



Sustained lymphocyte decreases after treatment for early breast cancer



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The role of adaptive immunity in long-term outcomes in early breast cancer is increasingly recognised. Standard (neo)adjuvant chemotherapy can have adverse effects on immune cells. We conducted a retrospective longitudinal study of full blood counts (FBC) of 200 patients receiving (neo)adjuvant chemotherapy for early breast cancer at a single institution. FBC results at four time points from pre-treatment to 12 months post-chemotherapy were analysed. Flow cytometry was performed for patients with matched pre- and post-chemotherapy peripheral blood mononuclear cell samples. A significant decrease in absolute lymphocyte count at 12 months post-chemotherapy was observed ($p < 0.01$), most pronounced in pre-menopausal patients ($n = 73$; $p < 0.01$), patients receiving dose-dense chemotherapy regimens ($n = 60$; $p < 0.01$) and patients receiving adjuvant radiotherapy ($n = 147$, $p < 0.01$). In pre-menopausal patients, significant changes in CD4⁺ T cells subsets post-chemotherapy were observed. Further investigation, including long-term clinical outcomes, is needed to meaningfully improve long-term anti-tumour immunity.

The advent of immune checkpoint inhibitors (ICI) has led to a renewed focus on immunity in the treatment of breast cancer. Breast cancer has historically been considered a non-immunogenic tumour type; however, higher levels of tumour-infiltrating lymphocytes (TILs) are known to be highly prognostic in breast cancer, particularly in early-stage disease^{1,2}. Recently, the addition of ICI (specifically, monoclonal antibodies against programmed cell death protein-1 and its ligand [anti-PD-(L)1 to neoadjuvant chemotherapy has become a standard treatment for stage II-III triple-negative breast cancers (TNBC), and is being investigated in other breast cancer subtypes³⁻⁹. Recognition of the prognostic implications of adaptive immunity and the increasing role of ICI in the treatment of early breast cancer brings attention to the immunosuppressive consequences of (neo) adjuvant chemotherapy and radiotherapy, given its routine use for high-risk early breast cancer. Grade 3-4 lymphopenia has been noted in up to 69% of patients receiving dose-dense anthracycline-cyclophosphamide followed by taxane-based (AC-T) chemotherapy regimens that are the current standard of care in high-risk early breast cancer; however, the long-term consequences of this have not been well studied in the era of anti-PD-(L)1 therapy where the aim of treatment is to increase lymphocyte proliferation and expand anti-tumour immune populations¹⁰. It is also

known that adjuvant radiotherapy can have detrimental effects on peripheral lymphocyte counts¹¹.

In contrast to the relatively rapid regeneration of innate immune cells following the haematopoietic stress of chemotherapy, restoration of lymphocytes, in particular T cells, is more complex¹². Chemotherapy alters prohaematopoietic signalling in the bone marrow to favour myeloid precursors, induces thymic atrophy, and depletes thymic epithelial cells (TECs) required for T cell education, all of which contribute to impaired de novo T cell regeneration¹³⁻¹⁵. T cell restoration can occur through increased thymic output of naïve T cells, or by homeostatic expansion of peripheral, predominately memory T cells with restricted T cell receptor diversity. As the thymus involutes with age from puberty, the capacity for adults to restore the CD4⁺ naïve T cell pool following chemotherapy is reduced and is increasingly dependent on peripheral clonal expansion of T cells¹². Even from early adulthood, thymic export accounts for as little as 30% of T cell production, decreasing to approximately 5% by the age of 55¹⁶⁻¹⁹. Thus, it is possible that the quality and diversity of T cells may never be fully restored in adults following chemotherapy, and recovery could be affected by patient sex-steroid hormone levels, as well as age, both of which are important prognostic factors in breast cancer^{20,21}.

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Inhibition of sex steroids has been investigated as a strategy to increase thymic production of T cells. There is evidence from animal models that sex-steroid ablation enhances thymopoiesis and immune regeneration, and the use of gonadotrophin-releasing hormone (GnRH) agonists has been associated with enhanced adaptive immune recovery following bone marrow transplant^{22–24}. As GnRH are routinely used for many pre-menopausal women receiving chemotherapy (for fertility preservation during chemotherapy, and for ongoing ovarian function suppression in hormone-receptor-positive breast cancer), we hypothesised that this may ameliorate post-chemotherapy lymphocyte recovery.

Effective adaptive immunity is essential for the efficient recognition of tumour antigens and plays a crucial role in long-term anti-cancer immune surveillance. This is supported by the established efficacy of anti-PD-(L)1 inhibitors, which improve response rates and long-term clinical outcomes when combined with chemotherapy in TNBC^{3,4,25}. In patients with metastatic breast cancer, both lymphopenia and reduced T cell receptor diversity are associated with significantly reduced overall survival^{26,27}. Importantly, baseline and persistent post-treatment lymphopenia both independently predict shorter disease-free survival in early breast cancer²⁸. Whilst a small number of studies, predominately in the setting of now superseded chemotherapy regimens, have shown persistent reduction in lymphocyte counts for at least 1–5 years post-chemotherapy for early breast cancer, comprehensive data in the context of contemporary chemotherapy regimens are lacking^{29–33}.

To investigate the long-term effect of chemotherapy on lymphocyte counts, we conducted a retrospective analysis of serial full blood counts (FBC) before, on completion of, 3–6 months post- and 12 or more months post-chemotherapy in patients who received (neo)adjuvant chemotherapy for early breast cancer at a single institution between 2015 and 2020. For nine patients with matched pre- and post-chemotherapy whole blood samples available, we performed flow cytometric analysis of peripheral blood mononuclear cells (PBMC) to assess the effect of chemotherapy on different T cell subsets.

Results

Patients

The study was conducted in accordance with the Declaration of Helsinki and local ethics regulations following approval by the Peter MacCallum Cancer Centre Human Research Ethics Committee (approval number 22_65R), and a low-risk waiver of consent was granted. Informed, written consent was obtained prior to collection of PBMC samples for these nine patients, which was approved under the SEGMENT program (HREC number 13/123)³⁴. Two hundred consecutive female patients who received chemotherapy for early breast cancer were included. Demographic and clinical characteristics are summarised in Table 1. Due to the availability of FBC records, the number of patients with available data for each FBC parameter varied at each time point, as shown in Table 1. The median age was 52.3 years (range 24.9–76.4 years), and 40.0% of patients were pre-menopausal at diagnosis. Approximately half of the pre-menopausal patients ($n = 41/80$, 51.25%) received a GnRH agonist (goserelin was used in all patients who received a GnRH agonist). The most common breast cancer phenotype was hormone-receptor positive (HR+), HER2-negative ($n = 118$, 59.0%). The most frequently prescribed chemotherapy regimen was anthracycline and cyclophosphamide, followed by a taxane (AC-T) (86.5%), and 30% of patients received a dose-dense regimen.

Absolute cell counts in the overall population

The median values for WCC, ANC and ALC in all patients are summarised in the Supplementary Data (Supplementary Table 1). Full recovery, compared to baseline, was not achieved at the 12-month time point in any parameter (Fig. 1). No significant trend in neutrophil-to-lymphocyte ratio over the study time period was observed (Supplementary Table 1).

Table 1 | Patient characteristics

	<i>n</i> (%)
<i>N</i>	200
Baseline	180 (90%)
End of treatment	177 (88.5%)
3–6 m post chemo	103 (51.5%)
>12 m post chemo	87 (43.5%)
Median age (min-max), years	52.3 (24.9–76.4)
Menopausal status, <i>n</i> (%)	
Pre-menopausal	80 (40.0)
Zoladex	41 (20.5)
Post-menopausal	120 (60.0)
Median tumour size (mm)	28.0 (3.0–141.0)
T stage, <i>n</i> (%)	
1	49 (24.5)
2	116 (58.0)
3	24 (12.0)
4	7 (3.5)
N stage, <i>n</i> (%)	
0	60 (30.0)
1	106 (53.0)
2	28 (14.0)
3	13 (6.5)
Hormone status, <i>n</i> (%)	
Positive	125 (62.5)
Negative	75 (37.5)
HER2 status	
Positive	16 (8.0)
Negative	184 (92.0)
Neoadjuvant treatment, <i>n</i> (%)	26 (13.0)
Chemotherapy regimen, <i>n</i> (%)	
AC-T	173 (86.5)
AC	23 (11.5)
TC	3 (1.5)
AC-T-Carbo	1 (0.5)
CMF	1 (0.5)
Trastuzumab	17 (8.5)
Trastuzumab/Pertuzumab	6 (3.0)
Dose-dense regimen, <i>n</i> (%)	
Yes	60 (30.0)
No	140 (70.0)
Other adjuvant therapy ^a , <i>n</i> (%)	12 (6.0)
Radiotherapy, <i>n</i> (%)	
Yes	163 (81.5)
No	37 (18.5)
Recurrence, <i>n</i> (%)	26 (13.0)

AC doxorubicin plus cyclophosphamide, *T* taxane, *Carbo* carboplatin, *TC* docetaxel plus cyclophosphamide, *CMF* cyclophosphamide, methotrexate and 5-fluorouracil.

^aOther adjuvant therapy following standard neoadjuvant chemotherapy: adjuvant capecitabine in 2 patients, adjuvant PARP inhibitor or placebo on trial in 2 patients, adjuvant CDK4/6i or placebo on trial in 2 patients adjuvant immunotherapy in 5 patients, adjuvant trastuzumab-emtansine in 1 patient.

Impact of menopausal status on lymphocyte recovery

Given the previously reported link between sex-steroid inhibition and increased thymic output, and the importance of menopausal status on breast cancer outcomes, we assessed lymphocyte recovery according to menopausal

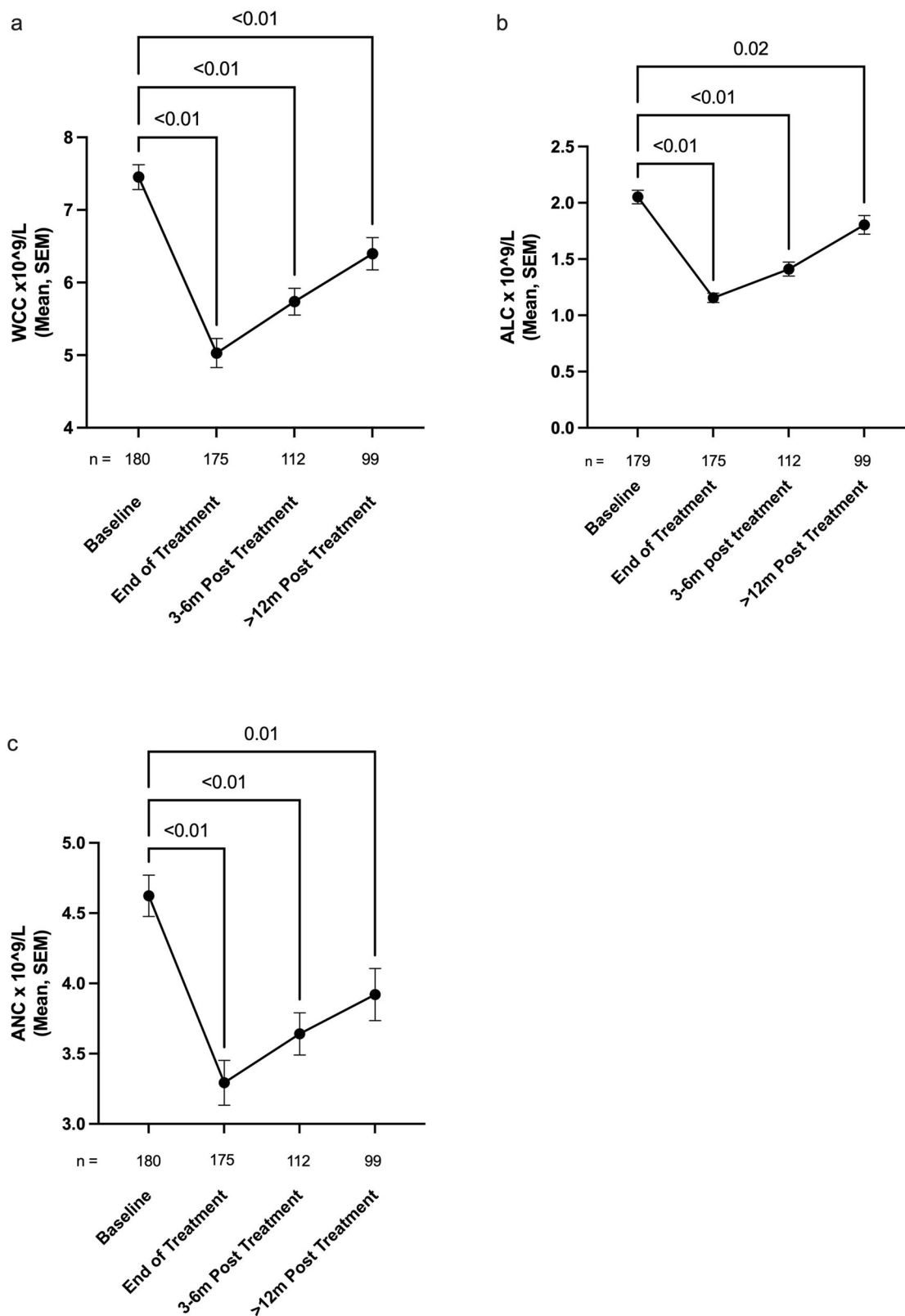


Fig. 1 | Mean white cell counts in all patients. Dots represent the mean value at each time point, and error bars represent standard error of the mean (SEM). Units for all parameters are $\times 10^9$ per litre of blood. *P*-value shown for multiple pairwise

comparisons. WCC white cell count (a), ALC absolute lymphocyte count (b), and ANC absolute neutrophil count (c) are shown.

status and use of a GnRH agonist among pre-menopausal patients. At baseline, there was no significant difference in mean lymphocyte count between post-menopausal versus pre-menopausal women (Mann–Whitney $p = 0.85$), or between those pre-menopausal patients who received a GnRH

agonist versus those who did not (Mann–Whitney $p = 0.13$). In the mixed effects test for trend, there was a significant negative trend from baseline to ≥ 12 months seen in both pre- and post-menopausal patients. The steepest and most significant deterioration in lymphocyte count was seen in pre-

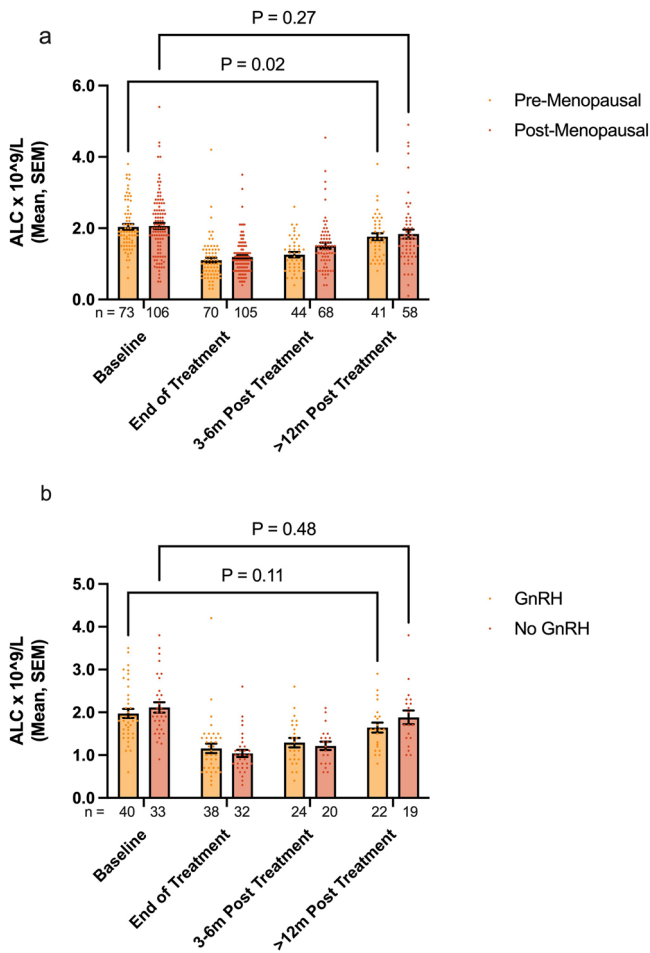


Fig. 2 | Multiple pairwise comparisons of mean absolute lymphocyte count (ALC) at each time point, according to menopausal status. In pre-menopausal patients, a significantly lower lymphocyte count persists at 12 months post-chemotherapy for pre-menopausal patients but not post-menopausal patients (a). When pre-menopausal patients were evaluated according to the use of gonadotropin-releasing hormone agonist (GnRH), the reduction in lymphocyte count at 12 months compared to baseline did not meet significance in either group (b). Individual absolute lymphocyte count values are represented by coloured dots, the mean is represented by the bar, and error standard error of the mean (SEM) is represented by the error lines.

menopausal patients (Table 3). In multiple pairwise comparisons, the difference in lymphocyte count compared to baseline remained statistically significant at ≥ 12 months in pre-menopausal women but not in post-menopausal women (Fig. 2a). In pre-menopausal patients, a similar, significant negative decrease in lymphocyte count was seen regardless of the use of a GnRH agonist, noting that data regarding duration of GnRH agonist (with chemotherapy only, or continued after chemotherapy) was not available (Fig. 2b).

Impact of age on lymphocyte recovery

The mixed effects linear test for trend was applied to the ALC in different age groups (grouped by tertile), showing a significant decrease in the youngest (age < 43 years) and middle age groups (age 43–61 years), but not in the older (age > 61 years) age group. Multiple pairwise comparisons showed a significantly lower ALC compared to baseline, maintained at ≥ 12 months in young (aged < 43) and middle-aged (aged 43–61 years) patients (Table 2).

Impact of chemotherapy choice and schedule on lymphocyte recovery

There was a statistically significant negative decrease in ALC in patients who received dose-dense chemotherapy regimens and in those who received

both anthracycline and taxane with either dose-dense or standard schedule (Table 2). Of all of the treatment and patient factors, the greatest magnitude and significance of the decrease in lymphocyte count was seen in patients who received a dose-dense regimen (Table 2). In the multiple pairwise comparisons, there remained a statistically significant decrease in ALC at ≥ 12 months compared to baseline in patients who received dose-dense regimens ($n = 60, p < 0.01$), but not in those who received non-dose-dense therapy ($n = 119, p = 0.46$) (Fig. 3a).

Impact of radiotherapy on lymphocyte recovery

A statistically significant negative decrease in lymphocyte count was seen in patients who received adjuvant radiotherapy (Table 2). In contrast, for patients who did not receive radiotherapy, the mean ALC at ≥ 12 months or more post-chemotherapy was actually higher than at baseline, although the trend was not significant (Table 2). Multiple pairwise comparisons showed a statistically significant reduction in lymphocyte count at all time points for patients who received adjuvant radiotherapy, including at ≥ 12 months post-chemotherapy (Fig. 3b).

Effect of chemotherapy on T cell differentiation

For nine patients (five pre-menopausal, four post-menopausal), matched pre- and post-chemotherapy PBMC samples were available for flow cytometric analysis. The mean time from the last chemotherapy administration to collection of the post-chemotherapy sample was 6.8 months (range 1–12 months). Additional clinical characteristics of these patients are provided in the Supplementary Table 2. For one post-menopausal patient, there were insufficient cells available from the baseline sample. Results from the post-chemotherapy sample for this patient is shown in the figures; however, this result was excluded from paired statistical analysis. Although there was no significant difference in the absolute numbers or percentages of CD4⁺ or CD8⁺ T cells, or the CD4⁺/CD8⁺ ratio, in pre-menopausal patients, there was a significant decrease in central memory CD4⁺ T cells and a compensatory increase in effector memory CD4⁺ T cells (Fig. 4b). Furthermore, a substantial numerical decrease in naïve CD4⁺ T cells in pre-menopausal patients is notable, although this did not meet significance due to the small sample size. A similar trend was observed in post-menopausal patients and the overall population (Supplementary Fig. 2). There was a numerical increase in CD4⁺/FOXP3⁺/CD25⁺ T regulatory cells in both pre- and post-menopausal patients and no significant change in CD8⁺/T regulatory cell ratio (Fig. 4d).

We also assessed the effect of chemotherapy treatment on the quantity of recent thymic emigrants (RTE), represented by CD31⁺/CD45RA⁺/CD4⁺ T cells, as a surrogate for thymic immune reconstitution³². We noted a significant increase in RTEs among both pre- and post-menopausal patients, with more notable increase in pre-menopausal compared to post-menopausal patients, with an increase in mean RTEs from 39.3% to 62.2% (+22.9%) of CD4⁺ T cells post-chemotherapy in pre-menopausal patients, versus 36.5% to 47.3% (+10.8%) in post-menopausal patients (Fig. 4c).

Discussion

Consistent with previous studies evaluating the effect of older chemotherapy regimens on immune cells, this study showed that a reduction in immune cells, including total WCC, ANC and ALC, is sustained for at least 12 months following chemotherapy. In the pre-anthracycline era, ANC and total WCC (but not ALC) were found to be depressed up to 24 months following treatment with melphalan with or without methotrexate²⁹. In another study of early breast cancer patients who received anthracycline-based chemotherapy with 5-fluorouracil (5-fluorouracil, epirubicin and cyclophosphamide, FEC) in addition to radiotherapy, a greater depression in WCC and ALC was noted compared to those patients who received radiotherapy alone, and this had not recovered to baseline at 12 months³⁰.

The majority of patients in our study received now standard sequential anthracycline and taxane-based chemotherapy regimens. We observed a more pronounced, sustained decline in peripheral lymphocytes in patients who received longer, dose-dense chemotherapy regimens and those regimens containing anthracycline and a taxane. It is interesting to note a significant

Table 2 | Trend in absolute lymphocyte count according to patient and treatment characteristics

	Mean absolute lymphocyte count ($\times 10^9/L$)				Test for trend	
	Baseline	End of treatment	3–6 m post chemo	>12 m post chemo	Slope	<i>p</i> -value
Menopausal status (<i>n</i>)						
Post-menopausal	2.06 (106)	1.19 (105)	1.51 (68)	1.84 (58)	−0.0785	0.04
Pre-menopausal	2.04 (73)	1.10 (70)	1.26 (44)	1.75 (41)	−0.1179	<0.01
GnRH agonist	1.97 (40)	1.16 (38)	1.29 (24)	1.64 (22)	−0.1279	0.02
No GnRH agonist	2.11 (33)	1.04 (32)	1.22 (20)	1.88 (19)	−0.1064	0.05
Age (<i>n</i>)						
<43	2.08 (44)	1.16 (40)	1.28 (25)	1.70 (24)	−0.1499	<0.01
43–61	2.03 (91)	1.11 (93)	1.34 (60)	1.72 (55)	−0.1078	<0.01
>61	2.06 (44)	1.25 (42)	1.69 (27)	2.15 (20)	0.0065	0.93
Chemotherapy regimen (<i>n</i>)						
AC-T	2.09 (158)	1.13 (153)	1.36 (104)	1.77 (86)	−0.1249	<0.01
AC without taxane	1.83 (18)	1.35 (18)	2.02 (8)	2.03 (12)	0.1085	0.36
Non-anthracycline	1.30 (3)	1.25 (4)	− (0)	2.1 (10)	0.2714	0.54
Dose dense	2.03 (60)	1.11 (58)	1.16 (40)	1.40 (25)	−0.2509	<0.01
Standard dose	2.06 (119)	1.18 (117)	1.55 (72)	1.94 (74)	−0.0343	0.31
Radiotherapy (<i>n</i>)						
Yes	2.05 (147)	1.16 (145)	1.37 (100)	1.68 (82)	−0.1325	<0.01
No	2.05 (32)	1.12 (30)	1.72 (12)	2.40 (17)	0.1051	0.16

Units for all lymphocyte values are $\times 10^9$ per litre of blood. Slope and *p*-value are derived from mixed effects linear test for trend, cut-off for significance, *p* < 0.05 (in bold). GnRH agonist gonadotropin-releasing hormone (goserelin in all cases), AC anthracycline with cyclophosphamide, T taxane.

negative trend in all cell counts, including neutrophils, in patients receiving dose-dense regimens despite the routine use of granulocyte colony-stimulating factors (G-CSF) to prevent acute neutropenia with these regimens. A prior study of 88 patients also reported delayed lymphocyte recovery in patients who received AC-T with G-CSF, compared to those who received AC without taxane or G-CSF, despite a greater initial depletion seen in patients who did not receive a taxane or G-CSF³². Whilst dose-dense chemotherapy regimens have become common in recent years due to the availability of G-CSF support, it is important to consider whether this will be the best approach in combination with immune-oncology strategies, given longer-term immunosuppression is a persistent issue, even with the use of G-CSF agents.

Although not a specific focus of this study, a lympho-ablative effect of radiotherapy is well established and is further supported by our findings¹¹. A previous study has shown significantly reduced naïve CD4⁺ cells compared to healthy controls in patients with early-stage breast cancer who received radiotherapy alone, although this difference was greater and more sustained in those who also received chemotherapy^{30,35}. Modelling supports that radiation-induced lymphopenia may be minimised by reducing the dose and volume of tissue irradiated³⁶. Recent high-level evidence supports omitting radiotherapy in selected cases of early breast cancer^{37–39}. Lymphopenia can also be minimised by reducing the volume of tissue irradiated⁴⁰. These strategies warrant further investigation in order to reduce the risk of lymphopenia following radiotherapy treatment.

Our study showed a significant decline in peripheral lymphocytes for younger and mid-aged patients, but not for older patients. Given reduced thymic output in older age, a greater sustained depression of lymphocytes might be expected post-chemotherapy in older patients. However, older patients are also less likely to receive dose-dense chemotherapy regimens. We observed a numerically lower baseline lymphocyte count in older patients, as would be expected. Lower levels of circulating naïve cells and expansion of memory populations in older adults may result in less net effect of chemotherapy on the composition of the T cell compartment. Several studies have evaluated the impact of age on recovery of specific T cell subsets following chemotherapy and have shown impaired reconstitution of immunity in older patients, particularly with respect to the CD4⁺ compartment^{33,41}.

The impact of menopausal status on lymphocyte recovery in our data is intriguing. We did not observe a statistically significant difference in the pattern of lymphocyte recovery between pre-menopausal patients who did and did not receive a GnRH agonist. The effect of GnRH agonists may be reduced by concurrent chemotherapy-induced menopause amongst all pre-menopausal patients, and these results may also be limited by our statistical power. In contrast to the expected protective effect of GnRH agonism on the thymus, pre-menopausal patients who received GnRH agonist actually had the greatest numerical suppression of ALC at 12 months compared to baseline, and the lowest absolute lymphocyte count at the 12 months post-treatment time point. These results suggest that in spite of sex-steroid inhibition, younger, pre-menopausal patients may be more susceptible to long-term lymphopenia than their older counterparts.

These findings are also reflected in the flow cytometric analysis, which, whilst limited by small numbers, show marked reductions in certain T cell subsets in pre-menopausal patients. Whilst there was no statistically significant difference in total CD4⁺ or CD8⁺ populations in either pre- or post-menopausal patients after chemotherapy, there was a near-halving of the absolute numbers and proportion of circulating CD4⁺ naïve cells, along with a significant reduction in the proportion of CD4⁺ central memory subsets, accompanied by a significant increase in CD4⁺ effector memory cells in pre-menopausal women. Previous work has shown that adaptive immune cell populations are sensitive to environmental factors, as well as age, sex and genetics. In healthy adults, cytomegalovirus (CMV) seropositivity is associated with significant increases in memory T cells, particularly CD4⁺ T effector memory cells, independent of age⁴². When adjusted for CMV seropositivity, Patin et al. showed only a modest increase in CD4⁺ memory cells with age, suggesting that the higher levels of memory cells seen in older adults can be driven by environmental factors. The changes in the CD4⁺ cell naïve and memory subpopulations observed post-chemotherapy in matched pairs, especially in younger, pre-menopausal patients in this study, are thus particularly noteworthy. This predominant impact of chemotherapy on the CD4⁺ compartment, with relatively unaffected CD8⁺ compartment, has also been observed in previous flow cytometric studies of peripheral immune cells post-treatment for early breast cancer^{30–32}. Whilst CD8⁺ effector T cells are considered responsible for the direct anti-tumour immune response, the critical

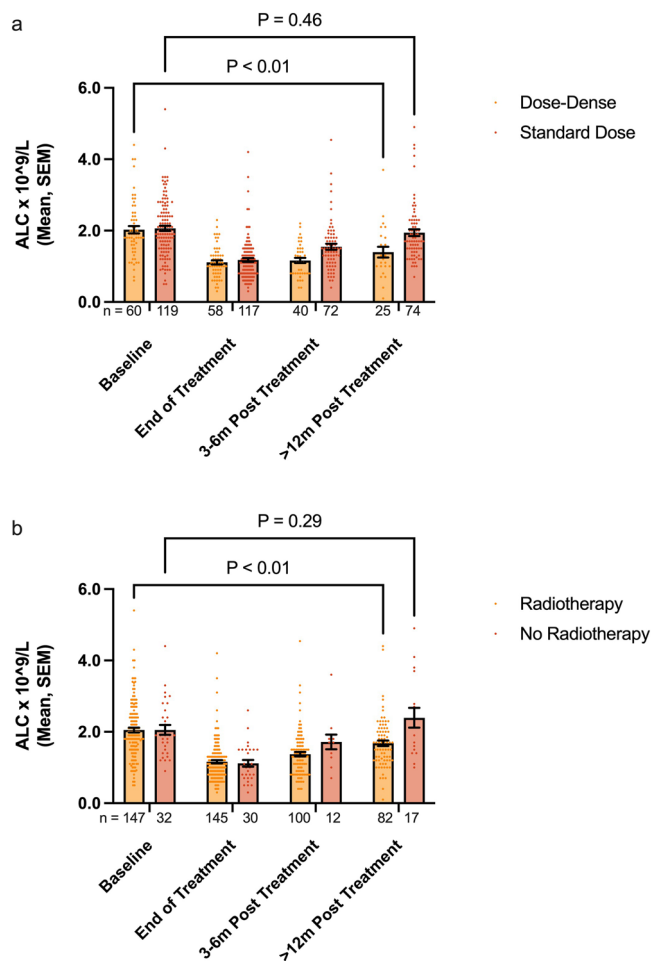


Fig. 3 | Multiple pairwise comparisons of mean absolute lymphocyte count (ALC) at each time point, according to treatment. In patients who received dose-dense chemotherapy regimens, there was a persistent, statistically significant reduction in mean absolute lymphocyte count at 12 months compared to baseline, but not in patients who received standard dose regimens (a). Similarly, there was a persistent significant reduction in absolute mean lymphocyte count at 12 months compared to baseline in patients who received radiotherapy, and not in those who did not (b). Individual absolute lymphocyte count values are represented by coloured dots, the mean is represented by the bar, and error standard error of the mean (SEM) is represented by the error lines.

helper role of CD4⁺ T cells in driving response to anti-PD-(L)1 therapy is increasingly recognised, raising the possibility that chemotherapy-induced suppression of CD4⁺ T cells could have an impact on clinical responses to combination or sequential chemo-immuno-oncology strategies^{43,44}.

Decreases in the CD4⁺ compartment appear to be partially compensated by increases in the effector memory cells subsets, as well as by a significant increase in RTE post-chemotherapy in both pre- and post-menopausal patients. The RTE increase is numerically greater in pre-menopausal patients than post-menopausal patients, consistent with greater thymic reserve in younger patients. Thymic output has been shown to decrease with age, and markers of thymic output correlate with levels of circulating naïve CD4⁺ and CD8⁺ T cells⁴⁵. In a previous study of immune cell recovery post high-dose chemotherapy followed by autologous stem cell transplantation for breast cancer, almost no CD4⁺ or CD8⁺ naïve T cell regeneration occurred in patients older than 55 years of age³³. The persistent marked reduction in naïve CD4⁺ T cells post-chemotherapy in pre-menopausal patients suggests increased thymic output may be inadequate to fully restore pre-chemotherapy immunity, even in younger patients. The effect of reduced naïve T cell pool on T cell receptor diversity in this setting requires dedicated evaluation.

The main limitation of this study relates to its retrospective nature and dependence on availability of peripheral blood count results and clinical

records at each time point. Clear documentation of adjuvant versus neoadjuvant administration of chemotherapy was not available. It is possible that the timing of chemotherapy relative to surgery and associated haematopoietic stress could further affect immune cell recovery, and we were unable to assess this. The flow cytometric analysis is limited by the small number of patients with matched pre- and post-PBMC samples, for whom treatment factors are variable. Despite these limitations, we have demonstrated a significant reduction of peripheral immune counts that persist for at least 12 months in patients receiving chemotherapy for early breast cancer, with greater suppression of lymphocytes in younger, pre-menopausal patients and with certain chemotherapy regimens. Our findings support ongoing efforts to refine the ideal chemotherapy backbone to partner with immune checkpoint inhibition in breast cancer patients.

Methods

Patient data

The study was conducted in accordance with the Declaration of Helsinki and local ethics regulations following approval by the Peter MacCallum Cancer Centre Human Research Ethics Committee ([HREC] approval number 22_65R), and a low-risk waiver of consent was granted by this committee based on the low-risk, observational nature of the study. Informed, written consent was obtained prior to the collection of PBMC samples for the nine patients, which was approved under the SEGMENT program (HREC number 13/123) as previously reported³⁴.

Patients who received at least one cycle of adjuvant or neoadjuvant chemotherapy for early breast cancer at the Peter MacCallum Cancer Centre in Melbourne, Australia, were identified from prescribing records for the previous 5 years. Demographic data and clinical parameters, including breast cancer phenotype, menopausal status, size, T stage, N stage, menopausal status, type of chemotherapy and radiotherapy, were obtained from the digital medical record. Full blood counts (FBC) from patients at baseline (prior to cycle 1, day 1), at the end of treatment ([EOT] prior to the final cycle of chemotherapy), 3–6 months following the final dose of chemotherapy and 12 months or more from the final dose of chemotherapy were recorded.

Flow cytometry analysis

Cryopreserved PBMC samples were thawed at 37 °C, resuspended in RF10 media (RPMI + 10% fetal bovine serum), counted and plated in a 96-well U-bottom plate at a concentration of 2.5 × 10⁶ cells per 100 µL of warm RF10 per well. For assessment of cell surface and intracellular markers, cells were stimulated with phorbol12-myristate13-acetate (PMA) at 1:1000 and ionomycin at 1:10 (BD Biosciences). Golgi Plug (BD Biosciences) and Golgi Stop (BD Biosciences), both at 1:10, were added to stimulated (+PMA) and unstimulated (–PMA) cells and incubated for 4 h at 37 °C, then washed twice and resuspended in phosphate-buffered saline (PBS). Cells were divided and stained with 100 µL zombie red viability dye (ZR-PE Texas Red) diluted to 1/800 in PBS and incubated at room temperature for 10 min at room temperature. After washing, 50 µL of FcR block (BD Biosciences) diluted to 1/20 in FACS wash buffer (2% FBS, 1 mM EDTA, 1 x PBS) was applied and incubated for 10 min at room temperature. After washing, 50 µL of surface antibody cocktail was applied and incubated for 20 min at 37 °C before washing with FACS buffer and fixing and permeabilising cells with Fix/Perm solution from the eBioscience™ Foxp3/transcription factor staining buffer set for 20 min at room temperature. After washing twice with serum free permeabilization wash buffer (Perm Wash), the intracellular antibody cocktail was added to cells and incubated for 20 min at room temperature before washing twice with Perm Wash and resuspending in 200 µL of 1% paraformaldehyde (PFA) containing FACS buffer. The composition of the surface antibody and intracellular antibody panels are provided in Table 3. Ultra Compensation beads (eBioSciences) were stained with each cell surface and intracellular antibody (25 µL beads with 5 µL antibody diluted to 1:20 for single colour controls).

Analysis was performed on the BD LSRFortessa flow cytometer. Instrument compensation was adjusted by the acquisition of the fully stained cells, followed by single colour controls. Further analysis was carried

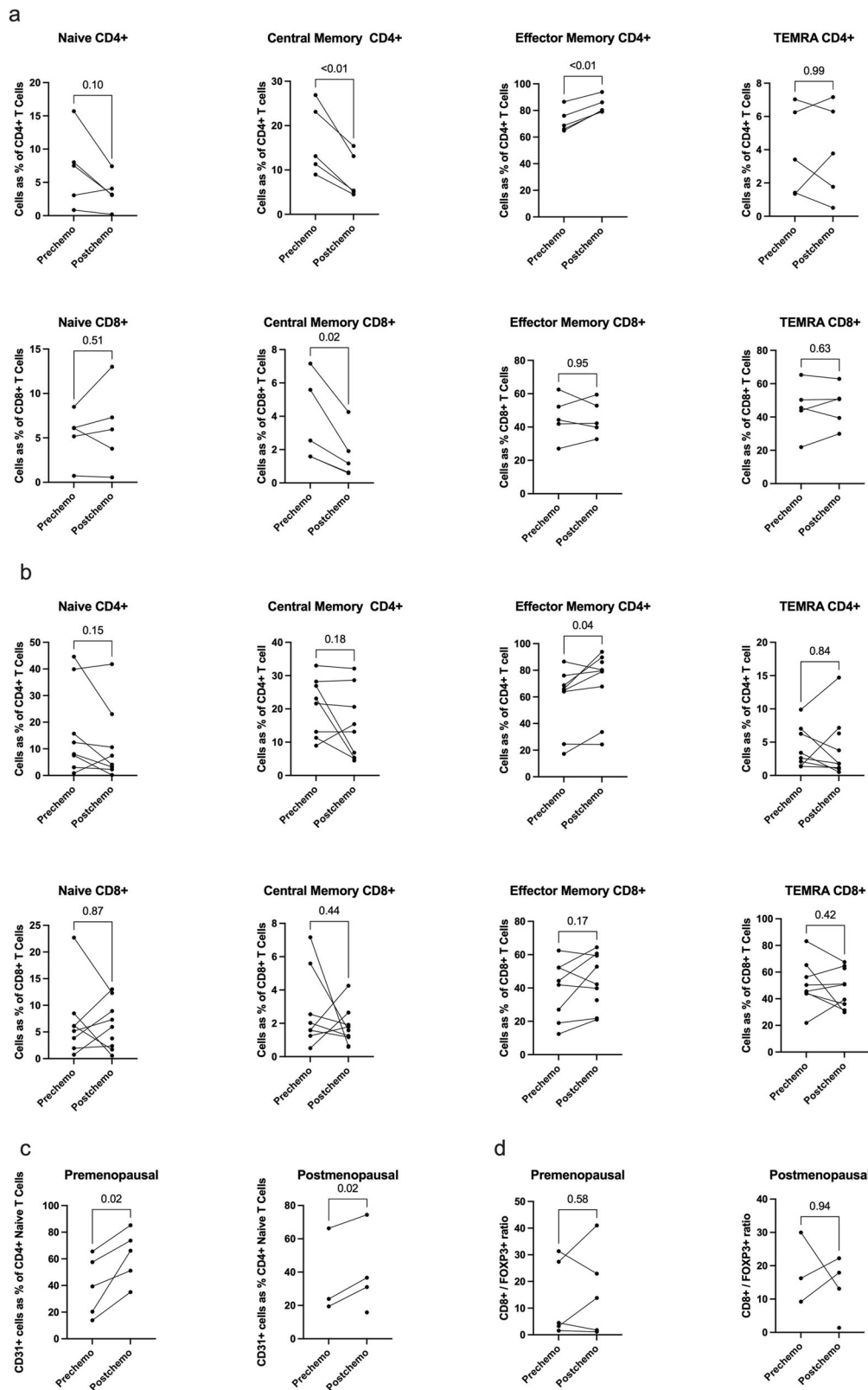


Fig. 4 | Effect of chemotherapy on CD4⁺ and CD8⁺ T cell subsets. Paired *t*-test show differences in between post- and pre-chemotherapy T cell subsets in matched pairs from pre-menopausal (a) and all patients (b). Post-chemotherapy change in recent thymic emigrant (CD31⁺CCR7⁺CD45RA⁺) CD4⁺ T cells for pre- and post-

menopausal patients is shown in (c), and post-chemotherapy change in CD8⁺/CD25⁺FOXP3⁺ T regulatory ratio in pre- and post-menopausal patients is shown in (d). Individual dots represent values for individual patient values. *P*-value shown for paired *t*-tests. TEMRA terminally differentiated memory cells.

Table 3 | Antibody panels for flow cytometric analysis

Surface stain	Fluorophore	Dilution	Supplier/reference number
Live/dead	PE Texas Red	1:800	Biolegend #423110
CD4	PE	1:200	Biolegend #317410
CD3	AF700	1:100	Biolegend #344822
CD8	BV510	1:100	Biolegend #344732
CD45RA	BV711	1:100	Biolegend #304138
CCR7	PE-Cy7	1:100	Biolegend #353226
PD-1	BV605	1:100	Biolegend #329924
CD31	APC-Cy7	1:100	BD Bioscience #563653
CD25	BUV395	1:100	BD Bioscience #564034
Intracellular stain			
IFN-gamma	AF488	1:100	Biolegend #502515
TNF-alpha	BV785	1:100	Biolegend #502948
CTLA-4	APC	1:100	Biolegend #349908
FOXP3	BV421	1:50	Biolegend #320124
IL2	BB700	1:100	BD Biosciences #566406

out on forward and side-scatter profile, live (ZR⁻) CD3⁺, CD4⁺ and CD8⁺ cells. Single-positive CD4⁺ and CD8⁺ cells were further classified into naïve (CCR7⁺, CD45RA⁺), effector memory (CCR7⁻/CD45RA⁻), central memory (CCR7⁺/CD45RA⁻) and terminally differentiated memory (CCR7⁻/CD45RA⁺) cells. Naïve CD4⁺ cells were stained with CD31 to identify recent thymic emigrants (RTEs: CCR7⁺/CD45RA⁺/CD31⁺) and T regulatory cells (CD4⁺/CD25⁺/FOXP3⁺) (Supplementary Fig. 1a). Assessment of intracellular cytokines was performed following short-term in vitro stimulation as described above, and non-stimulated cells were used as control. Post-acquisition, analysis was performed on FlowJo Software version 10.8.1. Intracellular cytokine-producing cells were analysed based on standard forward and side-scatter profiling, live (ZR⁻) CD3⁺, CD4⁺ and CD8⁺ T cell lineages (Supplementary Fig. 1c–e). Single-positive CD4⁺ and CD8⁺ T cells were further sub-classified into naïve (CCR7⁺, CD45RA⁺), effector memory (CCR7⁻/CD45RA⁻), central memory (CCR7⁺/CD45RA⁻) and terminally differentiated memory (CCR7⁻/CD45RA⁺) cells. The effector memory (CCR7⁻/CD45RA⁻) pool of CD4⁺ and CD8⁺ T cells was particularly examined for the individual expression of IFN- γ and TNF- α cytokines and assessed for co-expression of IFN- γ and TNF- α .

Statistical methods

Descriptive statistics were used to characterise the total white cell count (WCC), absolute neutrophil count (ANC), absolute lymphocyte count (ALC) and neutrophil-to-lymphocyte ratio (NLR) at each time point. To account for missing values, a mixed effects model (assuming values to be missing at random), was used to test the trend in FBC parameters from baseline through to 12 months or more post-treatment for the overall population and according to menopausal status (pre- or post-menopausal), age group (age <43 years, 43–61 years or >61 years), use of a gonadotrophin-releasing hormone (GnRH) agonist, chemotherapy regimen and use of radiotherapy. Multiple pairwise comparisons were performed to determine the difference compared to baseline for each parameter in the mixed effects model. Mann–Whitney test was used to compare baseline WCC, ALC and ANC according to baseline patient characteristics. For normally distributed data, a paired *t*-test was used to compare paired pre-chemotherapy and post-chemotherapy flow cytometric data. Where data was non-normally distributed, non-parametric (Wilcoxon) test was used for paired data, and Mann–Whitney test was used for non-paired data. Statistical analysis was performed using GraphPad Prism version 9.2.0. Two-sided *p*-values < 0.05 were considered statistically significant.

Data availability

The datasets analysed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

Study oversight and supervision were provided by S.L. J.D.D. analysed and interpreted patient and haematological data. Preparation of samples for analysis by flow cytometry was performed by J.D.D., K.C. and B.V. and analysis was conducted by J.D.D. and B.V. Review of statistical methods was provided by S.M. All authors read and approved the final manuscript.

Competing interests

J.D.D. has received travel and accommodation support for conferences from Novartis, MSD and Pierre Fabre and has received an honorarium from Gilead Life Sciences. S.L. receives research funding to institution from Novartis, Bristol Myers Squibb, MSD, Eli Lilly, Nektar Therapeutics, AstraZeneca/Daiichi Sankyo and Seattle Genetics. S.L. has acted as consultant to Seattle Genetics, Novartis, Bristol Myers Squibb, MSD, AstraZeneca/Daiichi Sankyo, Eli Lilly, Pfizer, Gilead Therapeutics, and Roche-Genentech. S.L. has acted as consultant or advisory board to Novartis, GlaxoSmithKline, Roche-Genentech, AstraZeneca/Daiichi Sankyo, Gilead Sciences, Seattle Genetics, MSD, Eli Lilly and Bristol Myers Squibb. S.J.L. receives research funding to institution from AstraZeneca, Beigene, Novartis, Roche, and SpringWorks Therapeutics. R.S. has served on advisory board and provided lectures for BMS, Roche, Exact Sciences, Daichii Sankyo and AstraZeneca and has received institutional research funding from Roche, Puma and MSD. P.F. has received an honorarium from Eli Lilly and Amplity Health.

Additional information

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