Sicilian semi- and supercentenarians: identification of age-related T cell immunophenotype to define longevity trait

Mattia Emanuela Ligotti[1], Giulia Accardi[1], Anna Aiello[1], Stefano Aprile[1,2], Anna Calabrò[1], Rosalia Caldarella[3], Calogero Caruso[1*], Marcello Ciaccio[3,4], Anna Maria Corsale[5,6], Francesco Dieli[5,7], Marta Di Simone[5,6], Giovanni Maurizio Giammanco[8], Chiara Mascarella[8], Arne N Akbar[9,§], Serena Meraviglia[5,7,§], Giuseppina Candore[1,§],*

[1]Laboratory of Immunopathology and Immunosenescence, Department of Biomedicine, Neurosciences and Advanced Diagnostics, University of Palermo, Palermo, Italy; [2]Unit of Transfusion Medicine, San Giovanni di Dio Hospital, Agrigento, Italy.[3]Department of Laboratory Medicine, University Hospital "P. Giaccone", Palermo, Italy; [4]Section of Clinical Biochemistry, Clinical Molecular Medicine and Clinical Laboratory Medicine Department of Biomedicine, Neurosciences and Advanced Diagnostics, University of Palermo, Palermo, Italy; [5]Central Laboratory of Advanced Diagnosis and Biomedical Research, University Hospital "P. Giaccone", Palermo, Italy;[6]Department of Health Promotion Sciences, Maternal and Infant Care, Internal Medicine and Medical Specialties "G. D'Alessandro", University of Palermo, Palermo, Italy; [7]Department of Biomedicine, Neurosciences and Advanced Diagnostics, University of Palermo, Palermo, Italy; [8]Section of Microbiology, Department of Health Promotion Sciences, Maternal and Infant Care, Internal Medicine and Medical Specialties "G. D'Alessandro", University of Palermo, Palermo, Italy; [9]Division of Medicine, University College London, London, UK.

[*]Corresponding authors (calogero.caruso@unipa.it, mattiaemanuela.ligotti@unipa.it)
[§]These authors contributed equally to this work as senior authors.

Abstract

The analysis of immunophenotype in semi- and supercentenarians could provide important information about their ability to adapt to factors associated with immune changes, including ageing *per se* and chronic Cytomegalovirus (CMV) infection. We investigated variations in percentages and absolute numbers of immune cell subsets, focusing on T cells, and pro-inflammatory parameters in an ageing cohort that included semi- and supercentenarians. We observed variability in hallmarks of immunosenescence related to age and CMV serological status. Oldest centenarians, *i.e.*, semi- and supercentenarians, seem to show the lowest percentages of naïve T cells, due to their age, and the highest percentages of effector memory cells re-expressing CD45RA (TEMRA) cells, according to their CMV status, and the highest levels of pro-inflammatory parameters. However, some of them, despite their age and CMV positivity, show CD8 naïve and TEMRA percentages, exhaustion/pro-inflammatory markers comparable to the younger ones. Overall, our study supports the suggestion that immune ageing, especially of semi- and super-centenarians, exhibit great variability that is not attributable to a single contributor but should be the full result of a favourable combination of several factors. Everyone ages differently because he/she is unique in genetics and experience of life and this applies even more to the immune system; everybody, indeed, has had a different immunological history during his/her life. Furthermore, our findings on inflammatory markers, TEMRA and CMV seropositivity in centenarians discussed in the light of the most recent literature, suggest that these changes are not unfavourable for centenarians, and in particular for the oldest ones.

Key words: CMV, immune ageing, immunophenotype, longevity, semi-supercentenarians, supercentenarians,

1

Introduction

Semi-supercentenarians (≥105 years old) and supercentenarians (≥110 years old) are a selected population, characterised by individuals who, to date, have survived two world wars and a plethora of environmental and microbial insults. A living witness to more than 100 years of history, they represent an invaluable source of information. However, their study is complicated by practical factors since they are a rare population (only one centenarian out of a thousand becomes supercentenarian [Accardi *et al*., 2020]), and ethical factors since they are particularly fragile subjects given their venerable age. As of 1 January 2021, there were 1,111 over 105 years old individuals living in Italy, of these, 17 women reached and exceeded 110 years of age [Istat, 2021]. Furthermore, within this population, no significant increase in deaths has been observed during 2020, the first year of the COVID-19 pandemic, in contrast to the other age groups of the older population [Istat, 2021]. This would seem to contrast with the profound changes in the immune system with ageing, collectively known as immunosenescence, which make people more susceptible to infections and less responsive to vaccines [Aiello *et al*., 2019; Garnica *et al.,* 2022]. So, it is reasonable to deduce that the immune system of these individuals has peculiar characteristics that enable them to reach the extreme limits of human life. This claim was recently supported by Hashimoto *et al*., [2019]. They have analysed at single-cell resolution blood lymphocytes of supercentenarians identifying CD4+ T cells with cytotoxic characteristics. So, the authors suggested that this might represent an essential adaptation to achieve exceptional longevity by sustaining immune responses to infections.

Several factors contribute to the complexity of the longevity trait, including genetics, epigenetics, lifestyle, environment, and stochasticity [Gentilini *et al*., 2013; Accardi *et al.,* 2018; Garagnani *et al*., 2021]. It is now evident that immunosenescence is not a programmed process exclusively related to ageing but a consequence of a series of events (including immunobiography) that culminates in a reduction in immune performance [Franceschi *et al*., 2017; Caruso *et al*., 2022]. For example, one of the hallmarks of immunosenescence is the alteration of the number and composition of different types of peripheral lymphocytes, *i.e*., the reduction in the number of

2

peripheral blood naïve cells, with a relative increase in the frequency of memory cells [Aiello *et al*., 2019]. Among adaptive immune cells, T cells are dramatically affected by ageing, with changes in their numbers, percentages, relative subset composition and functionality.

Human Cytomegalovirus (CMV) seropositivity has been associated with many of these T cell changes [Pawelec *et al*., 2005]. CMV is a β-herpes virus that infects different types of cells, especially monocytes and dendritic cells, establishing persistent latent infection [Reeves and Sinclair, 2008]. Cycles of viral reactivation cause the expansion of CMV-specific CD8 T cells [Karrer *et al*., 2008], substantially enriched in the late phenotype cells [Appay *et al*., 2002]. Indeed, persistent CMV infection results in chronic stimulation of CD8+ T cells, which expand clonally showing a late stage differentiated effector memory phenotype [Wertheimer *et al*., 2014], while the decrease of naïve CD4 and CD8 depends more on thymic involution [Caruso *et al*., 2022].

Although hallmarks of immunosenescence have been well defined [Aiello *et al*., 2019], most studies do not consider the extreme limit of ageing, represented by the semi- and supercentenarians (in this study we referred to them as the oldest centenarians), and is not still clear whether the CMV infection has long-term beneficial or deleterious immunological effects [Jergović *et al*., 2019]. In a previous study, we investigated the percentages of circulating lymphocyte subsets of 41 Sicilian donors, aged between 25 and 111 years, focusing on T and natural killer cells. Blood cells from a subgroup of 27 healthy donors, including the oldest living Italian supercentenarian at the time of recruitment, were used for a more complete dissection of T cell subsets. We did not record the increase in the rate of inversion of the CD4/CD8 *ratio*, frequently reported as being associated with ageing in literature. But at a subset level, as expected, we observed a shift in the distribution of T cells from naïve to effector memory phenotype. The supercentenarian showed a unique immunophenotypic signature regarding the relative percentages of her T cell subsets, with CD4+ and CD8+ T cell percentages and CD4+ naive T cell values in line with those recorded for younger subjects, despite seropositivity for CMV [Ligotti *et al*., 2021].

To explore the immunophenotypic trait of the oldest centenarians, in this paper we analysed both percentages and absolute numbers of immune cell subsets, focusing on T cells, and the inflammatory status in a cohort of Sicilian healthy individuals. For the assessment of inflammatory status, in addition to serum interleukin(IL)-6 and C-reactive protein (CRP), we also analysed neutrophil-to-lymphocyte *ratio* (NLR) and platelet-lymphocyte *ratio* (PLR) that recently emerged as informative markers of inflammation [Gasparyan *et al*., 2019; Luo *et al*., 2019]. This cohort included 11 long-living individuals (LLIs), *i.e.,* people aged over 90 years old (including 6 centenarians <105 years old), six semi-supercentenarians and two supercentenarians, one of whom was, at the time of recruitment, the oldest living man in Italy at age of 108 years [The Italian Centenarian Database]. Data were also analysed according to gender, as well as to CMV seropositivity. We did not analyse the Epstein Barr Virus (EBV) seropositivity because the accumulation of late-stage T cells is predominantly observed in CMV-seropositive older people, but not in older people infected with other persistent herpesviruses, such as EBV [Derhovanessian *et al*., 2011]. Moreover, in our previous study [Ligotti *et al*., 2021], all recruited subjects were EBV-positive.

Semi-supercentenarians and supercentenarians provide excellent evidence that it is possible to age successfully since they have a relative resistance to age-related diseases, having overcome the acute causes of death [Accardi *et al*., 2020]. Thus, the study of the immune system of these exceptional individuals may allow a better understanding of how to reach the extreme limits of the human lifespan

Materials and Methods

Study cohort

Subjects participating in the "Discovery of molecular and genetic/epigenetic signatures underlying resistance to age-related diseases and comorbidities (DESIGN, 20157ATSLF)" project funded by the Italian Ministry of Education, University and Research were used for the present investigation. Detailed study design and participant recruitment have been previously described

[Aiello et al., 2021]. For the present study, a total of 54 Sicilian participants (females = 28; males = 26) aged between 19 and 110 years were enrolled between 2020 and 2022, selected based on the absence of health issues. LLIs and the oldest centenarians were relatively healthy. Subjects had been excluded from the enrolment if they had been diagnosed with chronic and acute diseases, such as neoplastic and autoimmune diseases, as well as severe dementia. Another exclusion criteria was the use of immunomodulatory drugs within the previous 6 months. The subjects participated voluntarily and written informed consent was obtained from all of them (or from their children). The study protocol was conducted following the Declaration of Helsinki and its amendments. The Ethics Committee of Palermo University Hospital approved the study (Nutrition and Longevity, No. 032017). For comparative analyses, we divided our whole cohort into four age groups (Table 1), to better understand trends between each range age.

A database was created to deal with the collected information and all donors were identified with an alphanumeric code to respect privacy. The participants underwent venipuncture in the morning after a fasting period of 12 h. The blood was collected in specific tubes containing ethylene diamine tetraacetic acid or no additives. The serum was separated by blood centrifugation of dry tubes and stored at −80°C before use at the Laboratory of Immunopathology and Immunosenescence of the Department of Biomedicine, Neurosciences and Advanced Diagnostics of University of Palermo.

Haematological and biochemical parameter analysis

Whole blood was used for automated differential leukocyte counts of all donors, expressed as a percentage (in relationship to the total leukocytes), or as an absolute value. Serum levels of immunoglobulins A, G, and M (IgA, IgG, IgM), C-reactive protein (CRP), and interleukin (IL)-6 were also measured. Haematological and biochemical parameter analyses were performed at the Department of Laboratory Medicine, "P. Giaccone" University Hospital, Palermo, on an XN-2000 automated haematology (Sysmex) and Cobas® 8000 series e801 (Roche diagnostics) analysers.

Flow cytometry analysis

Flow cytometry analysis was performed in fresh whole blood samples using the following antibodies: CD3-FITC, CD45-PerCP/Cy5.5, CD4-PE/Cy7, CD19-APC, CD8-APC/Cy7), CD3(REA613)-FITC, CD4(SK3)-PerCP/Cy5.5, CD4(RPAT4)-APC, CD8(HIT8a)-PE, CD27(O323)-PE/Cy7, CD28(CD28.2)-PE/Cy5, CD45RA(HI100)-PE, CD45RA(T6D11)-PerCP, PD1(PD1.3.1.3)-APC (from BD Bioscience, Miltenyi and Biolegend). Flow cytometry analyses were performed on FACS Canto II (BD) at the Central Laboratory of Advanced Diagnosis and Biomedical Research, "P. Giaccone" University Hospital, Palermo. Lymphocytes, monocytes and granulocytes were identified through forward-(FSC) and side-scatter(SSC), and further identified in the SSC/CD45 dot-plot. An exemplificative schematic representation of the applied gating strategy is displayed in Figure 1. After setting the lymphocytes region in the CD45+/SSC-A low gate, events were gated in the CD3/CD4 and CD3/CD8 dot-plots to define both subsets. These were expressed as a fraction of the parental gated population (lymphocytes) and reported as percentages in the graphics. Based on the surface marker CD27 and CD45RA, CD4 and CD8 T cell populations were further divided into CD27+/CD45RA+ Naïve, CD27+/CD45RA- central memory (TCM), CD27-/CD45RA- effector-memory (TEM) and terminally differentiated CD27-/CD45RA+ (TEMRA). CD28 and PD1 positivity were also evaluated in CD4 and CD8 T cell populations respectively for their central role in T cell activation and their meaning as exhaustion markers.

Absolute numbers of CD3, CD4, and CD8 T cells were calculated using the lymphocyte absolute count from the haematological analysis.

CMV serology

Serum anti-CMV immunoglobulin (Ig)G values were determined by chemiluminescence immunoassay using the LIAISON® CMV IgG II kit (DiaSorin), respectively, according to the manufacturer's indications. The threshold for CMV seropositivity was 14 U/ml and the range upper limit was set at 180 U/ml. CMV serology analysis was performed at Section of Microbiology, Department of Health Promotion, Mother and Child Care, Internal Medicine and Medical Specialties, University of Palermo.

Statistical analysis

The NLR was calculated by dividing neutrophil count by lymphocyte count. The PLR was calculated by dividing platelet count by lymphocyte count. To analyse the percentages of T lymphocytes, flow cytometry data were analysed using FlowJo version 10.5.3 (Tree Star, Inc., Ashland, OR, USA) and statistical analysis was performed with GraphPad Prism version 9.3.1 (GraphPad Software, San Diego, CA, USA). Correlation between the number/percentage of cells and age in all individuals and males and females was examined using simple linear regression analysis. Figures were plotted as scatter plots with a linear regression line and 95% confidence bands. Analysis of inflammatory parameters between age groups was performed by Tukey's multiple comparison test. For each statistical analysis, only p-values < 0.05 were considered significant.

RESULTS

Analysis of leukocyte parameters, inflammatory markers, immunoglobulin levels and CMV serological status.

The number of blood monocytes, neutrophils, and lymphocytes as well as platelets were analysed according to age and gender. Correlation analysis showed that neither age nor gender affected neutrophil (Figure 2c,d) and lymphocyte (Figure 2e,f) counts. Based on data obtained from automated absolute leukocyte counts, we observed, instead, age-related increased monocyte numbers in all individuals (R^2=0.076, p=0.043), confirmed in males (R^2=0.359, p=0.001) but not in females (Figure 2a,b). Figure 2g,h showed that a progressive and significant decline in platelet number during ageing was also observed (R^2=0.126, p=0.008). Gender stratification showed that the age-related male group platelet count remained relatively stable but decreased dramatically in females (R^2=0.388, p=0.0004).

Concerning the inflammatory markers NLR and PLR, the linear regression analysis performed on our cohort showed a positive association between NLR and age (Figure 3a, R^2=0.084, p=0.033), although was confirmed only in the male population (Figure 4b, R^2=0.205, p=0.020) after gender

7

stratification. No significant differences were found in PLR, even if there is a trend to decrease with age in females (Figure 3d).

Analysis of serum pro-inflammatory markers showed a strong increase in IL-6 serum concentration up to age 105, then decreased slightly in the oldest centenarians. As shown in Table 2 the age-related increment of IL-6 is extremely significant in adults *vs.* LLI (p<0.0001) and old *vs* LLI (p=0.0001), while is very significant in adults *vs.* oldest centenarians (p=0.001) and significant in old *vs.* oldest centenarians (p=0.014). This data is confirmed by the linear regression analysis, showing an increase with ageing in IL-6 levels in all individuals (Figure 3a, R^2=0.337, p<0.0001) and in both genders (Figure 4b, R^2_F=0.242, p_F= 0.007; R^2_M=0.475, p_M <0.0001), although the greatest span is observed in the oldest centenarian group, where some individuals showed IL-6 levels comparable to adults, while others showed the highest levels in the cohort (Figure 4a,b). Similarly, serum concentrations of CRP increase with age, achieving significance when comparing adults and LLIs (Table 2, p=0.026), and decrease dramatically in the over 105 age group. This is reflected in a lack of significance in the total cohort in the linear regression analysis (Figure 3c), although we observed a significant age-associated increase in CRP levels in males (R^2=0.176, p=0.032) (Figure 3d).

No age-related changes in Immunoglobulin levels have been observed.

Finally, serum anti-CMV IgG levels were measured and 60% (n = 12/20) of adults, 73% (n = 11/15) of old and 100% of LLIs and oldest centenarians were found to be CMV seropositive. Comparing the CMV seroprevalence in these groups, we observed a significant difference between adults and LLIs (p=0.028), even though all oldest centenarians were also CMV-positive.

Analysis of T cell subsets according to age and gender

Total T cell counts, and percentages appeared to be unaffected by age and gender (Supplementary Figure 1), as well as the percentages and counts of CD4 and CD8 T cells (Supplementary Figures 2 and 3). Consequently, the reversal of the CD4/CD8 *ratio* was not observed (Supplementary Figure 4). Much of this maintenance in the absolute counts seems to be explained by an expansion of the TEMRA with a concomitant decrease of the naïve T cells. Indeed, both naïve

8

CD4 (Figure 5a, R^2=0.095, p=0.024) and CD8 T cells (Figure 5b, R^2=0.235, p=0.0002) exhibited an inverse relationship with age, with a higher decrease rate observed for CD8 naïve T cells. Although the decrease was evident, inter-individual variability can be seen within the various age decades, including the more extreme ones. Indeed, people over 100 years old showed some (most of) values below the trend line, while others were like those of the younger. CD4 TEM (Figure 5e, R^2=0.075, p=0.046) and TEMRA cells (Figure 5g, R^2=0.223, p=0.0003) increased linearly with age, as well as CD8 TEMRA (Figure 5h, R^2=0.343, p<0.0001) although at a much faster rate than CD4 TEMRA, which might explain why CD8 TEM remained rare (Figure 5f). Within the CD8 T cell subsets we observed a predominance of TEMRA over effector T cells with age, whereas in CD4 T cells there was a predominance of effectors over TEMRA. Stratification by gender produced the same significant differences in age-related T subset alterations between males and females, except for naïve and TEM CD4 (Supplementary Figure 5a and e, respectively), where the significant decrease observed in the total population was lost when observing individual genders. Furthermore, a greater decrease in naïve CD8 was observed counteracted by a greater increase in TEMRA CD8 in the female population than in the male population (Supplementary Figure 5b and h, respectively).

Due to its pivotal role in T-cell activation, we also investigated the expression of the CD28 marker in T cells. We observed a significant inverse correlation between age and the expression of CD28 in CD4 (Figure 6a, R^2=0.224, p=0.0003) and, mostly, CD8 T cells (Figure 6c, R^2=0.242, p=0.0002), confirmed in both sexes, with a greater tendency to decrease in the female population, probably due to the disproportion in numbers between the two genders in oldest centenarians. The lowest values of CD28 expression in both T populations were recorded at the extreme age limits. However, once again, the oldest centenarians showed variability in their values, most evident in the expression of CD28 in CD4 T cells as the values in the other age groups remained close to the trend line, while their values deviate more from it.

We also observed a strong correlation between age and the expression of programmed cell death protein 1 (PD-1), an exhaustion marker, in both CD4 (Figure 7a, R^2=0.393, p<0.0001) and CD8

(Figure 7c, R^2=0.083, p=0.035) T cells and age, confirmed in both genders only for CD4+ (Figure 7b, R^2_F=0.302, p_F= 0.002; R^2_M=0.479, p_M <0.0001). As with CD28, PD-1 expression also shows wide variability between individuals, more pronounced in CD8 T cells.

Analysis of T cell subsets according to age and CMV status

To better understand this variability, we investigated the expression of different markers at the individual level, correlating these values with CMV serological status represented through the size of the dots in the Figure 8. The relationship between the decrease in CD4 naïve and the increase in CD4 TEMRA and age was previously detected using a classic scatterplot. In Figure 8a, we aimed to observe whether these trends also correlated to CMV status, but no correlation was possible to hypothesize since CMV-negative individuals (smallest bubbles) showed values comparable to CMV-positive individuals (larger bubbles). In the CD8 compartment (Figure 8b), there was a clear correlation between the reduction in naïve and the increase in TEMRA cells, as can be seen from the movement of the bubbles from the right (higher values of naïve) to the left (lower values of naïve) at the top (higher values of TEMRA). Following this movement (from right to top left) we also observed an increase in the size of the bubbles, an index of an increase in the anti-CMV IgG titre, although some CMV-negative individuals showed naïve CD8 percentages comparable to those of seropositive individuals. Oldest centenarians (yellow bubble) seemed to cluster in the quadrant corresponding to the lowest percentages of naïve (due to their age) and highest percentages of TEMRA (CMV seropositive), except for some oldest centenarians who, despite their age and CMV positivity, showed CD8 naïve and TEMRA percentages comparable to the younger ones.

Then, we wanted to check the relationship between TEMRA, exhaustion/activation markers, and CMV seropositivity within our aged cohort.

The highest CD4 (Figure 8c, from 45% upwards) and CD8 PD1+ (Figure 8d, from 70% upwards) percentages were found in over 69 years old CMV-positive individuals. For lower percentages, however, an almost homogenous distribution was observed regardless of CMV status. Again, although CD8 TEMRA T cells were lower in uninfected individuals, the inverse relationship

10

was not always true, as some CMV-positive individuals showed similar low percentages of terminally differentiated cells.

Most of the higher percentages of CD4 CD28+ seem to be clustered on lower percentages of CD4 TEMRA (Figure 8e), but much more evident was the linear relationship between the decrease in expression of the costimulatory molecule CD28 and the increase in TEMRA in CD8 cells (Figure 8f). There was a clear contribution of CMV in the reduction of CD28 expression, as the highest CD8 CD28+ percentages were found in CMV-negative individuals or those with lower antibody anti-CMV IgG titres. The prevalence of yellow/green bigger bubbles in the upper left of the chart indicates that age and long-term CMV infection combined to increase in TEMRA with low CD28 expression. Surprisingly, the oldest centenarian individual placed in the highest percentages of CD28+ and the lowest percentages of TEMRA CD8 T cells.

In Figures 8g and h, no relation seems to exist between increased IL-6 level and CMV infection, as the lowest cut-off (1.5 pg/mL) was recorded in different individuals regardless of bubble size (IgG titre) and TEMRA percentages. On the other hand, the highest IL-6 values were recorded in centenarians and oldest centenarians (green-yellow bubble) creating a dependency of its concentrations with advancing age. However, some oldest centenarians showed IL-6 levels below the reference cut-off (7 pg/mL), again indicating a lack of absolute homogeneity in the oldest centenarian population.

DISCUSSION

In a context such as immunosenescence, where numerous factors contribute to the responses of an aged immune system, it is difficult to define which factors are net positive and which factors are net negative effects. For example, there is still debate as to the true influence of one of the major contributors to immunosenescence, *i.e.,* CMV seropositivity [Jergović *et al*., 2019; Caruso *et al*., 2022]. Studies on the oldest centenarians could provide help in interpreting the role of these factors

and/or their combinations and we believe that it is crucial to incorporate the analysis of their immunophenotype in immunosenescence studies.

Concerning blood cell analysis, no age-related changes in counts of neutrophils, a critical component of innate immunity [Rosales, 2018; Aiello *et al*., 2022], were observed. Likewise, despite the reported decline in absolute lymphocyte count and percentage during ageing [Valiathan *et al*., 2016], there was no age-related change in their absolute counts. Monocytes are key components of the innate immune system and are involved in pathogen recognition, secretion of pro-inflammatory cytokines, antigen presentation, and other important immune effector functions [Shi and Pamer, 2011; Aiello *et al*., 2022]. We observed a marked increase of these cells in males but not in females, confirming a previous report [Beenakker *et al*., 2020]. Monocyte-specific inflammatory *loci* appear to be more activated in males than in females [Márquez *et al*., 2020]. This should be linked to the increased pro-inflammatory status in men compared to women [Caruso *et al*., 2022].

Finally, no age-related changes in Ig levels were observed, although a previous study conducted on a larger cohort of subjects (166, 20-106 years old) showed an age-related increase in IgG, significant only in men, and IgA levels [Listì *et al*., 2006].

The analysis of the inflammatory profile confirmed the expected increase of the inflammatory markers with advancing age [Fagiolo *et al*, 1993; Franceschi *et al*., 2000; Alberro *et al*., 2021]. Specifically, we analysed NLR and PLR, which have been shown to be significantly associated with the presence and progression of several inflammatory diseases [Zinellu and Mangoni, 2023], and serum levels of the acute phase protein CRP and IL-6, that increase in response to inflammatory stimuli [Tanaka *et al*., 2014; Mantovani and Garlanda, 2023]. Most older individuals develop inflamm-ageing, a condition characterized by elevated levels of blood inflammatory markers such as IL-6 [Franceschi *et al*., 2000], particularly marked in males, that carries high susceptibility to chronic morbidity, disability, frailty, and premature death [Ferrucci and Fabbri, 2018]. In our cohort, we observed a highly significant increase in IL-6 with advancing age in both genders (more significant in males), and an age-related increase in CRP and NLR in all individuals and the male population,

but not in females. Some oldest centenarian showed monocyte count, NLR, IL-6 and CRP levels comparable to younger subjects, while others showed the highest values. Inflamm-ageing is known to be still present in centenarians, who are relatively healthy because they are suggested to have a complex and peculiar balance between pro-inflammatory and anti-inflammatory characteristics [Cevenini *et al*., 2013]. About this, deleterious effects of inflamm-ageing in centenarians might be neutralized by IL-19, of which high serum levels could contribute to dampen a Th1-driven, inflammatory response [Pinti *et al*., 2023]. Concerning oldest centenarians, it has been concluded that inflammation is an important malleable driver of ageing up to extreme old age [Arai *et al*., 2015]. Furthermore, our findings on some miRNAs, obtained in centenarians, including the oldest ones, suggest a possible epigenetic modulation, with anti-inflammatory effects, which may confer protection against tissue damage (Aiello *et al*., 2021; Accardi *et al.,* 2022). Thus, the overall observation confirmed the presence of inflammatory status in old people but there is no indication that excessive inflammation can be detrimental in the oldest individuals.

Among the main hallmarks of immunosenescence, there are a decrease in naïve and an increase in memory/effector T cells.These processes are a consequence respectively of thymic involution and pathogen stimulation, especially by persistent CMV infection [Aiello *et al*. 2019; Caruso *et al.*, 2022; Covre *et al*., 2020; Ligotti *et al*., 2021]. In our study, according to the progressive thymic involution and the relative reduction in thymic output with age, both naïve CD4 and CD8 T cells exhibited an inverse relationship with age. Although there was wide variability in these percentages, most of the values of the individuals over 100 years old (including the oldest ones) clustered below the trend line, while some were notable for having higher values compared to those of the younger giving them protection against new antigens. As a result of this, the average CD4 and CD8 naïve T cell values were lower in nonagenarians than in those over 100. Linear regression analysis revealed a significant increase in proportions of TEM CD4T cells but not significant changes in TEM CD8 T cells in aged individuals. We also observed a positive association between increasing age and a higher proportion of TEMRA T cells, particularly pronounced in the CD8+ compartment,

as reported in the literature [Callender *et al*., 2018; Añé-Kourí et al., 2022]. Concerning CD4 TEM and CD8 TEMRA, where linear regression analysis showed the highest significance, most values of the individuals over 100 years old (including the oldest ones) clustered above the trend line, while some were notable for having lower values compared to those of the younger. Recently, it was not found any significant association between TEMRA levels and the onset of a diverse set of chronic diseases (*e.g*., hypertension, cardiovascular disease, type 2 diabetes, and chronic kidney disease) after 65 years old (Salumets *et al*., 2022). On the other hand, the majority of non-naïve phenotype CD4+ and CD8+ T cells in the blood of LLIs and oldest centenarians are TEM or TEMRA; since most of them resist re-infections quite well, it would be reasonable to assume that these recirculating populations are playing an important role [Jameson and Masopust, 2018]. So, TEMRA are not necessarily unfavourable for very old age.

Highly differentiated T cells typically lose expression of the costimulatory receptor CD28 molecule, needed for T cell activation. With ageing, it is reported an accumulation of CD8+CD28- T cells that, functionally, have reduced proliferative capacity and increased activation of signalling pathways involved in cellular senescence [Effros *et al.,* 1994; Fagnoni *et al*., 1996; Weng et al., 2009]. According to this, we observed a significant age-related reduction in CD28 expression on both CD4 and CD8 T cells, confirmed in both genders, although, some over 100 years old individuals showed higher percentages of CD4+ and CD8+CD28+ T cells than individuals not only from the same group cohort but also from younger ones.

We also investigated the exhausted status of T cells, using the PD-1 marker. PD-1 is an important inhibitory receptor expressed on the surface of activated T cells and involved in the regulation of CD8 T cell exhaustion during chronic viral infection. It is also transiently expressed by activated CD8 T cells during the acute phase of viral infection [Petrovas *et al*., 2006; Henson *et al*., 2012; Wherry and Kurachi, 2015]. As with CD28, PD-1 expression on CD4 and CD8 T cells showed wide variability between individuals, although the net result was an increase in PD1+ CD4+ and CD8+ with ageing.

So, the heterogeneity of ageing-associated remodelling of the immune system in the oldest centenarians is confirmed in the T cells analysis. Concerning the role of gender, it must be stressed that in our cohort there was only one male semi-supercentenarian since women are more resilient and statistically more likely to achieve exceptional longevity [Caruso *et al*., 2013; Accardi *et al*., 2020], therefore the relative results are influenced by gender disproportionality in favour of oldest centenarian women.

We also explored the contribution of CMV infection to immune ageing within our cohort and whether this influence showed qualitative and quantitative variation between different individuals of the same age group. In our study, although the evident and expected shift from naïve to terminally differentiated T cell phenotype with ageing, some oldest centenarians were characterized by having values that were far from those expected, especially those closely related to age. Indeed, a high (but not the highest) percentage of naïve T cells was found among these individuals, giving them protection against new antigens. We also found a linear relationship between the decrease in CD28 expression and the increase in CD8 TEMRA percentages, and both age and CMV infection seemed to combine to increase TEMRA with low CD28 expression. Despite its age and anti-CMV IgG titre, an oldest centenarian individual showed high percentages of CD28+ and low percentages of TEMRA CD8 T cells. It was proposed that latent CMV infection causes large clonal expansions of terminally differentiated CD8+ T cells, in agreement with the age-related increase of these cells found in our cohort. However, individuals over 100 years old again showed wide variability, despite all of them being CMV-positive. As reported in the literature [Salumets *et al*., 2022; Caruso *et al*., 2022], no relation seemed to exist between increased IL-6 serum level and CMV infection, since there is great variability in these values among CMV seropositive individuals.

It has been argued that CMV is epidemiologically associated with certain chronic diseases, mainly cardiovascular disease and cancer with ageing and therefore would have harmful effects. Other studies have shown that human coevolution with CMV includes potentially positive impacts on adaptive immunity in late life. CMV, to ensure sustained viral life within the host, appears to

exercise great care to help its host survive [Nikolich-Žugich *et al*., 2020]. The oldest centenarians studied in the present paper seem to testify to the latter possibility. Support comes, moreover, from data showing that fewer older centenarians, almost certainly CMV-positive, died than the rest of the older population in the 2020 COVID-19 pandemic [Poulain *et al*., 2021; Caruso *et al*., 2023 a,b]. In agreement, three Brazilian supercentenarians recovered from COVID-19, showing robust IgG levels and neutralizing titers versus SARS-CoV-2 [de Castro *et al*., 2022].

Overall, our study suggests that immune parameters, especially of semi- and super-centenarians, exhibit great variability. This is not surprising because everyone ages differently because he/she is unique in genetics and experience of life and this applies even more to the immune system [Caruso *et al.,* 2022]. Everybody, indeed, had a different immunological history during his/her life because of the different antigenic stimuli encountered, called "immunobiography" [Franceschi *et al*., 2017]. Furthermore, our findings on age-related variations on inflammatory markers, naïve and TEMRA T cells, and CMV seropositivity in centenarians, discussed in the light of the most recent literature, suggest that these changes are not unfavourable for centenarians, and in particular for the oldest ones. This because they may be neutralized (inflammatory markers) or possibly useful or at least not unfavorable (CMV seropositivity and TEMRA) for the achievement of longevity and successful ageing.

Therefore, the immune system is expected to play a very important role in longevity, and the wide variability in its changes observed in centenarians is further evidence of immune adaptation to specific intrinsic and extrinsic conditions.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval
The Institutional Ethics Committee ("Paolo Giaccone", University Hospital) approved the DESIGN study protocol (Nutrition and Longevity, No. 032017). The study was conducted in accordance with the Declaration of Helsinki and its amendments.

Statement of human and animal rights

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from the participants or by their children

References

Accardi, G., Aiello, A., Vasto, S., Caruso, C. (2019). Chance and Causality in Ageing and Longevity. In: Caruso, C. (eds) Centenarians. Springer, Cham. https://doi.org/10.1007/978-3-030-20762-5_1

Accardi G, Aiello A, Aprile S, Caldarella R, Cammarata G, Carru C, Caruso C, Ciaccio M, Colomba P, Galimberti D, Gambino CM, Davinelli S, De Vivo I, Ligotti ME, Vasto S, Zinellu A, Candore G. The Phenotypic Characterization of the Cammalleri Sisters, an Example of Exceptional Longevity. Rejuvenation Res. 2020 Dec;23(6):476-484. doi: 10.1089/rej.2019.2299. Epub 2020 May 11. PMID: 32242495.

Accardi G, Bono F, Cammarata G, Aiello A, Herrero MT, Alessandro R, Augello G, Carru C, Colomba P, Costa MA, De Vivo I, Ligotti ME, Lo Curto A, Passantino R, Taverna S, Zizzo C, Duro G, Caruso C, Candore G. miR-126-3p and miR-21-5p as Hallmarks of Bio-Positive Ageing; Correlation Analysis and Machine Learning Prediction in Young to Ultra-Centenarian Sicilian Population. Cells. 2022 Apr 30;11(9):1505. doi: 10.3390/cells11091505. PMID: 35563810; PMCID: PMC9099697.

Aiello A, Accardi G, Aprile S, Caldarella R, Cammarata G, Carru C, Caruso C, Ciaccio M, De Vivo I, Gambino CM, Ligotti ME, Vasto S, Candore G. Pro-inflammatory status is not a limit for longevity: case report of a Sicilian centenarian. Aging Clin Exp Res. 2021 May;33(5):1403-1407. doi: 10.1007/s40520-020-01628-7. Epub 2020 Jun 23. PMID: 32577916.

Aiello A, Farzaneh F, Candore G, Caruso C, Davinelli S, Gambino CM, Ligotti ME, Zareian N, Accardi G. Immunosenescence and Its Hallmarks: How to Oppose Aging Strategically? A Review

of Potential Options for Therapeutic Intervention. Front Immunol. 2019 Sep 25;10:2247. doi: 10.3389/fimmu.2019.02247. PMID: 31608061; PMCID: PMC6773825.

Aiello A, Accardi G, Aprile S, Caldarella R, Carru C, Ciaccio M, De Vivo I, Gambino CM, Ligotti ME, Vasto S, Zinellu A, Caruso C, Bono F, Candore G. Age and Gender-related Variations of Molecular and Phenotypic Parameters in A Cohort of Sicilian Population: from Young to Centenarians. Aging Dis. 2021 Oct 1;12(7):1773-1793. doi: 10.14336/AD.2021.0226. PMID: 34631220; PMCID: PMC8460304.

Aiello A, Ligotti ME, Garnica M, Accardi G, Calabrò A, Pojero F, Arasanz H, Bocanegra A, Blanco E, Chocarro L, Echaide M, Fernandez-Rubio L, Ramos P, Piñeiro-Hermida S, Kochan G, Zareian N, Farzaneh F, Escors D, Caruso C, Candore G. How Can We Improve Vaccination Response in Old People? Part I: Targeting Immunosenescence of Innate Immunity Cells. Int J Mol Sci. 2022 Aug 31;23(17):9880. doi: 10.3390/ijms23179880. PMID: 36077278; PMCID: PMC9456428.

Alberro A, Iribarren-Lopez A, Sáenz-Cuesta M, Matheu A, Vergara I, Otaegui D. Inflammaging markers characteristic of advanced age show similar levels with frailty and dependency. Sci Rep. 2021 Feb 23;11(1):4358. doi: 10.1038/s41598-021-83991-7. PMID: 33623057; PMCID: PMC7902838.

Añé-Kourí AL, Ledón N, Ramos MB, González A, Pereira K, Rodríguez M, Vidal A, Rodríguez Y, Bicet YC, Llanez-Gregorich E, Lorenzo-Luaces P, Suárez GM, Silva A, Crombet T, Saavedra D, Lage A. Association among Terminally Differentiated T Cells, Frailty, and Dependency in a Group of Cuban Centenarians. Gerontology. 2022 Jun 21:1-10. doi: 10.1159/000525062. Epub ahead of print. PMID: 35728563.

Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, Ogg GS, King A, Lechner F, Spina CA, Little S, Havlir DV, Richman DD, Gruener N, Pape G, Waters A, Easterbrook P, Salio M, Cerundolo V, McMichael AJ, Rowland-Jones SL. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med. 2002 Apr;8(4):379-85. doi: 10.1038/nm0402-379. PMID: 11927944.

Arai Y, Martin-Ruiz CM, Takayama M, Abe Y, Takebayashi T, Koyasu S, Suematsu M, Hirose N, von Zglinicki T. Inflammation, But Not Telomere Length, Predicts Successful Ageing at Extreme Old Age: A Longitudinal Study of Semi-supercentenarians. EBioMedicine. 2015 Jul 29;2(10):1549-58. doi: 10.1016/j.ebiom.2015.07.029. PMID: 26629551; PMCID: PMC4634197.

Beenakker KGM, Westendorp RGJ, de Craen AJM, Chen S, Raz Y, Ballieux BEPB, Nelissen RGHH, Later AFL, Huizinga TW, Slagboom PE, Boomsma DI, Maier AB. Men Have a Stronger Monocyte-Derived Cytokine Production Response upon Stimulation with the Gram-Negative Stimulus Lipopolysaccharide than Women: A Pooled Analysis Including 15 Study Populations. J

Innate Immun. 2020;12(2):142-153. doi: 10.1159/000499840. Epub 2019 Jun 21. PMID: 31230049; PMCID: PMC7098282.

Callender LA, Carroll EC, Beal RWJ, Chambers ES, Nourshargh S, Akbar AN, Henson SM. Human CD8+ EMRA T cells display a senescence-associated secretory phenotype regulated by p38 MAPK. Aging Cell. 2018 Feb;17(1):e12675. doi: 10.1111/acel.12675. Epub 2017 Oct 12. PMID: 29024417; PMCID: PMC5770853.

Caruso C, Accardi G, Virruso C, Candore G. Sex, gender and immunosenescence: a key to understand the different lifespan between men and women? Immun Ageing. 2013 May 16;10(1):20. doi: 10.1186/1742-4933-10-20. PMID: 23680476; PMCID: PMC3737094.

Caruso C, Accardi G, Aiello A, Calabrò A, Ligotti ME, Candore G. Centenarians born before 1919 are resistant to COVID-19. Aging Clin Exp Res. 2023a Jan;35(1):217-220. doi: 10.1007/s40520-022-02287-6. Epub 2022 Nov 1. PMID: 36319938; PMCID: PMC9628492.

Caruso C, Marcon G, Accardi G, Aiello A, Calabrò A, Ligotti ME, Tettamanti M, Franceschi C, Candore G. Role of Sex and Age in Fatal Outcomes of COVID-19: Women and Older Centenarians Are More Resilient. International Journal of Molecular Sciences. 2023b; 24(3):2638. https://doi.org/10.3390/ijms24032638

Caruso C, Ligotti ME, Accardi G, Aiello A, Candore G. An immunologist's guide to immunosenescence and its treatment. Expert Rev Clin Immunol. 2022 Sep;18(9):961-981. doi: 10.1080/1744666X.2022.2106217. PMID: 35876758

Cevenini E, Monti D, Franceschi C. Inflamm-ageing. Curr Opin Clin Nutr Metab Care. 2013 Jan;16(1):14-20. doi: 10.1097/MCO.0b013e32835ada13. PMID: 23132168.

Covre LP, De Maeyer RPH, Gomes DCO, Akbar AN. The role of senescent T cells in immunopathology. Aging Cell. 2020 Dec;19(12):e13272. doi: 10.1111/acel.13272. Epub 2020 Nov 9. PMID: 33166035; PMCID: PMC7744956.

de Castro MV, Silva MVR, Naslavsky MS, Scliar MO, Nunes K, Passos-Bueno MR, Castelli EC, Magawa JY, Adami FL, Moretti AIS, de Oliveira VL, Boscardin SB, Cunha-Neto E, Kalil J, Jouanguy E, Bastard P, Casanova JL, Quiñones-Vega M, Sosa-Acosta P, Guedes JS, de Almeida NP, Nogueira FCS, Domont GB, Santos KS, Zatz M. Correction: The oldest unvaccinated Covid-19 survivors in South America. Immun Ageing. 2022 Dec 7;19(1):61. doi: 10.1186/s12979-022-00319-3. Erratum for: Immun Ageing. 2022 Nov 16;19(1):57. PMID: 36476248; PMCID: PMC9727865.

Derhovanessian E, Maier AB, Hähnel K, Beck R, de Craen AJM, Slagboom EP, Westendorp RGJ, Pawelec G. Infection with cytomegalovirus but not herpes simplex virus induces the accumulation of late-differentiated CD4+ and CD8+ T-cells in humans. J Gen Virol. 2011 Dec;92(Pt 12):2746-2756. doi: 10.1099/vir.0.036004-0. Epub 2011 Aug 3. PMID: 21813708.

Effros RB, Boucher N, Porter V, Zhu X, Spaulding C, Walford RL, Kronenberg M, Cohen D, Schächter F. Decline in CD28+ T cells in centenarians and in long-term T cell cultures: a possible cause for both in vivo and in vitro immunosenescence. Exp Gerontol. 1994 Nov-Dec;29(6):601-9. doi: 10.1016/0531-5565(94)90073-6. PMID: 9435913.

Fagiolo U, Cossarizza A, Scala E, Fanales-Belasio E, Ortolani C, Cozzi E, Monti D, Franceschi C, Paganelli R. Increased cytokine production in mononuclear cells of healthy elderly people. Eur J Immunol. 1993 Sep;23(9):2375-8. doi: 10.1002/eji.1830230950. PMID: 8370415.

Fagnoni FF, Vescovini R, Mazzola M, Bologna G, Nigro E, Lavagetto G, Franceschi C, Passeri M, Sansoni P. Expansion of cytotoxic CD8+ CD28- T cells in healthy ageing people, including centenarians. Immunology. 1996 Aug;88(4):501-7. doi: 10.1046/j.1365-2567.1996.d01-689.x. PMID: 8881749; PMCID: PMC1456634.

Ferrucci L, Fabbri E. Inflammageing: chronic inflammation in ageing, cardiovascular disease, and frailty. Nat Rev Cardiol. 2018;15:505-22.

Franceschi C, Bonafè M, Valensin S, Olivieri F, De Luca M, Ottaviani E, De Benedictis G. Inflamm-aging. An evolutionary perspective on immunosenescence. Ann N Y Acad Sci. 2000 Jun;908:244-54. doi: 10.1111/j.1749-6632.2000.tb06651.x. PMID: 10911963.

Franceschi C, Salvioli S, Garagnani P, de Eguileor M, Monti D, Capri M. Immunobiography and the Heterogeneity of Immune Responses in the Elderly: A Focus on Inflammaging and Trained Immunity. Front Immunol. 2017 Aug 15;8:982. doi: 10.3389/fimmu.2017.00982. PMID: 28861086; PMCID: PMC5559470.

Garagnani P, Marquis J, Delledonne M, Pirazzini C, Marasco E, Kwiatkowska KM, Iannuzzi V, Bacalini MG, Valsesia A, Carayol J, Raymond F, Ferrarini A, Xumerle L, Collino S, Mari D, Arosio B, Casati M, Ferri E, Monti D, Nacmias B, Sorbi S, Luiselli D, Pettener D, Castellani G, Sala C, Passarino G, De Rango F, D'Aquila P, Bertamini L, Martinelli N, Girelli D, Olivieri O, Giuliani C, Descombes P, Franceschi C. Whole-genome sequencing analysis of semi-supercentenarians. Elife. 2021 May 4;10:e57849. doi: 10.7554/eLife.57849. PMID: 33941312; PMCID: PMC8096429.

Garnica M, Aiello A, Ligotti ME, Accardi G, Arasanz H, Bocanegra A, Blanco E, Calabrò A, Chocarro L, Echaide M, Kochan G, Fernandez-Rubio L, Ramos P, Pojero F, Zareian N, Piñeiro-Hermida S, Farzaneh F, Candore G, Caruso C, Escors D. How Can We Improve the Vaccination Response in Older People? Part II: Targeting Immunosenescence of Adaptive Immunity Cells. Int J Mol Sci. 2022 Aug 29;23(17):9797. doi: 10.3390/ijms23179797. PMID: 36077216; PMCID: PMC9456031.

Gasparyan AY, Ayvazyan L, Mukanova U, Yessirkepov M, Kitas GD. The Platelet-to-Lymphocyte Ratio as an Inflammatory Marker in Rheumatic Diseases. Ann Lab Med. 2019 Jul;39(4):345-357. doi: 10.3343/alm.2019.39.4.345. PMID: 30809980; PMCID: PMC6400713.

Gentilini D, Mari D, Castaldi D, Remondini D, Ogliari G, Ostan R, Bucci L, Sirchia SM, Tabano S, Cavagnini F, Monti D, Franceschi C, Di Blasio AM, Vitale G. Role of epigenetics in human aging and longevity: genome-wide DNA methylation profile in centenarians and centenarians' offspring. Age (Dordr). 2013 Oct;35(5):1961-73. doi: 10.1007/s11357-012-9463-1. Epub 2012 Aug 25. PMID: 22923132; PMCID: PMC3776126..

Hashimoto K, Kouno T, Ikawa T, Hayatsu N, Miyajima Y, Yabukami H, Terooatea T, Sasaki T, Suzuki T, Valentine M, Pascarella G, Okazaki Y, Suzuki H, Shin JW, Minoda A, Taniuchi I, Okano H, Arai Y, Hirose N, Carninci P. Single-cell transcriptomics reveals expansion of cytotoxic CD4 T cells in supercentenarians. Proc Natl Acad Sci U S A. 2019 Nov 26;116(48):24242-24251. doi: 10.1073/pnas.1907883116. Epub 2019 Nov 12. PMID: 31719197; PMCID: PMC6883788.

Henson SM, Macaulay R, Franzese O, Akbar AN. Reversal of functional defects in highly differentiated young and old CD8 T cells by PDL blockade. Immunology. 2012 Apr;135(4):355-63. doi: 10.1111/j.1365-2567.2011.03550.x. PMID: 22211948; PMCID: PMC3372750.

Istat. Available online: https://www.istat.it/it/files//2022/06/STAT-TODAY_CENTENARI-2021.pdf

Italian supercentenarians database [in Italian]. Available at: https://www.supercentenariditalia.it/persone-viventi-piu-longeve-in-italia

Jameson SC, Masopust D. Understanding Subset Diversity in T Cell Memory. Immunity. 2018 Feb 20;48(2):214-226. doi: 10.1016/j.immuni.2018.02.010. PMID: 29466754; PMCID: PMC5863745.

Jergović M, Contreras NA, Nikolich-Žugich J. Impact of CMV upon immune aging: facts and fiction. Med Microbiol Immunol. 2019 Aug;208(3-4):263-269. doi: 10.1007/s00430-019-00605-w. Epub 2019 Apr 19. PMID: 31004198; PMCID: PMC6635032.

Karrer U, Sierro S, Wagner M, Oxenius A, Hengel H, Koszinowski UH, Phillips RE, Klenerman P. Memory inflation: continuous accumulation of antiviral CD8+ T cells over time. J Immunol. 2003 Feb 15;170(4):2022-9. doi: 10.4049/jimmunol.170.4.2022. PMID: 12574372.

Ligotti ME, Aiello A, Accardi G, Aprile S, Bonura F, Bulati M, Gervasi F, Giammanco GM, Pojero F, Zareian N, Caruso C, Farzaneh F, Candore G. Analysis of T and NK cell subsets in the Sicilian population from young to supercentenarian: The role of age and gender. Clin Exp Immunol. 2021 Aug;205(2):198-212. doi: 10.1111/cei.13606. Epub 2021 Jun 17. PMID: 33866541; PMCID: PMC8274165.

Listì F, Candore G, Modica MA, Russo M, Di Lorenzo G, Esposito-Pellitteri M, Colonna-Romano G, Aquino A, Bulati M, Lio D, Franceschi C, Caruso C. A study of serum immunoglobulin levels in elderly persons that provides new insights into B cell immunosenescence. Ann N Y Acad Sci. 2006 Nov;1089:487-95. doi: 10.1196/annals.1386.013. PMID: 17261791.

Luo H, He L, Zhang G, Yu J, Chen Y, Yin H, Goyal H, Zhang GM, Xiao Y, Gu C, Yin M, Jiang X, Song X, Zhang L. Normal Reference Intervals of Neutrophil-To-Lymphocyte Ratio, Platelet-To-Lymphocyte Ratio, Lymphocyte-To-Monocyte Ratio, and Systemic Immune Inflammation Index in Healthy Adults: a Large Multi-Center Study from Western China. Clin Lab. 2019 Mar 1;65(3). doi: 10.7754/Clin.Lab.2018.180715. PMID: 30868857.

Mantovani A, Garlanda C. Humoral Innate Immunity and Acute-Phase Proteins. N Engl J Med. 2023 Feb 2;388(5):439-452. doi: 10.1056/NEJMra2206346. PMID: 36724330.

Márquez EJ, Chung CH, Marches R, Rossi RJ, Nehar-Belaid D, Eroglu A, Mellert DJ, Kuchel GA, Banchereau J, Ucar D. Sexual-dimorphism in human immune system aging. Nat Commun. 2020 Feb 6;11(1):751. doi: 10.1038/s41467-020-14396-9. PMID: 32029736; PMCID: PMC7005316.

Nikolich-Žugich J, Čicin-Šain L, Collins-McMillen D, Jackson S, Oxenius A, Sinclair J, Snyder C, Wills M, Lemmermann N. Advances in cytomegalovirus (CMV) biology and its relationship to health, diseases, and aging. Geroscience. 2020 Apr;42(2):495-504. doi: 10.1007/s11357-020-00170-8. Epub 2020 Mar 11. PMID: 32162210; PMCID: PMC7205956.

Pawelec G, Akbar A, Caruso C, Solana R, Grubeck-Loebenstein B, Wikby A. Human immunosenescence: is it infectious? Immunol Rev. 2005 Jun;205:257-68. doi: 10.1111/j.0105-2896.2005.00271.x. PMID: 15882359.

Petrovas C, Casazza JP, Brenchley JM, Price DA, Gostick E, Adams WC, Precopio ML, Schacker T, Roederer M, Douek DC, Koup RA. PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. J Exp Med. 2006 Oct 2;203(10):2281-92. doi: 10.1084/jem.20061496. Epub 2006 Sep 5. PMID: 16954372; PMCID: PMC2118095.

Pinti M, Gibellini L, Lo Tartaro D, De Biasi S, Nasi M, Borella R, Fidanza L, Neroni A, Troiano L, Franceschi C, Cossarizza A. A Comprehensive Analysis of Cytokine Network in Centenarians. Int J Mol Sci. 2023 Feb 1;24(3):2719. doi: 10.3390/ijms24032719. PMID: 36769039; PMCID: PMC9916918.

Poulain M, Chambre D, Pes GM. Centenarians exposed to the Spanish flu in their early life better survived to COVID-19. Aging (Albany NY). 2021 Sep 27;13(18):21855-21865. doi: 10.18632/aging.203577. Epub 2021 Sep 27. PMID: 34570724; PMCID: PMC8507269.

Reeves M, Sinclair J. Aspects of human cytomegalovirus latency and reactivation. Curr Top Microbiol Immunol. 2008;325:297-313. doi: 10.1007/978-3-540-77349-8_17. PMID: 18637513.

Rosales C. Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? Front Physiol. 2018 Feb 20;9:113. doi: 10.3389/fphys.2018.00113. PMID: 29515456; PMCID: PMC5826082.

Salumets A, Tserel L, Rumm AP, Türk L, Kingo K, Saks K, Oras A, Uibo R, Tamm R, Peterson H, Kisand K, Peterson P. Epigenetic quantification of immunosenescent CD8+ TEMRA cells in human blood. Aging Cell. 2022 May;21(5):e13607. doi: 10.1111/acel.13607. Epub 2022 Apr 9. PMID: 35397197; PMCID: PMC9124311.

Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. Nat Rev Immunol. 2011 Oct 10;11(11):762-74. doi: 10.1038/nri3070. PMID: 21984070; PMCID: PMC3947780.

Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol. 2014 Sep 4;6(10):a016295. doi: 10.1101/cshperspect.a016295. PMID: 25190079; PMCID: PMC4176007.

Valiathan R, Ashman M, Asthana D. Effects of Ageing on the Immune System: Infants to Elderly. Scand J Immunol. 2016 Apr;83(4):255-66. doi: 10.1111/sji.12413. PMID: 26808160.

Weng NP, Akbar AN, Goronzy J. CD28(-) T cells: their role in the age-associated decline of immune function. Trends Immunol. 2009 Jul;30(7):306-12. doi: 10.1016/j.it.2009.03.013. Epub 2009 Jun 18. PMID: 19540809; PMCID: PMC2801888.

Wertheimer AM, Bennett MS, Park B, Uhrlaub JL, Martinez C, Pulko V, Currier NL, Nikolich-Žugich D, Kaye J, Nikolich-Žugich J. Aging and cytomegalovirus infection differentially and jointly affect distinct circulating T cell subsets in humans. J Immunol. 2014 Mar 1;192(5):2143-55. doi: 10.4049/jimmunol.1301721. Epub 2014 Feb 5. PMID: 24501199; PMCID: PMC3989163.

Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. Nat Rev Immunol. 2015 Aug;15(8):486-99. doi: 10.1038/nri3862. PMID: 26205583; PMCID: PMC4889009.

Zinellu A, Mangoni AA. Neutrophil-to-lymphocyte and platelet-to-lymphocyte ratio and disease activity in rheumatoid arthritis: A systematic review and meta-analysis. Eur J Clin Invest. 2023 Feb;53(2):e13877. doi: 10.1111/eci.13877. Epub 2022 Sep 24. PMID: 36121342.

Table 1. Age and gender of Sicilian cohort healthy donors.

	Adult (n=20)	Older (n=15)	LLI (n=11)	Oldest centenarians (n=8)
Age (years)				
Mean±SD	39±12.5	74.7±6.2	99.8±3.5	108.2±1.6
Range	19.5-63.6	68.5-87.3	93.3-104.7	105.7-110.3
Gender n (%)				
Female	10 (50)	7 (46.6)	4 (36.4)	7 (87.5)
Male	10 (50)	8 (53.4)	7 (63.6)	1 (12.5)

Age is reported in years and months. LLI=long-lived individuals; n= number; SD=standard deviation. Oldest centenarians refer to semi- and supercentenarians.

Table 2. Some immunological characteristics and CMV serological status of the study population, divided into four age groups for comparative analysis (see materials and methods).

	Adult (n=20)	Older (n=15)	LLI (n=11)	Oldest centenarians (n=8)	Age group comparison	p-value
CMV+ n (%)	12 (60)	11 (73)	11 (100)	8 (100)	Adults vs LLI	**0.028**
Anti-CMV IgG titre range (U/ml)	25.6->180	63.6->180	110.2->180	150->180		
IL-6 (pg/mL)	2.1±1.2	3.2±2.4	12.5±7.9	9.8±7.3	Adults vs LLI	**<0.0001**
					Adults vs Oldest cent.	**0.0016**
					Older vs LLI;	**<0.0001**
					Older vs Oldest cent.	**0.0143**
CRP (mg/L)	1.24±1.22	2.8±3.4	6.4±9.7	1.3±1	Adults vs LLI	**0.0268**
IgA (mg/dL)	238.3±84.3	269.8±107.8	280.5±200.2	322.1±121.1	ns	ns
IgG (mg/dL)	1051±208.1	994.1±187.4	1235±363.6	1111±235.8	ns	ns
IgM (mg/dL)	104.1±41.31	92.35±60.80	163±189.3	74.96±40.58	ns	ns

IL-6, PCR and Ig values are shown as mean±standard deviation per group. CRP=C-reactive protein; Ig=immunoglobulin; IL-6=interleukin-6; LLI=long-lived individual; ns=not significant. For CMV serological status, p-values obtained from the χ^2 test, and for inflammatory parameters and Ig titre, p-values obtained from the one-way ANOVA test are reported. p-values >0.05 is not significant.
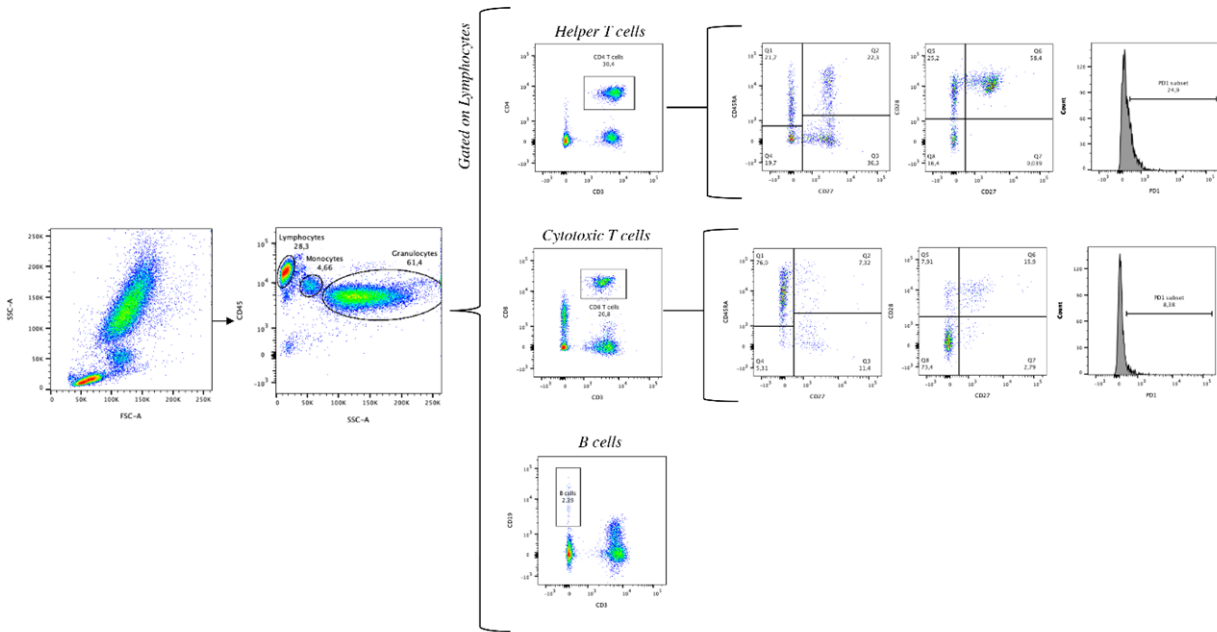
Figure 1. **Gating strategy for identification of CD4+, CD8+ T cells and their relative subsets** (and B cells). The doublet exclusion on forward-scatter height (FCS-H) *versus* forward-scatter area (FCS)-A followed by side-scatter height (SSC-H) versus side-scatter area (SSC-A) is not shown. A representative histogram showing PD-1 staining intensity on T cells is shown on the right. A representative donor is presented.
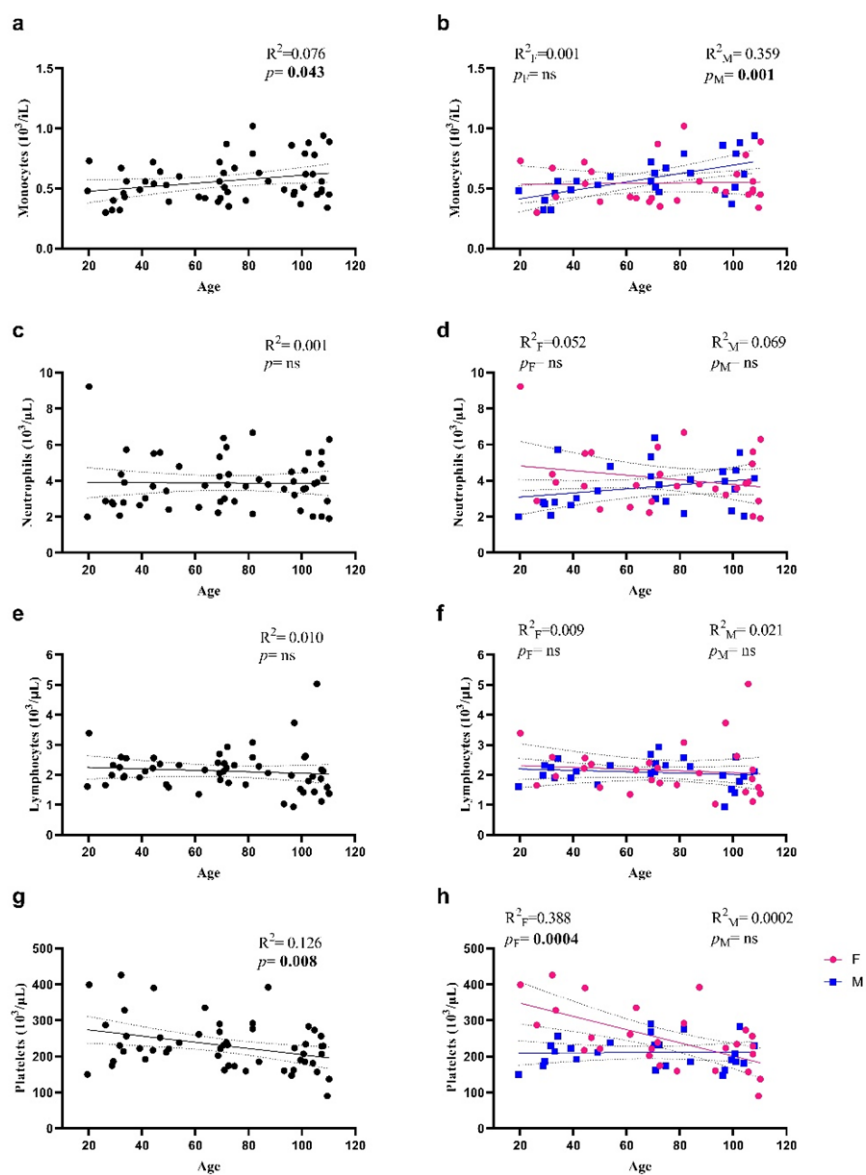
Figure 2. Correlations between blood cells and age. Linear regression analysis shows the relationship between absolute numbers of blood cells and age in all individuals (n = 54) (black line, a,c,e,g), males (n = 26) (blue line) and females (n = 28) (pink line) (b,d,f,h). Each point represents data from an individual healthy donor. The coefficient of determination and *p*-values are shown on the graphs. F = female; M = male; ns = not significant; R^2 = R squared.
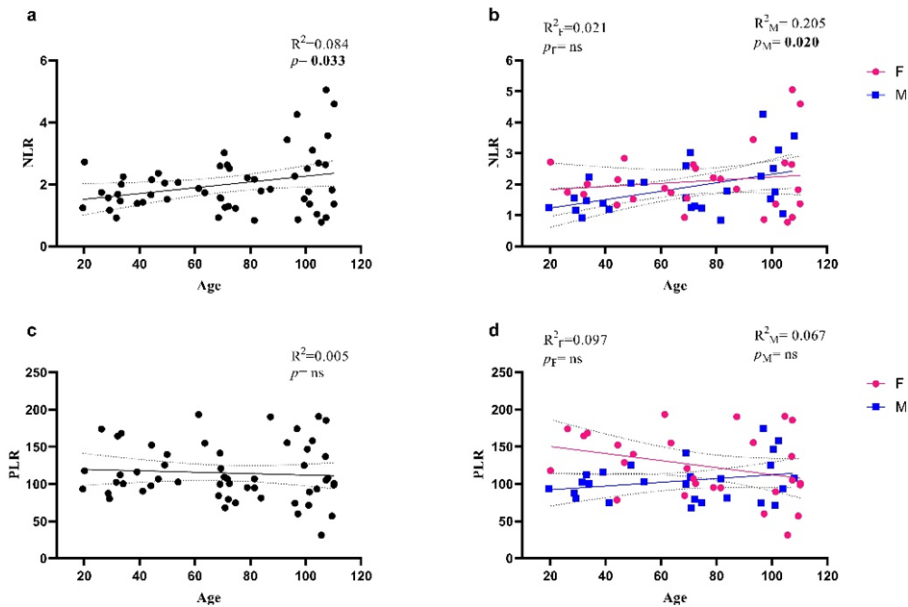
Figure 3. Correlations between NLR and PLR and age. Linear regression analysis showing the relationship between NLR (a,b) and PLR (c,d) and age in all individuals (n = 54) (black line), males (n = 26) (blue line) and females (n = 28) (pink line). Each point represents data from an individual healthy donor. The coefficient of determination and *p*-values are shown on the graphs. F= female; M= male; NLR= neutrophil-to-lymphocyte ratio; ns= not significant; PLR= platelet-lymphocyte ratio; R^2= R squared.
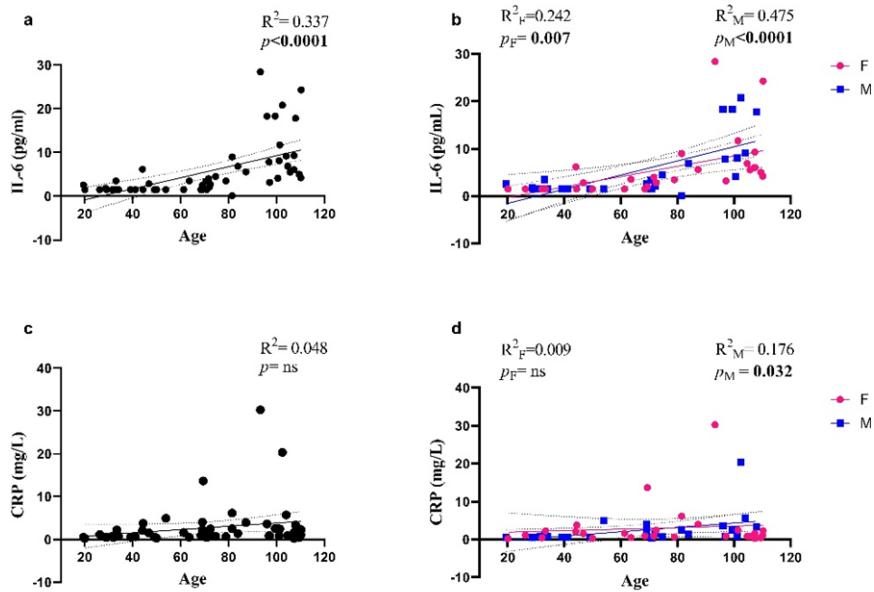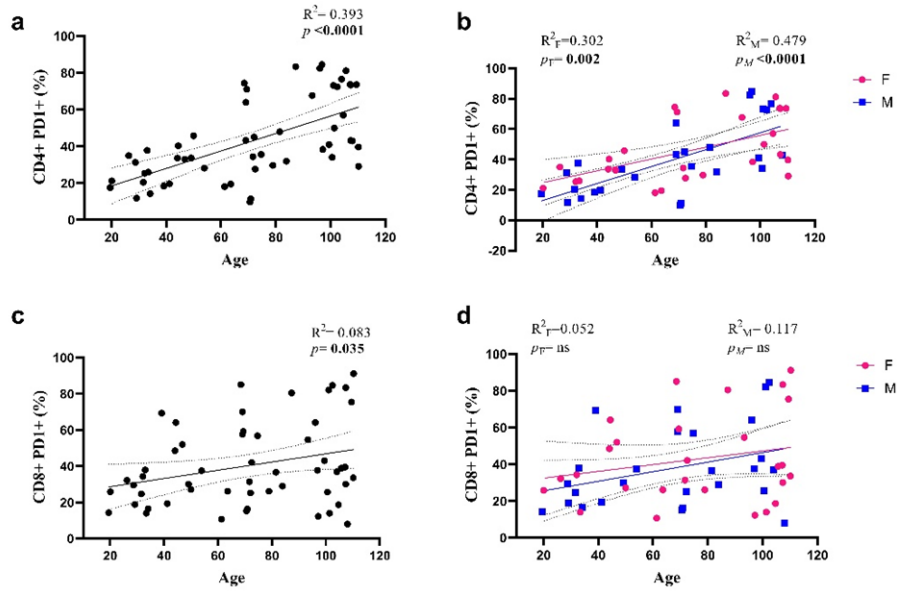
Figure 4. Correlations between inflammatory markers and age. Linear regression analysis showing the relationship between IL-6 (a,b) and CRP (c,d) and age in all individuals (n = 54) (black line), males (n = 26) (blue line) and females (n = 28) (pink line). Each point represents data from an individual healthy donor. The coefficient of determination and *p*-values are shown on the graphs. CRP= C-reactive protein; F= female; IL-6= interleukin-6; M= male; R^2= R squared; ns= not significant.
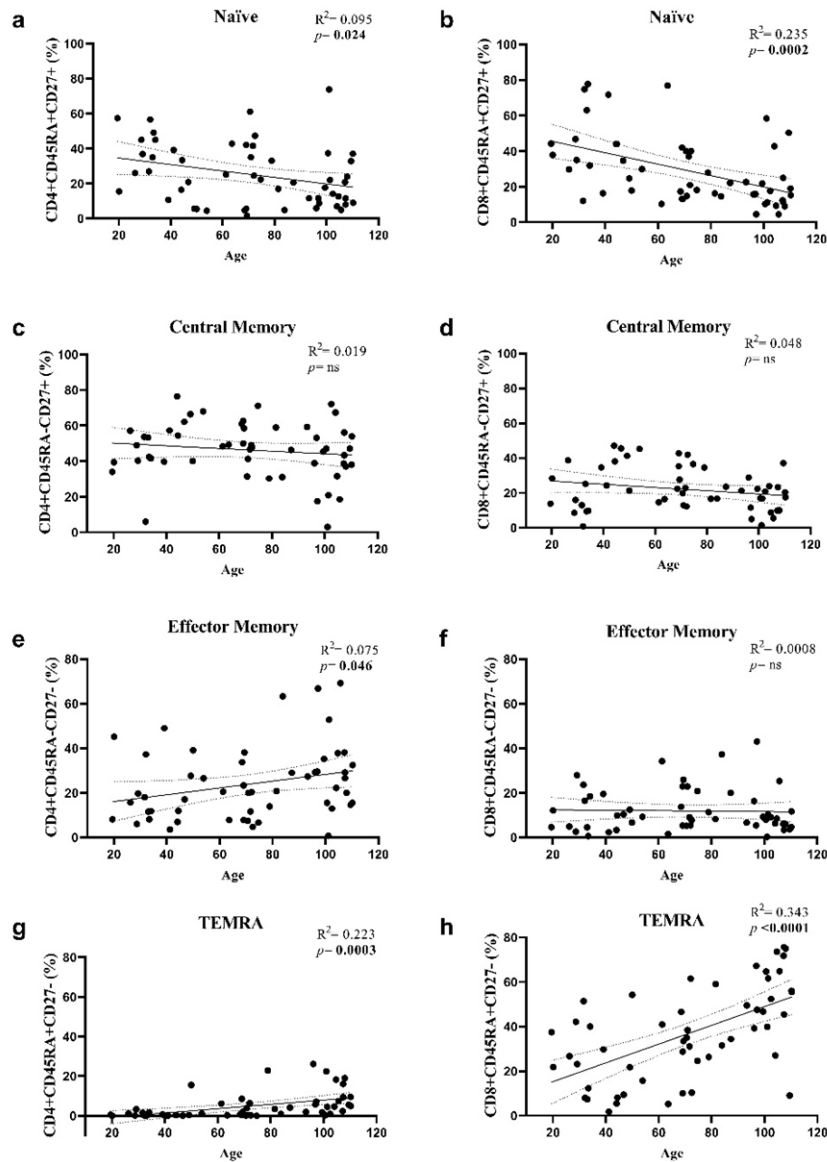
Figure 5. Age-related changes in T cell subsets. Linear regression analysis showing the relationship between CD4 and CD8 T cell subsets and age in all individuals (n = 54). Each point represents data from an individual healthy donor. The coefficient of determination and *p*-values are shown on the graphs. ns= not significant; R^2= R squared; TEMRA, terminally differentiated effector memory.
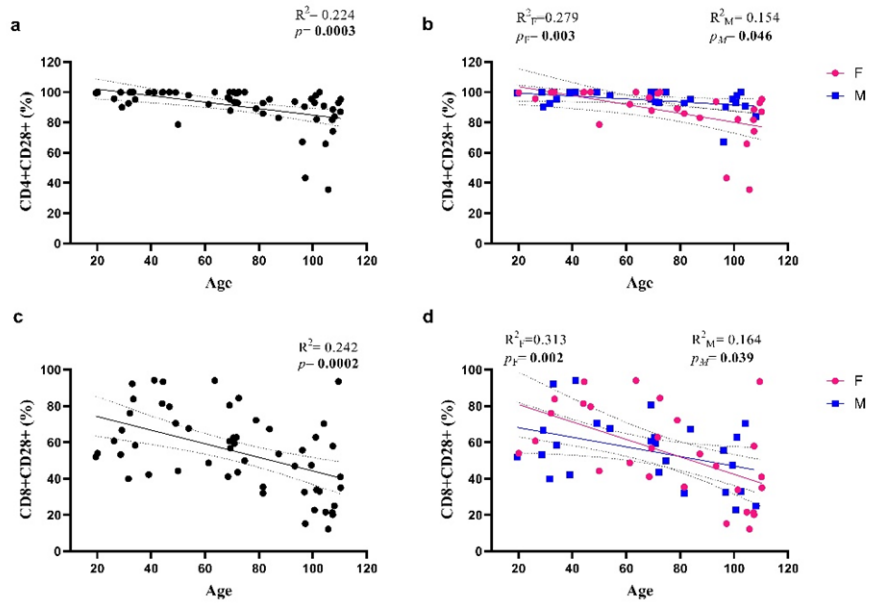
Figure 6. Correlations between CD28 expression on T cells and age. Linear regression analysis showing the relationship between CD4+CD28+ percentages (a,b), CD8+CD28+ percentages (c,d) and age in all individuals (n = 54) (black line), males (n = 26) (blue line) and females (n = 28) (pink line). Each point represents data from an individual healthy donor. The coefficient of determination and p-values are shown on the graphs. F= female; M= male; R^2= R squared.

Figure 7. Correlations between PD1 expression on CD4 and CD8 T cells and age. Linear regression analysis showing the relationship between percentages of CD4+ PD1+ (a,b) and CD8+PD1+ (c,d) and age in all individuals (n = 54) (black line), males (n = 26) (blue line) and females (n = 28) (pink line). Each point represents data from an individual healthy donor. The coefficient of determination and *p*-values are shown on the graphs. F = female; M = male; n.s. = not significant; PD-1= programmed death-1; R^2 = R squared.
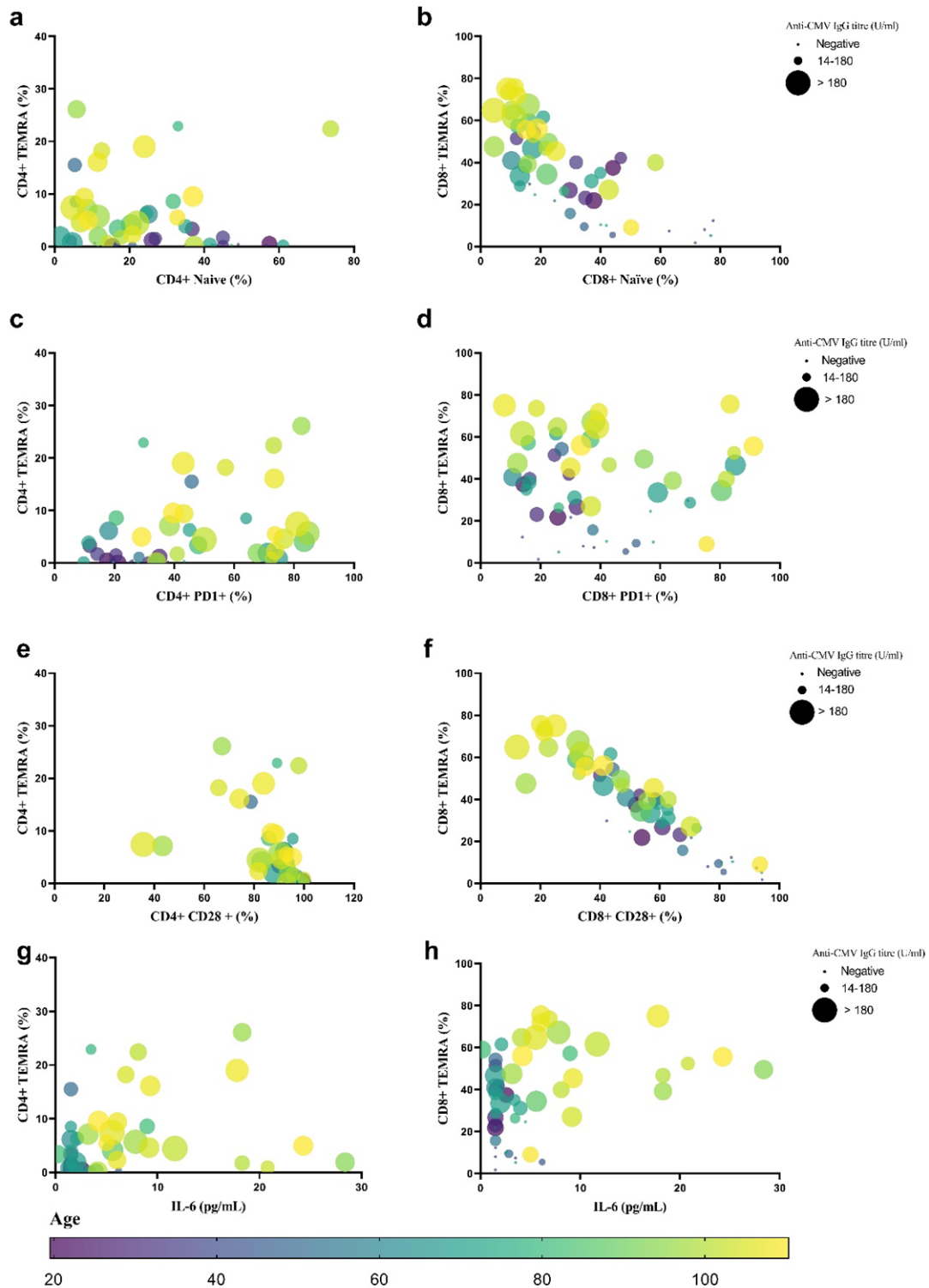
Figure 8. Bubble plot showing the relationship between age, anti-CMV IgG titre and percentages of specific T cell populations. The size of each symbol is proportionate to the IgG titre (U/mL), while the symbol's colour represents the individual's age on a continuous scale. Each point represents data from an individual healthy donor. %, percentages; IL-6=interleukin-6; PD1, programmed cell death protein 1; TEMRA, terminally differentiated effector memory.
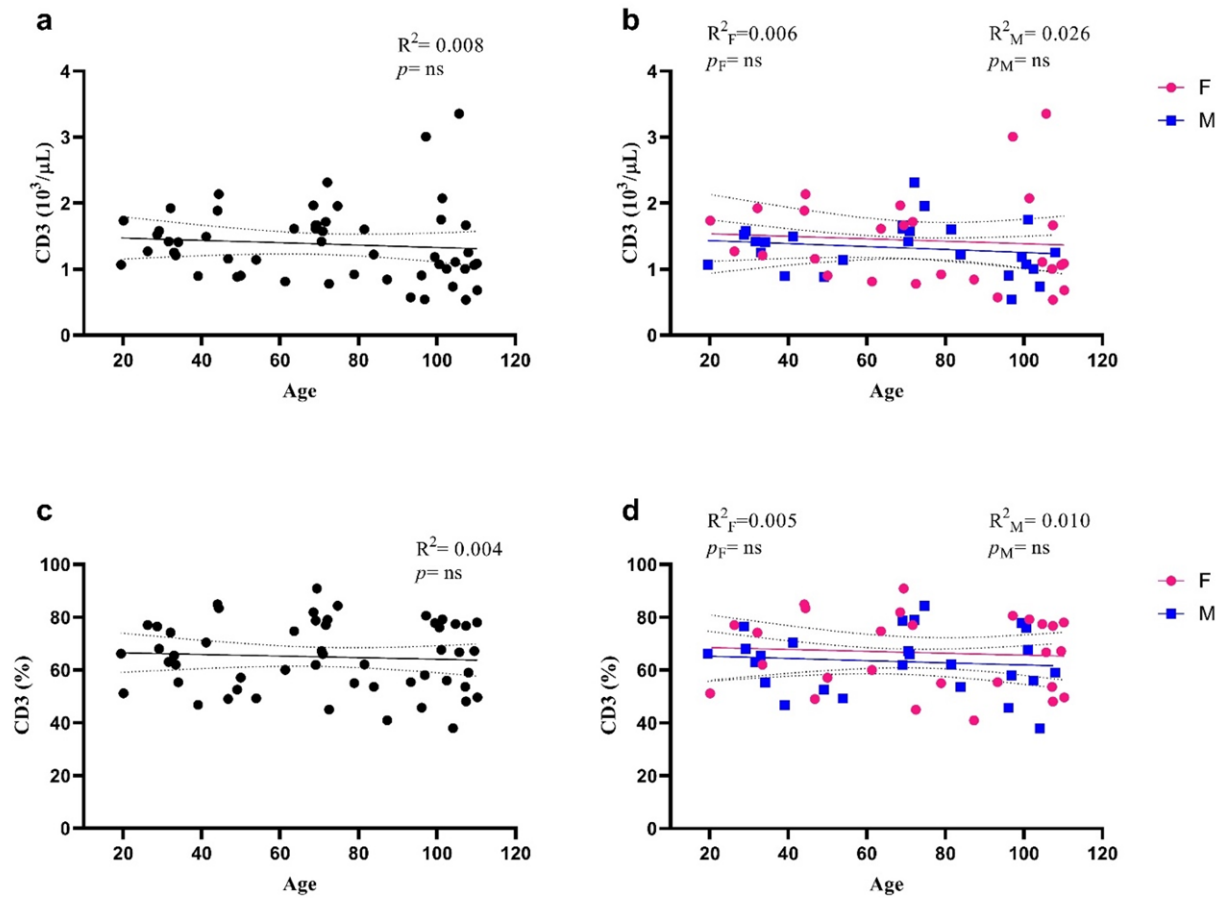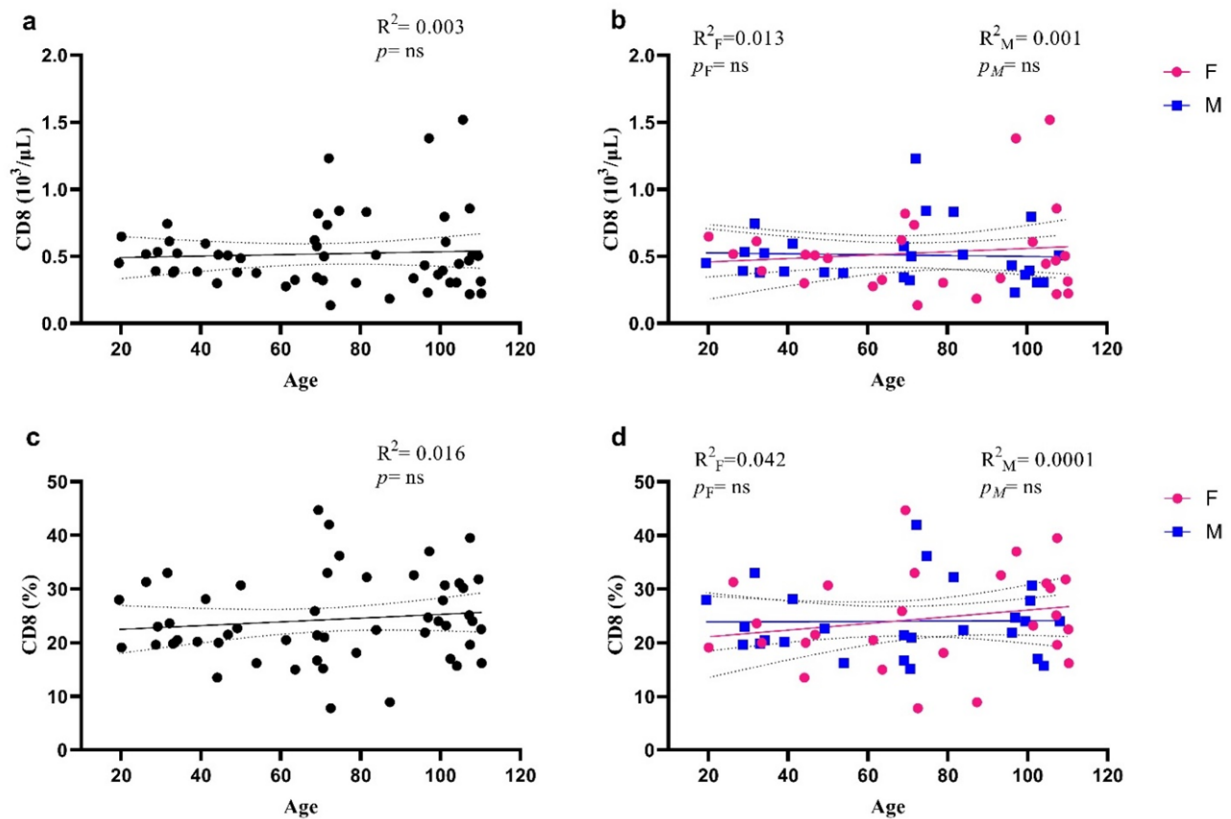
Supplementary Figure 1. Correlations between counts and percentages of CD3 T cells and age.
Linear regression analysis showing the relationship between lymphocyte CD3+ counts (a,b) and percentages (c,d) and age in all individuals (N = 56) (black line), males (N = 26) (blue line) and females (N = 28) (pink line). Each point represents data from an individual healthy donor. The coefficient of determination and p-values are shown on the graphs. F = female; M = male; n. = not significant; R2 = R squared.
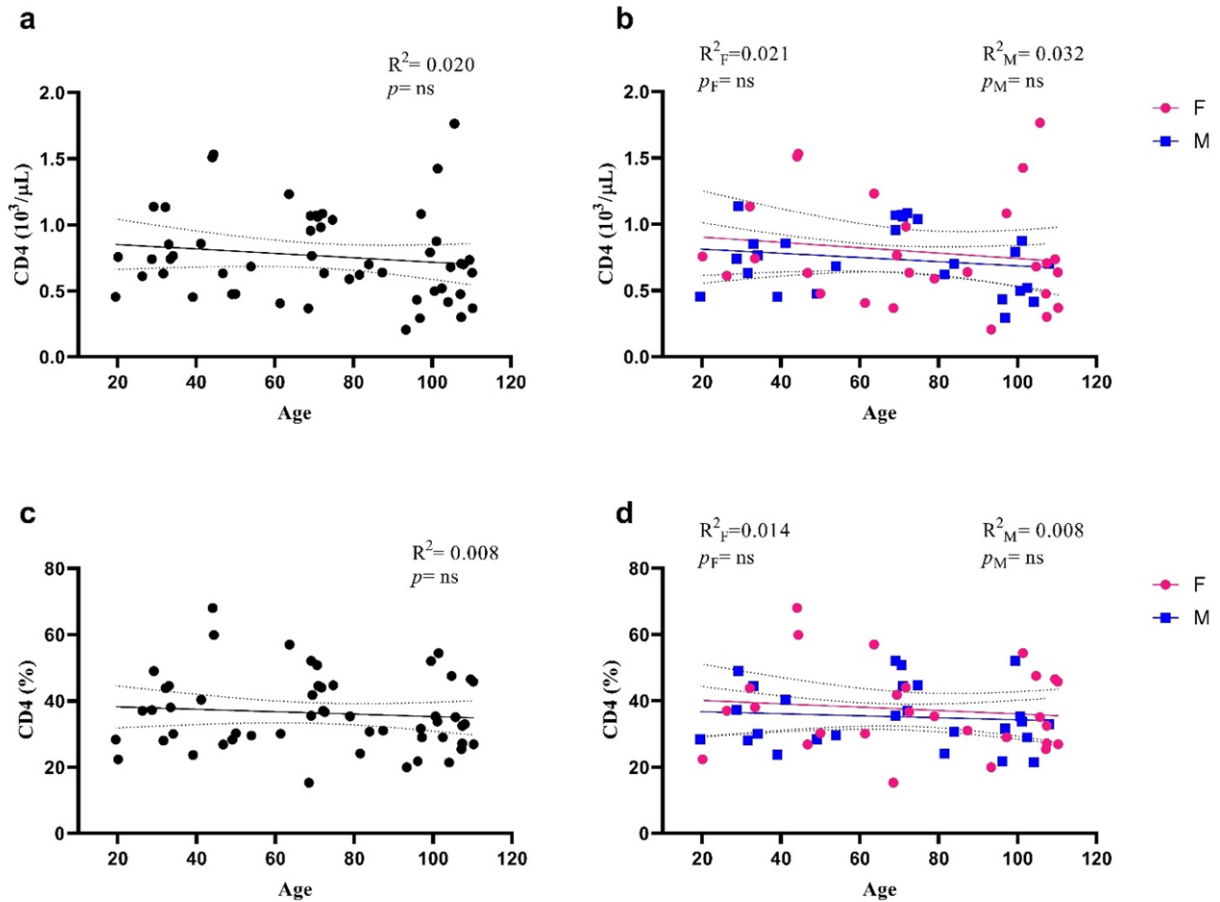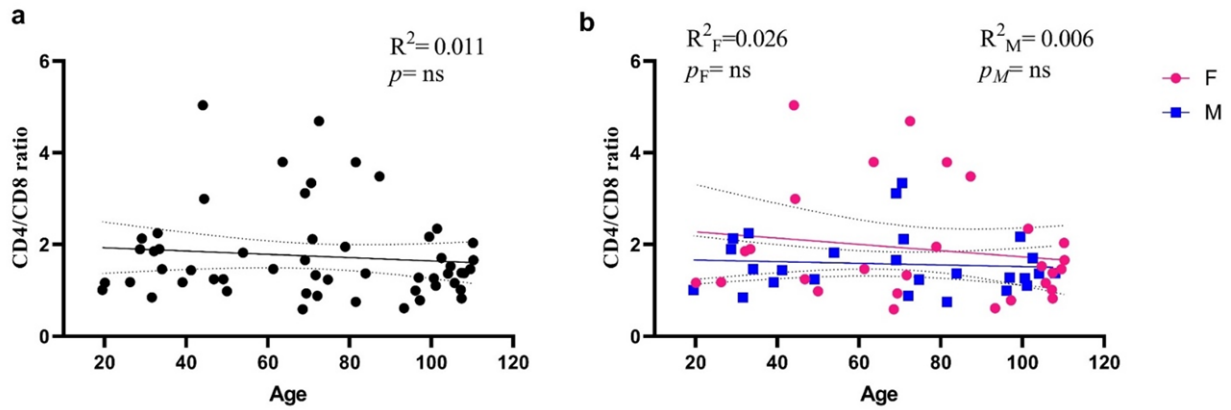
Supplementary Figure 2. Correlations between counts and percentages of CD4 T cells and age. Linear regression analysis showing the relationship between lymphocyte CD4+ counts (a,b) and percentages (c,d) and age in all individuals (N = 56) (black line), males (N = 26) (blue line) and females (N = 28) (pink line). Each point represents data from an individual healthy donor. The coefficient of determination and p-values are shown on the graphs. F = female; M = male; ns = not significant; R^2 = R squared.
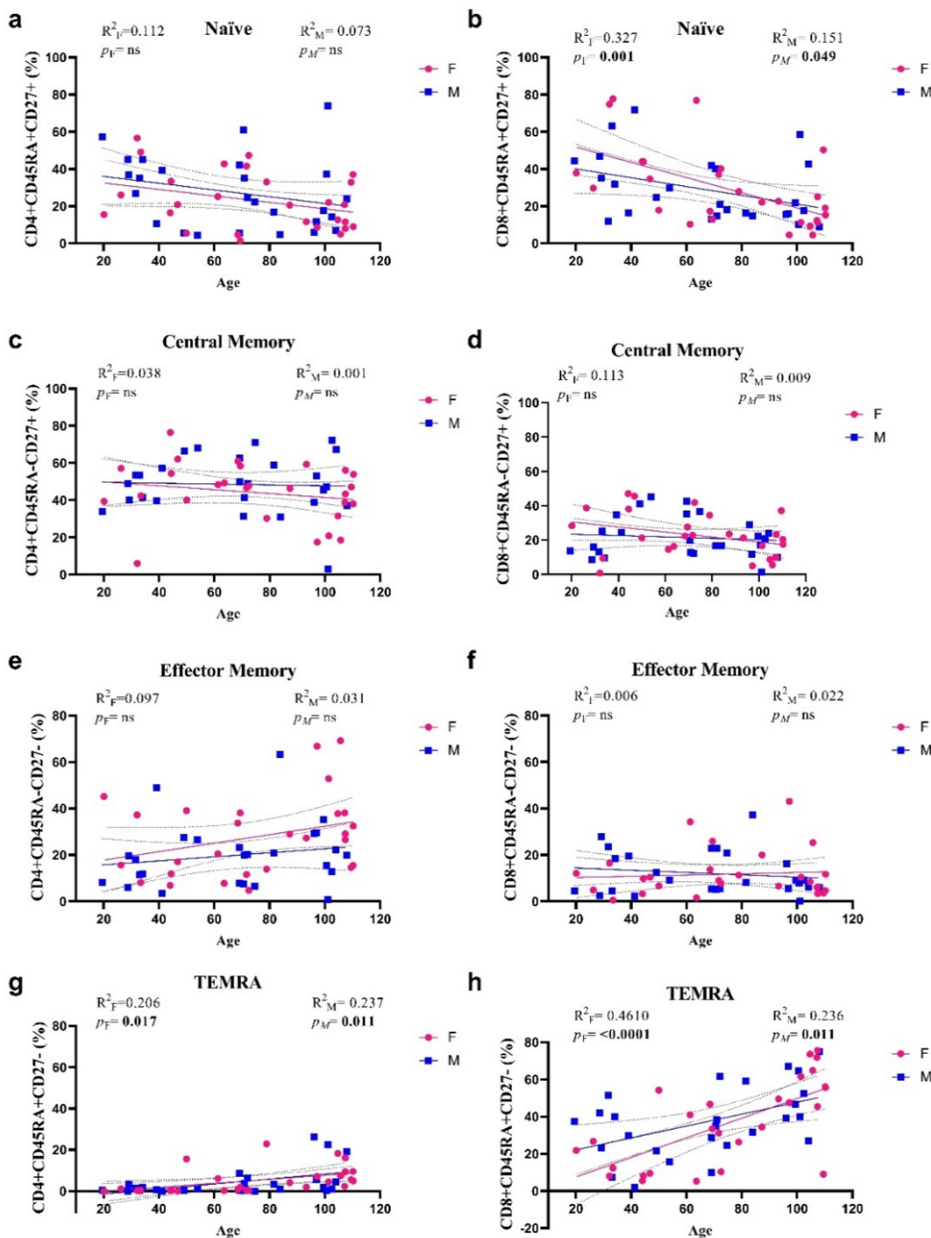
Supplementary Figure 3. Correlations between counts and percentages of CD8 T cells and age. Linear regression analysis showing the relationship between lymphocyte CD8+ counts (a,b) and percentages (c,d) and age in all individuals (N = 56) (black line), males (N = 26) (blue line) and females (N = 28) (pink line). Each point represents data from an individual healthy donor. The coefficient of determination and p-values are shown on the graphs. F = female; M = male; ns = not significant; R^2 = R squared.

Supplementary Figure 4. Correlations between CD4/CD8 T cell *ratio* and age. Linear regression analysis showing the relationship between lymphocyte CD4/CD8 *ratio* and age in (a) all individuals (N = 56) (black line), (b) males (N = 26) (blue line) and females (N = 28) (pink line). Each point represents data from an individual healthy donor. The coefficient of determination and *p*-values are shown on the graphs. F = female; M = male; ns = not significant; R^2 = R squared.

Supplementary Figure 5. Age-related changes in T cell subsets. Linear regression analysis showing the relationship between CD4 and CD8 T cell subsets and age in males (n = 26) (blue line) and females (n = 28) (pink line). Each point represents data from an individual healthy donor. The coefficient of determination and *p*-values are shown on the graphs. ns = not significant; R^2 = R squared; TEMRA, terminally differentiated effector memory.