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# Red Cell Distribution Width and Absolute Lymphocyte Count Associate With Biomarkers of Inflammation and Subsequent Mortality in Rheumatoid Arthritis

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***ABSTRACT.*** ***Objective.*** Morbidity and mortality in rheumatoid arthritis (RA) is partly mitigated by maintaining immune and hematologic homeostasis. Identification of those at risk is challenging. Red cell distribution width (RDW) and absolute lymphocyte count (ALC) associate with cardiovascular disease (CVD) and mortality in the general population, and with disease activity in RA. How these variables relate to inflammation and mortality in RA was investigated.

***Methods.*** In a retrospective single Veterans Affairs (VA) Rheumatology Clinic cohort of 327 patients with RA treated with methotrexate (MTX)+/- a tumor necrosis factor (TNF) inhibitor (TNFi), we evaluated RDW and ALC before and during therapy and in relation to subsequent mortality. Findings were validated in a national VA cohort (n = 13,914). In a subset of patients and controls, we evaluated inflammatory markers.

***Results.*** In the local cohort, high RDW and low ALC prior to MTX treatment was associated with subsequent mortality over 10 years (both $P < 0.001$). The highest mortality was observed in those with both high RDW and low ALC. This remained after adjusting for age and comorbidities and was validated in the national RA cohort. In the immunology cohort, soluble and cellular inflammatory markers were higher in patients with RA than in controls. ALC correlated with age, plasma TNF receptor II, natural killer HLA-DR mean fluorescence intensity, and CD4CM/CD8CM HLA-DR/CD38%, whereas RDW associated with age and ALC. MTX initiation was followed by an increase in RDW and a decrease in ALC. TNFi therapy added to MTX resulted in an increase in ALC.

***Conclusion.*** RDW and ALC before disease-modifying antirheumatic drug therapy are associated with biomarkers of monocyte/macrophage inflammation and subsequent mortality. The mechanistic linkage between TNF signaling and lymphopenia found here warrants further investigation.

*Key Indexing Terms:* absolute lymphocyte count, biomarkers, cardiovascular disease, immunity, red cell distribution width

Rheumatoid arthritis (RA) is the most common autoimmune arthritis, characterized by joint inflammation and joint destruction.[1] RA is associated with increased risk of coronary artery disease (CAD),[2] with up to a 50% increased risk of cardiovascular (CV) mortality (equivalent to that conferred by diabetes mellitus [DM]) and 59% increased risk of CV disease (CVD).[3] This increased risk is at least in part attributable to traditional CV risk factors,[4] but also to the chronic inflammatory state, reflected by C-reactive protein (CRP), erythrocyte sedimentation rate (ESR),[5] rheumatoid factor (RF), and cyclic citrullinated peptide (CCP). Potential mediators include endothelial dysfunction, elevated levels of cytokines, lipid mediators (ie, triglycerides,

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oxidized low-density lipoprotein, inflammatory high-density lipoprotein, lysophosphatidic acid), LL-37, insulin resistance, anticitrullinated protein antibodies, and monocyte activation.[5,6]

Increased red cell distribution width (RDW) reflects both states of anemia and immature red cell production. In some cases, this can be driven by variables of systemic inflammation.[7,8] RDW is also associated with increased risk of CVD in the general population, independent of anemia.[9] It has therefore been proposed that RDW be considered predictive of CVD in patients with RA.[10] Another biomarker of morbidity and mortality in inflammatory and noninflammatory diseases is the absolute lymphocyte count (ALC). Lymphopenia in the general population is associated with reduced longevity independent of age and clinical risk factors.[11] How RDW and ALC relate to inflammatory variables, CVD, and mortality in RA is currently unclear.

## METHODS

*Study population.* The retrospective chart review and prospective immunologic sampling components of this study were approved by the Institutional Review Board of the Cleveland Louis Stokes Veteran's Administration Medical Center (VAMC), protocol 17046-H35. All local VAMC electronic medical records were reviewed for RA diagnoses based on International Classification of Diseases (ICD), 9th and 10th revisions, made in the Veterans Affairs (VA) Rheumatology Clinic, with an initial methotrexate (MTX) prescription fill between January 1, 2006, and December 31, 2019, with at least 90 days' supply of MTX filled and a MTX medication possession ratio of at least 75% (Supplementary Figure S1, available with the online version of this article). A total of 521 unique patients met these criteria and charts were individually reviewed for verification of MTX start (as opposed to continuation when changing medical care system and associated new VA prescriptions), resulting in a total of 343 remaining patients. Of these, records were reviewed for complete blood count (CBC) laboratories in the year prior to MTX start, 3 to 12 months after start of MTX, and 3 to 12 months after the addition or start of tumor necrosis factor (TNF) inhibitor (TNFi) medications. Other RA medications prescribed were recorded, and CBCs performed while patients were on RA medications other than MTX, TNF, hydroxychloroquine (HCQ), sulfasalazine, or prednisone were excluded. These exclusions left 327 patients. RF and CCP status were determined from a laboratory database pull and checked for values above or below the reference range. Additional clinical information and laboratory data were obtained as described below.

A subgroup of the parent RA patient cohort (n = 64) followed during 2018 to 2019 in the Rheumatology Clinic was approached for the collection of blood samples (referred to here as an immunology cohort). A comparator control group included patients lacking a diagnosis of any inflammatory arthritis. Those seen by their providers at the VAMC General Medicine Clinics in 2018 to 2019 were also enrolled for blood sampling (n = 37).

Considering a nationwide cohort of patients with RA with MTX starts between 2006 and 2019, we applied similar inclusion/exclusion criteria, but notably without performing manual chart reviews, to confirm new MTX starts (Supplementary Figure S1, available with the online version of this article; n = 13,689). Data were extracted from the VA Corporate Data Warehouse and accessed using the VA Informatics and Computing Infrastructure. With this larger cohort, we replicated our survival models to assess the effects of RDW and ALC on time to all-cause mortality.

*Data extraction.* Clinical and administrative databases were queried to extract demographic information, lipid profiles (high- and low-density lipoproteins and total cholesterol), statin use, smoking history, diagnosis of hypertension (HTN), systolic and diastolic blood pressure (BP), diagnosis of CAD, diagnosis of DM, RDW, and ALC. In both the local and national cohorts, we used the ICD codes described by Quan et al,[12] looking for presence in the active problem list and recent (prior year) inpatient or multiple recent outpatient diagnoses. For the local cohort that was manually chart reviewed, the comorbidities were further confirmed during the chart review process, matching diagnoses with the medication list. The initial and main reference point of this study is from the timepoint of applying first-line standard of care therapy for RA, specifically MTX initiation. Patient mortality was extracted after this time of reference from both VA databases and a Vital Status File that incorporates the Beneficiary Identification Records Locator Subsystem Death file, Medicare Vital Status File, and the Social Security Administration Death Master File.

When sufficient data were available, the atherosclerotic cardiovascular disease (ASCVD) 10-year risk score was calculated as described by Stone et al.[13] The Charlson Comorbidity Index (CCI) was calculated based upon aggregating the diagnoses described above present in the year prior to MTX start as described by Charlson et al.[14]

*Plasma markers of immune activation.* Plasma collected from the RA and control groups was assessed for the following inflammatory markers: soluble CD14 (sCD14), sCD163, interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), TNF receptor II (TNFR2), pentraxin 3 (PTX3), and CRP (R&D Systems for all) by ELISA.

*Flow cytometry.* Peripheral blood mononuclear cells were labeled with fluorochrome conjugated monoclonal antibodies and flow cytometry was performed as shown in Supplementary Figure S2 (available with the online version of this article).

*Statistical analysis.* Associations between continuous variables were assessed using the Spearman rank correlation test. Paired *t* tests were used to assess differences in ALC and RDW values before vs after initiation of treatments. Survival analysis methods, including log-rank tests and Cox proportional hazard models, were used to assess mortality across biomarker levels while adjusting for covariates of interest. Cut-off points for ALC and RDW in survival analyses used here (1.2 k/cmm for ALC and 14.5% for RDW) were based upon prior cohort survival analyses.[15,16] Those with missing pretreatment data were excluded from the survival analysis and the numbers at risk in the survival curves indicate the remaining subjects. No data imputation was performed. All *P* values presented are 2-sided and unadjusted. Statistical analyses were performed using SPSS for Windows version 27.0 (IBM Corp.) and R Statistical Software version 4.0.5 (R Core Team).

## RESULTS

*Study cohort characteristics at time of MTX start.* Demographics and laboratory features of the 327 patients with RA at our single VA center at the time of start of MTX therapy are shown in Table 1. As expected for a VA cohort, the majority were male (93%), commonly had DM (33%), HTN (64%), or CAD (11%), were on statin therapy (62%), or were current or former smokers (68%). Overall, 63% of the patients were seropositive for RF and/or CCP (when tested for both), all the patients were started on MTX therapy (as per required inclusion criteria), and 82 (25%) were subsequently prescribed a TNFi. Demographics and clinical features of the national VA RA cohort (n = 13,689), as well as the local RA cohort subgroup (n = 64) with comparative controls (n = 37) in which immunologic sampling was performed, are also shown in Table 1, and are similar to those of the overall local RA cohort. Before MTX therapy start, age was negatively correlated with ALC (Spearman ρ –0.16, $P = 0.006$) and positively correlated with RDW (ρ 0.21, $P < 0.001$). HCQ use (> 90 days of medication refill in the year prior to MTX start, 16% of the local cohort) was not associated with ALC or RDW. Within the national cohort, the mean age of women

*Table 1.* National, local, and immunology cohort characteristics.

| | Retrospective Chart-Review Cohort | | | | | Nationwide Database Cohort | | | | | Immunology Cohort | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | All Patients | Pretreatment RDW > 14.5 | Pretreatment RDW ≤ 14.5 | Pretreatment ALC ≥ 1.2 | Pretreatment ALC < 1.2 | All Patients | Pretreatment RDW > 14.5 | Pretreatment RDW ≤ 14.5 | Pretreatment ALC ≥ 1.2 | Pretreatment ALC < 1.2 | Controls | RA |
| Patients, n | 327 | 89 | 229 | 253 | 59 | 13,688 | 3245 | 9057 | 11,220 | 2101 | 37 | 64 |
| Age, yrs | 65 (60-71) | 68 (62-72) | 64 (58-71) | 65 (59-70) | 68 (62-74) | 65 (58-71) | 66 (60-73) | 64 (57-70) | 64 (57-70) | 68 (61-75) | 65 (54-71) | 69 (61-73) |
| Male, n (%) | 304 (93) | 83 (93) | 214 (93) | 235 (93) | 57 (97) | 11919 (87) | 2856 (88) | 7915 (87) | 9667 (86) | 1922 (91) | 33 (89) | 65 (94) |
| Race/ethnicity, n (%) | | | | | | | | | | | | |
| White | 276 (84) | 72 (81) | 196 (86) | 219 (87) | 44 (75) | 10806 (79) | 2349 (72) | 7393 (82) | 8818 (79) | 1711 (81) | 28 (76) | 51 (80) |
| African American | 36 (11) | 12 (13) | 23 (10) | 26 (10) | 8 (14) | 1876 (14) | 674 (21) | 1001 (11) | 1575 (14) | 244 (12) | 9 (24) | 10 (15) |
| Other/unknown/missing | 10 (3) | 4 (5) | 6 (2) | 5 (2) | 5 (9) | 1006 (7) | 222 (7) | 663 (7) | 827 (7) | 146 (7) | | |
| Hispanic | 5 (2) | 1 (1) | 4 (2) | 3 (1) | 2 (3) | 656 (5) | 128 (4) | 439 (5) | 560 (5) | 75 (4) | 0 | 3 (5) |
| Charlson Comorbidity Index, mean (SD) | 1.92 (1.7) | 2.27 (1.8) | 1.78 (1.6) | 1.84 (1.6) | 2.31 (1.9) | 1.57 (1.6) | 1.92 (1.8) | 1.44 (1.5) | 1.51 (1.6) | 1.89 (1.8) | | |
| Statin, n (%) | 203 (62) | 63 (71) | 132 (58) | 158 (62) | 36 (61) | 7331 (54) | 1720 (53) | 4858 (54) | 6058 (54) | 1087 (52) | 20 (54) | 40 (58) |
| CAD, n (%) | 35 (11) | 12 (13) | 23 (10) | 29 (11) | 5 (8) | 1062 (8) | 277 (9) | 656 (7) | 848 (8) | 191 (9) | 5 (14) | 19 (30) |
| DM, n (%) | 107 (33) | 37 (42) | 66 (29) | 86 (34) | 16 (27) | 3482 (25) | 999 (31) | 2146 (24) | 2891 (26) | 505 (24) | | |
| HTN, n (%) | 210 (64) | 70 (79) | 135 (59) | 163 (64) | 41 (69) | 8415 (61) | 2122 (65) | 5459 (60) | 6899 (61) | 1306 (62) | | |
| Total cholesterol, mg/dL | 158 (138-182) | 152 (127-170.8) | 163 (144-184.5) | 157 (138-181) | 160 (137.5-183.8) | 168 (143-196) | 161 (136-189) | 170 (145-197) | 169 (144-197) | 162 (140-188) | 181 (148-201) | 148 (123-176) |
| LDL, mg/dL | 95 (76-114) | 88 (71-108.4) | 98 (78-115) | 95 (76-113) | 94 (71.5-116.5) | 98 (77-122) | 92 (73-116) | 99 (78-123) | 98.6 (77-123) | 94 (75-114.1) | 105 (77-116) | 74 (62-106) |
| HDL, mg/dL | 39 (33-48) | 39 (32-49) | 40 (33-48) | 39 (33-48) | 39 (30-48.2) | 43 (36-53) | 43 (35-53) | 43 (36-53) | 43 (35.9-53) | 44 (37-56) | 47 (37-65) | 39 (34-54) |
| Hgb | 13.7 (12.7-14.6) | 12.7 (11.7-13.6) | 14.2 (13-14.8) | 13.9 (12.7-14.7) | 13.2 (12.4-14.5) | 13.8 (12.7-14.8) | 13.1 (11.9-14.2) | 14 (13-14.9) | 13.9 (12.8-14.8) | 13.3 (12.2-14.3) | | |
| Albumin | 3.7 (3.4-4) | 3.5 (3.1-3.7) | 3.8 (3.5-4) | 3.7 (3.4-3.9) | 3.6 (3.3-4) | 3.9 (3.6-4.2) | 3.8 (3.5-4.1) | 3.9 (3.7-4.2) | 3.9 (3.6-4.2) | 3.8 (3.5-4.1) | | |
| ASCVD 10-yr score | 22.2 (12.8-32.5) | 26.6 (17.6-39.7) | 19.4 (11.7-28.7) | 22.2 (12.6-31.9) | 22.8 (15.6-34.4) | 17.4 (9.2-28.4) | 20 (11.5-31.5) | 16.8 (8.8-27.2) | 17.1 (8.9-27.6) | 20.4 (11.9-32.4) | 20 (8-30) | 26 (19-37) |
| Smoker, current or former, n (%) | 221 (68) | 68 (76) | 147 (64) | 180 (71) | 31 (53) | 7631 (56) | 1949 (60) | 5116 (56) | 6364 (57) | 1047 (50) | 22 (59) | 37 (58) |
| Nonsmoker or missing, n (%) | 106 (32) | 21 (24) | 82 (36) | 73 (29) | 28 (47) | 6057 (44) | 1296 (40) | 3941 (44) | 4856 (43) | 1054 (50) | 15 (41) | 27 (42) |
| Seropositive for RF or CCP, % | 63 | 69 | 61 | 60 | 84 | 63 | 70 | 61 | 62 | 64 | | 88 |
| Received TNFi with MTX, n (%) | 82 (25) | 18 (20) | 62 (27) | 69 (27) | 8 (14) | 2860 (21) | 643 (20) | 1979 (22) | 2444 (22) | 350 (17) | | |
| ALC | | | | | | | | | | | | |
| Median (IQR) | 1.71 (1.3-2.19) | 1.74 (1.23-2.26) | 1.71 (1.31-2.16) | 1.86 (1.53-2.35) | 1.07 (0.9-1.15) | 1.7 (1.32-2.2) | 1.61 (1.2-2.12) | 1.76 (1.4-2.2) | 1.86 (1.5-2.3) | 1 (0.8-1.1) | 1.8 (1.4-2.0) | 1.6 (1.2-1.9) |
| Range | 0.5-4.4 | 0.5-3.7 | 0.61-4.4 | 1.2-4.4 | 0.5-1.19 | 0.1-5 | 0.1-5 | 0.2-5 | 1.2-5 | 0.1-1.19 | | |
| RDW | | | | | | | | | | | | |
| Median (IQR) | 13.8 (13.3-14.8) | 15.3 (15-16) | 13.5 (13.1-14) | 13.8 (13.3-14.8) | 14.1 (13.2-15.05) | 13.7 (13.1-14.6) | 15.4 (14.9-16.4) | 13.4 (12.9-13.9) | 13.7 (13.1-14.5) | 14.1 (13.2-15.1) | 13.8 (13.3-14.5) | 14.1 (13-15.3) |
| Range | 11.7-21.2 | 14.6-21.2 | 11.7-14.5 | 11.7-21.2 | 11.9-18.9 | 10.6-29.1 | 14.6-29.1 | 10.6-14.5 | 10.6-29.1 | 11.1-27.3 | | |

Clinical characteristics are shown for local retrospective, national and immunology subgroup cohorts. For local and national cohorts, characteristics are shown for the group as well as for low and high RDW and ALC subgroups. Unless otherwise stated, medians and IQRs are shown for each variable. RF and CCP were tested in 66% and 63% of the local and national cohorts, respectively. When tested for both RF and CCP, the proportion (%) seropositive for either is shown. ALC: absolute lymphocyte count; ASCVD: atherosclerotic cardiovascular disease; CAD: coronary artery disease; CCP: cyclic citrullinated peptide; DM: diabetes mellitus; HDL: high-density lipoprotein; Hgb: hemoglobin; HTN: hypertension; LDL: low-density lipoprotein; MTX: methotrexate; RA: rheumatoid arthritis; RDW: red cell distribution width; RF: rheumatoid factor; TNFi: tumor necrosis factor inhibitor.

was 53.7 years compared to 64.8 years in men. Adjusting for age and allowing for an age-by-sex interaction, we found no sex differences nor age-by-sex interaction effects on RDW. We did, however, find that women had higher ALCs than men, a result similar to that previously published by others evaluating the National Health and Nutrition Examination Survey database.[17]

*High RDW and low ALC prior to start of MTX therapy are associated with subsequent mortality.* We next evaluated the relationship between ALC, RDW, and traditional cardiac risk factor variables at the start of MTX therapy and subsequent mortality. High RDW and low ALC prior to treatment were associated with subsequent mortality over a 10-year period (log-rank test, $P < 0.001$ for both; Figure 1). These findings remained after adjusting for age and comorbidities (Table 2). Specifically, high RDW and low ALC remained associated with mortality after adjusting for age, gender, race, CAD, HTN, DM, statin use, systolic BP, diastolic BP, total cholesterol, smoking status, and treatment prior to MTX start (Table 2). Of the latter variables, age, and diastolic BP were also associated with mortality in the adjusted model ($P = 0.004$, $P < 0.001$, and $P = 0.03$, respectively).

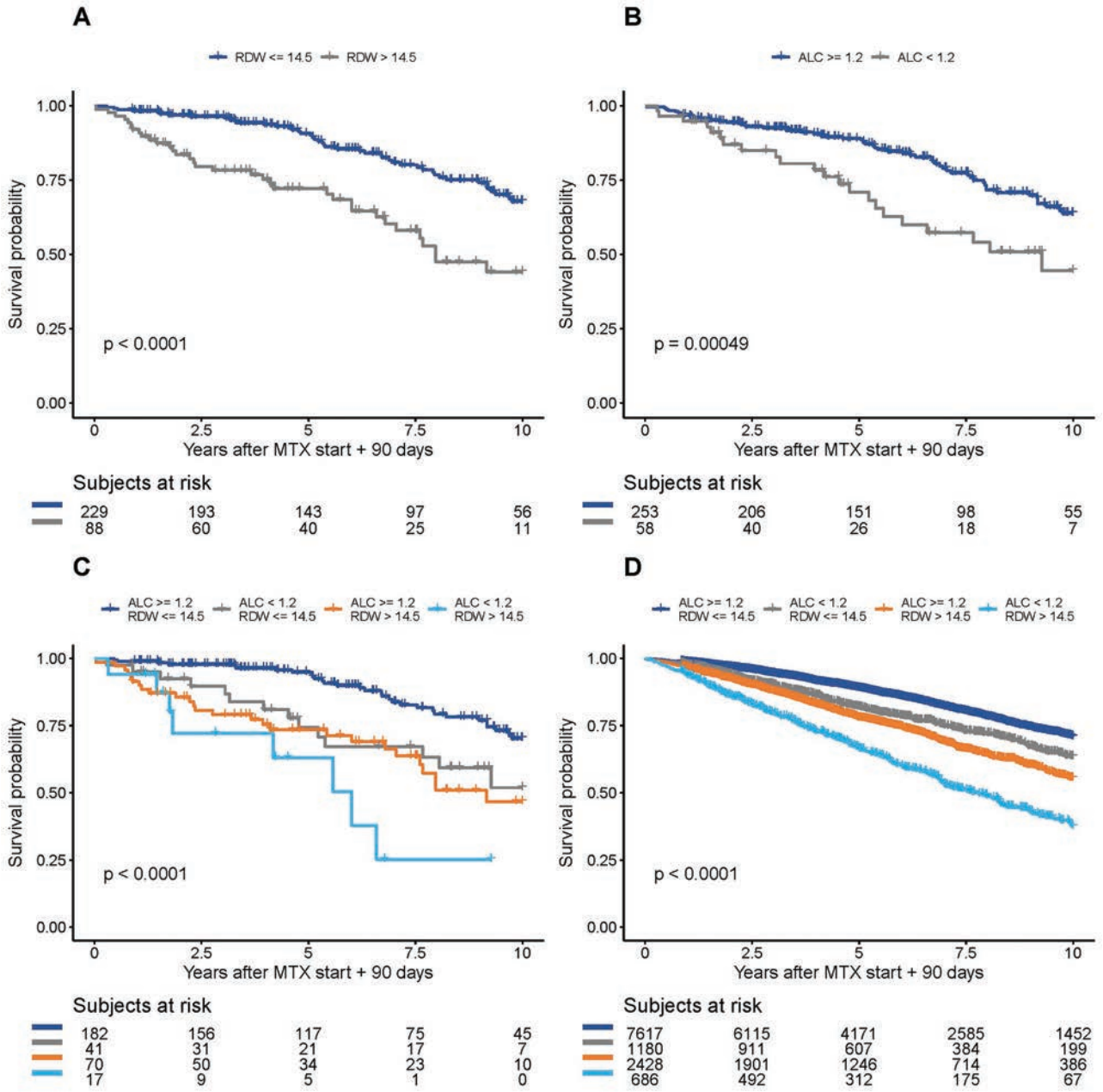


*Figure 1.* Lower survival in patients with RA with high RDW or low ALC prior to MTX start. (A) Survival probability in patients in local cohort with high RDW (> 14.5%) vs without high RDW. (B) Low ALC (< 1.2 K/cmm) vs high ALC. (C) Patients with low RDW/high ALC vs low RDW/low ALC vs high RDW/high ALC vs high RDW/low ALC. (D) National Cohort survival by low RDW/high ALC vs low RDW/low ALC vs high RDW/high ALC vs high RDW/low ALC. ALC: absolute lymphocyte count; MTX: methotrexate; RA: rheumatoid arthritis; RDW: red cell distribution width.

*Table 2.* Local and national cohort survival model, including all variables in adjusted models.

| | Local | | | | National | | | |
|---|---|---|---|---|---|---|---|---|
| | Unadjusted | | Adjusted | | Unadjusted | | Adjusted | |
| | HR (95% CI) | *P* | HR (95% CI) | *P* | HR (95% CI) | *P* | HR (95% CI) | *P* |
| High RDW | 2.75 (1.78-4.24) | < 0.001 | 2.23 (1.37-3.61) | 0.001 | 1.92 (1.77-2.08) | < 0.001 | 1.75 (1.60-1.92) | < 0.001 |
| Low ALC | 2.45 (1.53-3.92) | < 0.001 | 1.85 (1.05-3.24) | 0.03 | 1.55 (1.41-1.70) | < 0.001 | 1.14 (1.03-1.27) | 0.02 |
| Age | | | 1.05 (1.02-1.09) | 0.000 | | | 1.07 (1.07-1.08) | < 0.001 |
| Gender (female vs male) | | | 0.85 (0.26-2.80) | 0.79 | | | 0.53 (0.42-0.68) | < 0.001 |
| Race: Black vs White | | | 0.75 (0.34-1.64) | 0.47 | | | 0.78 (0.67-0.91) | 0.001 |
| Race: Other vs White | | | – | – | | | 1.44 (1.23-1.68) | < 0.001 |
| CAD at MTX | | | 0.61 (0.24-1.56) | 0.30 | | | 0.68 (0.57-0.81) | < 0.001 |
| HTN at MTX | | | 1.17 (0.65-2.10) | 0.59 | | | 1.095 (0.99-1.22) | 0.09 |
| DM at MTX | | | 0.95 (0.56-1.64) | 0.86 | | | 1.26 (1.15-1.39) | < 0.001 |
| Statin at MTX | | | 1.13 (0.65-1.99) | 0.67 | | | 1.05 (0.95-1.16) | 0.39 |
| Systolic BP | | | 1.003 (0.99-1.02) | 0.71 | | | 1.00 (0.997-1.003) | 0.95 |
| Diastolic BP | | | 0.97 (0.94-0.996) | 0.03 | | | 0.99 (0.98-0.99) | < 0.001 |
| Total cholesterol | | | 1.003 (0.996-1.01) | 0.43 | | | 0.999 (0.998-1.001) | 0.36 |
| Smoking: Current/ former vs never/NA | | | 1.37 (0.74-2.53) | 0.31 | | | 1.46 (1.33-1.60) | < 0.001 |
| LR[a] | | 0.71 | | 0.79 | | 0.11 | | 0.23 |

[a] LR test of addition of interaction between ALC and RDW to model above. ALC: absolute lymphocyte count; BP: blood pressure; CAD: coronary artery disease; DM: diabetes mellitus; HR: hazard ratio; HTN: hypertension; LR: likelihood ratio; MTX: methotrexate; NA: not applicable; RDW: red cell distribution width.

We next sought to validate our local cohort findings in a larger national VA cohort. In a cohort of 13,689 national VA patients with RA diagnosis (Supplementary Figure S1, available with the online version of this article), the same relationships were observed between ALC, RDW, and mortality (Figure 1D). Further, significant differences in mortality were observed after adjustment for the same comorbidities (Table 2), and there was no interaction observed between RDW and ALC and relationships with mortality (likelihood ratio test of addition of interaction, $P \geq 0.11$; Table 2). In this larger cohort, in addition to RDW and ALC, many of the other variables in the model were each associated with mortality as well, as expected.

*Association between ALC and RDW with inflammatory markers.* Because the added risk of CVD is thought to be mediated through RA pathogenesis-related inflammation, we next performed a prospective analysis of plasma markers of inflammation in a subset of our RA cohort (living) in 2019 (Table 1, immunology cohort). Comparing participants with RA to controls, participants with RA had higher plasma sCD14, sCD163, TNFR2, IL-6, and MCP-1 levels, as well as T cell subset HLA-DR/CD38 expression and monocyte subset HLA-DR expression (Supplementary Figure S3, available with the online version of this article), and tended to have lower ALC ($P = 0.06$). In some cases, alteration in inflammatory markers (eg, MCP-1) associated with a type of RA therapy, whereas in other cases, markers differed from controls regardless of therapy subgroup (eg, TNFR2; Supplementary Figure S3E).

Within the RA group, RDW positively correlated with age and negatively correlated with ALC, albumin, and mean platelet volume (MPV). Additionally, within the RA group, ALC levels negatively correlated with RDW, age, TNFR2, CD16+56dim NK%, and HLA-DR/CD38 expression on CD4CM and CD8CM, and positively correlated with HLA-DR expression on NK cells, tissue factor on monocytes, RF, and CRP (Figure 2).

*MTX initiation results in an increase in RDW and a decrease in ALC, whereas addition of TNFi to MTX therapy results in an increase in ALC and little or no change in RDW.* Because MTX is first-line standard of care for treatment of RA, we first evaluated the effect of MTX on RDW and ALC. MTX is also known to inhibit purine synthesis and thus is an inhibitor of DNA synthesis occurring in rapidly proliferating cells.[18] Here, after MTX initiation, a decrease in ALC was observed (paired *t* test, $P < 0.001$; Figure 3A) comparing pretreatment to 3 to 12 months after MTX start. We next sought to understand whether change in ALC was associated with baseline ALC. MTX start was associated with the lowering of ALC primarily in those with higher ALC prior to MTX (Figure 3A). Because TNF related pathway marker (*s*TNFRII) levels often reflect TNF signaling, and *s*TNFRII levels here negatively correlated with ALC (Figure 2), we next sought to specifically evaluate the mechanistic linkage between TNF signaling and immunohematologic homeostasis by examining the effect of TNF blocker therapy on ALC. In patients with RA on stable MTX therapy, we observed an increase in ALC after starting TNF blocker therapy ($P = 0.01$; Figure 3B). The ALC increase was primarily observed in those with lower ALC at baseline ($P < 0.01$) and not those with higher baseline ALC ($P = 0.17$).

MTX use is associated with low grade anemia and increased RDW.[19,20] Here, as expected, MTX initiation was associated with an increase in RDW (paired *t* test, $P < 0.001$; Supplementary Figure S4, available with the online version of this article). In contrast to ALC, an increase in RDW was observed in both those with low and high RDW at baseline (Supplementary Figure S4A). Further, in contrast to effects observed on ALC,

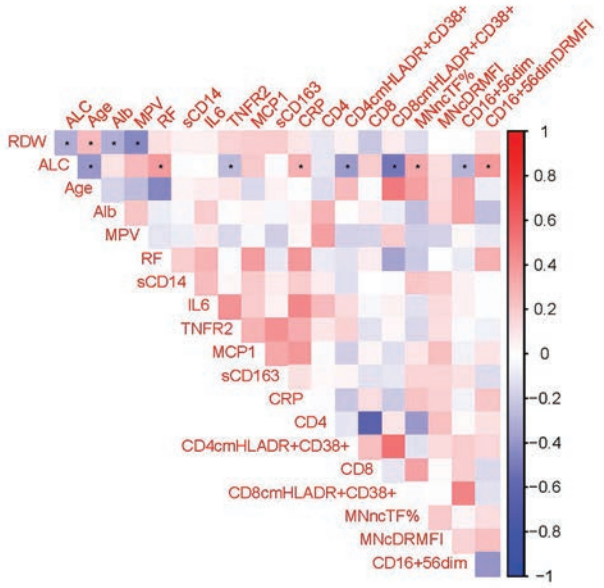


*Figure 2.* ALC is associated with age, RF, TNFR2, CRP, HLA-DR expression on NK cells, and HLA-DR/CD38 expression on CD4CM and CD8CM, whereas RDW is associated with age, ALC, albumin, and MPV. Heat map representation of Spearman rank correlations using complete pairwise data for correlations when there were missing data for participants with RA (n = 64), with significance at 0.05 indicated only for ALC and RDW. * $P < 0.05$. Alb: albumin; ALC: absolute lymphocyte count; CRP: C-reactive protein; HLA-DR: human leukocyte antigen–DR isotype; IL-6: interleukin 6; MCP-1: monocyte chemoattractant protein-1; MNcDRMFI: xxxx; MNncTF%: xxxx; MPV: mean platelet volume; NK: natural killer; RA: rheumatoid arthritis; RDW: red cell distribution width; RF: rheumatoid factor; sCD14: soluble CD14; sCD163: soluble CD163; TNFR2: tumor necrosis factor receptor II.

addition of TNF blocker therapy in the presence of MTX therapy did not result in any change in RDW (Supplementary Figure S4B).

Among a subset of patients in the present cohort with both pre-MTX and post-MTX (MTX without TNFi therapy) laboratory values present (n = 242), we looked at pretreatment values and the change in ALC and RDW. We did not detect an effect from the changes in ALC or RDW on the relation between pretreatment laboratory values and mortality in an adjusted model (data not shown).

## DISCUSSION

Understanding the immunological factors that both contribute to and are markers of morbidity and mortality in patients with RA is needed to better guide clinical care in this aging population. Both traditional risk factors and inflammatory parameters are thought to contribute to elevated CVD risk in RA. In this regard, we find here that prior to MTX therapy, higher RDW and lower ALC—variables associated with mortality in other cohorts—are associated with subsequent mortality independent of each other and independent of traditional CV risk factors in both local and national VA RA cohorts. When we sought to understand how pathogenic RA inflammation is related to RDW and ALC, we found ALC is negatively correlated with age, plasma TNFR2, HLA-DR/CD38 expression on CD4CM and CD8CM, and CD16+56dim NK%, and positively correlated with HLA-DR expression on NK cells, CRP, RF, and monocyte tissue factor expression. At the same time, RDW is positively correlated with age and negatively correlated with ALC, albumin, and MPV (Figure 2). Notably, in some cases, alteration in inflammatory markers (eg, MCP-1) was also associated with RA therapy status, whereas in other cases, markers differed from

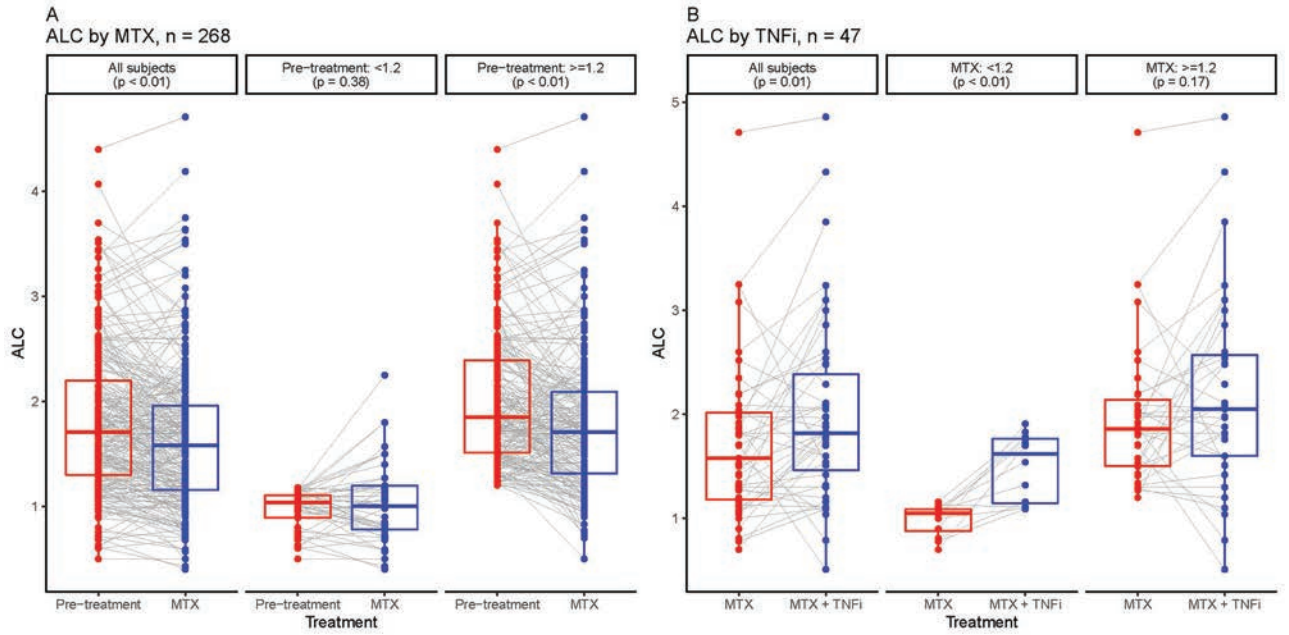


*Figure 3.* ALC decreases on MTX therapy in those with high baseline ALC, and ALC increases upon addition of TNFi therapy to those on MTX primarily in those with low baseline ALC. (A) ALC before and after start of MTX and in low and high ALC subgroups. (B) ALC before and after start of TNF blocker therapy in patients on MTX. ALC: absolute lymphocyte count; MTX: methotrexate; TNFi: tumor necrosis factor inhibitor.

controls regardless of therapy subgroup. This led us to evaluate the effect of MTX and TNF blockade on RDW and ALC. MTX therapy raised RDW levels and lowered ALC levels, whereas TNFi therapy raised ALC levels. When we further focused on the selectivity of these effects, we found MTX-related lowering of ALC occurred primarily in those with higher ALC levels at baseline, whereas TNF-mediated increase in ALC occurred primarily in persons with lower ALC levels on MTX therapy. These data are consistent with a model in which TNF signaling can contribute to the lowering of ALC levels, TNF inhibition can restore immune homeostasis and ALC, and ALC is selectively lowered with MTX in those with higher ALC because of inhibition of DNA synthesis and the consequent effect upon rapidly proliferating cells present in uncontrolled RA.

Several bacterial and viral infections induce lymphopenia of variable severity, ranging from the severe lymphopenia caused by HIV and lymphopenia observed after chemotherapy or radiotherapy, to milder more transient ones. Lymphopenia may develop with aging as well.[21] The administration of TNF reduces levels of circulating T cells,[22] whereas some data in the setting of RA indicate TNF inhibition may increase naïve CD4 cell numbers.[23] Few studies have evaluated ALC as a prognostic biomarker of all-cause mortality, though recent data indicate ALC may predict all-cause mortality in a manner independent of RDW, age, and traditional cardiac risk factors.[11] Although it has been appreciated that RA disease-modifying antirheumatic drug (DMARD) therapy may affect overall immune function, this is the first report to our knowledge of lymphopenia in the setting of RA, prior to MTX therapy, associating with all-cause mortality. Our evaluation of ALC over the course of both MTX and TNFi therapy provides controlled insight into this mortality relationship independent of, and dependent on, DMARD therapy.

TNF-$\alpha$ is a proinflammatory cytokine that plays a central role in inflammation and has been directly implicated in the pathogenesis of RA.[24] TNF-$\alpha$ is also known to contribute to foam cell formation, and blockade of this cytokine is thought to improve CVD.[25] TNFR2 is shed at times of cellular activation and/or TNF signaling. The TNFR2 gene locus has been associated with both enhanced TNF pathway engagement and insulin resistance, which can lead to the development of severe HTN accompanied by atherosclerosis through an augmented inflammatory process.[26] Additionally, serum soluble TNFR2 levels have been correlated with disease activity and severity in RA.[27] Moreover, elevated levels of TNFR2 have been associated with a significantly increased risk of mortality, independent of other markers for RA activity and severity.[28] Here we provide possible linkage between TNFR2, TNFi therapy initiation, and ALC levels in the setting of treated RA. Specifically, we observed that elevated TNFR2 levels are correlated with lower ALC levels during RA, and that TNF inhibition can lead to an increase in ALC, primarily in persons with lower ALC levels. Our observation that ALC positively correlates with monocyte tissue factor and NK cell HLA-DR expression may be consistent with a model in which antigen presenting cell and/or innate cell activation is associated with, and may contribute to, T cell activation and proliferation. Although our sample size is small to adequately evaluate change in ALC or RDW in relation to mortality, we looked at pretreatment values and change in ALC and RDW among a subset of patients in the present cohort with both pre-MTX and post-MTX (MTX without TNFi therapy) laboratory values present (n = 242). We did not detect an effect from the changes in ALC or RDW on the relation between pretreatment laboratory values and mortality in an adjusted model. This may suggest changes in ALC or RDW do not affect the relation between pretreatment ALC or RDW and mortality. Further investigation on the effects of TNF signaling on ALC levels and the relation between these factors and mortality is warranted in larger cohorts.

Results with plasma cytokines in the smaller local cohort indicated that IL-6 levels were higher in RA than in the control groups. Serum sCD14 concentrations have been reported as elevated in RA.[29] The same was observed in our data set. It has been shown that levels of sCD14 in RA are positively and significantly correlated with ESR and high-sensitivity CRP.[30] Plasma levels of CD163 have been shown to rise in acute and chronic inflammatory conditions associated with macrophage activation.[30] Here, patients with RA had higher sCD163 levels than controls. MCP-1 is produced by monocytes, endothelial cells, and vascular smooth muscle cells.[31] Prolonged elevation of systemic MCP-1 levels, as noted in the patients with RA when compared to controls, could reflect increased atherosclerotic burden. Here, patients with RA had higher levels of MCP-1 compared to controls. Together, these inflammatory markers involved in the pathogenesis of RA may provide insight into linkages between RDW, ALC, and mortality.

RDW is an automated measure of the heterogeneity of red blood cell sizes and it is routinely performed as part of a CBC in clinical settings.[32] RDW has been proposed as a useful parameter for evaluating both CVD risk and risk of all-cause mortality in the noninstitutionalized US population, independent of anemia.[33] Inflammation may alter erythropoiesis, red blood cell circulation half-life, and red cell membrane deformability.[7] Anemia of chronic disease is characterized by increased RDW, independent of iron status.[34] RDW may reflect nutritional deficiency (eg, iron, vitamin B12, or folic acid), bone marrow depression, and/or chronic inflammation.[35] In the setting of RA, RDW level has been found to be elevated compared with levels found in osteoarthritis, positively correlated with disease activity score in 28 joints (DAS28) and CRP levels, independent of anemia,[3,36] and positively associated with an increased risk of cardiac events.[10] Here, we find that in patients with RA, higher RDW was associated with greater all-cause mortality independent of traditional CV risk factors. These findings are consistent with a model in which pathogenic inflammation that contributes to T cell activation and peripheral T cell subset depletion/recompartmentalization may be linked to red cell homeostasis. Certainly, inflammatory mediators can worsen red blood cell survival, lead to erythropoietin resistance, and stimulate the production of hepcidin.[7] Each of these mechanisms may impair red blood cell production and increase RDW. Potential players include MCP-1 produced by monocytes, endothelial cells, and

vascular smooth muscle cells. However, we did not observe a relationship between MCP-1 and RDW.

Our study was limited by a number of factors. Our large cohort analysis was retrospective, we were unable to directly comment on quantitative measures of RA activity such as DAS28 or DAS28-CRP, and assessment of accuracy of new start of MTX was not verified by chart review as it was in our local cohort. In regard to the latter, we performed a sensitivity analysis, where we excluded a randomly chosen subset (as many as 34% with prior MTX found in our local cohort) and repeated this step 1000 times to examine the distribution of the hazard ratios and *P* values observed in the full nationwide cohort. These results were consistent with the results of the model on the full population, indicating the effects observed in the national cohort are not detected based on a large sample including potential MTX continuations. Our prospective study component evaluating plasma inflammatory markers was not of sufficient size to determine significant contributions of specific DMARD use or duration of RA to inflammatory marker levels. However, as an exploratory analysis of linkages between RDW, ALC, and RA inflammatory markers, this analysis accomplished the goal of identifying targets to evaluate in larger prospective studies powered to control for covariates and potential confounding factors. Our single-center RA cohort is within the VAMC care system and this, of course, biases the patient demographic to males, not representing female-specific RA issues. The ASCVD 10-year risk score calculator has an upper age limit of 79 years, and those patients older than this may not have an accurate traditional CV risk factor assessment, though in our adjusted analysis we considered traditional CV risk factors and not the ASCVD score. Finally, the cause of death was difficult to determine in many of the patients with mortality events, owing to medical record imprecision. The latter prohibits us to fully appreciate CAD and non-CAD related death. However, the latter is counterbalanced by our verification of the observed primary relationships in a substantially larger national VA cohort, providing assurance that the main relationships observed here are reproducible and robust.


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## ONLINE SUPPLEMENT

Supplementary material accompanies the online version of this article.

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