

**Title:** Relationship of blood monocytes with chronic lymphocytic leukemia aggressiveness and outcomes: a multi-institutional study

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**Abstract**

Monocyte-derived cells, constituents of the cancer microenvironment, support chronic lymphocytic leukemia (CLL) cell survival *in vitro* via direct cell-cell interaction and secreted factors. We hypothesized that circulating absolute monocyte count (AMC) reflects the monocyte-derived cells in the microenvironment, and that higher AMC is associated with increased CLL cell survival *in vivo* and thus inferior CLL patient outcomes. We assessed the extent to which AMC at diagnosis of CLL is correlated with clinical outcomes, and whether this information adds to currently used prognostic markers. We evaluated AMC, clinically used prognostic markers, and time to event data from 1,168 CLL patients followed at the Mayo Clinic, the Duke University Medical Center, and the Durham VA Medical Center. Elevated AMC was significantly associated with inferior clinical outcomes, including time to first therapy (TTT) and overall survival (OS). AMC combined with established clinical and molecular prognostic markers significantly improved risk-stratification of CLL patients for TTT. As an elevated AMC at diagnosis is associated with accelerated disease progression, and monocyte-derived cells in the CLL microenvironment promote CLL cell survival and proliferation, these findings suggest that monocytes and monocyte-derived cells are rational therapeutic targets in CLL.

## Introduction

An altered tumor microenvironment is recognized as a hallmark of cancer [1]. In chronic lymphocytic leukemia (CLL), an incurable malignancy with a spectrum of clinical aggressiveness, numerous laboratory-based studies have supported the concept that mononuclear phagocytes (monocytes and macrophages), derived from inflammatory peripheral blood monocytes or tissue resident macrophages [2], in the tumor microenvironment modulate CLL cell viability, survival, and resistance to drug-induced apoptosis [3-6]. These data suggest that circulating monocytes may contribute to the population of CLL-associated macrophages in lymph nodes, spleen, and bone marrow. Elevated level of circulating monocytes could promote CLL cell survival and proliferation, conferring worse clinical outcomes for CLL patients.

In various solid and hematologic malignancies, the circulating absolute monocyte count (AMC) at the time of diagnosis has prognostic significance [7-13]. In CLL, such work has not fully evaluated the relevance of AMC with other routinely used prognostic markers in the context of large patient cohorts. Thus, it is currently unclear the extent to which AMC adds information to other routinely measured markers in CLL, and whether it should be used by clinicians.

To assess the extent to which AMC is an *in vivo* reflection the *in vitro* protective effect of monocyte-derived cells in the CLL microenvironment, we reviewed AMC values at the time of CLL diagnosis, using retrospective data from two large CLL cohorts at the Mayo Clinic and the Duke University and Durham VA Medical Centers. We sought to determine the prognostic significance of AMC at diagnosis in terms of overall survival (OS) and time to first therapy (TTT) in CLL, as well as to evaluate AMC together with other prognostic markers.

## Methods

### Patients

Patients with a diagnosis of CLL [14], either evaluated at the Mayo Clinic Rochester, the Duke University Medical Center, or the Durham VA Medical Center, were enrolled in IRB approved protocols at their respective institutions to collect clinical data. Data obtained included date of diagnosis, Rai stage, treatment history, gender, age, and molecular prognostic factors, specifically immunoglobulin heavy chain variable region (IGHV) gene mutation status, ZAP70 status (>20% considered positive), CD38 status (>30% considered positive), and cytogenetic aberrations by interphase fluorescence in situ hybridization (FISH) testing.

The AMC was defined as the monocyte count measured on the day of diagnosis  $\pm$  3 months, prior to the administration of any CLL-directed therapy. CLL patients in whom AMC values were not available were not included in this analysis. The monocyte count was determined either via automated or manual differential.

### Statistical Considerations

The primary outcome was TTT, which was defined as time from date of CLL diagnosis to date of first therapy or date of last follow-up if untreated. Prognostic analyses were conducted using Cox proportional hazards models [15]. The resulting hazard ratios (HR), 95% confidence intervals (CI), and p-values are reported. These analyses were adjusted for age (log<sub>10</sub> transformed), gender, and Rai stage, which was categorized as 0, 1 or 2, and 3 or 4, and were stratified by site between the Mayo Clinic and Duke University/Durham VA Medical Centers.

Analyses were based on all available patients with complete baseline data. Missingness of baseline covariates was considered non-informative. AMC was treated as a continuous variable, while CD38, ZAP70, IGHV and FISH statuses were treated as binary variables (reference values were CD38-, ZAP70-, IGHV mutated, and FISH negative for 17p and 11q deletions, respectively). We used multivariate Cox proportional hazards models to test for interactions between AMC and other prognostic markers in models adjusted for age, gender and Rai stage and stratified by site.

To determine if AMC added information to other routinely measured markers, we used analysis of deviance [15] to compare multivariate Cox proportional hazards models. A model including AMC and CD38, ZAP70, IGHV, and FISH statuses was compared to a nested model lacking AMC as a predictor. Both models were adjusted for age, gender and Rai stage and stratified by site. As an illustration of the contribution of AMC, HR, 90% CI and p-values are reported for all predictors in the full model. These time to event analyses were also used applied to the secondary outcome of OS, defined as time from date of CLL diagnosis to death date or date of last follow-up.

Conditional inference trees [16] were used in a TTT model with AMC (but without other covariates) stratified by site, to determine the AMC value of maximum separation of TTT, defining “high” or “low” AMC. This cut-off value was used to visualize the association of AMC and the time to event outcomes in Kaplan–Meier (KM) [15] plots of TTT and OS. This analysis was used for visualization only, rather than model-building purposes. For consistency, the same cut-off value was used for both the TTT and OS KM plots. To illustrate the use of AMC in

concert with other routinely measured markers in risk stratification of patients, KM plots of TTT were generated with separate curves for each combination of dichotomized AMC and selected risk factors.

Statistical analyses were conducted at the Duke Cancer Institute. We used the R software environment for statistical computing and graphics [17] and the survival [18], party [19], and coin [20] extension packages to conduct the statistical analyses and to generate the figures. P-values and confidence interval estimates were not adjusted for multiple testing.

## Results

### CLL patient characteristics

We evaluated data collected from CLL patients at the Mayo Clinic (Mayo; n = 947) and the Duke University and Durham VA Medical Centers (Duke/VA; n = 221). Demographic, clinical, and molecular prognostic factors from both cohorts are outlined in Table I. The Mayo Clinic cohort patients were older at the time of diagnosis, included more women, and had a lower fraction of patients with unfavorable risk prognostic factors. The combined dataset (n = 1,168) included patients with high-risk prognostic features such as Rai stage of 3 or 4 (6%), unmutated IGHV (26%), and unfavorable interphase cytogenetics (17pdel or 11qdel by FISH; 9%). We analyzed the combined dataset to evaluate associations between AMC at diagnosis and clinical outcomes.

### Elevated AMC at diagnosis is associated with inferior clinical outcomes

The AMC at diagnosis ranged from 0 to  $23.72 \times 10^3$  cells/mm<sup>3</sup> (median 0.55) in the Mayo cohort, and 0 to  $7.63 \times 10^3$  cells/mm<sup>3</sup> (median 0.64) in the Duke/VA cohort (p-value = 0.0007). In the combined dataset, AMC values ranged from 0 to  $23.72 \times 10^3$  cells/mm<sup>3</sup> (median 0.58). Controlling for age, gender, and Rai stage at diagnosis, elevated AMC evaluated as a continuous variable was significantly associated with a shorter time to first therapy (HR 1.13, 95% CI 1.08 – 1.19, p-value < 0.0001). In addition, elevated AMC was significantly associated with inferior overall survival (HR 1.09, 95% CI 1.01 – 1.16, p-value = 0.0240). The results of the individual and combined analyses, stratified by site, are shown in Table II.

To graphically visualize and assess the clinical impact of AMC, we used conditional inference tree analysis that identified  $1.545 \times 10^3$  cells/mm<sup>3</sup> (94<sup>th</sup> percentile across all patients in the combined dataset) as an AMC cut-off of maximum separation between prognostic subgroups for TTT. Patients with an AMC equal to or above this level at diagnosis had a shorter time to therapy (HR 2.94, 95% CI 2.13 – 4.06, p-value < 0.0001), after controlling for age, gender, and Rai stage and stratifying by site. This cut-off also identified patients with an inferior overall survival (HR 1.75, 95% CI 1.14 – 2.67, p-value = 0.0104). These results are shown in Table II and Figure 1. Both as a continuous variable and when considered as a dichotomous variable, elevated AMC at diagnosis was associated with inferior patient outcomes.

Elevated AMC provides added risk-stratification when combined with routinely used clinical and molecular prognostic factors

As seen in Table II, commonly used molecular prognostic markers (CD38 expression, IGHV mutation status, and interphase cytogenetics by FISH) were prognostic for TTT and OS in the

combined cohort, after controlling for Rai stage, age, and gender, and cohort. We evaluated the contribution of multiple clinical and molecular markers for their prognostic significance, using a multivariate Cox proportional hazards model. AMC, as a continuous measure, retained prognostic significance for TTT within this context (Table III). In addition, using analysis of deviance to compare this full model to a reduced model without AMC demonstrated a significant difference between the models ( $p = 0.0005$ ), indicating that AMC contributes prognostic information about TTT in these cohorts, beyond that contributed by other currently used prognostic factors. However, AMC was not significantly associated with OS in a similar multivariate model.

As shown above, patients with AMC greater than or equal to  $1.545 \times 10^3$  cells/mm<sup>3</sup> at diagnosis comprise a high-risk group (Figure 1). We found no significant interactions between AMC and any of the established prognostic markers (data not shown). Elevated AMC appeared to further risk-stratify patient groups defined by clinically used molecular prognostic markers (Figure 1). For example, CLL patients with low AMC and mutated IGHV appeared to have the best prognosis, while patients with high AMC/mutated IGHV have a similar prognosis to patients with low AMC/unmutated IGHV. Similarly, patients with low AMC and low-risk cytogenetics (absence of 17p or 11q deletions) appeared to have prognoses superior to those of patients with high AMC and presence of high-risk cytogenetics. A similar pattern was observed for CD38 and ZAP70 expression (data not shown). These results suggest that when combined with other prognostic markers, a high AMC identifies CLL patients at risk for rapid progression and need for treatment.



## Discussion

This study defines the AMC as a robust prognostic factor in newly diagnosed CLL. From the perspective of prognostication and risk-stratification, we note that AMC at diagnosis is an easily measured marker, has prognostic significance across two cohorts representing three medical centers, and defines high and low risk groups of CLL patients even within the context of other commonly used prognostic markers (such as interphase cytogenetics and IGHV mutation status). Higher AMC at diagnosis is associated with inferior clinical outcomes, specifically time to first therapy and overall survival. A similar association has been observed in two smaller CLL cohorts [12, 13].

The findings presented here suggest that circulating blood monocytes reflect the lymph node and bone marrow microenvironment. Supportive macrophages or nurse-like cells have been established as important constituents of this microenvironment, favoring CLL cell viability and providing protection from numerous CLL directed therapies [3, 5, 6, 21]. In other malignancies, an elevated AMC is associated with higher density of tumor associated macrophages [8]. Thus, in CLL, a higher AMC could reflect a higher level of monocytes and macrophages in the tumor microenvironment, which as a protective feature for CLL cells would confer inferior outcomes for patients.

Alternatively, it has also been suggested that a subset of the circulating monocytes are myeloid derived suppressor cells (MDSC; CD14<sup>+</sup>HLA<sup>lo/neg</sup>) that function to suppress the immune system and induce tolerance [22]. Thus, an elevated AMC levels may be a surrogate for an increase in MDSCs, which could allow CLL cells to evade immune surveillance. In turn, this would be

associated with inferior patient outcomes. In fact, one report identified an association between elevated MDSC levels and inferior prognosis in CLL [23].

Most recently, it has been found that monocyte expansion occurs in leukemic E $\mu$ -TCL1 mice, and monocytes appear to be necessary for the development and progression of CLL in this mouse model [24, 25]. Interestingly, a therapeutic approach to reduce the number of monocytes delayed the onset of leukemia when E $\mu$ -TCL1 cells were adoptively transferred to wildtype or CD4<sup>+</sup> T-cell depleted mice [25]. These results suggest that monocyte depletion could be an effective CLL therapy.

Our study includes data from over 1,000 patients, with broad applicability to patients at other medical centers. However, we note several details that could affect our conclusions. First, there was variability between the cohorts in this study that, despite the use of stratification and controlling for certain covariates, could affect the results obtained. For example, compared to the Duke/VA cohort, the Mayo Clinic cohort patients had a lower fraction of patients with unfavorable risk prognostic factors such as unmutated IGHV and presence of 17pdel or 11qdel. Second, results in the two cohorts could be affected by different durations of follow-up or by missing baseline covariates (which were assumed to be non-informative for the purposes of these analyses). Third, selecting this cohort of patients from the entire Mayo Clinic and Duke/VA patient populations may have introduced confounders that could alter the prognostic power of AMC or other established prognostic factors. Last, generalizing the dichotomized AMC to other cohorts may be difficult since the cut-off value of  $1.545 \times 10^3$  cells/mm<sup>3</sup> represented the 94<sup>th</sup> percentile, and thus may be easily skewed by variations in ranges and outliers in AMC between

populations. In fact, this cut-off value is higher than those reported in earlier publications [12, 13].

One potential concern about the reproducibility across centers is related to different methods of measuring AMC, whether it be by manual or automated differential, or the type of automated blood count instrument. In the Duke/VA cohort, no differences in AMC were observed between Duke and the VA even though automated differentials were consistently used to determine AMC for VA patients while manual differentials were typically used for Duke patients (data not shown). In the Mayo clinic cohort, slight but significant differences were seen in median AMC determined with manual or automated methods ( $0.52$  vs.  $0.57 \times 10^3$  cells/mm<sup>3</sup>,  $p = 0.01$ ).

However, in a subset of patients in the Mayo clinic cohort, the AMC was determined by both manual and automated differentials on the same day. In these patients, no significant differences in AMC were observed. Importantly, regardless of site (Mayo, Duke, or Durham VA), AMC at diagnosis remained a strong predictor of clinical outcomes.

Collectively, these findings add to the growing body of evidence demonstrating that extrinsic factors, particularly those related to the tumor microenvironment, promote CLL progression and provide valuable prognostic information. The AMC is inexpensive, easily interpreted, universally available, and identifies patients at high-risk of disease progression, particularly when used in conjunction with conventional prognostic factors. As monocyte-derived cells promote CLL progression via cell-cell interactions, secretion of trophic factors, and suppression of host immunity, the observation that an elevated AMC is associated with more rapid disease progression may have significant therapeutic implications.

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**Conflict of Interest**

The authors declare no conflicts of interest.

**Author contributions**

D.R.F and R.A.W. conceived the study, designed and performed research, analyzed data, and wrote the paper; A.B. S., K.O., K.G.C., and S.S. performed research, analyzed data and assisted with manuscript preparation; N.E.K, C.A.H, W.D., T.D.S., and J.B.W. assisted with research design and analysis, and critically reviewed the manuscript.

## References Cited

1. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57-70.
2. Perdiguero EG, Geissmann F. The development and maintenance of resident macrophages. *Nat Immunol* 2015;17:2-8.
3. Burger JA, Tsukada N, Burger M, et al. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. *Blood* 2000;96:2655-2663.
4. Kurtova AV, Balakrishnan K, Chen R, et al. Diverse marrow stromal cells protect CLL cells from spontaneous and drug-induced apoptosis: development of a reliable and reproducible system to assess stromal cell adhesion-mediated drug resistance. *Blood* 2009;114:4441-4450.
5. Nishio M, Endo T, Tsukada N, et al. Nurselike cells express BAFF and APRIL, which can promote survival of chronic lymphocytic leukemia cells via a paracrine pathway distinct from that of SDF-1alpha. *Blood* 2005;106:1012-1020.
6. Seiffert M, Schulz A, Ohl S, et al. Soluble CD14 is a novel monocyte-derived survival factor for chronic lymphocytic leukemia cells, which is induced by CLL cells in vitro and present at abnormally high levels in vivo. *Blood* 2010;116:4223-4230.
7. Eo WK, Jeong da W, Chang HJ, et al. Absolute monocyte and lymphocyte count prognostic score for patients with gastric cancer. *World J Gastroenterol* 2015;21:2668-2676.
8. Kumagai S, Marumo S, Shoji T, et al. Prognostic impact of preoperative monocyte counts in patients with resected lung adenocarcinoma. *Lung Cancer* 2014;85:457-464.
9. Matsuo K, Hom MS, Moeini A, et al. Significance of monocyte counts on tumor characteristics and survival outcome of women with endometrial cancer. *Gynecol Oncol* 2015;138:332-338.

10. Wilcox RA, Ristow K, Habermann TM, et al. The absolute monocyte and lymphocyte prognostic score predicts survival and identifies high-risk patients in diffuse large-B-cell lymphoma. *Leukemia* 2011;25:1502-1509.
11. Wilcox RA, Ristow K, Habermann TM, et al. The absolute monocyte count is associated with overall survival in patients newly diagnosed with follicular lymphoma. *Leuk Lymphoma* 2012;53:575-580.
12. Herishanu Y, Kay S, Sarid N, et al. Absolute monocyte count trichotomizes chronic lymphocytic leukemia into high risk patients with immune dysregulation, disease progression and poor survival. *Leuk Res* 2013;37:1222-1228.
13. Mazumdar R, Evans P, Culpin R, et al. The automated monocyte count is independently predictive of overall survival from diagnosis in chronic lymphocytic leukaemia and of survival following first-line chemotherapy. *Leuk Res* 2013;37:614-618.
14. Hallek M, Cheson BD, Catovsky D, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood* 2008;111:5446-5456.
15. Therneau TM, Grambsch PM. *Modeling Survival Data: Extending the Cox Model*. New York: Springer; 2000.
16. Hothorn T, Hornik K, Zeileis A. Unbiased Recursive Partitioning: A Conditional Inference Framework. *Journal of Computational and Graphical Statistics* 2006;15:651- 674.
17. R Core Team. *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing; 2015.
18. Therneau T. *A Package for Survival Analysis in S*. 2015.

19. Hothorn T. A Laboratory for Recursive Partytioning. 2015.
20. Hothorn T, Hornik K, van de Wiel MA, Zeileis A. Implementing a Class of Permutation Tests: The coin Package. *Journal of Statistical Software* 2008;28:1-23.
21. Pedersen IM, Kitada S, Leoni LM, et al. Protection of CLL B cells by a follicular dendritic cell line is dependent on induction of Mcl-1. *Blood* 2002;100:1795-1801.
22. Jitschin R, Braun M, Buttner M, et al. CLL-cells induce IDOhi CD14+HLA-DRlo myeloid-derived suppressor cells that inhibit T-cell responses and promote TRegs. *Blood* 2014;124:750-760.
23. Liu J, Zhou Y, Huang Q, Qiu L. CD14HLA-DR expression: A novel prognostic factor in chronic lymphocytic leukemia. *Oncol Lett* 2015;9:1167-1172.
24. Hanna BS, McClanahan F, Yazdanparast H, et al. Depletion of CLL-associated patrolling monocytes and macrophages controls disease development and repairs immune dysfunction in vivo. *Leukemia* 2015.
25. Kocher T, Asslaber D, Zaborsky N, et al. CD4+ T cells, but not non-classical monocytes, are dispensable for the development of chronic lymphocytic leukemia in the TCL1-tg murine model. *Leukemia* 2015.

## Table Legends

Table I: Patient characteristics in the Mayo Clinic and Duke/Durham VA cohorts and for the combined cohort. FISH unfavorable = presence of 17pdel or 11qdel. FISH favorable = absence of 17pdel and 11qdel. NA = not available.

Table II: Univariate analysis of absolute monocyte count (AMC) as a continuous variable, AMC as a dichotomized variable (dichotomized at  $1.545 \times 10^3$  cells/mm<sup>3</sup>), and established molecular prognostic markers, after controlling for age, gender, and Rai stage at diagnosis. Hazard ratio (HR), 95% confidence intervals (CI) and p-value provided by site and for the combined cohort (stratified by site), both for time to therapy and for overall survival. UM = unmutated. FISH unfavorable = presence of 17pdel or 11qdel.

Table III: Multivariate Cox proportional hazards model of clinical and molecular markers for time to therapy. The number of patients with complete data for this analysis = 449, and the number of events = 147. Hazard ratio (HR), 95% confidence intervals (CI) and p-value provided for the combined cohort (stratified by site). Rai Stage is collapsed to three levels (0; 1 or 2; 3 or 4). UM = unmutated. FISH unfavorable = presence of 17pdel or 11qdel.



## Figure Legends

Figure 1: Elevated AMC, dichotomized at  $1.545 \times 10^3$  cells/mm<sup>3</sup> is associated with inferior time to therapy (A) and overall survival (B) in the combined cohort. Total number of patients and number of events for each curve as follows: For TTT, the Low AMC curve had 1098 and 299, respectively, and the High AMC curve had 70 and 45, respectively. For OS, the Low AMC curve had 1098 and 233, respectively, and the High AMC curve had 70 and 26, respectively. Elevated AMC in combination with clinically used molecular prognostic markers, such as IGHV mutational status (C) or chromosomal aberrations measured by FISH (D), provide additional risk-stratification in terms of time to therapy. UM = unmutated; M = mutated; FISH + = presence of 17pdel or 11qdel; FISH - = absence of 17pdel or 11qdel. In (C), the total number of patients and number of events for each curve are as follows: M and Low AMC with 386 and 81, respectively; M and High AMC with 19 and 12, respectively; UM and Low AMC with 266 and 119, respectively; and UM and High AMC with 32 and 26 respectively. In (D), the total number of patients and number of events for each curve are as follows: FISH - and Low AMC with 552 and 114, respectively; FISH - and High AMC with 43 and 24, respectively; FISH + and Low AMC with 89 and 39, respectively; FISH + and High AMC with 12 and 11, respectively.

Table I

|   | Mayo Clinic<br>(n = 947) | Duke/Durham VA<br>(n = 221) | Combined Cohort<br>(n = 1168) | p-value |
|---|--------------------------|-----------------------------|-------------------------------|---------|
| Age at diagnosis (in years), median (range)           | 65 (25 – 97)             | 62 (30 – 90)                | 64.5 (25 – 97)                | 0.0002  |
| Gender (%)  |                          |                             |                               | 0.0083  |
| Female  | 327 (35)                 | 55 (25)                     | 382 (33)                      |         |
| Male  | 620 (65)                 | 164 (74)                    | 784 (67)                      |         |
| NA  | 0 (0)                    | 2 (1)                       | 2 (0)                         |         |
| Rai Stage at diagnosis (%)                            |                          |                             |                               | 0.6412  |
| 0   | 481 (51)                 | 122 (55)                    | 603 (52)                      |         |
| 1 or 2  | 410 (43)                 | 77 (35)                     | 487 (42)                      |         |
| 3 or 4  | 50 (5)                   | 18 (8)                      | 68 (6)                        |         |
| NA  | 6 (1)                    | 4 (2)                       | 10 (1)                        |         |
| CD38 (%)  |                          |                             |                               | 0.0229  |
| Neg   | 602 (64)                 | 168 (76)                    | 770 (66)                      |         |
| Pos   | 261 (28)                 | 48 (22)                     | 309 (26)                      |         |
| NA  | 84 (9)                   | 5 (2)                       | 89 (8)                        |         |
| ZAP70 (%)   |                          |                             |                               | 0.0002  |
| Neg   | 417 (44)                 | 105 (48)                    | 522 (45)                      |         |
| Pos   | 197 (21)                 | 93 (42)                     | 290 (25)                      |         |
| NA  | 333 (35)                 | 23 (10)                     | 356 (30)                      |         |
| IGHV (%)  |                          |                             |                               | 0.1043  |
| Mutated   | 284 (30)                 | 121 (55)                    | 405 (35)                      |         |
| Unmutated   | 226 (24)                 | 72 (33)                     | 298 (26)                      |         |
| NA  | 437 (46)                 | 28 (13)                     | 465 (40)                      |         |
| FISH (%)  |                          |                             |                               | 0.0585  |
| Favorable   | 460 (49)                 | 135 (61)                    | 595 (51)                      |         |
| Unfavorable   | 69 (7)                   | 32 (14)                     | 101 (9)                       |         |
| NA  | 418 (44)                 | 54 (24)                     | 472 (40)                      |         |
| Time to therapy (in years), median (range)            | 6.28 (0 – 10.30)         | 5.95 (0 – 18.47)            | 6.17 (0 – 18.47)              | 0.8921  |
| Patients who required therapy (%)                     | 235 (25)                 | 109 (49)                    | 344 (29)                      |         |
| Overall survival (in years), median (range)           | 9.35 (0 – 10.31)         | 11.79 (1.16 – 23.83)        | 10.54 (0 – 23.83)             | <0.0001 |
| Patients who died (%)                                 | 184 (19)                 | 75 (34)                     | 259 (22)                      |         |
| Time to follow up or death (in years), median (range) | 3.77 (0 – 10.31)         | 8.68 (1.16 – 23.83)         | 4.57 (0 – 23.83)              | <0.0001 |

Table II

| Time to therapy    |             |      |             |          |                |      |             |          |                                      |      |             |          |
|--------------------|-------------|------|-------------|----------|----------------|------|-------------|----------|--------------------------------------|------|-------------|----------|
|                    | Mayo Clinic |      |             |          | Duke/Durham VA |      |             |          | Combined Cohort (stratified by site) |      |             |          |
|                    | n (events)  | HR   | 95% CI      | p-value  | n (events)     | HR   | 95% CI      | p-value  | n (events)                           | HR   | 95% CI      | p-value  |
| AMC (continuous)   | 941 (232)   | 1.12 | 1.06 – 1.18 | < 0.0001 | 215 (107)      | 1.36 | 1.09 – 1.70 | 0.0059   | 1156 (339)                           | 1.13 | 1.08 – 1.19 | < 0.0001 |
| AMC (dichotomized) | 941 (232)   | 3.67 | 2.48 – 5.44 | < 0.0001 | 215 (107)      | 1.92 | 1.09 – 3.37 | 0.0246   | 1156 (339)                           | 2.94 | 2.13 – 4.06 | < 0.0001 |
| CD38 +             | 858 (214)   | 1.88 | 1.42 – 2.48 | < 0.0001 | 210 (103)      | 1.33 | 0.85 – 2.09 | 0.2171   | 1068 (317)                           | 1.71 | 1.36 – 2.16 | < 0.0001 |
| ZAP70 +            | 612 (139)   | 1.29 | 0.92 – 1.81 | 0.1446   | 192 (88)       | 1.30 | 0.85 – 1.99 | 0.2336   | 804 (227)                            | 1.29 | 0.99 – 1.69 | 0.0584   |
| IGHV UM            | 509 (138)   | 2.43 | 1.69 – 3.50 | < 0.0001 | 189 (98)       | 2.87 | 1.87 – 4.41 | < 0.0001 | 698 (236)                            | 2.60 | 1.97 – 3.42 | < 0.0001 |
| FISH unfavorable   | 526 (135)   | 2.01 | 1.32 – 3.07 | 0.0012   | 165 (81)       | 1.46 | 0.87 – 2.43 | 0.1509   | 691 (216)                            | 1.84 | 1.33 – 2.55 | 0.0002   |

| Overall survival   |             |      |             |          |                |      |             |         |                                      |      |             |          |
|--------------------|-------------|------|-------------|----------|----------------|------|-------------|---------|--------------------------------------|------|-------------|----------|
|                    | Mayo Clinic |      |             |          | Duke/Durham VA |      |             |         | Combined Cohort (stratified by site) |      |             |          |
|                    | n (events)  | HR   | 95% CI      | p-value  | n (events)     | HR   | 95% CI      | p-value | n (events)                           | HR   | 95% CI      | p-value  |
| AMC (continuous)   | 941 (181)   | 1.08 | 1.00 – 1.16 | 0.0550   | 215 (74)       | 1.14 | 0.85 – 1.52 | 0.3784  | 1156 (255)                           | 1.09 | 1.01 – 1.16 | 0.0240   |
| AMC (dichotomized) | 941 (181)   | 1.82 | 1.10 – 3.02 | 0.0206   | 215 (74)       | 1.50 | 0.67 – 3.33 | 0.3225  | 1156 (255)                           | 1.75 | 1.14 – 2.67 | 0.0104   |
| CD38 +             | 858 (157)   | 1.58 | 1.14 – 2.18 | 0.0062   | 210 (72)       | 1.18 | 0.69 – 2.02 | 0.5459  | 1068 (229)                           | 1.49 | 1.13 – 1.96 | 0.0049   |
| ZAP70 +            | 612 (64)    | 1.15 | 0.68 – 1.94 | 0.6051   | 192 (57)       | 1.59 | 0.93 – 2.69 | 0.0889  | 804 (121)                            | 1.40 | 0.97 – 2.00 | 0.0688   |
| IGHV UM            | 509 (62)    | 2.57 | 1.50 – 4.43 | 0.0007   | 189 (71)       | 2.17 | 1.31 – 3.58 | 0.0026  | 698 (133)                            | 2.32 | 1.61 – 3.33 | < 0.0001 |
| FISH unfavorable   | 526 (72)    | 3.08 | 1.86 – 5.12 | < 0.0001 | 165 (49)       | 1.81 | 0.94 – 3.46 | 0.0743  | 691 (121)                            | 2.58 | 1.74 – 3.84 | < 0.0001 |

Table III

|                  | Combined Cohort (stratified by site) |             |          |
|------------------|--------------------------------------|-------------|----------|
|                  | HR                                   | 95% CI      | p-value  |
| Age (log10)      | 1.09                                 | 0.17 – 6.90 | 0.9299   |
| Gender (male)    | 1.97                                 | 1.30 – 2.99 | 0.0014   |
| Rai Stage        | 2.99                                 | 2.29 – 3.89 | < 0.0001 |
| CD38 +           | 1.31                                 | 0.90 – 1.89 | 0.1543   |
| ZAP70 +          | 1.16                                 | 0.82 – 1.63 | 0.4087   |
| IGHV UM          | 2.33                                 | 1.59 – 3.41 | < 0.0001 |
| FISH unfavorable | 1.33                                 | 0.87 – 2.03 | 0.1922   |
| AMC              | 1.18                                 | 1.09 – 1.27 | < 0.0001 |

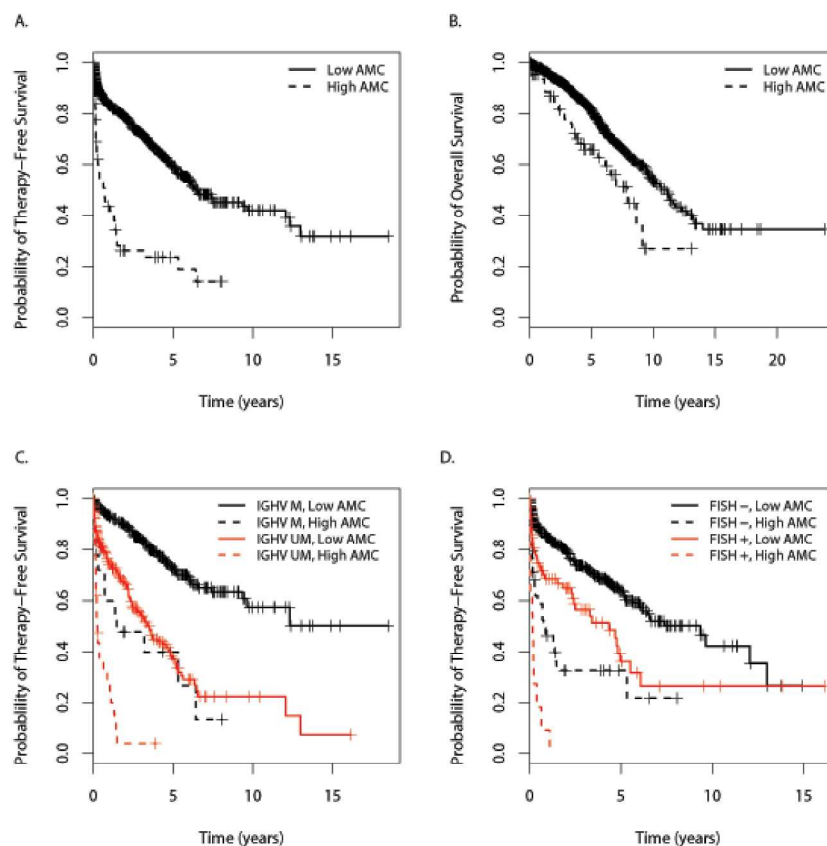


Figure 1: Elevated AMC, dichotomized at  $1.545 \times 10^3$  cells/mm<sup>3</sup> is associated with inferior time to therapy (A) and overall survival (B) in the combined cohort. Total number of patients and number of events for each curve as follows: For TTT, the Low AMC curve had 1098 and 299, respectively, and the High AMC curve had 70 and 45, respectively. For OS, the Low AMC curve had 1098 and 233, respectively, and the High AMC curve had 70 and 26, respectively. Elevated AMC in combination with clinically used molecular prognostic markers, such as IGHV mutational status (C) or chromosomal aberrations measured by FISH (D), provide additional risk-stratification in terms of time to therapy. UM = unmutated; M = mutated; FISH + = presence of 17pdel or 11qdel; FISH - = absence of 17pdel or 11qdel. In (C), the total number of patients and number of events for each curve are as follows: M and Low AMC with 386 and 81, respectively; M and High AMC with 19 and 12, respectively; UM and Low AMC with 266 and 119, respectively; and UM and High AMC with 32 and 26 respectively. In (D), the total number of patients and number of events for each curve are as follows: FISH - and Low AMC with 552 and 114, respectively; FISH - and High AMC with 43 and 24, respectively; FISH + and Low AMC with 89 and 39, respectively; FISH + and High AMC with 12 and 11,

respectively.  
279x361mm (300 x 300 DPI)

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