensitive and specific for detecting CMML.[2] In addition, large studies using targeted sequencing panels have identified recurrent somatic mutations in >90% of patients with CMML,[3] providing a further potential tool for diagnosis. The presence of a *TET2* mutation, in combination with a *SRSF2* (or *ZRSR2*) mutation, has been shown to be highly specific for a myelomonocytic phenotype[4]; these, along with *ASXL1*, are the most frequently mutated genes within this disease group.[3] Although the 2016 WHO diagnostic criteria have stated that these mutations can support a diagnosis of CMML, no study has directly assessed the use of this technology in a diagnostic setting. The aim of this study was to determine whether mutational analysis and flow cytometry can provide confirmatory evidence of disease and predict outcome in patients presenting with a monocytosis.

Methods

Patients and samples

The research was undertaken within the Haematological Malignancy Diagnostic Service (HMDS), a fully integrated laboratory that serves a population of ~6 million and is the benchmark for hematopathology services within the United Kingdom. All

consecutive samples (PB or bone marrow [BM]) received between July 2014 and July 2016 from patients ≥18 years old for the investigation of monocytosis were included. Patients with a confirmed myeloid diagnosis prior to July 2014 were excluded. The decision to investigate was at the discretion of the referring clinician; therefore, the study cohort reflects the variety of samples received in a routine laboratory for the investigation of a monocytosis. An absolute monocyte count was determined for all PB samples when received at HMDS (Table 1) using flow cytometry (see "Flow cytometry"). Interestingly, the absolute count was calculated to be $<1 \times 10^9$/L in a proportion of samples (11%); however, the vast majority were very close to this threshold, and review of local blood count parameters and clinical details confirmed the presence of a PB monocytosis and clinical suspicion of CMML. This highlights the recognized variation in monocyte counts between laboratories and the difficulty when applying arbitrary cutoffs as diagnostic criteria.

A total of 283 patients was referred during this time period (Table 1), of which 121 and 162 had an initial PB or BM sample, respectively (Figure 1). A confirmed diagnosis was only made on those cases with an ultimate BM sample (n = 207). All samples were processed according to gold standard techniques and were double reported, meaning the diagnosis was agreed upon by 2 experienced hematopathologists. Those with a confirmed diagnosis were classified in accordance with the WHO 2008 classification. Those failing to fulfill the morphological and genetic WHO 2008 criteria, as agreed by 2 hematopathologists, were classified as "nondiagnostic."

All samples were taken with fully informed patient consent for investigation of a suspected hematological disorder. This study had local Institutional Review Board approval (REC reference-16/NE/0105) and was performed in accordance with the Declaration of Helsinki.

Flow cytometry

All samples for immunophenotypic analysis were processed within 24 hours. Numerical studies and assessment of monocytic CD56 expression were performed on BM or PB samples following a stain–lyse–wash procedure (FACS Lyse; Becton Dickinson; supplemental Figures 1-2; supplemental Table 1, available on the *Blood* Web site). There was a strong correlation between monocyte CD56 expression in the PB and BM (supplemental Figure 5), enabling analysis using samples from either source.

PB CD14/CD16 "classical" monocytic subset studies were performed on samples following NH_4Cl lysis of erythrocytes using a lyse–stain–wash procedure. A minimum of 10^5 leukocytes was acquired on a single cytometer (FACSCanto II; Becton Dickinson) for all cases. Monocytes were identified using a combination of CD64, CD45, and scatter characteristics, and a single operator (M.C.) performed all analyses (supplemental Figures 3-4).

DNA extraction and targeted amplicon sequencing

In parallel with the above analyses, samples were subjected to targeted high-throughput sequencing. Referring clinicians and hematopathologists were blinded to the results of this analysis to exclude reporting or treatment bias.

DNA was extracted from fresh blood or BM mononuclear cells using a QIAamp DNA Mini Kit (QIAGEN, Manchester, UK). Targeted gene sequencing of 27 genes recurrently mutated in myeloid malignancies was performed on a MiSeq System (Illumina, Chesterford, UK). Panel design, validation, and variant filtering criteria are included in supplemental Methods and supplemental Tables 2 and 3. The mean coverage of identified variants was 1514× (range, 52-5605×).

Clinical follow-up

All follow-up BM assessments were performed as clinically indicated by the referring clinician. These samples were also processed according to gold standard techniques and underwent targeted sequencing in parallel, as described above. Any new diagnoses were recorded.

Survival data were available for all patients and censored on the date of extraction (8 August 2017). Additional clinical information, including serial full blood count data, was collected on a subcohort of patients (n = 182), directly from the referring hospital or through the HMRN (n = 85).[5]

Statistical analysis

Survival curves were produced using the Kaplan-Meier method, and simple differences in survival were assessed with the log-rank test. The impact of abnormalities on overall survival (OS) and risk of progression were estimated using Cox regression; in cases in which variable selection was required to arrive at a multivariable regression, the lasso was used for variable selection, and results were reported for the corresponding relaxed lasso model.

Sensitivity, specificity, and positive and negative predictive values were calculated using 2×2 contingency tables.

Comparison between flow cytometric parameters in the main cohort was performed using the Mann-Whitney *U* test. Correlation among CD56 expression, M1 monocyte, and mutational analysis was performed using logistic and Poisson regression.

The effect of mutations on longitudinal blood counts was assessed using random effects models. Four models were fitted using a full-factorial interaction between time and mutation status: (1) a random intercept model, (2) a random intercept and slope model with uncorrelated random effects, (3) a random intercept and slope model with correlated random effects, and (4) a random intercept and slope model with correlated random effects, additionally adjusted for age and sex. For each mutation/blood count relationship, the best-fitting model was chosen according to a likelihood ratio test. To limit any potential effect from periods of acute illness or intensive treatment, blood count trajectory analysis was restricted to patients with <40 measurements over >100 days.

Results

Somatic mutations are detected at high frequency in patients with a monocytosis, irrespective of diagnosis

To define the mutation spectrum in patients referred with a monocytosis, targeted sequencing results were analyzed for the total cohort and correlated with the final diagnosis in those who underwent BM sampling. Of the total 283 patients, ≥1 mutation was detected in 78% of samples (the spectrum is presented in Figure 2A; see also supplemental Table 4). Of these patients,

Table 1. Patient characteristics

Characteristic	Distribution in cohort		
No. of patients	283		
Males/females, n	174/109		
Age, median (range), y	76 (24-96)		
Final diagnosis, n			
PB only	76		
CMML	114		
AML	11		
MPN	9		
MDS	4		
Other	4		
Nondiagnostic	65		
	CMML	**Other hematologic malignancy**	**Nondiagnostic**
Age, median (range), y	76 (24-91)	76 (42-93)	73 (34-93)
Blood count parameters: median (range)			
Hemoglobin (g/L)	105.5 (38-161)	108 (53-174)	122 (84-163)
White cell count ($\times 10^9$/L)	13.6 (4-104.9)	10.6 (3.9-83.4)	7.9 (4.2-38.2)
Platelets ($\times 10^9$/L)	90 (1-442)	154 (39-1085)	150 (8-499)
Monocytes ($\times 10^9$/L)*	2.69 (0.47-23.59)	1.71 (0.23-9.57)	1.29 (0.72-4.08)
Mutation frequency: no. of patients (%)			
TET2	72 (63)	9 (32)	31 (48)
SRSF2	48 (42)	9 (32)	14 (22)
ASXL1	39 (34)	13 (46)	10 (15)
NRAS	17 (15)	7 (25)	5 (8)
RUNX1	16 (14)	6 (21)	4 (6)
DNMT3A	9 (8)	5 (18)	5 (8)
CBL	18 (16)	1 (4)	4 (6)
KRAS	9 (8)	3 (11)	2 (3)
SETBP1	7 (6)	2 (7)	1 (2)
JAK2	3 (3)	7 (25)	1 (2)
EZH2	8 (7)	2 (7)	1 (2)
SF3B1	6 (5)	2 (7)	0 (0)

AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm.

*Monocyte count was determined by flow cytometry (see "Methods").

207 underwent BM assessment for a definitive diagnosis. HMDS provides a centralized integrated hematopathology service, and all BMs were reviewed independently by 2 hematopathologists to ensure consistent and high-quality BM reporting for this purpose.

In those with a confirmed myeloid malignancy (142/207 cases; 69%), a mutation was almost invariably detected (140/142; 99% of cases). Of the 2 mutation-negative cases, 1 had a complex karyotype, including inv3 (involving *MECOM*), leaving only 1 case with no demonstrable clonal abnormality. The significant majority of diagnostic cases (80%; 114/142) were classified as CMML. The remaining samples were classified with a spectrum of myeloid malignancies, although, importantly, 11 patients were diagnosed with acute myeloid leukemia (AML) (n = 11), highlighting the importance of a BM assessment in patients referred with a monocytosis.

Somatic mutations were also detected at a high frequency in nondiagnostic samples. At least 1 mutation was detected in 37 of 65 patients (57%) with indeterminate features. The spectrum of mutations in this group mirrored those detected in the diagnostic group, with *TET2*, *SRSF2*, and *ASXL1* being the most frequently mutated (Figure 2B). The most notable differences in the nondiagnostic group were the absence of high-risk mutations, including *TP53*, *FLT3*, and *NPM1*, as well as those associated with specific morphological abnormalities, such as *SF3B1*, which correlates strongly with the presence of ring sideroblasts.[6] The median and mean number of mutations were higher in those with a confirmed diagnosis (median, 3; range, 0-8; mean, 3) vs those without (median, 1; range, 0-6; mean, 2) (Figure 2C). However, in patients with a confirmed mutation, the number of mutations did not differ significantly between diagnostic and nondiagnostic groups ($P = .62$).

The median variant allele fraction (VAF) for all variants was 39% (range, 5.2-100%; supplemental Figure 6), and there was no difference between the VAF in diagnostic and nondiagnostic cases ($P = .33$). In those patients with an isolated mutation, the

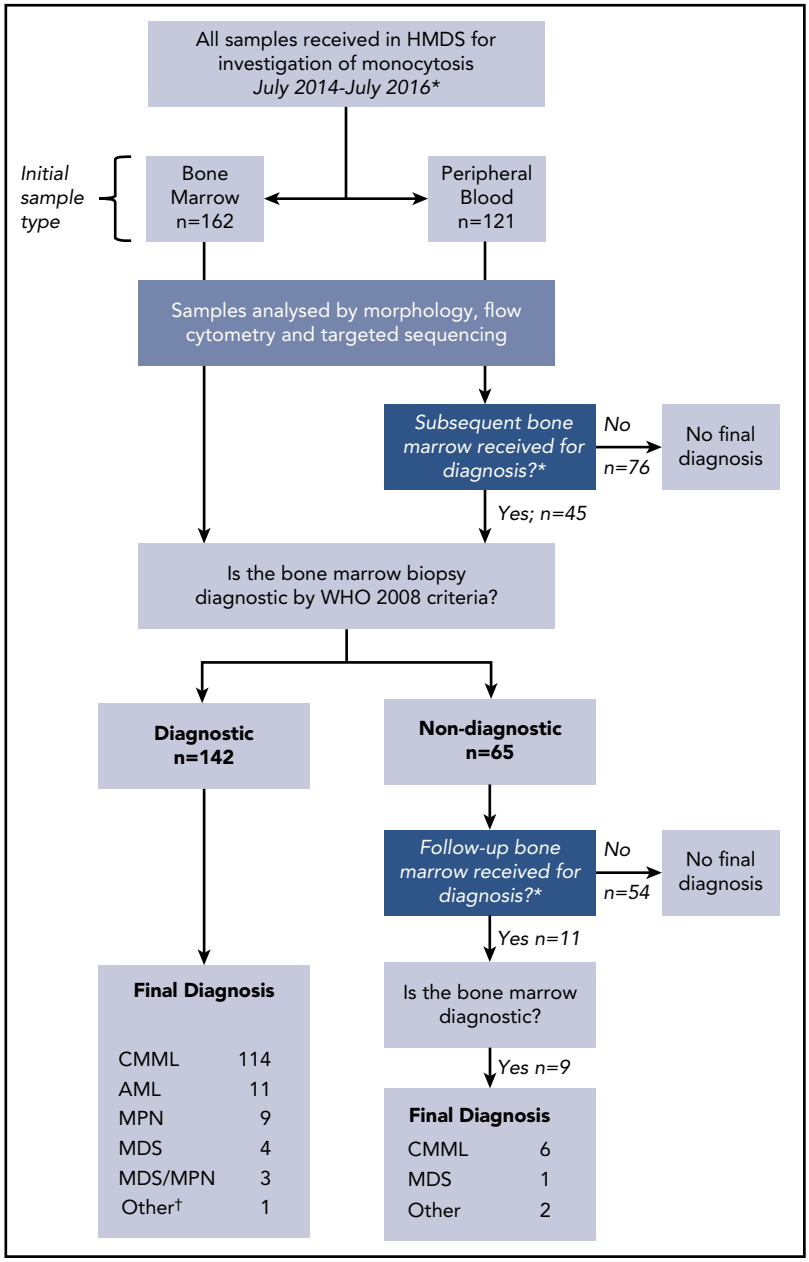


Figure 1. Summary of samples included in the study. Flowchart of cases referred to HMDS for investigation of a monocytosis. *Decision to investigate was at the discretion of the referring clinician. †Focal area of diffuse large B-cell lymphoma noted in BM, likely co-occurring with CMML.

median VAF was also noted to be high (38.2%; range, 6.3-97.1%), with only 2 variants having VAF $< 10\%$.

Therefore, mutations are found at a very high frequency with a high clonal burden in patients with a monocytosis, and they involve a similar spectrum of genes, irrespective of diagnosis.

OS and blood count trajectory correlate strongly with mutation profile

To understand the long-term clinical impact of detecting these mutations, objective outcome measures, including OS and longitudinal blood count analysis, were assessed in the total cohort and correlated with the final diagnosis.

The median survival of all patients from the time of first sampling was 35.2 months (95% confidence interval [CI] 25 months-not reached; Figure 3A). Survival correlated strongly with the number of mutations. Those without a mutation had a significantly better OS, and even the presence of a single mutation resulted in a significant reduction in survival ($P = .004$; Figure 3B). On univariate analysis, age was strongly associated with survival. For mutations occurring in $>5\%$ of subjects, *ASXL1*, *CBL*, *DNMT3A*, *NRAS*, and *RUNX1* were all strongly associated with survival, as were *EZH2* and *STAG2* among the less frequently mutated genes. To investigate multivariate significance, all genes mutated in $>5\%$ subjects were entered into a lasso survival regression. Taking the 1SE shrinkage parameter, age, *ASXL1*, *CBL*, *DNMT3A*, *NRAS*, and *RUNX1* were selected by the lasso and retained significance in a relaxed lasso regression (supplemental Table 5).

In those patients who proceeded to a BM biopsy, survival correlated with the final morphological diagnosis. Those without a confirmed diagnosis had a significantly better OS than did

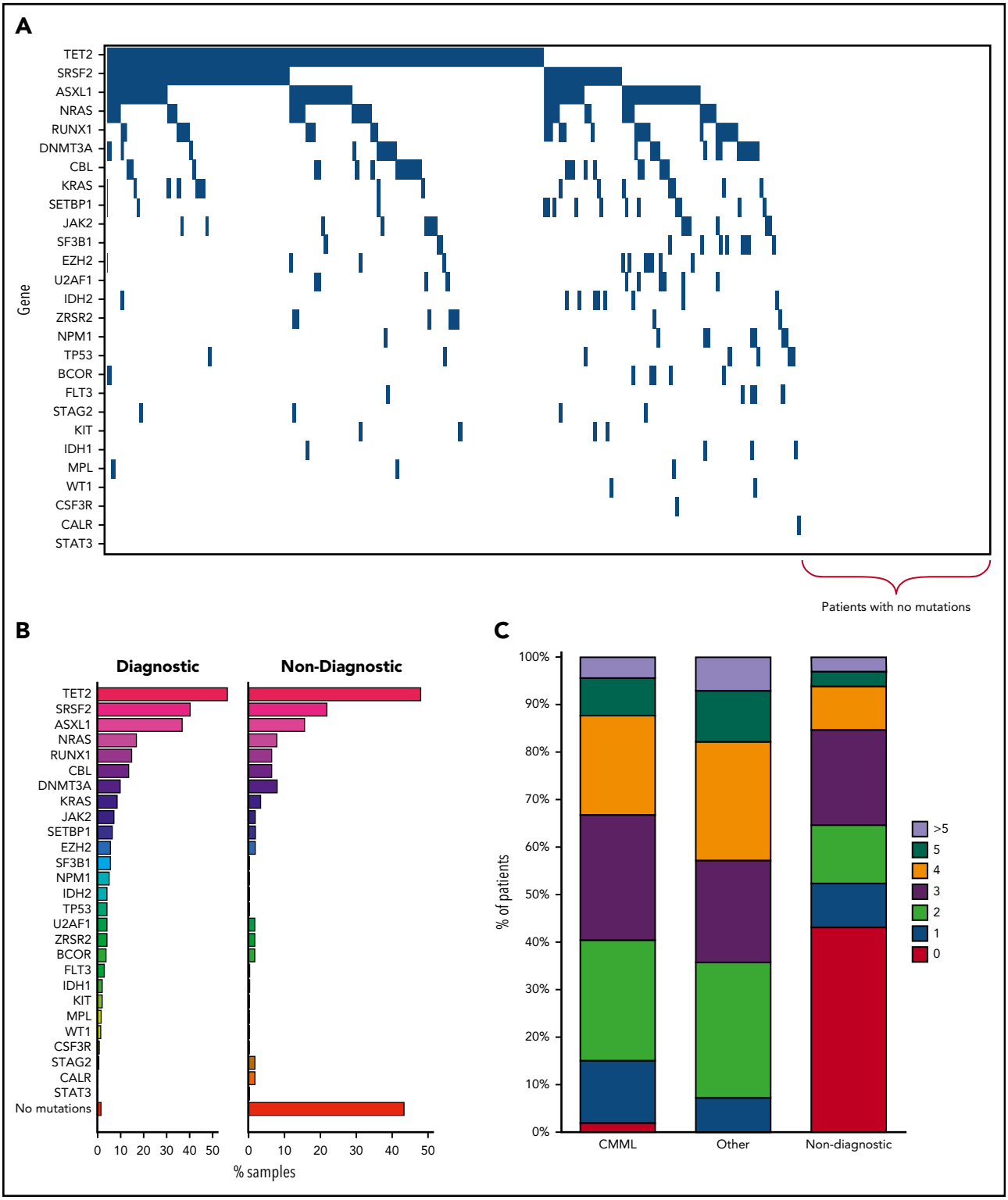


Figure 2. Characteristics of mutations detected in patient samples. (A) Spectrum of mutations detected across all patients in the study (N = 283). (B) Comparison of mutations detected in those with a diagnostic BM sample (n = 142) vs a nondiagnostic BM sample (n = 65). (C) Distribution of the number of mutations according to final diagnostic category. "Other" denotes those patients with an alternative hematological malignancy.

those with CMML or another myeloid malignancy. However, this survival benefit was retained only in those without a demonstrable mutation (P = .0002), with mutated patients having a similar survival to CMML patients (P = .118; not statistically significant) (Figure 4).

Longitudinal blood count data were available for 182 patients, although they were restricted to those with <40 measurements over >100 days (n = 133) to exclude periods of acute hospital admissions (due to periods of acute illness/infection) or intensive chemotherapy (median follow-up, 465 days; range, 119-996).

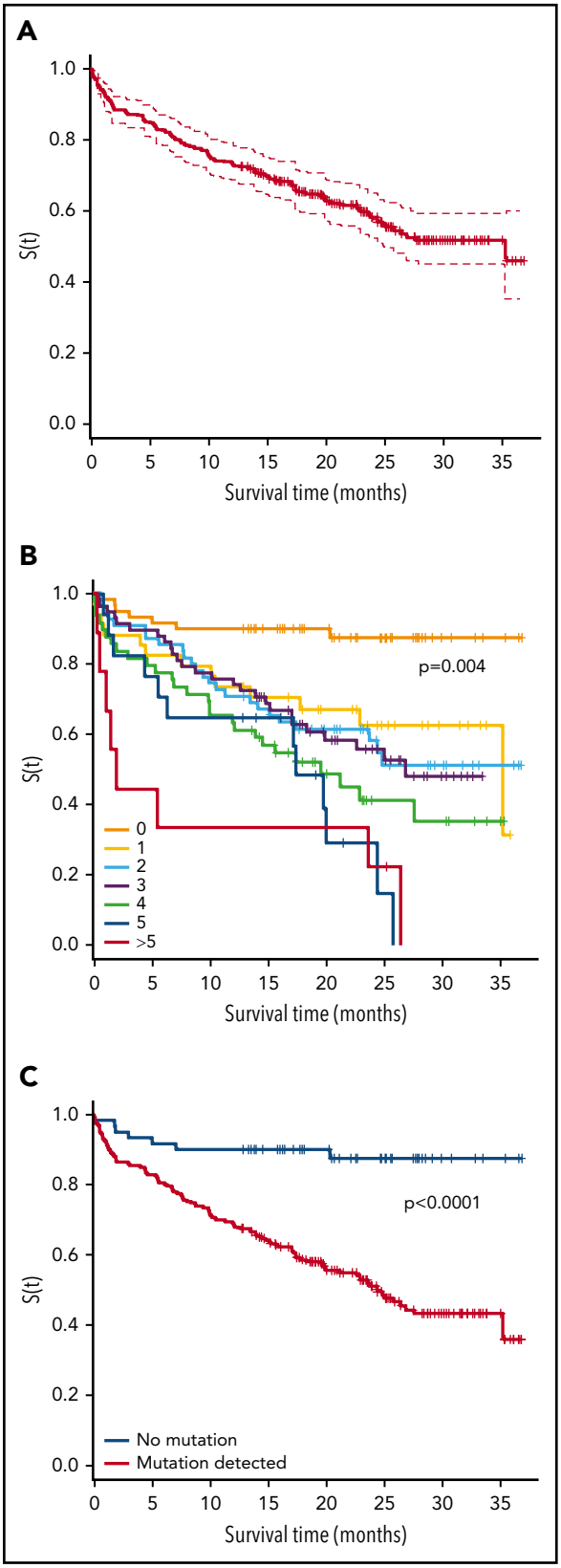


Figure 3. OS according to mutation number. (A) OS in total cohort from time of initial sample. (B) OS in total cohort by number of mutations detected at the time of initial sample. The *P* value was determined using the log-rank test to compare subjects without a mutation and subjects with a single mutation. (C) OS in total cohort by the presence or absence of a mutation. $P < .0001$, log-rank test.

The presence of a mutation was associated with a significantly lower hemoglobin and platelet count and a higher monocyte count relative to those without a mutation, which persisted over time and followed a divergent trajectory (Figure 5). With respect to individual mutations, certain mutations were associated with increasing or declining blood count parameters over time (supplemental Table 6). Monocyte counts were found to increase over time in subjects with *TET2*, *SRSF2*, *ASXL1*, *NRAS*, or *RUNX1* mutations relative to nonmutated subjects; similarly, white blood counts increased in subjects with *ASXL1*, *NRAS*, and *DNMT3A* mutations, and platelet levels decreased in subjects with *ASXL1*, *CBL*, and *RUNX1* mutations relative to nonmutated subjects.

In those without a confirmed diagnosis, follow-up BM biopsies were received from 11 patients. Importantly, of those with a subsequent diagnosis of CMML, all had a confirmed mutation on the original sample. In total, 7 of 37 (19%) nondiagnostic mutated patients had a confirmed diagnosis (6 CMML, 1 myelodysplastic syndrome [MDS]). Furthermore, none of the mutation-negative cases went on to develop CMML; however, 2 patients had confirmed alternative hematological diagnoses: diffuse large B-cell lymphoma and Rosai-Dorfman disease.

These findings confirm that the presence of a mutation has a significant impact on outcome with respect to survival and blood count parameters.

PB mutation profiling is predictive of a BM diagnosis

PB mutational analysis has been shown to correlate strongly with BM analysis in MDS, providing a potential alternative to BM sampling. To determine whether this is also true in CMML, matched PB and BM samples were analyzed. A total of 121 PB samples was received as the initial sample, and somatic mutations were detected in 66% (80/121). Forty-five of 121 patients (37%) had a subsequent BM biopsy performed for diagnosis. Sequencing failed on 2 of the matched BM samples. Of the 124 variants detected in the remaining 43 patients, there was high concordance between PB and BM (96%), with only 5 discordant results. Importantly, these were low-level variants at the limit of detection for the test or variants detected at areas of poor coverage (*SRSF2/ASXL1*). All 9 mutation-negative cases were fully concordant.

The presence of a mutation in the PB was highly predictive of diagnosing a myeloid malignancy in BM, with all but 1 case with a demonstrable mutation having a subsequent diagnosis (positive predictive value, 0.97; negative predictive value, 1.0; supplemental Figure 7). Of note, none of the mutation-negative ($n = 11$) cases had a subsequent confirmed diagnosis.

Immunophenotypic features correlate strongly with the presence of a mutation and a subsequent diagnosis

Flow cytometry has been proposed as a potential diagnostic tool in the investigation of patients with a monocytosis. To determine whether immunophenotyping can predict for the presence of a mutation or a BM diagnosis, flow cytometric analysis was performed alongside sequencing.

First, a comparison was made between the immunophenotypic features in the BM of those patients with a confirmed diagnosis

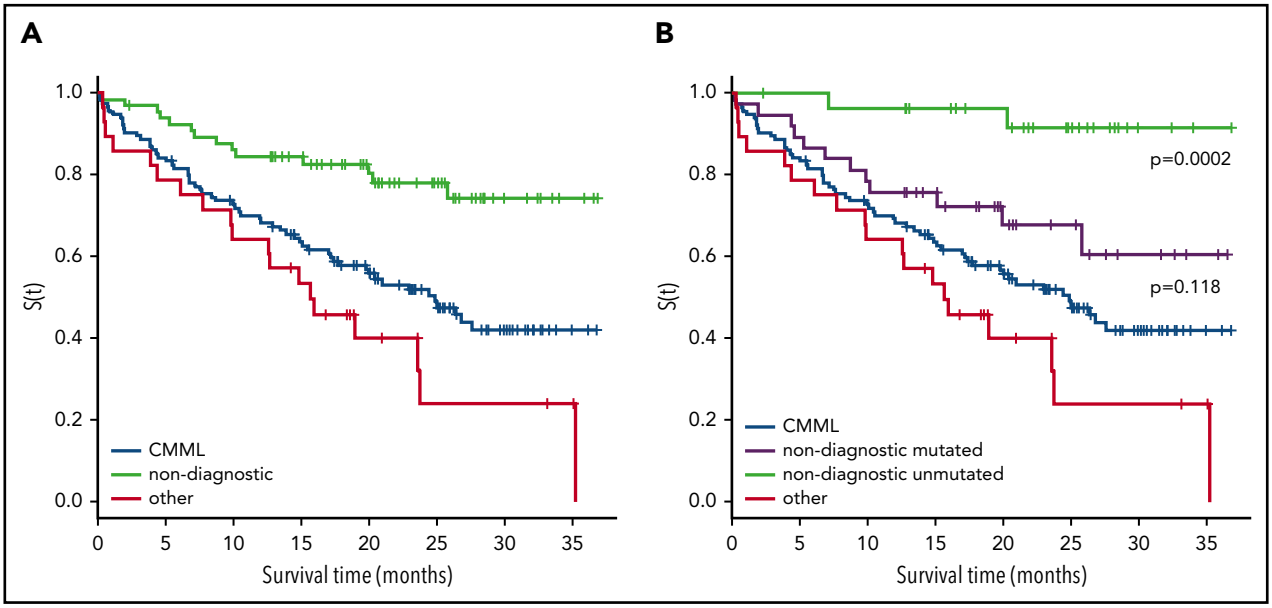


Figure 4. OS according to final diagnosis. (A) OS by diagnosis on BM sample (n = 207). (B) OS by diagnosis with nondiagnostic samples separated by the presence or absence of a mutation. $P = .0002$, CMML vs nondiagnostic unmutated patients, log-rank test; $P = .118$, CMML vs nondiagnostic mutated patients, log-rank test.

of CMML vs nondiagnostic samples. Importantly, nondiagnostic mutated patients had immunophenotypic features indistinguishable from CMML with respect to increased CD64$^+$ monocytes, reduced CD14 expression, and aberrant CD56 expression on monocytes (Figure 6). This was most pronounced with regard to CD56 expression (in PB or BM), which was found almost exclusively in those with a mutation. With respect to individual mutations, aberrant expression of CD56 was strongly associated with *TET2* mutations (odds ratio [OR], 4.0; 95% CI 2.4-6.8; $P < .0001$).

PB monocyte subsets and CD56 expression are predictive of a somatic mutation

The presence of >94% classical (M1) monocytes has been shown to be highly sensitive and specific for a diagnosis of CMML.[2] PB monocyte subset analysis was not available for every patient in the main cohort; therefore, to analyze the relationship among M1 monocytes, CD56 expression, and the mutation profile, a separate cohort of 135 patients was investigated. Of these 135 patients, 95 underwent a subsequent BM biopsy for definitive diagnosis (CMML = 28, MDS = 23, myeloproliferative neoplasm = 9, nondiagnostic = 27, other = 8). The presence of aberrant CD56 was again strongly associated with the presence of a mutation (OR, 43.9; 95% CI, 8.9-793.9; $P = .0003$). This was also noted, to a lesser extent, in association with having >94% M1 monocytes (OR, 3.9; 95% CI, 1.8-8.7; $P = .0007$) (supplemental Table 7). There was some correlation between the presence of CD56 expression and >94% M1 monocytes ($r = 0.17$; $P = .039$), and combining both produced a stronger effect (OR, 8.5; 95% CI, 3.9-19.5; $P < .00001$). Importantly, combining these phenotypic aberrancies did not capture all patients with a mutation. Although CD56 was highly specific for the presence of a mutation (98%), sensitivity was only 48%. Similarly, the presence of >94% M1 monocytes had a specificity of 75% for detecting a mutation, but the sensitivity was only 56%.

With respect to a confirmed diagnosis, CD56 expression (OR, 4.9; 95% CI, 1.9-13; $P = .001$) and >94% M1 monocytes (OR, 4.2; 95% CI, 1.7-11.5; $P = .003$) were associated with a final diagnosis of CMML; however, of note, 4 patients with CMML did not have either of these phenotypic aberrancies.

Discussion

This is the first study to formally examine the use of mutational analysis of patients presenting with a monocytosis. This was performed in combination with current gold standard techniques, including recently described flow cytometric analyses, in a large patient cohort. By analyzing sequential samples referred to a regional diagnostic laboratory, this study has investigated the typical patient population encountered in routine hematology practice. The use of objective outcome measures (longitudinal blood counts and OS) and an unselected patient population have minimized bias and ensured that the results are applicable in the "real-world" setting. Using a targeted sequencing panel of recurrently mutated genes, this study confirms that somatic mutations are identified in virtually all patients with a morphological diagnosis of CMML, as well as in a significant proportion of patients with a monocytosis and nondiagnostic features. It is possible that the proportion of nondiagnostic samples with detectable mutations was inflated as a result of referral bias and a high pretest probability of disease in those undergoing testing; however, these patients had a mutation spectrum, immunophenotype, and outcome indistinguishable from CMML. The presence of a mutation significantly impacted on survival, irrespective of the final diagnosis.

A number of technical limitations of this study should be highlighted. First, because these were routine samples referred for investigation, a corresponding germline sample was not available for analysis. The absence of reference material means that the distinction between germline variants or private single nucleotide polymorphisms and somatic variants is challenging; however, sequencing was limited to well-documented driver genes, and the landscape of mutations in these genes is well

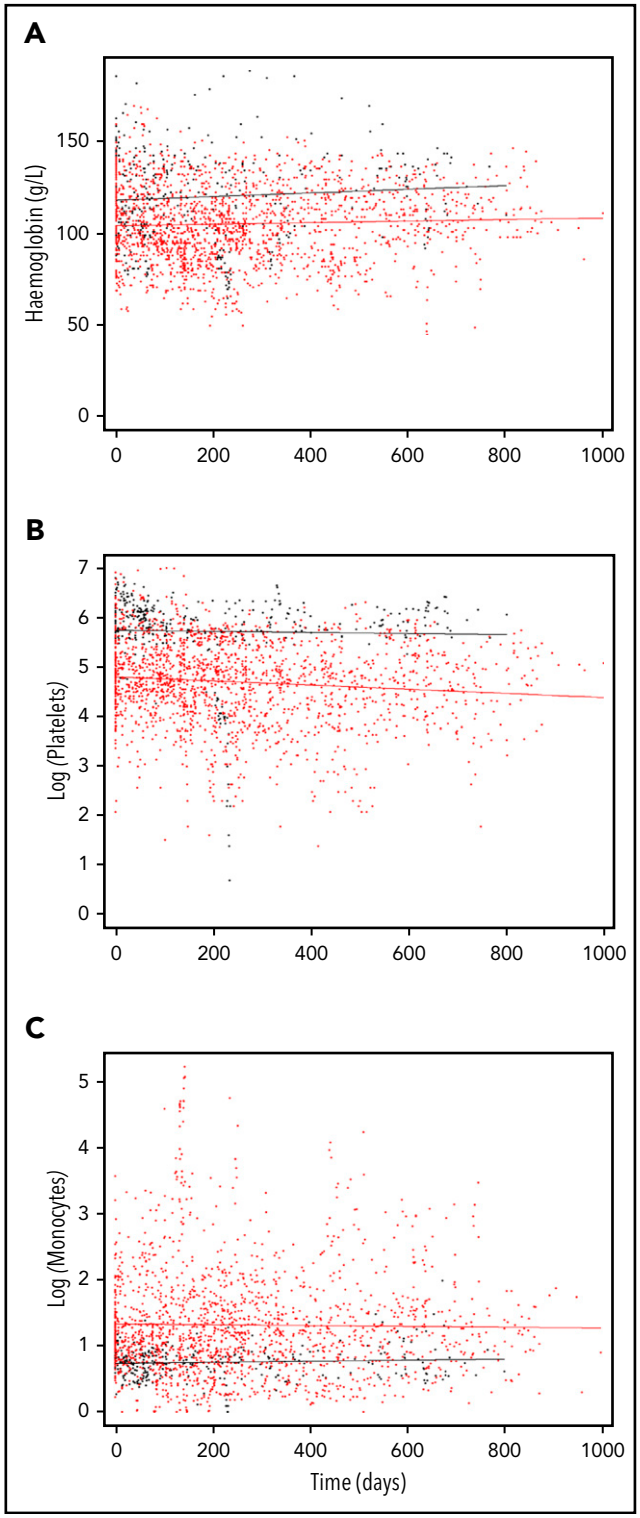
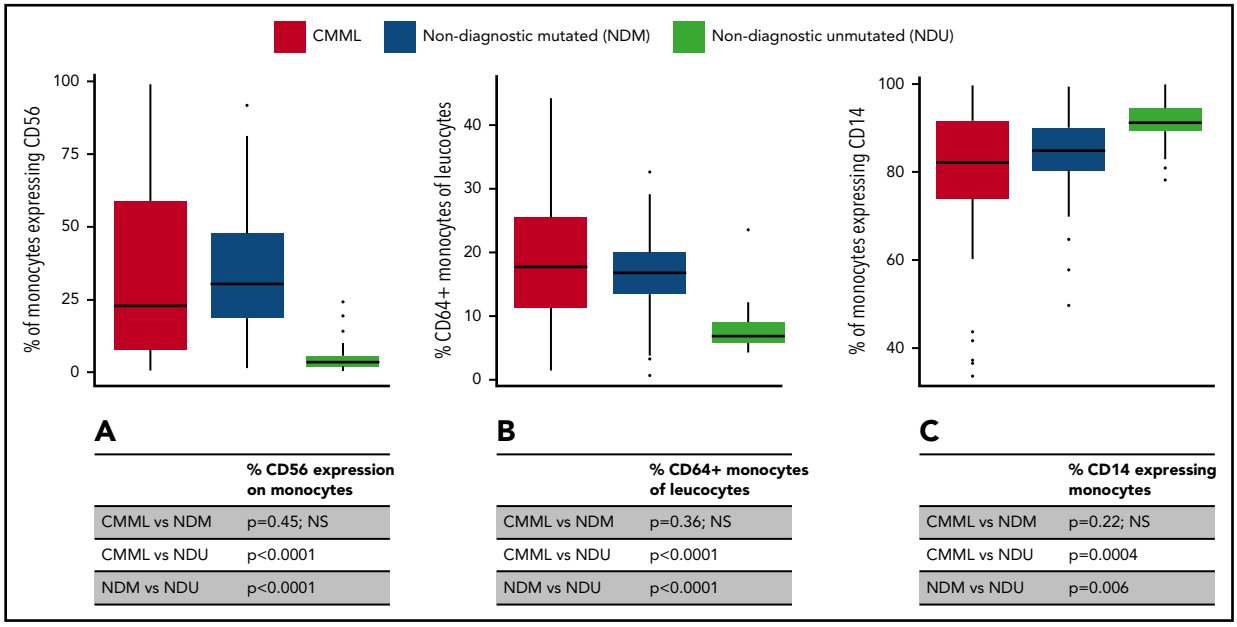


Figure 5. Longitudinal blood count trajectories in relation to mutation status. Plots of all blood count trajectories averaged between mutated (red) and unmutated (black) groups with overlaid linear regression line. Hemoglobin (g/L) (A), platelet count (log transformed) (B), and monocyte count (log transformed) (C) in patients with or without a detectable mutation.

established. Strict filtering criteria were applied (see supplemental Methods) to ensure that only high-confidence variants were included. Second, the sequencing analysis used amplicon-based library preparation, which has recognized limitations with respect to polymerase chain reaction errors and false-positive results, particularly at low VAF; however, the panel was validated internally and externally (see supplemental Methods), and only reproducible variants were included if detected at low VAF or in areas of low coverage. Therefore, the results are, to the best of our ability, accurate. In the future, deeper sequencing should enable more accurate variant calling at low VAF.

The findings of this study will be key to refining future diagnostic algorithms in the investigation of patients referred with a monocytosis. Mutational analysis has been incorporated into the recent amendment of the WHO diagnostic criteria, which now state that the presence of a mutation can support a diagnosis of CMML. However, concerns have been raised regarding the use of mutational analysis in this setting because of reports of frequent somatic mutations in aging healthy individuals.[7-10] As a result, the WHO has stated that the presence of a mutation in CMML or MDS should not be used alone as proof of disease.[1] However, our study has shown that, even in the absence of morphological features, those patients with a mutation had a clinical phenotype and genotype indistinguishable from CMML and a comparably poor outcome. Distinguishing features were also noted between the variants reported in healthy individuals and the mutations detected in our study group. The VAF or clone size of the mutations in our study was significantly higher than in healthy individuals (median 39.2% vs 9%-10%), and this was demonstrated across diagnostic and nondiagnostic samples. This finding has also been described in patients with unexplained cytopenias, and several studies have shown that VAF $>$ 10% and the presence of co-occurring mutations can distinguish clinically significant cytopenias from healthy individuals.[11-13] Although the higher VAF is replicated in our patient group, importantly, our study has shown that even isolated mutations have a significant impact on survival in patients with a monocytosis. These findings provide strong evidence that, in those subjects without diagnostic morphological features, the presence of a mutation, irrespective of mutation number, could be disease defining. At the very minimum, it is imperative that these patients are identified and monitored closely.

It has become increasingly feasible to perform mutational analysis in routine clinical practice, and this study has demonstrated how modest-sized gene panels can provide significant diagnostic and prognostic information. The panel used in the study targeted genes implicated in myeloid malignancies and was incorporated into the routine workload and performed in "real time" in a cost-effective manner. The genetic profile in CMML is now well established and is noted to be relatively homogeneous, involving only a restricted number of genes. Mutation frequencies in $>$90% of patients have been consistently reported using varying panel sizes, including as few as 19 genes.[3,14-16] The mutation profile in our cohort mirrored that reported in the literature; despite the restricted panel, the mutation frequency was high, and a significant impact on outcome was demonstrated. The recognized poor prognostic impact of *ASXL1* mutations[3,14,17-20] was also replicated across this data set. Therefore, mutational analysis is viable in a routine diagnostic laboratory. It is also likely that a proportion of these patients will have additional mutations in genes not sequenced in this study. To further investigate this would require more extensive sequencing on much larger patient populations.



	% CD56 expression on monocytes
CMML vs NDM	p=0.45; NS
CMML vs NDU	p<0.0001
NDM vs NDU	p<0.0001

	% CD64+ monocytes of leucocytes
CMML vs NDM	p=0.36; NS
CMML vs NDU	p<0.0001
NDM vs NDU	p<0.0001

	% CD14 expressing monocytes
CMML vs NDM	p=0.22; NS
CMML vs NDU	p=0.0004
NDM vs NDU	p=0.006

Figure 6. Relationship between immunophenotype and mutations. Box-and-whisker plots comparing immunophenotypic features of CMML, nondiagnostic mutated cases (NDM), and nondiagnostic unmutated cases (NDU). (A) Percentage of monocytes expressing CD56. (B) Percentage of CD64$^+$ monocytes among leukocytes. (C) Percentage of CD14-expressing monocytes. The *P* values were determined using the Mann-Whitney *U* test. NS, not statistically significant.

The potential for PB to be used as a screening tool for monocytosis has also been addressed in this study. This is an attractive option, particularly in a disease commonly presenting in the older patient population. Using flow cytometry, the presence of >94% M1 monocytes in the PB was reported to be highly sensitive and specific for CMML.[2] Subsequent studies have validated these findings and also confirmed the ability to distinguish CMML from MDS and myeloproliferative neoplasm cases presenting with a monocytosis.[21,22] However, these studies are centered on morphological diagnoses, and mutational analyses have not been performed consistently. Although our study has shown a strong correlation between skewed monocyte subsets and a diagnosis of CMML, this did not capture all patients and was neither sensitive nor specific for the presence of a mutation. In contrast, aberrant CD56 expression was highly specific for the presence of a mutation (98%), particularly involving *TET2*. CD56 expression has been reported to be highly sensitive and specific for a diagnosis of CMML (100% and 67%, respectively) when combined with other immunophenotypic features, including reduced expression of myeloid antigens and ≥20% immature monocytes[23]; however, subsequent studies raised concerns regarding the overexpression of CD56 in reactive conditions.[24] Our data show that CD56 expression at diagnosis is invariably associated with the presence of a somatic mutation, although sensitivity was low (48%). Therefore, flow cytometry could provide a screening tool for the investigation of PB monocytes; however, ultimately, mutational analysis will be required to identify patients who require clinical follow-up.

Importantly, there was high concordance between PB and BM mutational analysis, and the presence of a PB mutation was highly predictive of a subsequent BM diagnosis. This suggests that screening of the PB may be a suitable method for identifying or excluding significant mutations; however, this could lead to an increase in inappropriate referrals and a significant burden on laboratory personnel. Furthermore, the small proportion of mutated patients in our cohort with other hematological malignancies in the BM, including AML, highlights the importance of a baseline BM assessment to definitively classify the disease. In contrast, the negative predictive value of PB screening was 100%, suggesting that those without a mutation should not undergo BM assessment. In the first instance, PB screening would be a practical option in those patients unfit for BM assessment or potentially to monitor for treatment response or disease evolution. The latter would require further investigation in a prospective study.

In conclusion, this study has confirmed that mutations are commonly detected in patients referred with a persistent monocytosis. The presence of a mutation impacts significantly on outcome, irrespective of diagnosis, and patients with a mutation who fail to meet WHO criteria have CMML disease characteristics. These findings validate the inclusion of somatic mutations in the diagnostic criteria for CMML, and, at the very minimum, those without a confirmed diagnosis require close clinical follow-up. Although PB can be confidently used to detect mutations, a baseline BM biopsy is required for definitive disease classification in patients fit for treatment. Immunophenotypic assessment of monocytes may provide a potential screening tool to detect those with a mutation; however, it will miss a proportion of mutated patients. Ultimately, early identification of patients could provide an opportunity for intervention in this patient group, and this requires further investigation.

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Authorship

Contribution: C.C. and S.C. designed the study and wrote the manuscript; C.C., M.C., M.S., S.V.H., and P.E. performed the research; and C.C., M.C., J.T., P.G., A.S., and S.C. analyzed the data.

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Footnotes

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