Dynamic alterations of immunosenescence-related genes in older women with breast cancer receiving chemotherapy: A prospective study

Qi Wu [a], Barbara Brouwers [a], Bruna Dalmasso [b], Cindy Kenis [c,d,e], Peter Vuylsteke [f], Guy Debrock [g], Ann Smeets [h], Annouschka Laenen [i], Hans Wildiers [a,c,e,1,*], Sigrid Hatse [a,1,**]

[a] *Department of Oncology, Laboratory of Experimental Oncology (LEO), KU Leuven, Herestraat 49, Leuven 3000, Belgium*
[b] *IRCCS Ospedale Policlinico San Martino, Genetics of Rare Cancers, Genoa, Italy*
[c] *Department of General Medical Oncology, University Hospitals Leuven, Leuven, Belgium*
[d] *Department of Geriatric Medicine, University Hospitals Leuven, Leuven, Belgium*
[e] *Department of Public Health and Primary Care, Academic Centre for Nursing and Midwifery, KU Leuven - University of Leuven, Leuven, Belgium*
[f] *Department of Medical Oncology, UCLouvain, CHU UCL Namur, Site Sainte-Elisabeth, Place Louise Godin 15, Namur 5000, Belgium*
[g] *Department of Medical Oncology, Ziekenhuizen Oost Limburg (ZOL), Schiepse Bos 6, Genk 3000, Belgium*
[h] *Multidisciplinary Breast Center, University Hospitals Leuven, Herestraat 49, Leuven 3000, Belgium*
[i] *Interuniversity Centre for Biostatistics and Statistical Bioinformatics, Leuven, Belgium*

ARTICLE INFO

ABSTRACT

Background: The exact impact of chemotherapy on the immune system of older patients with breast cancer is not well known. A longitudinal study was performed investigating the evolution of the blood immune profile during and after chemotherapy in this population.

Patients and Methods: The study included 39 patients receiving adjuvant chemotherapy (chemotherapy group, CTG) and 32 patients receiving only hormone therapy (control group, CG). A 10-gene panel associated with immunosenescence was measured in peripheral blood mononuclear cells (PBMC) before (T1), at 3 months (T2) and at 12 months (T3) after initiation of adjuvant therapy. Nutrition status was assessed by using a mini nutritional assessment scale. Linear mixed model analyses were performed for trajectory evolution, with or without adjusting for age, tumor stage, breast cancer phenotype, and/or corresponding baseline gene levels.

Results: Six genes relating to T cell activation (*CD28, CD27, CD86, LCK, GRAP, LRRN3*), and two genes relating to oxidative stress (*PRDX6, HMOX1*) exhibited a significant group-by-time effect, even after adjusting covariates(p≤ 0.01). In CTG, the T cell activation genes substantially declined from T1 to T2 and bounced back to a level higher than baseline at T3 (p<0.03), which was not observed in CG (p>0.26). Patients with malnutrition detected at T1 experienced more pronounced perturbation regarding *CD27, LCK, CD69, VAMP5*, and *LRRN3* (p<0.05).

Conclusion: Chemotherapy leads to transient perturbation of immune-related gene expression and potentially stimulates immunity in the long term. Well-nourished patients experience less impact of chemotherapy on immune-related gene expression profiles.

Background

Female breast cancer is the leading cause of cancer incidence and mortality among females worldwide, representing 24.5% of all cancer cases and 15.5% of all cancer deaths. Approximately 45% of female breast cancers are diagnosed in patients aged 65 years and older, of which 19% are above the age of 75 [1]. As the population of older individuals is increasing, breast cancer in older individuals has and will continue to become more prevalent.

Despite the marked expansion of an older population with breast cancer, the safety, efficacy, and necessity of different therapeutic modalities in this population remain under-studied. Older patients with breast cancer are less likely to receive standard chemotherapy because of increased risk of detrimental side effects [2]. Besides acute toxicity events, repeated cycles of chemotherapy-induced exhaustion and subsequent repopulation of the hematopoietic compartment may lead to accelerated aging of the immune system, also known as immunosenescence. Immune aging leads to a progressive deterioration of the ability to develop adapted immunity against new antigens, concurrent with a gradual increase in non-specific systemic inflammation. Hence, the understanding of immune alterations associated with chemotherapy in older patients with breast cancer is of paramount importance, most particularly when a combination with immunotherapy is considered. The monitoring of immunosenescence parameters could therefore be beneficial for planning and optimizing the combination of anti-cancer treatments.

One of the pronounced characteristics of immunosenescence, which is believed to be responsible for the impaired immune responsiveness with increasing age, is the loss of important T cell stimulatory coreceptors, including CD28 and CD27 [3]. This is coupled with an associated decrease in downstream molecules of the T cell receptor (TCR) signaling pathway, such as lymphocyte-specific protein tyrosine kinase

(LCK) [3], and the growth factor receptor-bound protein 2 (GRB2)-related adaptor protein (GRAP) which propagates TCR activation [4]. As a result, the ability of effector T cells to recognize and destroy pathogens or malignant cells is impaired. While senescent T cells are dysfunctional, they also show a significantly higher reactive oxygen species (ROS) level [5], which potentially explains the decrease in circulating naïve T lymphocyte numbers with age [6,7]. These alterations lead to an accumulation of dysfunctional T cells with altered capacity for antigen recognition and effector activation, further causing a decreased capacity to respond to new threats.

There is increasing interest in cellular biomarkers of immunosenescence. Based on several large-scale studies, examining genome-wide alterations of age-related gene expression profiles in peripheral blood samples by microarray analysis, we constructed a 10-gene panel involved in immunosenescence to study the impact of adjuvant chemotherapy on the immune system in older women with breast cancer. The 10-gene panel mainly covers the two aspects of immunosenescence discussed above: (1) diminished T cell activation, reflected by decreased expression of CD28 [8], CD27 [9–11], LCK [8], GRAP [9], CD69 [8,12], and leucine-rich repeat neuronal protein 3 (LRRN3 [9,11], a naïve T cell marker), and increased expression of CD86 [8] and vesicle-associated membrane protein 5 level (VAMP5 [9,10,13], trafficking of TCRs to the immune synapse). (2) higher ROS level, highlighted by decreased peroxiredoxin level [8] (PRDX6, removes ROS [5, 14]), and increased haem oxygenase-1 level [8] (HMOX1, induced by ROS generation, which in turn removes ROS as well [15]) (Fig. 1)

The goal of this study was to explore the dynamics of immune-related gene expression in peripheral blood from older patients with breast cancer receiving systemic therapy, either consisting of a classical chemotherapy regimen or solely anti-hormone therapy as a control. Using the above-mentioned 10-gene panel and real-time qPCR, we aimed to analyze serial blood samples obtained from patients with
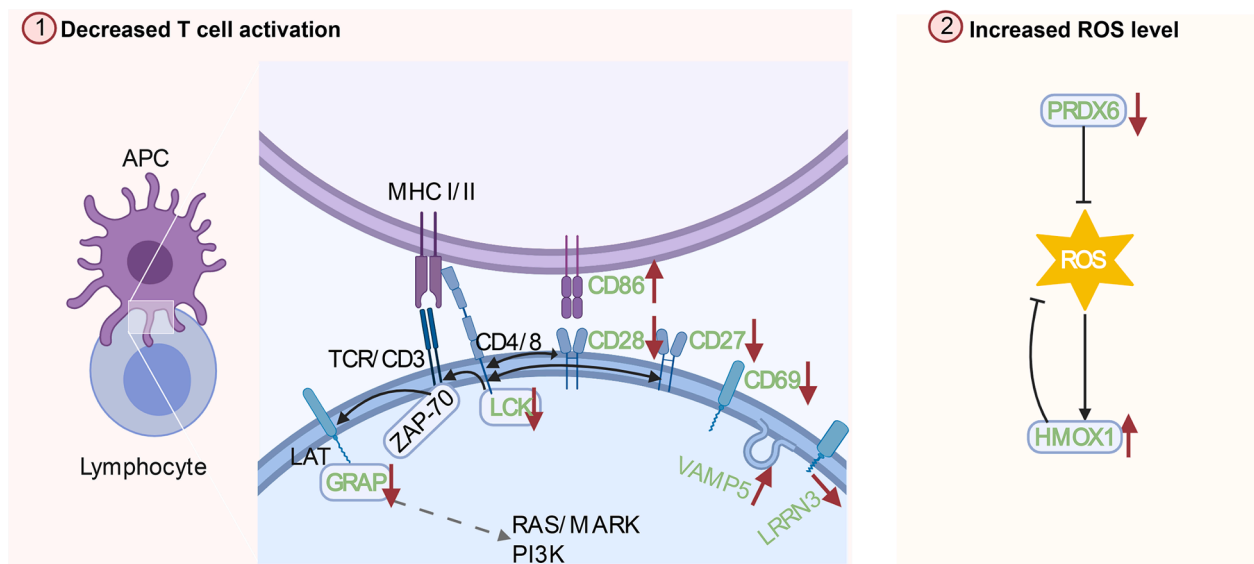

Fig. 1. Schematic of age-associated immunosenescence genes. This figure is an integrative view of the network related to immunosenescence, mainly focusing on T cell activation and ROS. Red arrows denote the increase/decrease during aging. Genes/biomarkers highlighted in green were included in our study. ①In brief, during T cell activation, cells are activated initially through their TCRs by antigen/MHC complexes expressed by antigen-presenting cells. Subsequent signals such as CD28, CD27, or other costimulatory receptors (such as CD69), are required for T cell activation. After TCR engagement, LCK phosphorylates the intracellular TCR domain, enabling the recruitment and activation of ZAP70, which in turn phosphorylates the adapter LAT. Phosphorylated LAT acts as a scaffold, recruiting and binding other adapters, such as GRAP, driving downstream signal amplification and diversification, leading to T cell activation. Of note, most genes related to TCR are downregulated during aging. LRRN3, a marker of naïve T cells, is decreased during aging, whereas VAMP5, which plays a role in the trafficking of receptors to the immune synapse, is increased during aging. ②Increased ROS level is observed during aging, with decreased PRDX6 level that normally remove ROS, and increased HMOX1 as a result of increased ROS, in return also removes ROS, i.e. via heme. Abbreviations: APC: antigen presenting cell; GRAP: growth factor receptor-bound protein 2 (GRB2)-related adaptor protein; HMOX1: haem oxygenase-1; LCK: lymphocyte-specific protein tyrosine kinase; LRRN3: leucine-rich repeat neuronal protein 3; MHC-: major histocompatibility complex-; PRDX6: peroxiredoxin 6; TCR: T cell receptors; VAMP5: vesicle-associated membrane protein 5; ZAP-: leucine-rich repeat neuronal protein-. Figure is drawn using tools in Biorender.com

breast cancer after surgery and during follow-up. The underlying aim is to gain insights into the immune effects of standard treatment for older women with breast cancer.

Methods

Participants

This is a sub-study from a previously reported prospective clinical study evaluating the effect of adjuvant chemotherapy on clinical and biological aging parameters in older female patients with breast cancer (≥70 years) (trial registration number NCT00849758, ClinicalTrials. gov) [16]. In brief, patients with breast cancer were recruited from 2 academic and 3 regional hospitals in Belgium from 2009 until 2012. Eligibility criteria included: woman older than 70 years old, received primary surgery for early breast cancer, pathological stage I to III, scheduled for adjuvant systemic therapy. Clinical characteristics were assessed through hospital medical records. Surrogate BC molecular phenotypes were defined based on the 2011 St. Gallen criteria: (i) luminal A-like (estrogen receptors (ER)-positive and/or progesterone receptors (PR)-positive, human epidermal growth factor receptor 2 (HER2)-negative, grade 1 or 2), (ii) luminal B-like (ER-positive and/or PR-positive, grade 3), (iii) HER2-like (ER-negative and PR-negative, and HER2-positive), and triple-negative breast cancer-like (TNBC, ER-negative, PR-negative, and HER2-negative). Two groups of patients were included based on the type of adjuvant systemic therapy received: adjuvant chemotherapy group (*CTG*) and adjuvant hormone therapy group (*CG*). In CTG, the scheduled chemotherapy consisted of docetaxel at a dose of 75 mg/m^2 and cyclophosphamide at 600 mg/m^2 every 3 weeks for a total of 4 cycles (TC scheme). Trastuzumab could be added to the TC scheme for HER2-positive breast cancer. Primary prophylaxis with G-CSF (granulocyte-colony stimulating factor) was administered as per standard practice guidelines. Antihormonal therapy was additionally foreseen if the breast tumor was positive for ER/PR receptor(s). In CG, an aromatase inhibitor was foreseen as the sole adjuvant systemic therapy. All patients received radiotherapy according to institutional guidelines (after chemotherapy in the CTG group). White blood cell growth factor support was recommended in the CTG arm during chemotherapy. All participants provided written informed consent.

Geriatric assessment A GA was performed at baseline as described [16]. Comorbidity was measured with the Charlson Comorbidity Index (CCI); Functional status by Katz's Activities of Daily Living (ADL), Lawton's Instrumental Activities of Daily Living (IADL) scales and Eastern Co-operative Oncology Group-Performance Status (ECOG-PS); cognitive status by the Mini-Mental State Examination (MMSE); mood with the 15-item Geriatric Depression Scale (GDS); nutritional status by the Mini Nutritional Assessment-Short Form (MNA-SF). Leuven Onco-geriatric Frailty Score (LOFS) was applied to summarize GA results in a more refined linear score ranging from 10 (very fit) to 0 (very frail) [16].

Blood sample collection

All blood samples were collected as part of the previous study [16]. In brief, we collected patients' blood at baseline, after 3 months and after 12 months. The first time point (*T1*) for a blood draw was typically between 3 and 6 weeks after surgery, and always before administration of the first TC cycle in the CTG group. The second time point (*T2*) was on the day of the fourth and last TC cycle (blood was taken immediately prior to chemotherapy administration) for patients in the CTG group and approximately 3 months after inclusion for patients in the CG group. The last time point (*T3*) was roughly 12 months after inclusion for both groups. For this gene expression study, 2.5ml of blood was collected in PAXgene Blood RNA tubes (PreAnalytiX, QIAGEN), which were stored at -80°C until further analysis. See overview in *Fig. 2*.

Gene expression analysis

Leukocyte RNA was isolated and purified from the PAXgene Blood RNA tube by the use of the PAXgene Blood RNA kit (PreAnalytiX, QIAGEN) according to the manufacturer's instructions. 500ng of total RNA from each sample was used for cDNA synthesis using the Invitrogen Superscript III kit (Invitrogen) according to the manufacture's manual. Real-time qPCR analysis in 96-well plates was performed on the Light-Cycler 480 platform (Roche), using Roche Realtime Ready custom panel assays, containing target-specific primers and a matching probe from the Universal Probe Library (UPL) for the following target genes: *CD28* (Gene ID: 940), *CD27* (Gene ID: 939), *CD86* (Gene ID: 942), *LCK* (Gene ID: 3932), *GRAP* (Gene ID: 10750), *CD69* (Gene ID: 969), *VAMP5* (Gene ID: 10791), *LRRN3* (Gene ID: 54674), *PRDX6* (Gene ID: 9588), and *HMOX1* (Gene ID: 3162). The expression of target genes was normalized relative to three chosen reference genes: *GAPDH* (Gene ID: 2597), *B2M* (Gene ID: 567), *YWHAZ* (Gene ID: 7534). The RealTime Ready assays comply with the Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) guidelines [17] and include internal controls for RNA degradation, efficiency of cDNA synthesis and presence of genomic DNA. A reference cDNA sample was also included on each
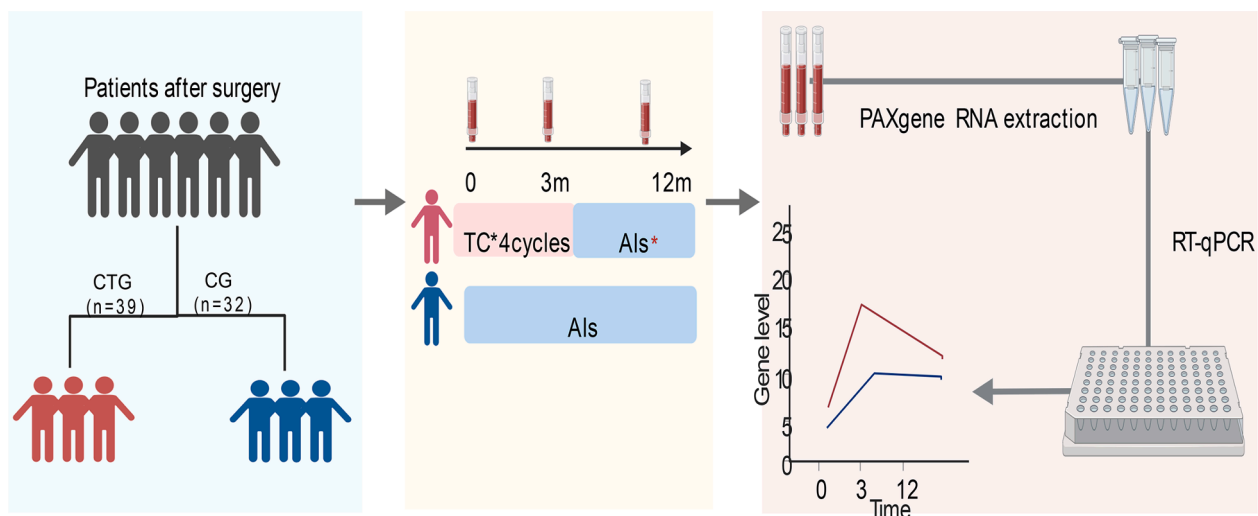
Fig. 2. Study design. Gene expression analysis was performed on peripheral blood samples collected after surgery and before initiation of systemic therapy (T1), 3 months after initiation of systemic therapy or before the 4th cycle of TC (T2) and at 12 months (T3). Red * if patients were ER and or PR positive. *Abbreviations:* AIs, aromatase inhibitors; ER: estrogen receptors; PR: progesterone receptors; TC: docetaxel cyclophosphamide. Figure is drawn using tools in Biorender.com

plate to evaluate inter-plate variation. The 20-μL PCR reaction mix was composed of diluted cDNA and LightCycler 480 Probes Master (Roche Diagnostics). The reactions were run on the LightCycler 480 machine using the cycling program specified in the assay guidelines. The Light-Cycler 480 software, version 1.5.1 was used for calculation of the relative Ct values.

Plasma aging biomarkers

A large panel of other aging biomarkers was previously measured in plasma samples from the patient cohort of the initial study (*n*=107) and results were reported in the past [16]. For the present study, only patients with PAXgene blood samples available (*n*=71) were included. The previously obtained biomarker results on these 71 patients were also integrated in the current study, with a specific focus on interleukin 6 (*IL-6*), *IL-10*, tumor necrosis factor (*TNF-α*), monocyte chemoattractant protein-1 (*MCP1*, also known as CC-chemokine ligand 2, *CCL2*), regulated upon activation, normal t cell expressed and secreted (*RANTES*, also known as *CCL5*), insulin-like growth factor 1 (*IGF-1*), and leukocyte telomere length, measured by qPCR as the ratio of telomeric sequences vs. single-copy gene (T/S ratio).

Statistical analyses

Continuous variables were tested for normality of distribution by Shapiro-Wilk testing. For comparison of baseline characteristics for the CTG and CG, t-tests (normally distributed) or Wilcoxon rank-sum tests (non-normally distributed) were used for continuous variables, and chi-square (χ^2) tests were used for categorical variables. The primary aim of this study was to assess trajectories of change in the genes from T1 to T2 and from T1 to T3 using linear mixed modeling (LMM) for longitudinal data, controlling for important potential confounders (age, TNM-stage, phenotype, and/or corresponding baseline gene level). Spearman correlation was used to estimate correlations between the 10-gene panel and biological aging and CGA as well. Statistical computations were performed using SAS software (version 9.4; SAS Institute, Cary, NC), R software (version 4; www. r-project. org), and GraphPad Prism software (version 9.0; GraphPad Software, Inc). A two-sided *P*<0.05 was considered significant for overall group differences.

The LMM included as fixed effects time (0, 3, and 12 months treated as nominal), group (CTG & CG), group-by-time interaction, and baseline covariates: chronological age (recorded at diagnosis), TNM-stage (in two categories: I-II & III) and phenotype (in four categories: luminal A-like, luminal B-like, Her2+-like, TNBC-like). Gene expression level (log 10 transformed) was the response variable. A random intercept for patients was modelled to deal with clustering due to the longitudinal data structure. Estimation was performed using the restricted maximum likelihood method. Marginal adjusted mean differences were used to quantify the changes from T1 to T2 and from T1 to T3 by group per time point. To confirm our results, we also conducted an LMM that only include luminal B-like patients for both groups.

All analyses contained all available data. Missing data were not common, except for 2 patients from 1 center with missing TNM stage information, phenotype, and histology type and grade, as a result of an inability to contact participants by telephone, where we assumed they were missing completely at random.

Results

Patient population

The previously reported prospective clinical study included in total 109 participants [16]. Of these, 39 patients in the chemotherapy group (CTG) and 32 patients in the control group (CG) had PAXgene blood samples available at all 3 time points and were included in the present sub-study (*Fig. 2*). The remaining 38 patients with missing blood samples

were excluded. Almost all CTG patients completed their adjuvant chemotherapy, one stopped after the second cycle because of a severe allergic reaction, two patients stopped after the third cycle because of severe infection. The demographic data are displayed in *Table 1*. As a result of the study design (i.e. allocation of patients to CTG or CG according to the physician's decision whether or not chemotherapy was indicated), there were some differences between the groups with respect to age, breast cancer phenotype, and TNM-stage: as expected, patients in the CG group were older (*p*=0.0039), had a lower degree of tumor burden (TNM-stage, *p*=0.01), and a good prognosis-related phenotype (*p*<0.0001).

Impact of chemotherapy on immunosenescence-related genes in older patients with breast cancer

To investigate whether chemotherapy has an impact on the process of immunosenescence in older patients with breast cancer, a panel consisting of 10 genes attributable to immunosenescence was applied to monitor the evolution in the CTG and CG groups. All genes were measured at baseline (after surgery) prior to any systemic therapy (T1), at 3 months (T2), and at 12 months (T3) after starting the therapy.

At baseline, *CD28, CD86*, and *HMOX1* showed a significantly lower level in CTG compared with CG (*p*=0.02, *p*=0.03, *p*=0.04, respectively. *Fig. 3; Table 2*), even after correcting covariates (age, stage, and phenotype).

From T1 to T2, reflecting the short-term impact of chemotherapy, LMM analysis of the gene expression data revealed patients receiving chemotherapy showed a significant decline of *CD28, CD27, GRAP*, and *LRRN3* level (all *p*<0.001, even after correction for covariates, such as age, stage, phenotype, initial gene level) and to a lesser extent *CD86* and *LCK* (*p*<0.05). With regard to ROS level, *PRDX6* was significantly increased (p<0.001). In controls receiving hormone therapy only, short-term changes were clearly less pronounced (*Fig. 3; Table 2*).

From T1 to T3, reflecting the long-term effect of chemotherapy, minor increases in *CD28*, and *CD27* (*p*<0.05), moderate increases in *CD86*, and *LCK* (*p*<0.001), and intense increase in *HMOX1* (*p*<0.0001) could be observed. Conversely, no significant changes were observed in patients from CG (*Fig. 3;* Table 2).

To examine whether the evolution over time was different between both groups, group-by-time interaction was performed in the general cohort. The result indicated a significant interaction effect for *CD28, CD27, CD86, LCK, GRAP, LRRN3, PRDX6*, and *HMOX1*, even after adjusting all covariates including age, stage, phenotype, and initial gene level (all *p*<0.01)). Specifically, the T cell activation-related genes *CD28, CD27, CD86, LCK, GRAP*, and *LRRN3* decreased to a much larger extent in CTG compared with CG at 3 months. After 12 months, their levels were restored, and for some genes were even slightly higher than the baseline levels in CTG. Also, the ROS-related gene *PRDX6* dramatically increased in CTG at 3 months and then dropped back to baseline, whereas only minor changes occurred in CG (*Fig. 3;* Table 2).

To make sure that the different time evolution patterns between both groups were not simply caused by non-equal breast cancer subtype composition of CTG and CG, the luminal B-like subtype was extracted from the general cohort (Supplement Fig. S1). Similar results were observed as in the luminal B-like sub-cohort, i.e. more substantial alteration of *CD28, CD27, CD86, LCK, GRAP, LRRN3*, and *PRDX6* in CTG in comparison to CG at 3 months, and a significant increase in the ROS-related genes at 12 months in CTG. Also, time evolution patterns differed in two groups in terms of *CD28, CD86, GRAP, LRRN3, PRDX6*, and *HMOX1*.

Immunosenescence-related gene expression in relation to chronological age

We next examined whether the 10-gene panel correlated with biological age in the entire patient cohort (CTG + CG) at baseline (i.e. after surgery and prior to any systemic therapy) by performing Spearman
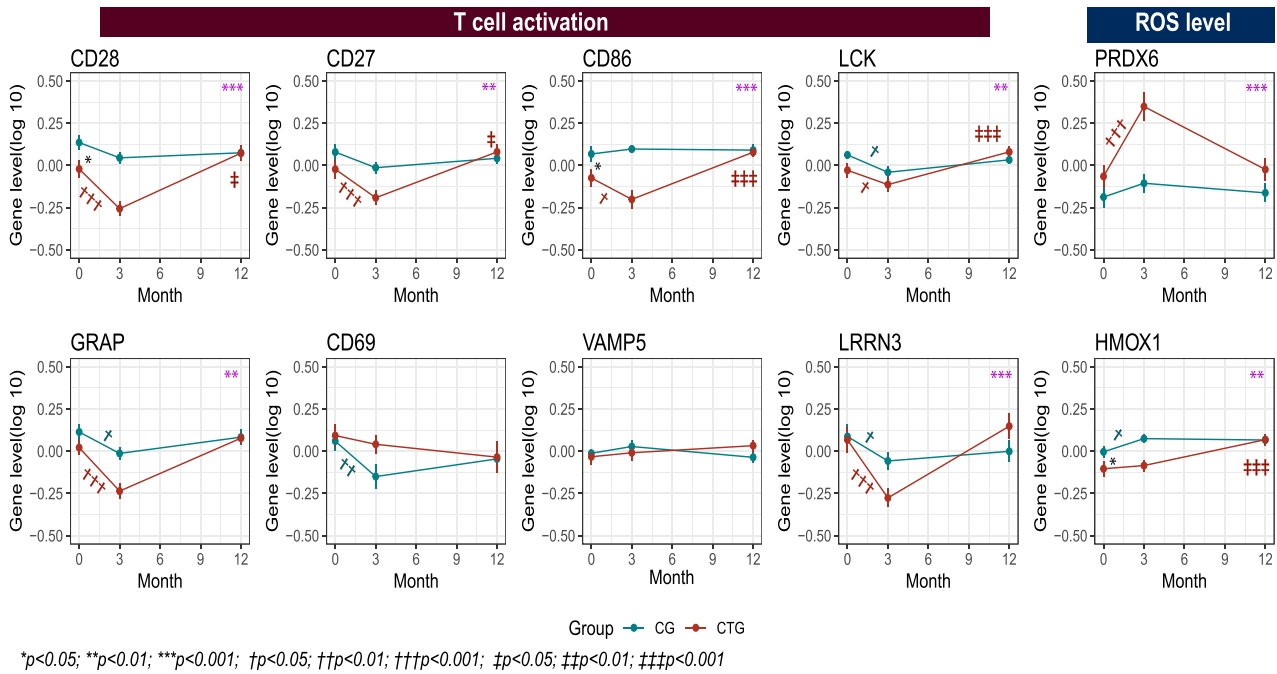
Fig. 3. Trajectory evolution of immunosenescence-related gene levels of patients receiving chemotherapy (red lines, *n*=39) **or not having chemotherapy** (blue lines, *n*=32) **at baseline** (T1), **3 months** (T2) and **12 months** (T3) **after the surgery.** Data are represented as mean ± standard error (SEM) of each timepoint for each group. * denotes comparison between CTG and CG (baseline comparison (black * at T1); group-by-time interaction (purple * at top right)); † denotes comparison between T2 and T1 in CTG (red) and CG (blue); ‡ denotes comparison between T3 and T1 in CTG (red) and CG (blue). All gene levels are log10 transformed. *Abbreviations:* GRAP: growth factor receptor-bound protein 2 (GRB2)-related adaptor protein; HMOX1: haem oxygenase-1; LCK: lymphocyte-specific protein tyrosine kinase; LRRN3: leucine-rich repeat neuronal protein 3; PRDX6: peroxiredoxin 6; ROS: reactive oxygen species; VAMP5: vesicle-associated membrane protein 5.

correlation analysis. Of these 10 genes, *LRRN3* tended to be negatively associated with age (r_s= -0.22, p=0.07), meaning that higher age is associated with lower *LRRN3* levels.

Plasma cytokines and leukocyte telomere length are associated with immunosenescence-related gene expression

Plasma cytokines and leukocyte telomere length are well-known aging biomarkers [18,19]. We therefore examined if these age-related
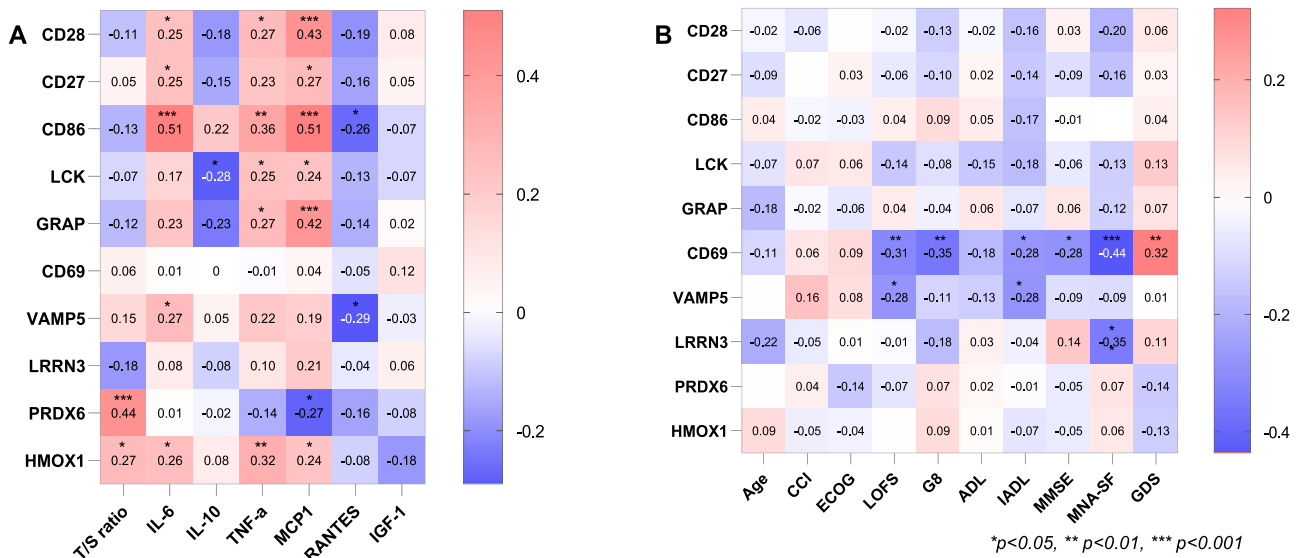

Fig. 4. Heatmap of association between immunosenescence-related genes and inflammaging (A) **and clinical frailty** (B). Color code represents the correlation strength from low (blue) to high (red). The * in the heatmap denoted statistical significance for correlation. Spearman rank correlation was applied to test the association between gene panel and inflammatory cytokines and frailty. *Abbreviations:* T/S ratio, telomere sequences vs. single-copy gene; IL-, interleukin-; TNF-, tumor necrosis factor-; MCP1/CCL2, *monocyte* chemotactic protein 1/chemokine (C-C motif) ligand 2; RANTES/CCL5, regulated upon activation, normal T cell expressed and presumably secreted/chemokine (C-C motif) ligand 5; IGF-1, insulin like growth factor-1; CCI, Charlson comorbidity index; ECOG, eastern cooperative oncology group; LOFS, Leuven oncogeriatric frailty score; ADL, activities of daily living; IADL, instrumental activities of daily living; MMSE, mini mental state examination; MNA-SF, mini nutritional assessment-short form; GDS, geriatric depression scale.

biomarkers also correlated with the 10 immunosenescence-related genes in the entire patient cohort (CTG + CG) at baseline (i.e. after surgery and prior to any systemic therapy) (*Fig. 4A*).

Interestingly, *PRDX6* (r_s = 0.44, *p*<0.001) and *HMOX1* (r_s= 0.27, *p*=0.04), which biologically are both involved in ROS level regulation, were positively correlated with leukocyte telomere length. On the other hand, several genes of the panel (i.e. *CD28, CD27, CD86, LCK, GRAP*, and *HMOX1*) were significantly correlated with the plasma cytokines *MCP1, IL-6,* and *TNF-α*. The link with pro-inflammatory plasma cytokines was most prominent for *CD86*.

Association of immunosenescence-related gene expression with clinical frailty as measured by GA

Aside from biological changes, clinical frailty is another, even more relevant manifestation of the aging process, which is frequent among older patients with breast cancer as well. Therefore, we explored in the entire patient cohort (CTG + CG) at baseline the relationship between the 10-gene panel and diverse components of the GA, which is routinely used in the clinic to estimate older patients' frailty status (*Fig. 4B*). Most strikingly, *CD69* was widely correlated with many GA tools: GDS (*r*= 0.32,*p*=0.008), MNA-SF (r_s = -0.44, *p*=0.0001), G8 (r_s = -0.35, *p*=0.003), LOFS (r_s = -0.31, *p*=0.009), IADL (r_s = -0.28, *p*=0.02), and MMSE (r_s = -0.28, *p*=0.02), which means the higher the *CD69* level, the more frail patients were.

Remarkably, multiple genes from the T cell activation domain conversely correlated with MNA-SF (i.e. *CD69*: r_s = -0.44, *p*=0.0001; *LRRN3*: r_s = -0.35, *p*=0.003) albeit non statistically significance observed among some genes(i.e. *CD28* and *CD27*)(*Fig. 4B*). In line with this, we split the cohort into "MNA-SF normal" (MNA-SF ≥12), and "MNA-SF abnormal" (MNA-SF <12) to investigate whether patients' nutrition status influenced those genes' evolution patterns (*Fig. 5*). Consistent with our hypothesis, less perturbation was observed in the "MNA normal" subgroup compared with the "MNA abnormal" group in

terms of *CD28, CD27, CD86, LCK, GRAP, LRRN3* these T cell activation-related genes. Thus, maintaining a good nutritional status is important to minimize the impact of systemic anti-cancer treatment on the immune compartment.

Predictive value of baseline immunosenescence-related gene expression for aging/frailty evolution in patients receiving chemotherapy

Given the observed correlations of immunosenescence-related genes with biological aging and clinical frailty markers, we next applied linear regression models to investigate whether baseline gene levels could predict the evolution of biological aging or clinical frailty markers under chemotherapy treatment.

Interestingly, *HMOX1* proved to be not only correlated with telomere length at baseline but also predicted its changes, especially in the short term (β (SE)= -0.36 (0.12), *p*=0.003 at T2; β (SE) -0.19 (0.10), *p*=0.08 at T3, corrected for initial telomere length, data not shown): the higher the initial *HMOX1* expression, the shorter the telomere length at 3 months and 12 months. On the other hand, higher baseline expression of several immunosenescence-related genes (*CD28, CD86,* and *HMOX1*) predicted lower plasma levels of the inflammaging marker *TNF-α* at 12 months (*p*=0.001 or lower, corrected for initial *TNF-α* level, data not shown). None of the genes showed predictive value at baseline with regard to the evolution of clinical frailty parameters.

Discussion

In this study, we applied a 10-gene panel to evaluate immunosenescence alterations in older patients with breast cancer either or not receiving adjuvant chemotherapy treatment. LMM analysis on gene expression data obtained with peripheral blood mononuclear cells revealed that most genes related to T cell activation were significantly downregulated at 3 months but had a robust recovery within 12 months after the start of systemic therapy. More substantial changes were
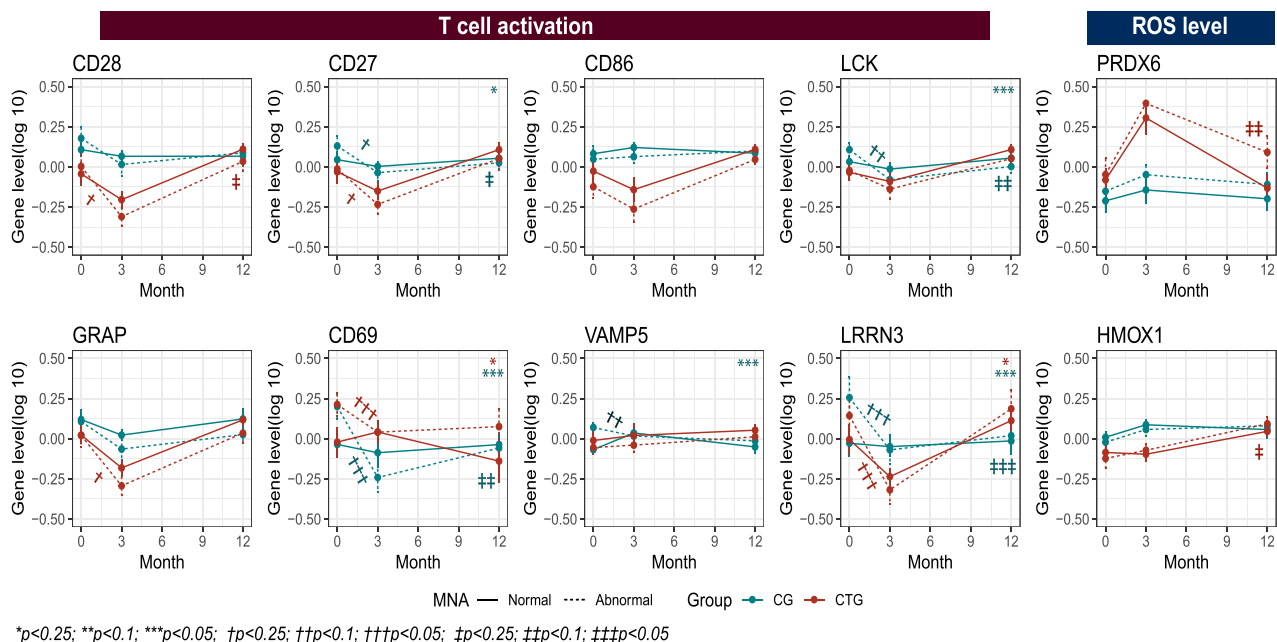

*p<0.25; **p<0.1; ***p<0.05; †p<0.25; ††p<0.1; †††p<0.05; ‡p<0.25; ‡‡p<0.1; ‡‡‡p<0.05

Fig. 5. Trajectory evolution of immunosenescence-related gene levels of patients receiving chemotherapy (red lines) **or not having chemotherapy** (blue lines) **in MNA normal** (solid lines, *n*=20 for CTG, and *n*=19 for CG) **or abnormal** (dashed lines, *n*=19 for CTG and *n*=13 for CG) **patients at baseline** (T1), **3 months** (T2) **and 12 months** (T3) **after the surgery**. * denotes group-by-time interaction analysis of MNA (normal & abnormal) and month (T1, T2, and T3) in CTG (red) and CG (blue); † denotes short-term comparison (T2-T1) between MNA normal and abnormal in CTG (red) and CG (blue) groups; ‡ denotes long-term comparison (T3-T1) between MNA normal and abnormal in CTG (red) and CG (blue) groups. All gene levels are log10 transformed. *Abbreviations:* GRAP: growth factor receptor-bound protein 2 (GRB2)-related adaptor protein; HMOX1: haem oxygenase-1; LCK: lymphocyte-specific protein tyrosine kinase; LRRN3: leucine-rich repeat neuronal protein 3; LumB, luminal B-like; PRDX6: peroxiredoxin 6; ROS: reactive oxygen species; VAMP5: vesicle-associated membrane protein 5.

observed among patients receiving chemotherapy compared with patients receiving only hormone therapy. Of note, eventual levels of CD28, CD27, CD86, LCK at 12 months were significantly higher than initial levels measured before the onset of adjuvant chemotherapy, which is not mirrored in patients receiving adjuvant hormone therapy only. Similar results were observed in the sub-analysis confined to the luminal B tumor subtype. Together, our data suggest that adjuvant systemic chemotherapy leads to substantially decreased T cell activation in the short term, while potentially triggering a long-term immune stimulation in older patients with breast cancer.

Docetaxel plus cyclophosphamide (TC) is among the most widely used chemotherapy regimens for breast cancer. However, older patients with breast cancer are often undertreated for fear of intolerability. Mounting studies indicated that chemotherapy may also stimulate the tumor immune response, which led to the combination of chemotherapy and immunotherapy to harness the synergistic effects [20,21]. Our previous studies revealed that the general compositional complexity of most circulating T cell subsets was restored by 12 months after adjuvant treatment of older patients with breast cancer [6,16]. Here we demonstrate that a set of genes attributable to T cell activation was deteriorated in the short term after receiving chemotherapy, but the activity bounced back to the baseline level and even higher. This suggests that chemotherapy in older women with breast cancer is well-tolerated and may actually stimulate immune responsiveness in the long term, as witnessed by significantly increased CD28, CD27, CD86, and LCK levels at 12 months. Our results support and extend the previous findings of Mozaffari et al [22], who demonstrated significantly reduced p56, CD3, and Zap70 levels in the short term in patients following chemotherapy. Furthermore, current findings complement our previous report on the transient influence of adjuvant chemotherapy on the immune compartment and on biological and clinical aging markers. They also reinforce the idea of immune induction by chemotherapy, which has been demonstrated by a synergistic effect of combining chemotherapy and immunotherapy in clinical trials [23–26]. It should be mentioned here that clinical drug combination trials are usually conducted among younger patients (>90% of participants are younger than 65 ys [24,25]). Our report for the first time suggests that a potential immune stimulation can also be observed among older breast cancer patients receiving chemotherapy. Obviously conclusive data are yet to be obtained in order to identify patients who would truly benefit from this strategy before putting it into clinical practice.

Clinical frailty is a state of decreased physiological reserves and vulnerability to external stressors that is frequent in older individuals with cancer [27–29] and can be assessed by geriatric assessment (GA). Our study revealed that CD69 conversely correlated with multiple CGA scales, reflecting the lower CD69 is, the healthier patients will be. Despite its classical role as an early marker of T cell activation, CD69 is recognized as a metabolic gatekeeper as well [12]. Earlier study indicate anti-CD69 antibodies triggered an increase of glucose uptake [30], might be a potential explanation for conversely related MNA scale. Yet the mechanism is not completely established especially regarding CD69 with other aspects of clinical frailty, such as conversely related G8, IADL, MMSE, and positively related GDS. Another interesting finding is that MNA-SF scale negatively correlated with multiple genes relating to T cell activation. When further classifying patients based on MNA-SF score, patients with preexisting malnutrition tended to experience a more pronounced gene level reduction at 3 months and a slower recovery at 12 months, as highlighted by CD28, CD27, CD69, and LCK, whereas well-nourished patients suffered less perturbation of T cell activation genes. This is in line with the findings that malnutrition in aging persons aggravates the already impaired immunity through a negative impact on T cell number, activation, proliferation, and cytokine production, as a result of decreased fuel supporting those processes [31–34]. Here we demonstrated for the first time that genes attributable to T cell activation were decreased to a larger extent in patients with malnutrition status compared with patients with normal nutrition status. Clinically, these data imply that screening for initial malnutrition in older breast cancer patients might be essential to identify patients who may benefit from nutritional intervention before, during, and after cancer care. However, a more detailed mechanistic understanding of the effect of nutrition on immunity is needed, as well as clinical studies demonstrating improved immunity when the nutritional status is improved.

Aside from declined expression of genes related to T cell activation, perturbation of genes linked to ROS level was also observed during the time course of chemotherapy. ROS has been shown to promote telomere shortening [35,36], which is generally recognized as a hallmark of aging. The antioxidant peroxiredoxin 6 (PRDX6), scavenging ROS, is enriched at telomeric DNA sites and PRDX6 loss causes preferential damage to the telomeres [37], which leads to accelerated telomere shortening. In our study, PRDX6 was dramatically increased during chemotherapy, as compared with a minor increase in patients not receiving chemotherapy. Although PRDX6 was previously shown to be decreased in the older population as a result of diminished promoter activity [5], older patients from our study cohort were still capable to reactivate PRDX6 expression to cope with increased ROS after systemic therapy, as indicated by other studies [38,39]. Chemotherapy itself will therefore not necessarily lead to an accelerated telomere shortening through ROS accumulation. This is in line with our earlier report where we showed comparable time evolution of leukocyte telomere length in CTG and CG [16].

Besides telomere shortening, accumulation of proinflammatory cytokines, such as IL-6, TNF-α, MCP1, is another hallmark of aging [40]. Interestingly, we have found a consistent positive correlation between T cell activation genes at baseline, most particularly CD86, and the proinflammatory cytokines IL-6, MCP-1 and TNF-α. Conversely, the anti-inflammatory cytokine IL-10 rather tended towards negative association with the 10-gene panel. The biological significance of these observations remains unclear.

Remarkably, our data did not reveal strong associations between the evaluated immunosenescence-related genes and chronological age. Only LRRN3, which emerged as an age-related gene in previous studies [9, 11], presented a weak relation to chronological aging in our cohort as well, albeit not statistically significant. The lack of correlation between the 10-gene panel and chronological age may be explained by the narrow age range covered in our study (70-90 years). Hence, the lack of association with chronological age in this cohort does not necessarily imply that these markers are not age-related.

Our study has some limitations. Firstly, only a limited selection of immunosenescence-related genes were studied in our research. It should be pointed out, though, that no universally accepted immunosenescence markers have been established so far. Secondly, the nonrandomized study set-up allowed physician bias in offering adjuvant chemotherapy to the recruited patients. By consequence, patients in the control group were older than patients in the chemotherapy group, and presented with earlier tumor stage and good-prognosis phenotype. Nevertheless, our results were not affected by adjusting for all the potential confounders. Moreover, a sub-analysis within the luminal B-like phenotype afforded similar results. Third, patients in the chemotherapy group received G-CSF support. However, all blood samples were taken at least 21 days after the last GCSF administration in view of published data that G-CSF acting time is reported to be 14 days [41]. Moreover, it has been shown that G-CSF reversed the effects from chemotherapy, preventing the loss of lymphatic progenitors and mature lymphocytes [42,43]. In line we would expect more changes from chemotherapy in the short term in the absence of GCSF, which will not bias the results we observed in this study. Last, we have to recognize this is a small size exploratory study, future studies assessing the immunological effect from chemotherapy among older patients with breast cancer might be of great value.

Conclusions

In conclusion, our immunosenescence-related gene profiling data suggest that in the long term, chemotherapy does not accelerate the decline of the immune system of older patients with breast cancer. Therefore, older age and fear of accelerated immune deterioration should not be a criterium to withhold chemotherapy from older patients if there is a potential benefit. On the contrary, chemotherapy may have the potential to improve immunity in older breast cancer patients in the long run. Secondly, our results show that patients with a normal nutrition status seem to experience less perturbation of immunosenescence-related gene profiles. A strong correlation between our 10-gene panel of immunosenescence and well-recognized biological and clinical aging biomarkers confirmed the validity of our gene panel to study immune aging and also underscores the biological relevance of our results.

Declarations

Ethics approval and consent to participate: This work was approved by University Hospital Leuven's ethics committees (S51518). All participants consented to participate in this work.

Consent for publication: Not applicable.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Funding

Authors' contributions

SH and HW contributed to the study conception and design. BB acquired the data. QW and AL analyzed the data. QW interpreted the data and wrote the manuscript drafts. All authors revised and approved the manuscript.

Acknowledgments

We thank all participants in our studies. We also thank Multidiscipline Breast Cancer Center at the University Hospital Leuven for recruiting and collecting health data.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101527.

Reference

[1] H. Sung, et al., Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 Countries, CA Cancer J. Clin. 71 (3) (May 2021) 209–249, https://doi.org/10.3322/CAAC.21660.

[2] K.S. Scher, A. Hurria, Under-representation of older adults in cancer registration trials: known problem, little progress, J. Clin. Oncol. 30 (17) (Jun. 2012) 2036–2038, https://doi.org/10.1200/JCO.2012.41.6727.

[3] J. Zhang, T. He, L. Xue, H. Guo, Senescent T cells: a potential biomarker and target for cancer therapy, EBioMedicine 68 (Jun. 2021), https://doi.org/10.1016/J.EBIOM.2021.103409.

[4] M.Y. Bilal, J.C.D. Houtman, GRB2 nucleates T cell receptor-mediated LAT clusters that control PLC-γ1 activation and cytokine production, Front. Immunol. 6 (MAR) (2015) 141, https://doi.org/10.3389/FIMMU.2015.00141/BIBTEX.

[5] E. Kubo, B. Chhunchha, P. Singh, H. Sasaki, D.P. Singh, Sulforaphane reactivates cellular antioxidant defense by inducing Nrf2/ARE/Prdx6 activity during aging and oxidative stress, Sci. Rep. 7 (1) (Dec. 2017), https://doi.org/10.1038/S41598-017-14520-8.

[6] J.K. Bailur, et al., Immune profiles of elderly breast cancer patients are altered by chemotherapy and relate to clinical frailty, *Breast Cancer Res.* 19 (1) (Feb. 2017) 1–10, https://doi.org/10.1186/S13058-017-0813-X.

[7] S. Gupta, T. Young, L. Yel, H. Su, S. Gollapudi, Differential sensitivity of naïve and subsets of memory CD4+ and CD8+ T cells to hydrogen peroxide-induced apoptosis, Genes Immun. 8 (7) (Aug. 2007) 560–569, https://doi.org/10.1038/sj.gene.6364416, 2007 87.

[8] T.K.D. Vo, et al., Transcriptomic biomarkers of human ageing in peripheral blood mononuclear cell total RNA, Exp. Gerontol. 45 (3) (Mar. 2010) 188–194, https://doi.org/10.1016/J.EXGER.2009.12.001.

[9] A.C. Holly, et al., Towards a gene expression biomarker set for human biological age, Aging Cell 12 (2) (2013) 324, https://doi.org/10.1111/ACEL.12044.

[10] L.W. Harries, et al., Human aging is characterized by focused changes in gene expression and deregulation of alternative splicing, Aging Cell 10 (5) (Oct. 2011) 868, https://doi.org/10.1111/J.1474-9726.2011.00726.X.

[11] M.J. Peters, et al., The transcriptional landscape of age in human peripheral blood, Nat. Commun. 6 (1) (Oct. 2015) 1–14, https://doi.org/10.1038/ncomms9570.

[12] D. Cibrián, F. Sánchez-Madrid, CD69: from activation marker to metabolic gatekeeper, Eur. J. Immunol. 47 (6) (Jun. 2017) 946–953, https://doi.org/10.1002/EJI.201646837.

[13] A. Onnis, C.T. Baldari, Orchestration of immunological synapse assembly by vesicular trafficking, Front. Cell Dev. Biol. 7 (Jul. 2019) 110, https://doi.org/10.3389/FCELL.2019.00110/BIBTEX.

[14] B. Chhunchha, P. Singh, W.D. Stamer, D.P. Singh, Prdx6 retards senescence and restores trabecular meshwork cell health by regulating reactive oxygen species, Cell Death Discov. 3 (1) (Dec. 2017) 17060, https://doi.org/10.1038/CDDISCOVERY.2017.60.

[15] R. Gozzelino, V. Jeney, M.P. Soares, Mechanisms of cell protection by heme Oxygenase-1, Annu. Rev. Pharmacol. Toxicol. 50 (2010) 323–354, https://doi.org/10.1146/annurev.pharmtox.010909.105600.

[16] B B, et al., The impact of adjuvant chemotherapy in older breast cancer patients on clinical and biological aging parameters, Oncotarget 7 (21) (May 2016) 29977–29988, https://doi.org/10.18632/ONCOTARGET.8796.

[17] S.A. Bustin, et al., The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin. Chem. 55 (4) (Apr. 2009) 611–622, https://doi.org/10.1373/CLINCHEM.2008.112797.

[18] A. Vaiserman, D. Krasnienkov, Telomere length as a marker of biological age: state-of-the-art, open issues, and future perspectives, Front. Genet. 11 (Jan. 2021) 1816, https://doi.org/10.3389/FGENE.2020.630186/BIBTEX.

[19] M.J. Yousefzadeh, et al., Circulating levels of monocyte chemoattractant protein-1 as a potential measure of biological age in mice and frailty in humans, Aging Cell 17 (2) (Apr. 2018), https://doi.org/10.1111/ACEL.12706.

[20] R. Cubas, et al., Chemotherapy combines effectively with Anti–PD-L1 treatment and can augment antitumor responses, J. Immunol. 201 (8) (Oct. 2018) 2273–2286, https://doi.org/10.4049/JIMMUNOL.1800275/-/DCSUPPLEMENTAL.

[21] M.L. Axelrod, et al., Changes in peripheral and local tumor immunity after neoadjuvant chemotherapy reshape clinical outcomes in patients with breast cancer, Clin. Cancer Res. 26 (21) (Nov. 2020) 5668–5681, https://doi.org/10.1158/1078-0432.CCR-19-3685.

[22] F. Mozaffari, et al., NK-cell and T-cell functions in patients with breast cancer: effects of surgery and adjuvant chemo- and radiotherapy, Br. J. Cancer 97 (1) (Jun. 2007) 105, https://doi.org/10.1038/SJ.BJC.6603840.

[23] L. Voorwerk, et al., Immune induction strategies in metastatic triple-negative breast cancer to enhance the sensitivity to PD-1 blockade: the TONIC trial, Nat. Med. 25 (6) (May 2019) 920–928, https://doi.org/10.1038/s41591-019-0432-4.

[24] P. Schmid, et al., Pembrolizumab for early triple-negative breast cancer, N. Engl. J. Med. 382 (9) (Feb. 2020) 810–821, https://doi.org/10.1056/NEJMOA1910549/SUPPL_FILE/NEJMOA1910549_DATA-SHARING.PDF.

[25] E.A. Mittendorf, et al., Neoadjuvant atezolizumab in combination with sequential nab-paclitaxel and anthracycline-based chemotherapy versus placebo and chemotherapy in patients with early-stage triple-negative breast cancer (IMpassion031): a randomised, double-blind, phase 3 trial, Lancet 396 (10257) (Oct. 2020) 1090–1100, https://doi.org/10.1016/S0140-6736(20)31953-X.

[26] M.A. Franzoi, E. Romano, M. Piccart, Immunotherapy for early breast cancer: too soon, too superficial, or just right? Ann. Oncol. 32 (3) (Mar. 2021) 323–336, https://doi.org/10.1016/J.ANNONC.2020.11.022.

[27] D.C. Grimberg, et al., Assessments of frailty in bladder cancer, Urol. Oncol. Semin. Orig. Investig. 38 (9) (Sep. 2020) 698–705, https://doi.org/10.1016/J.UROLONC.2020.04.036.

[28] A. Uslu, O. Canbolat, Relationship between frailty and fatigue in older cancer patients, Semin. Oncol. Nurs. 37 (4) (Aug. 2021) 151179, https://doi.org/10.1016/J.SONCN.2021.151179.

[29] V. Balzano et al., "Prevalence of Malnutrition and Frailty in Elderly Cancer Patients and Resulting Impact on Primary Treatment Recommendations in Colorectal and Breast Cancer," doi: 10.34175/jno202004002.

[30] M. Conde, et al., Anti-CD69 antibodies enhance phorbol-dependent glucose metabolism and Ca2+ levels in human thymocytes. Antagonist effect of cyclosporin A, J. Leukoc. Biol. 60 (2) (1996) 278–284, https://doi.org/10.1002/JLB.60.2.278.

[31] L. Soldati, L. Di Renzo, E. Jirillo, P.A. Ascierto, F.M. Marincola, A. De Lorenzo, The influence of diet on anti-cancer immune responsiveness, J. Transl. Med. 16 (1) (Mar. 2018) 1–18, https://doi.org/10.1186/S12967-018-1448-0/FIGURES/2.

[32] N.J. Maciver, R.D. Michalek, J.C. Rathmell, Metabolic regulation of T lymphocytes, Annu. Rev. Immunol. 31 (Mar. 2013) 259–283, https://doi.org/10.1146/ANNUREV-IMMUNOL-032712-095956.

[33] S. Cohen, K. Danzaki, N.J. MacIver, Nutritional effects on T-cell immunometabolism, Eur. J. Immunol. 47 (2) (Feb. 2017) 225, https://doi.org/10.1002/EJI.201646423.

[34] O. Nájera, C. González, G. Toledo, L. López, R. Ortiz, Flow cytometry study of lymphocyte subsets in malnourished and well-nourished children with bacterial infections, Clin. Diagn. Lab. Immunol. 11 (3) (May 2004) 577–580, https://doi.org/10.1128/CDLI.11.3.577-580.2004.

[35] J. Lin, E. Epel, Stress and telomere shortening: Insights from cellular mechanisms, Ageing Res. Rev. 73 (Jan. 2022) 101507, https://doi.org/10.1016/J.ARR.2021.101507.

[36] A. Lagnado, et al., Neutrophils induce paracrine telomere dysfunction and senescence in ROS-dependent manner, EMBO J. 40 (9) (May 2021), e106048, https://doi.org/10.15252/EMBJ.2020106048.

[37] F. Pacifici, et al., Prdx6 plays a main role in the crosstalk between aging and metabolic sarcopenia, Antioxidants 9 (4) (Apr. 2020), https://doi.org/10.3390/ANTIOX9040329.

[38] U.S. Srinivas, B.W.Q. Tan, B.A. Vellayappan, A.D. Jeyasekharan, ROS and the DNA damage response in cancer, Redox Biol. 25 (Jul. 2019), 101084, https://doi.org/10.1016/J.REDOX.2018.101084.

[39] H. Mizutani, S. Tada-Oikawa, Y. Hiraku, M. Kojima, S. Kawanishi, Mechanism of apoptosis induced by doxorubicin through the generation of hydrogen peroxide, Life Sci. 76 (13) (Feb. 2005) 1439–1453, https://doi.org/10.1016/J.LFS.2004.05.040.

[40] M. Michaud, et al., Proinflammatory cytokines, aging, and age-related diseases, J. Am. Med. Dir. Assoc. 14 (12) (Dec. 2013) 877–882, https://doi.org/10.1016/J.JAMDA.2013.05.009.

[41] E. Reyes, et al., Granulocyte colony-stimulating factor (G-CSF) transiently suppresses mitogen-stimulated T-cell proliferative response, Br. J. Cancer 80 (1) (Mar. 1999) 229–235, https://doi.org/10.1038/sj.bjc.6690344.

[42] G. Bonanno, et al., Effects of pegylated G-CSF on immune cell number and function in patients with gynecological malignancies, J. Transl. Med. 8 (Nov. 2010) 114, https://doi.org/10.1186/1479-5876-8-114.

[43] S. Rutella, F. Zavala, S. Danese, H. Kared, G. Leone, Granulocyte colony-stimulating factor: a novel mediator of T cell tolerance, J. Immunol. 175 (11) (Dec. 2005) 7085–7091, https://doi.org/10.4049/JIMMUNOL.175.11.7085.