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The effects of local irradiation on circulating lymphocytes in dogs receiving fractionated radiotherapy

Running Title: Lymphopenia in dogs receiving radiotherapy

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Abstract:

Localized radiation therapy can be an effective treatment for cancer but is associated with localized and systemic side effects. Several studies have noted changes in complete blood count (CBC) parameters including decreases in the absolute lymphocyte count (ALC) and increases in the neutrophil:lymphocyte ratio (NLR). These changes could reflect immunosuppression and may contribute to decreased efficacy of immunotherapies used to treat cancer. We hypothesized that dogs would demonstrate decreased ALCs during a course of radiotherapy. A retrospective study was conducted on 203 dogs receiving definitive-intent radiotherapy. Demographic information, CBC values and details of the radiotherapy protocol were collected. The mean lymphocyte count pre-treatment was 1,630.68 cells/μl (SD ± 667.56) with a mean NLR of 3.66 (SD ± 4.53). The mean lymphocyte count mid-treatment was 1,251.07 cells/μl (SD ± 585.96) and the mean NLR was 6.23 (SD ± 4.99). There was a significant decrease in the mean lymphocyte count by 351.41 lymphocytes/μl (SD ± 592.32) between pre-treatment and mid-treatment ($p<0.0001$), and a corresponding significant increase in the mean NLR of 0.93 ($p=0.02$). Lymphopenia grade increased in 33.5% of dogs and was significant ($p=0.03$). The ALC decrease was not correlated with the volume irradiated ($p=0.27$), but correlated with the irradiated volume: body weight ratio ($p=0.03$). A subset of patients (n=35) with additional CBCs available beyond the mid-treatment time point demonstrated significant and sustained

downward trends in the ALC compared to baseline. Although severe lymphopenia was rare, these decreases, especially if sustained, could impact adjuvant therapy for their cancer.

Keywords: Dog, canine, radiation therapy, lymphopenia, immunosuppression

Introduction:

While most side effects related to therapeutic irradiation occur in the volume of tissue treated, systemic effects can also be observed. These systemic effects can have a negative impact on patients such as weight loss, fatigue and anorexia. In rare instances systemic effects may have a positive effect leading to distant tumor regression[1]. Lymphocytes have long been known to be amongst the most radiosensitive cell types[2,3]. Among circulating cells in the blood, lymphocytes are thought to be the most sensitive followed by neutrophils, then monocytes, platelets and erythrocytes[3]. Lymphocytes are an important cell type in developing an immune response to cancer and treatment-related lymphopenia may confer a negative impact on anti-tumor immunity and on a patient’s prognosis in general[4,5].

In radiotherapy, the total prescribed radiation dose is often fractionated, i.e., delivered in multiple doses. Definitive-intent fractionation involves smaller doses per treatment, while hypofractionated radiation involves larger doses delivered over only a few treatments[6]. Several studies in human medicine reported decreases in the absolute lymphocyte count (ALC) and increases in the neutrophil:lymphocyte ratio (NLR) in patients undergoing both definitive intent and hypofractionated courses of localized radiotherapy[7,8]. The most common theory proposed for these changes relates to the irradiation of circulating lymphocytes and their subsequent apoptotic death over the course of radiotherapy. These changes can persist beyond the time a patient is irradiated and can persist for up to 12 months or longer after completion of a course of radiotherapy in humans[3,7,8]. Also of note, NLR has been used as a biomarker for subclinical inflammation. A recent metanalysis of human studies found that an increased pre-treatment NLR in patients undergoing radiotherapy was associated with a poorer survival rate[9].

While a veterinary toxicity scoring system exists for radiotherapy effects as well as for chemotherapy or biological antineoplastic therapies in dogs, neither system specifically addresses lymphopenia[10,11]. However, the Common Terminology Criteria for Adverse Events (CTCAE) v5.0 in humans grades lymphopenia as follows: grade 1, ALC between 800 cells/mm^3 and the lower limit of normal; grade 2, ALC from 500 cells/mm^3 to < 800 cells/mm^3; grade 3, ALC from 200 cells/mm^3 to < 500 cells/mm^3; and grade 4, ALC < 200cells/mm^3 [12].

Studies have demonstrated variable changes in ALC depending on the anatomic location and volume of tissue or tumor being irradiated[13,14]. Patients receiving chemotherapy or corticosteroids in addition to radiation showed an even greater decrease in the ALC[13]. Other factors affecting the ALC include the total dose of radiation, dose per fraction, number of fractions and radiotherapy technique[15]. Grade 3 and 4 lymphopenias were also associated with an increased rate of mortality in human cancer patients receiving radiation and chemotherapy[13].

Localized radiation-induced tumor cell death can initiate an adaptive host immune response against metastatic cancers through abscopal effects, although it is uncommon[16,17]. Possibly coupling a radiation-induced primed immune response along with immunotherapy could prove to be an effective combination therapy against cancer progression[18]. While this is an emerging field, the optimal dosing and timing of radiotherapy for use in such combination therapies is unknown. Check point inhibitors combined with radiotherapy may potentially increase the chance of inducing an abscopal effect and improve outcomes. Other combinations of immunotherapies combined with radiation therapy include anti-tumor vaccines, Adoptive T cell transfer, intratumoral CpG administration, intratumoral dendridic cells and natural killer cell therapies[19]. However these have not been fully investigated and negative effects can be seen. For example, human patients who demonstrated severe lymphopenia after a palliative course of radiotherapy for solid tumors with metastatic disease were more likely to have severe lymphopenia when starting adjuvant therapy, which was associated with an increased mortality with subsequent treatment of a PD-1 immune checkpoint inhibitor[20]. These findings support the importance of continued investigation of the impact of radiotherapy on circulating immune cell populations.

The primary objective of this retrospective study was to evaluate changes in the ALC and NLR in a series of dogs undergoing fractionated radiotherapy. We hypothesized that dogs would demonstrate a decreased ALC and an increased NLR during a course of radiotherapy. A secondary objective was to evaluate factors that may influence such changes.

Material and Methods

The electronic medical records system at the Veterinary Medical Teaching Hospital at the University of California Davis from October 2013 to November 2018 was searched for dogs receiving a definitive intent course of radiotherapy. Definitive intent radiation plans were defined as one of the following protocols: 2.3 Gy/fraction for 20 daily fractions, 2.5 Gy/fraction for 20 daily fractions, 3 Gy/fraction for 16 daily fractions or 3 Gy/fraction for 17 daily fractions. October 2013 was chosen as the start date for this study as this was the date that a new linear accelerator and record and verify system (Aria patient management system, Varian Oncology Systems, Palo Alto, CA, USA), were installed at the facility. Information abstracted from the medical records included demographic information, location of tumor, complete blood counts (CBC) including the date of assessment and absolute differential numbers, concurrent medications, type of radiation, total dose delivered, fractionation, volume irradiated, and radiation planning type. Exclusion criteria included concurrent chemotherapy, if they were started on a course of steroids within the two weeks prior to beginning radiotherapy, absence of CBC data, and lack of CBC data within two fractions of the mid-treatment time point.

Per standard practice at our facility, all dogs had a CBC done prior to beginning a course of radiotherapy and again half way through the course of radiotherapy as part of their general health screening and anesthesia assessment. In cases where the CBC was done at a referring veterinarian’s practice prior to starting radiotherapy, results were collected from the paper records. Subsequent CBCs after completing radiotherapy were done as clinically indicated and ordered by the attending clinicians. To evaluate effects over time in the subset of cases where more than two CBCs were available for review, CBC data for each subject was grouped according to pre-treatment, days 5-15, days 16-30, days 30-60 or days 60-140 after the start of radiotherapy. In cases where the original paper copy of the CBC could not be located, the referring veterinarian was contacted in order to obtain the original CBC data. The normal reference range for lymphocyte counts at our laboratory is 1,000 – 4,000 cells/μl. Lymphopenia was graded according to the CTCAE v.5.

Tumor location was defined by the area irradiated and was stratified into the following groupings: brain, head and neck, trunk, extremity, intracavitary (including intrathoracic, abdominal, and pelvic) and spinal. All dogs were treated on a linear accelerator (TrueBeam, Varian Oncology Systems, Palo Alto, CA, USA). For cases that were computer planned, volume irradiated was defined as volume of the PTV. The PTV volume was calculated using the measure volume function in the treatment planning software. For photon cases treated with manually calculated fields, the port film was exported into an image processing program (Image J, National Institute of Mental Health, Bethesda, Maryland, USA). The patient area within the field was outlined and the area calculated. This area value was multiplied by the prescribed depth in order to calculate a volume. For electron fields, the 80% isodose line for the chosen electron beam energy is commonly used to ensure target coverage at this institution. Therefore, the open area of the electron cone was calculated for these plans, and the area value was then multiplied by the depth of the 80% isodose line for the treatment beam energy.

Statistical analysis

Descriptive statistics were generated. To compare changes in ALC and NLR, a paired t-test was done to investigate for changes between the pre-treatment and mid-treatment values. To determine if changes in the ALC or NLR were dependent on factors such as irradiated volume, weight and the irradiated volume to weight ratio, a linear regression analysis was conducted. A Fischer’s exact test was done to determine changes in lymphopenia grade. In the subset of cases where more than two CBCs were available, linear regression was used to examine for changes in lymphocyte counts over time. Statistical analysis was done using a commercially available software program (Stata 14.2, Stata Corporation, College Station, TX). A p-value of <0.05 was set as the level for significance.

Results

A total of 203 dogs met inclusion criteria for the study. The initial search of the electronic medical records system identified 225 cases. Twenty-two cases were excluded because of the following reasons: undergoing concurrent chemotherapy (n = 4); absence of CBC data (n = 6); involvement in a clinical trial utilizing medications with potential effects on

CBC values (n = 6); and absence of mid-treatment CBC data (n = 6). The data that support the findings of this study are openly available in the supplemental data for this study[21].

Patient Demographics

The median age was 8.97 years (range 0.7 – 15.1 years). The sample included 18 intact males, 94 castrated males, 4 intact females, and 87 female spayed dogs. The median weight was 26.4 kg (range 2.8 – 66.5kg). A total of 53 mixed breed dogs were included in the study. The pure breed dogs were as follows: Labrador retrievers (n = 24), golden retrievers (n = 14), boxers (n = 9), pit bull terriers (n =7), English bull dogs (n = 5), Chihuahuas (n = 5), Australian cattle dogs (n =4), Boston terriers (n = 4), Australian shepherds (n = 3), dachshunds (n = 3), German shepherd dogs (n = 3), German shorthaired pointers (n = 3), great Danes (n = 3), miniature poodles (n = 3), miniature schnauzers (n = 3) and Siberian huskies (n = 3). There were 14 other breeds with two dogs each and 26 breeds with one dog each. 21 dogs were on steroids at the time of presentation, and by tumor location, included brain (n=6), head and neck (n=8), extremity (n=3), trunk (n=1), and spine (n=3).

Irradiation Procedures

Irradiated areas included intracranial (n=11), head and neck (n=75), extremities (n=68), trunk (n=22), spinal (n=5) and intracavitary (n=22). 69 cases were hand planned and 134 cases were computer planned. The median volume irradiated was 137.9cm^3 (range 6.43 - 2,507.6cm^3). The median irradiated volume to weight ratio was 6.38cm^3/kg (range 0.23 – 175cm^3/kg). The median prescribed dose was 48 Gy. One case received 45 Gy, one case received 46 Gy, 138 cases received 48 Gy, 41 cases received 50 Gy and 22 cases received 51 Gy. A median of 16 (range 12-20) fractions were delivered, with a median dose of 3 Gy (range 2.3 – 4 Gy) per fraction.

Changes in Lymphocyte Values

The values compared in the study were derived from the pre-treatment CBC (median 26 days before the start of radiotherapy) and the mid-treatment CBC (median of 10 days after the start of radiotherapy). The mean ALC from the pre-treatment CBCs was 1,630.68 cells/μl (SD ± 667.56) with a mean NLR of 3.66 (SD ± 4.53) (Figure 1). At the time of the pre-treatment CBC, 169 cases had an ALC that was at or above the lower end of the normal reference interval and 34 cases showed some degree of lymphopenia. The grade of lymphopenia from the pre-treatment CBCs are presented in Table 1. The mean ALC in the mid-treatment CBC was 1,251.07 cells/μl (SD ± 585.96) and the mean NLR was 6.23 (SD ± 4.99) (Figure 1). A mean decrease of 351.41 lymphocytes/μl (SD ± 592.32) observed for the ALC between the pre-treatment and mid-treatment time points was significant ($p<0.0001$), and a corresponding mean increase in the NLR of 0.93 was also significant ($p=0.02$) (Figure 2). The grade of lymphopenia from the mid-treatment CBCs is presented in Table 1. The one case with lymphocytosis on the pre-treatment CBC returned to the normal range by the mid-treatment CBC. Six cases had decreased severity of their lymphopenia grade by 2 grades, and

11 cases had decreased severity of their lymphopenia by 1 grade. A total of 117 cases had no change in their grade. In contrast, 30 cases had increased severity of their lymphopenia by 1 grade, 31 cases had increased severity of their lymphopenia by 2 grades, and 7 cases had increased severity of their lymphopenia by 3 grades. This corresponds to a decrease in lymphopenia grade for 8.87% of cases, no change in grade for 57.67% of cases, and an increase in grade for 33.5%. There was a significant increase in the grade of lymphopenia between the pre-treatment lymphocyte and mid-treatment lymphocyte counts ($p=0.03$).

The decrease in ALC was also compared with the anatomic location irradiated and no significant difference was found in the degree of the decreased ALC based on the region irradiated ($p=0.14$). Based on the volume irradiated, there was no correlation with the decrease in ALC ($p=0.61$) nor the increase in the NLR ($p=0.59$). However, comparison of increases in the irradiated volume: body weight ratio with decreases in ALC using linear regression showed a statistically significant but weak correlation ($p=0.04$, Coef 4.49, $R^2=0.03$), although no correlation was found when comparing this ratio with the NLR ($p=0.85$). An increasing irradiated volume: body weight ratio was correlated with the anatomic location irradiated ($p<0.0001$, $R^2=0.1861$) with the ratio increasing in the following order: spine, brain, head and neck, intracavitary, extremity, and trunk.

Patients with more than the two CBCs (n=35) were examined for longer term effects of radiotherapy on their ALC. The patients were divided based on the days of their CBCs after the start of radiotherapy into the following time frames: pre-treatment; n=35, mid-treatment; n=37, Day 16-30; n=11, Day 31-60; n=33, and Day 61-140; n=33. Statistical analysis showed significant downward trends in the ALC for mid-treatment (p=0.001, Coef.= -315.0533, 95% CI= -496.7952 – -133.3114), Day 16-30 (p=0.001, Coef.= -595.1221, 95% CI= -925.2996 - -264.9446), Day 31-60 (p<0.0001, Coef.= -683.3039, 95% CI= -954.8986 - -411.7092), and Day 61-140 (p<0.0001, Coef.= -511.6372, 95% CI= -747.6681 - -275.6063) compared to pre-tretament with no recovery to baseline within the study period of up to 140 days (Figure 3).

Discussion

Findings from this study shows that local irradiation can be associated with systemic decreases in circulating lymphocyte counts with the mean ALC significantly decreasing and the mean NLR increasing from the pre-treatment CBC to the mid-treatment CBC. It has long been known that whole body irradiation can lead to rapid and prolonged lymphopenia in dogs, whereas the systemic effects of localized radiotherapy on ALC has been less appreciated [22-24]. Although there was a significant decrease in the ALC seen, the mean ALC in the mid-treatment CBC was still within the reference range. Further, 108 cases showing an initial ALC within the normal range maintained a normal ALC based on the mid-treatment CBC. These findings are in contrast to the over 33% of cases that showed an increase or worsening in their lymphopenia grade between the two time points. These results are similar to the findings reported in human medicine and the one veterinary study that looked at changes in lymphocyte counts resulting from radiotherapy[25]. There is one veterinary study that looked at changes in CBC values at the midpoint and conclusion of radiotherapy compared to CBC values taken prior to starting radiotherapy. They found significant reductions in hematocrit, total white blood cell count, neutrophils, eosinophils, monocytes, lymphocytes and platelets occurred during definitive radiotherapy in 103 dogs[25]. The ALC significantly decreased and the mean lymphocyte count at the end of radiotherapy ($0.906 \pm 0.65 \times 10^3$ cell/μl) was below the lower limit of their laboratory's reference interval (1.1×10^3 cell/μl). This paper however was not focused on changes in lymphocyte counts, but rather myelosuppression, and did not provide detailed information beyond the mean ALC and standard deviations at the different time points evaluated. This study also did not try to evaluate the effects of radiotherapy on systemic lymphocyte counts beyond the last day of treatment.

While our study shows that severe lymphopenia is not common at least at the midpoint through radiotherapy, the findings suggest that radiotherapy may decrease the ALC which could have important implications for adjuvant therapies[13]. Furthermore, findings from a subset of dogs with additional CBCs available beyond the pre-treatment and mid-treatment timepoints revealed significant and prolonged decreases in the ALC. These results are similar to those of other human studies and suggest a protracted effect of irradiation on lymphocyte counts[3,7,8].

Other studies looking at changes in the NLR found that post-treatment NLR was more indicative of a poor clinical outcome as opposed to the pre-treatment NLR, often due to the decrease in ALC[8]. While we did find an increased NLR based on mid-treatment CBCs, assessment of clinical outcome was beyond the scope of this paper. Association of radiotherapy-induced changes in the NLR with prognosis warrants further evaluation.

A human study noted that the tumor volume irradiated in non-small cell lung carcinoma was significantly associated with a decrease in ALC[14]. In our study we found no association between tumor volume and changes in the either the ALC or NLR. Given the relatively large weight range in the dogs studied, we created an irradiated volume: body weight ratio to try to account for this variability. Although the irradiated volume: body weight ratio was significantly associated with the decrease in the ALC ($P=0.04$), the linear regression model yielded a low R^2 value (0.03), indicating a weak but positive relationship (Coef = 4.49). Results of our study did not show an association of the anatomic location irradiated with decreases in the ALC or increases in the NLR, although the local site irradiated may play a role in the systemic immune response after radiotherapy. Not surprisingly the volume irradiated did correlate with the anatomic location irradiated, whereby intracranial and spinal locations had smaller volumes irradiated than tumors located in other anatomic locations. It will be important for future research to explore if there are stronger correlations between changes in the ALC, anatomic location, and the irradiated volume: body weight ratio in dogs, and to determine how the location and ratio together may affect the ALC.

Dogs were included in the study if they were receiving corticosteroids for greater that two weeks prior to starting radiotherapy. This cutoff was chosen as anti-inflammatory doses of prednisone, while leading to decreases in the absolute lymphocyte count at two weeks, do not cause further drops in the ALC with further chronic administration[26]. Additionally it has been shown that anti-inflammatory doses of prednisone do not effect neutrophil counts when receiving them daily at two weeks[26].

Radiation therapy is known to have immunomodulating effects on the local area irradiated that could lead to an anti-tumor response. These effects include inducing an immunogenic tumor cell death, release of antigens for T-cell priming, attraction of T-cells to the local tumor, and destruction of immunosuppressive stromal cells. These effects have led to a theory that radiotherapy may act as an in-situ vaccination against a tumor, which has been clinically demonstrated as the abscopal effect[1]. The abscopal effect is described as a systemic immune response targeting distant metastasis after local irradiation of a tumor. This was first reported in the 1950s and given that there are approximately 21 such cases in the literature, this is a relatively rare event[16]. Combining radiation with other immunotherapies to more reliably induce an abscopal effect is a promising approach, and several veterinary studies have been published using this strategy[27,28].

Radiation may also have a differential effect on different classes of lymphocytes. Previous studies have shown that multiple immune cell subsets are decreased after irradiation including B lymphocytes, T lymphocytes (CD4+ and CD8+) and NK cells, while T regulatory cells appear to be more radioresistant than other classes of lymphocytes[13]. Preferential sparing of T regulatory cells could have a negative implication for effective anti-tumor immune responses and also argues for the use of immune check point inhibitors along with radiotherapy as some check point inhibitors may decrease the numbers of T regulatory cells. This point remains controversial. For example while T regulatory cells are known to express CTLA-4, some recent work shows that in mouse tumors anti-CTLA-4 immunotherapy leads to both increased T effector cells and decreased T regulatory cells while in humans CTLA-4 blockade only increased levels of T effector cells without affecting T regulatory cells[29]. As our study assessed the lymphocyte population as a whole, it will be necessary for future research to analyze the subpopulations of circulating lymphocytes and their response to irradiation.

The presumed systemic immunosuppression that results after localized radiotherapy, as has been shown by the decreases in circulating lymphocytes seen in this and other studies, may on its own limit the effectiveness of radiotherapy to elicit a tumor immune response. This immunosuppression also suggests that multi-modality immunotherapies may also be affected. Interestingly, lymphocyte counts have been shown to be a predictor of clinical benefit and overall survival in human patients with advanced refractory melanoma treated with ipilimumab, a monoclonal antibody that blocks CTLA-4. Those patients with an ALC <1000/μl had worse outcomes[30].

Due to our study being retrospective in nature, our sampled population often did not have follow-up CBC's after the midpoint of radiotherapy and none beyond 140 days after starting radiotherapy. This means we were not measuring the full effect of fractionated radiotherapy on lymphocytes; approximately half the total radiation dose was delivered at the time of the second measurement which may have underestimated the impact of radiation on the lymphocyte values. Many pre-treatment CBCs were done by the referring veterinarian and not at the same laboratory as the mid-treatment CBC. These laboratories likely used different equipment for completing the test, which is a limitation of this study. Further we only had limited cases with available lymphocytes values after the course of radiotherapy was completed, so the recovery trajectory for the lymphocyte values cannot be evaluated. We were also limited to CBC data that does not include information on changes in subsets of lymphocytes, which may be differentially affected by radiation treatment. In addition, we did not measure clinical outcomes in this study nor were dogs tested for immunosuppression making it hard to draw direct conclusions if the drops in lymphocyte counts seen actually resulted in immunosuppression or affected clinical outcome in the study group. Radiation impact on lymphocyte subsets has not been adequately explored and may have greater implications than just the absolute drops in the ALC. A prospective study evaluating the effects of irradiation long term will need to be done in order to evaluate these factors.

In conclusion we found significant decreases in the ALC and increases in the NLR associated with radiotherapy across a large cohort of canine patients. The impact of these changes in circulating lymphocyte numbers on clinical outcome and survival warrants further research and may also have implications for those patients undergoing adjuvant immunotherapy. Further, it still remains unknown as to which subclasses of lymphocytes are most impacted by definitive courses of radiotherapy, and the duration of these effects on the ALC that are seen in canine patients.

Conflicts of interest:

The authors declare no potential conflict of interest.

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Table 1

Pre-treatment CBC [†]	Change in Grade from Pre-treatment to Mid-treatment CBC[†]					
Lymphopenia Grade (N)	Lymphocytosis	WRI[‡]	Grade 1	Grade 2	Grade 3	Grade 4
Lymphocytosis (1)	0	1	0	0	0	0

WRI (168)	0	108	24	29	7	0
Grade 1 (19)	0	10	4	4	1	0
Grade 2 (13)	0	5	1	4	2	1
Grade 3 (1)	0	0	0	1	0	0
Grade 4 (1)	0	0	0	1	0	0
	Mid-treatment CBC[†] lymphopenia Grade (N)	124	29	38	11	1

Number of cases for each lymphopenia grade for both the pre-treatment and mid-treatment lymphocyte counts showing change in grade. CBC[†] - complete blood count, WRI[‡] - within reference interval.

Figure Legends:

Figure 1: Histograms of absolute lymphocyte counts from pre-treatment (A) and mid-treatment (C) Complete blood counts as well as neutrophil to lymphocyte ratios from pre-treatment (B) and mid-treatment (D) in dogs undergoing definitive fractionated radiotherapy. The vertical dashed lines represent the lower and upper limits of the reference interval.

Figure 2: Box plots for the changes in the absolute lymphocyte counts and neutrophil to lymphocyte ratios between pre-treatment and mid-treatment values for dogs undergoing definitive fractionated radiotherapy. These were both statistically significant ($p<0.0001$ and $p = 0.02$ respectively). The clear bars represent the median values. The upper and lower quartiles are represented by the ends of the boxes. The ends of the bars represent the range with outliers removed and the circles and triangles represent outliers

for the absolute lymphocyte count differences and neutrophil to lymphocyte count ratios respectively.

Figure 3: Box plots for the changes in the absolute lymphocyte counts between pre-treatment and later time period values for dogs undergoing definitive fractionated radiotherapy. The changes between the pre-treatment lymphocyte count and subsequent time period lymphocyte counts were statistically significant ($p=0.001$, $p=0.001$, $p<0.0001$, $p<0.0001$, respectively). The clear bars represent the median values. The upper and lower quartiles are represented by the ends of the boxes. The ends of the bars represent the range with outliers removed and the circles represent outliers.