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Research Article

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

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Abstract

The immunophenotype of oldest centenarians, i.e. 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 infection. We investigated, by flow cytometry, variations in percentages and absolute numbers of immune cell subsets, focusing on T cells, and pro-inflammatory parameters in a cohort of 28 women and 26 men (age range 19–110 years). We observed variability in hallmarks of immunosenescence related to age and Cytomegalovirus serological status. The eight oldest centenarians showed the lowest percentages of naïve T cells, due to their age, and the highest percentages of T-effector memory cells re-expressing CD45RA (TEMRA), according to their cytomegalovirus status, and high levels of serum pro-inflammatory parameters, although their means were lower than that of remaining 90+ donors. Some of them showed CD8 naïve and TEMRA percentages, and exhaustion/pro-inflammatory markers comparable to the younger ones. Our study supports the suggestion that immune ageing, especially of oldest centenarians, exhibits great variability that is not only attributable to a single contributor but should also be the full result of a 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 has had a different immunological history. 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 might be not unfavourable for centenarians, and in particular for the oldest ones.

Keywords: CMV, immune ageing, immunophenotype, longevity, semi-supercentenarians, supercentenarians

Abbreviations: CMV: human cytomegalovirus; COVID-19: coronavirus disease 2019; CRP: C-reactive protein; EBV: Epstein–Barr virus; *F*: females; Fsc: forward-scattered light; Ig: immunoglobulin; Il: interleukin; *M*: males; NLR: neutrophil-to-lymphocyte *ratio*; PD-1: programmed cell death protein 1; PLR: platelet-to-lymphocyte ratio; SSC: side-scattered light; Tcm: T central memory; Tem: T effector memory; Temra: Terminally differentiated T effector memory.

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 [1]), and ethical factors since they are

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particularly fragile subjects given their venerable age. As of 1 January 2021, there were 1111 over 105 years old individuals living in Italy, of these, 17 women reached and exceeded 110 years of age [2]. 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 [2]. 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 [3]. 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.* [4],. They have analysed single-cell resolution blood lymphocytes of Japanese 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. More recently, this datum has been confirmed in seven Caucasoid American centenarians (age range 100–109 years) [5].

Several factors contribute to the complexity of the longevity trait, including genetics, epigenetics, lifestyle, environment, and stochasticity [6–8]. 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 [9, 10]. 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 peripheral blood naïve cells, with a relative increase in the frequency of memory cells [3]. 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 [11]. CMV is a β-herpes virus that infects different types of cells, especially monocytes and dendritic cells, establishing persistent latent infection [12]. Cycles of viral reactivation cause the expansion of CMV-specific CD8 T cells [13], substantially enriched in the late phenotype cells [14]. Indeed, persistent CMV infection results in chronic stimulation of CD8+ T cells, which expand clonally showing a late-stage differentiated effector memory phenotype [15], while the decrease of naïve CD4 and CD8 depends more on thymic involution [10].

Although hallmarks of immunosenescence have been well defined [3], 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 [16]. 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 [17].

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-to-lymphocyte *ratio* (PLR) that recently emerged as informative markers of inflammation [18, 19]. This cohort included 11 long-living individuals (LLIs), i.e. people aged over 90 years old (including six 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 [20]. Moreover, in our previous study [17], 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 [1]. 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 [21]. 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 criterion 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). 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. The Ethics Committee of Palermo University Hospital approved the study (Nutrition and Longevity, No. 032017). For

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)
CMV serological status	(*n* = 19)	(*n* = 14)	(*n* = 11)	(*n* = 8)
CMV+ *n* (%)	12 (*63*)	11 (*78*)	11 (*100*)	8 (*100*)
Anti-CMV IgG titre range (U/ml)	25.6->180	63.6->180	110.2->180	150->180

Age is reported in years and months. The serological status of one adult and one older is missing. LLI = long-lived individuals; *n* = number; SD = standard deviation. Oldest centenarians refer to semi- and supercentenarians.

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 the 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 on an XN-2000 automated haematology (Sysmex) analyser. Serum levels of immunoglobulins A, G, and M (IgA, IgG, and IgM), CRP, and IL-6 were also measured on Roche Diagnostics cobas® 8000 modular analyser: IgG, IgA, IgM, and CRP values by an immunoturbidimetric assay on cobas® c 503 analytical unit, whereas IL-6 values by an immunoassay test using electrochemiluminescence technology on cobas® e 801 analytical unit. Haematological and biochemical parameter analyses were performed at the Department of Laboratory Medicine, "P. Giaccone" University Hospital, Palermo.

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, and 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-scattered light (SSC), and further identified in the SSC/CD45 dot-plot. An exemplificative schematic representation of the applied gating strategy is displayed in Supplementary Fig. S1. 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. Serological status according to age is reported in Table 1. CMV serology analysis was performed at the 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). The correlation between age and the number/percentage of cells, inflammatory markers, as well as immunoglobulins in all individuals and males and females, were 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, immunoglobulins, and T cells between age groups was performed by Tukey's multiple comparison test or one-way ANOVA test. For each statistical analysis, only *P*-values < 0.05 were considered significant.

Results

Analysis of leukocyte parameters, inflammatory markers, and immunoglobulin levels

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 (Fig. 1a and b) and lymphocyte (Fig. 1c and d) 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 (Fig. 1e and f). Fig. 1g and 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 the female group (R^2 = 0.388, P = 0.0004).

We also investigated the relationship between age and some inflammatory markers. Since 63% (n = 12/19) of adults, 78% (n = 11/15) of old and 100% of LLIs and oldest centenarians were found to be CMV seropositive (Table 1), we performed the same analyses including (Table 2) and excluding CMV-negative individuals (Supplementary Table S1).

Concerning the inflammatory markers NLR and PLR, the linear regression analysis performed on our cohort showed a positive association between NLR and age (Fig. 2a, R^2 = 0.084, P = 0.033), but was confirmed only in the male population (Fig. 2b, R^2 = 0.205, P = 0.020) after gender stratification. Although the upward trend is maintained, statistical significance is lost when comparing different age groups (Fig. 2c and Table 2), even when only CMV-positive individuals are included (Supplementary Table S1). No significant differences were found in PLR in all individuals (Fig. 2d–f and Table 2) and in CMV-positive individuals (Supplementary Table S1), even if there is a trend to decrease with age in females (Fig. 2e).

Analysis of serum pro-inflammatory markers showed a strong increase in IL-6 serum levels up to age 105, then decreased slightly in the oldest centenarians (Fig. 2g–i). As shown in Table 2 and Fig. 2i the age-related increment of IL-6 is extremely significant in adults *vs.* LLIs (P < 0.0001) and old *vs.* LLIs (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). These significant differences were confirmed also by comparing only CMV-positive individuals (Supplementary Table S1), except for the old *vs.* oldest centenarians comparison. This finding is confirmed by the linear regression analysis, showing an increase in ageing in IL-6 levels in all individuals (Fig. 2g, R^2 = 0.337, P < 0.0001) and in both genders (Fig. 2h, $R^2_F = 0.242$, 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 (Fig. 2g and h). Similarly, serum concentrations of CRP increase with age, achieving significance when comparing adults and LLIs (Table 2 and Fig. 2l, 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 (Fig. 2j), although we observed a significant age-associated increase in CRP levels in males (R^2 = 0.176, P = 0.032) (Fig. 2k). Nevertheless, comparison analysis among CMV-positive individuals showed no significant differences in CRP levels (Supplementary Table S1).

No age-related changes in Immunoglobulin levels have been observed (Table 2 and Supplementary Fig. S2), also after the exclusion of CMV-negative donors (Supplementary Table S1).

Analysis of T-cell subsets according to age and gender

Total T-cell counts and percentages appeared to be unaffected by age and gender (Fig. 3), as well as the percentages and counts of CD4 and CD8 T cells (Fig. 4). Consequently, the reversal of the CD4/CD8 *ratio* was not observed (Supplementary Fig. 3). No significant difference was also found when the different age groups were compared excluding CMV-negative donors (Supplementary Table 2).

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 CD4 (Fig. 5a, R^2 = 0.095, P = 0.024) and CD8 T cells (Fig. 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 was found 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. Comparison of means in the percentages of naïve T cells among different age groups in CMV-positive donors has shown a decreasing trend that was not statistically significant, likely due to the fact that so we eliminated most adults who had high values of naïve cells (Supplementary Table S2). We observed no differences in CD4 CM (Fig. 5c) and CD8 CM (Fig. 5d) with age. CD4 TEM (Fig. 5e, R^2 = 0.075, P = 0.046) and TEMRA cells (Fig. 5g, R^2 = 0.223, P = 0.0003) increased linearly with age, as well as CD8 TEMRA (Fig. 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 (Fig. 5f). Within the CD8 T cell subsets, we observed a predominance of TEMRA over effector T cells with age, confirmed in CMV-positive donors (Supplementary Table S2), 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 (Fig. 6a 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

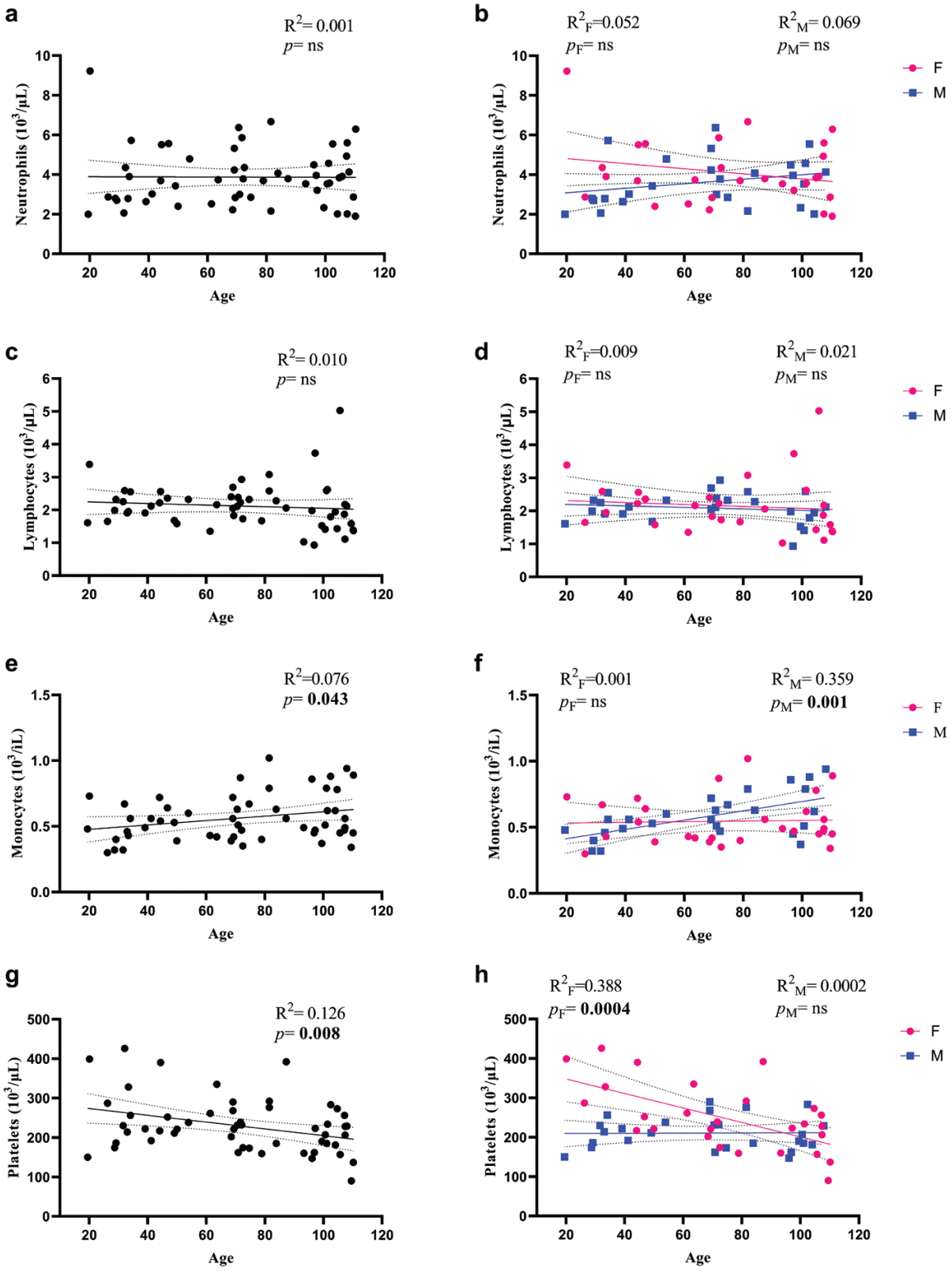


Figure 1. Correlations between blood cells and age. Linear regression analysis shows the relationship between absolute numbers of blood cells and age in all individuals (n = 54) (black line, a, c, e, and g), males (n = 26) (blue line) and females (n = 28) (pink line) (b, d, f, and h). Each point represents 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

Table 2. Some immunological characteristics 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
PLR	124 ± 33.2	103.6 ± 30.5	121.7 ± 45.9	102.9 ± 46.7	ns	ns	
NLR	1.7 ± 0.5	1.8 ± 0.7	2.2 ± 1.1	2.6 ± 1.7	ns	ns	
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; Oldest cent. = oldest centenarian. P-values obtained from the one-way ANOVA test are reported. P-values > 0.05 is not significant.

in TEMRA CD8 in the female population than in the male population (Fig. 6b 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 (Fig. 7a, R^2 = 0.224, P = 0.0003) and, mostly, CD8 T cells (Fig. 7c, R^2 = 0.242, P = 0.0002), confirmed in both sexes, with a greater tendency to decrease in the female population. 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. The decrease can also be observed by looking, in CMV-positive subjects, at the differences between the means of each age group (Fig. 7e) although not statistically significant. Interestingly, both the adult and older CMV-negative groups show higher percentages of CD28 expression than their CMV-positive counterparts. The oldest centenarians, whose CMV-negative counterpart is missing, show the highest standard deviation values within the cohort, confirming that although the mean value is the lowest observed, there is no homogenous trend within this age group.

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 (Fig. 8a, R^2 = 0.393, P < 0.0001) and CD8 (Fig. 8c, R^2 = 0.083, P = 0.035) T cells and age, confirmed in both genders only for CD4+ (Fig. 8b, $R^2_F = 0.302$, P_F = 0.002; $R^2_M = 0.479$, P_M < 0.0001). The differences between the younger group (adults) and the older ones (LLIs and Oldest centenarian) are also confirmed when comparing the averages of CD4+PD1+ percentages in CMV-positive individuals (Fig. 8e). The increase in PD1 expression with age is also observed from adults to older CMV-negative, more pronounced in CD8 T cells. However, the means of PD1 expression on CD4 appears to be slightly reduced compared to the adult and older CMV-positive counterparts (Fig. 8e). The high standard deviation values observed in the expression of both CD28 and PD-1, especially in the CMV-positive as they are numerically greater, are further evidence of the wide heterogeneity of these values within our cohort.

Analysis of T-cell subsets according to age and CMV serological 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 Fig. 9. The relationship between the decrease in CD4 naïve and the increase in CD4 effector memory (the CD4 subtype predominating with age) and age was previously detected using a classic scatterplot. In Fig. 9a, we aimed to observe whether these trends also correlated to CMV status. Most but not all CMV-negative donors (smallest bubbles) showed higher percentages of naïve and lower percentages of effector memory CD4 T cells. Most adults CMV-positive seem to have the same trend as their CMV-negative counterparts, but older CMV-positive appear overall to reduce the percentages of naïve and increase those of effector memory as anti-CMV titre (bubble size) increases. Although the simultaneous presence of the highest effector and lowest naïve values occur in older individuals, some oldest centenarians (yellow bubbles) show discrete CD4 naïve values with low effector memory values despite their high anti-CMV titre. In the CD8 compartment (Fig. 9b), 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. The 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

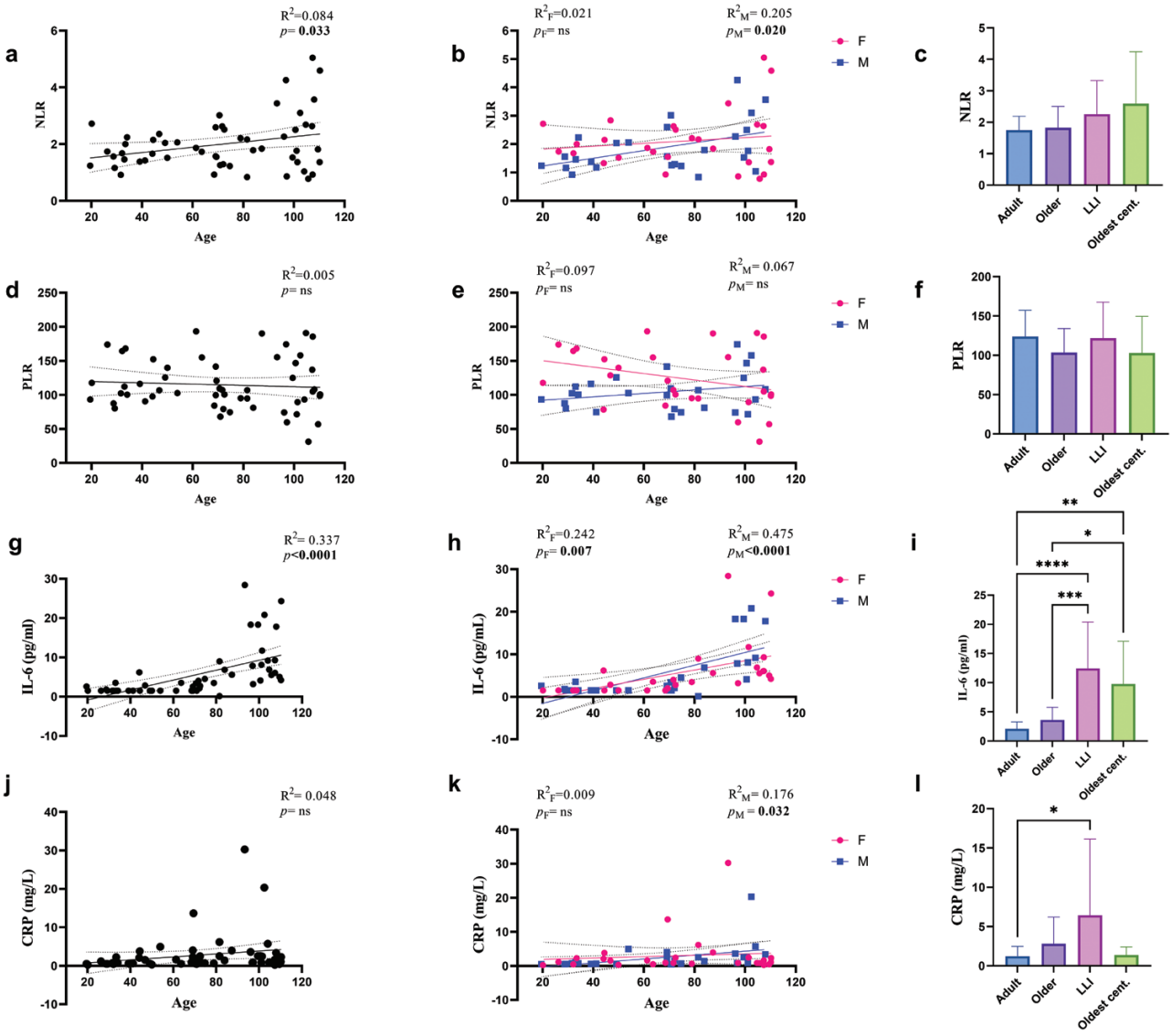


Figure 2. NLR and PLR, inflammatory markers and age. Linear regression analysis shows the relationship between NLR (a and b) and PLR (c and d), IL-6 (e and f), and CRP (g and h) and age in all individuals (n = 54) (black line), males (n = 26) (blue line) and females (n = 28) (pink line). Each point represents data from an individual healthy donor. Column bar graphs showing differences between the mean of the values of NLR (c), PLR (f), IL-6 (i), and CRP (l) from each aged group obtained by one-way ANOVA test. The coefficient of determination and P-values are shown on the graphs. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$; CRP = C-reactive protein; F = female; IL-6 = interleukin-6; M = male; NLR = neutrophils-to-lymphocytes ratio; PLR = platelets-to-lymphocytes ratio; R^2 = R squared; ns = not significant

check the relationship between effector memory/TEMRA, exhaustion/activation markers, and CMV seropositivity within our aged cohort.

The highest CD4 (Fig. 9c, from 45% upwards) and CD8 PD1+ (Fig. 8d, from 70% upwards) percentages were found in CMV-positive older, LLIs, and oldest centenarians. 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 was not always true, as some CMV-positive individuals showed similar low percentages of terminally differentiated cells.

In Fig. 9e, it can be observed that CMV-negative individuals had higher percentages of CD4 CD28+, as shown previously (Fig. 7a and e) but a wider distribution of CD4 effector memory percentages. In CMV-positive individuals, as age and antibody titre increased, a decrease in CD28 expression and an increase in CD4 effector memory were observed. However, there seems to be an overlap between the values of the LLIs with those of the oldest centenarians (yellow bubbles) (Fig. 9e). 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 (Fig. 9f). 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 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 Fig. 9g and h, no relation seems to exist between increased IL-6 level and CMV infection, as the lowest cut-off (1.5 pg/

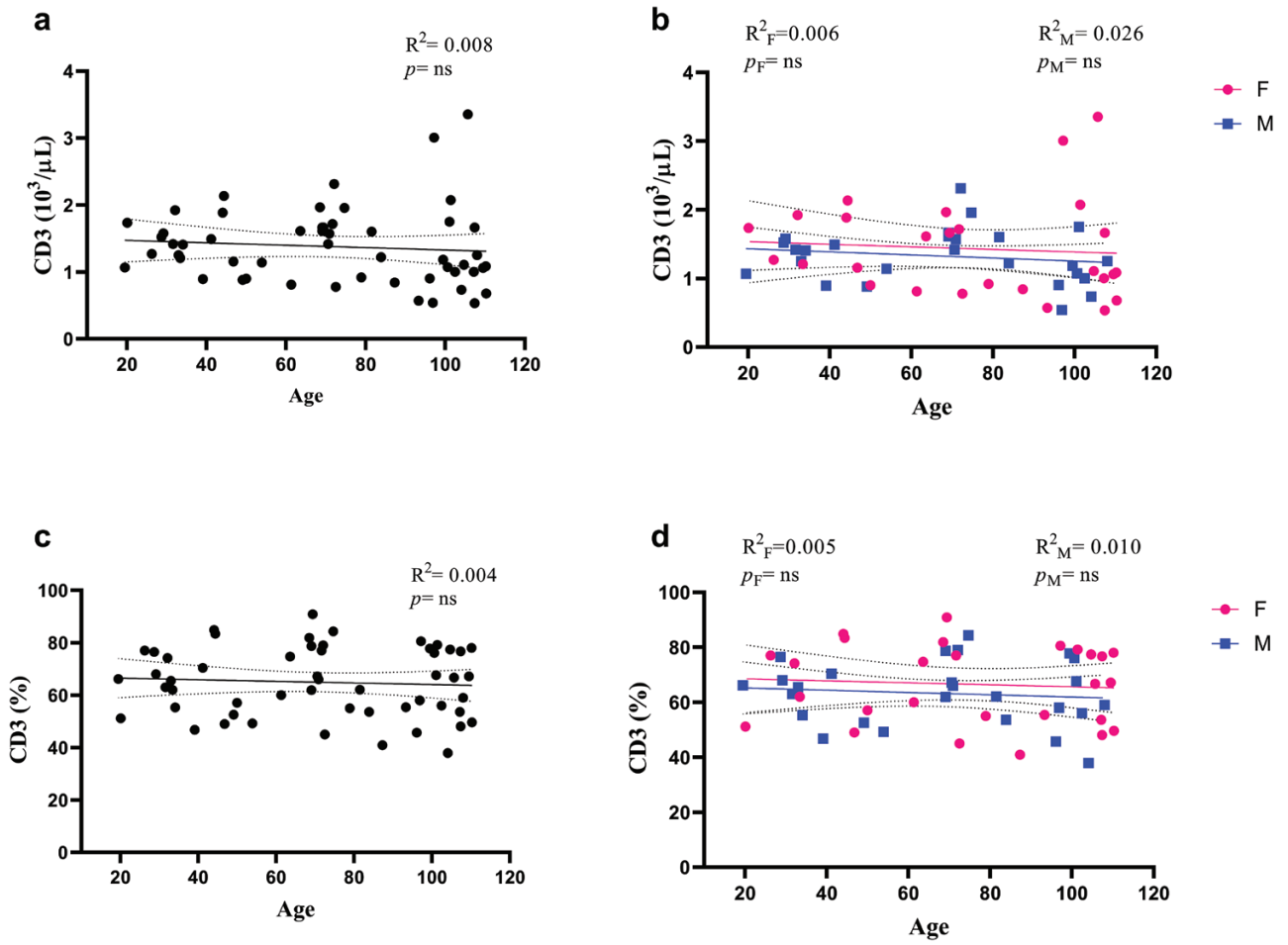


Figure 3. Correlations between counts and percentages of CD3 T cells and age. Linear regression analysis shows the relationship between lymphocyte CD3+ counts (a and b) and percentages (c and d) and age in all individuals (*n* = 56) (black line), males (*n* = 26) (blue line) and females (*n* = 28) (pink 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

ml) was recorded in different individuals regardless of bubble size (IgG titre) and effector memory/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 cutoff (7 pg/ml), again indicating a lack of absolute homogeneity in the oldest centenarian population.

Discussion

We analysed different haematological, immunological, and inflammatory markers in a cohort of 54 Sicilians, including eight semi and super-centenarians (mean age 108.2 years), focusing on T-cell immunophenotypes. Oldest centenarians showed the lowest percentages of naïve T cells, due to their age, and higher percentages of TEMRA cells, based on their CMV status, as well as high levels of serum inflammatory parameters, although the means were lower than that of LLIs. Some of them showed percentages of CD8 naïve and TEMRA, CD28– and PD1+ T cells as well as IL-6 and CRP values comparable to those of the younger subjects, hence exhibiting a large variability. 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 [1, 22], therefore some differences may have been affected by gender disproportionality in favour of oldest centenarian women.

Concerning blood cell analysis, no age-related changes in counts of neutrophils, a critical component of innate immunity [23, 24], were observed. Likewise, despite the reported decline in absolute lymphocyte count and percentage during ageing [25], there was no age-related change in their absolute counts. We observed a marked increase of monocytes, key components of the innate immune system [24, 26], in males but not in females, confirming a previous report [27]. Monocyte-specific inflammatory *loci* appear to be more activated in males than in females [28]. This should be linked to the increased pro-inflammatory status in men compared to women [10].

Finally, no age-related changes in Ig levels were observed, although a previous study conducted on 166 subjects (20–106 years old) showed an age-related increase in IgG, significant only in men, and IgA levels [29].

In accordance with previous studies [30–32], we observed an increase in inflammatory markers with advancing age. Specifically, we analysed NLR and PLR, which have

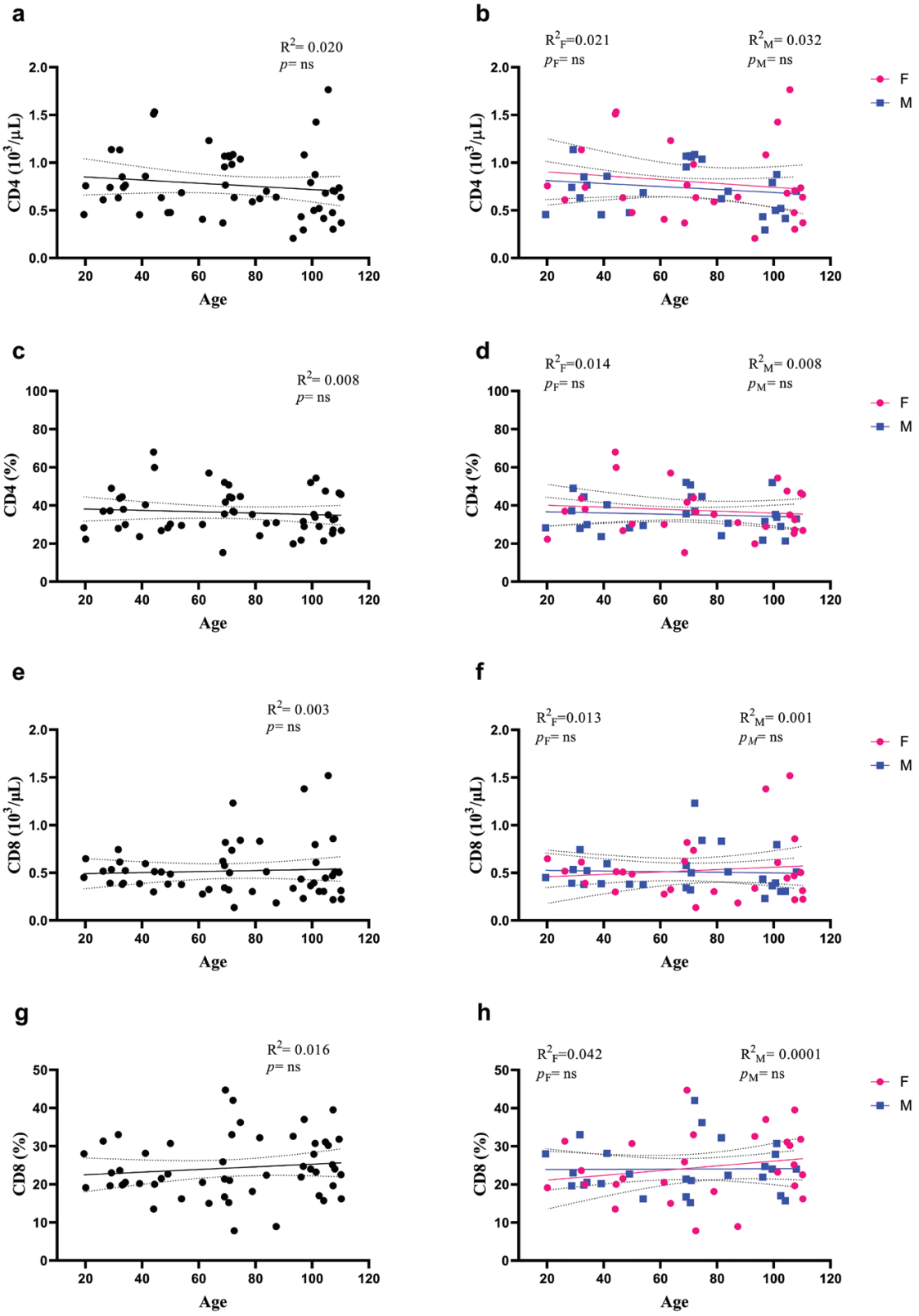


Figure 4. Correlations between counts and percentages of CD4 and CD8 T cells and age. Linear regression analysis shows the relationship between lymphocyte CD4+ and CD8+ counts and percentages and age in all individuals (n = 56) (black line), males (n = 26) (blue line) and females (n = 28) (pink line). 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

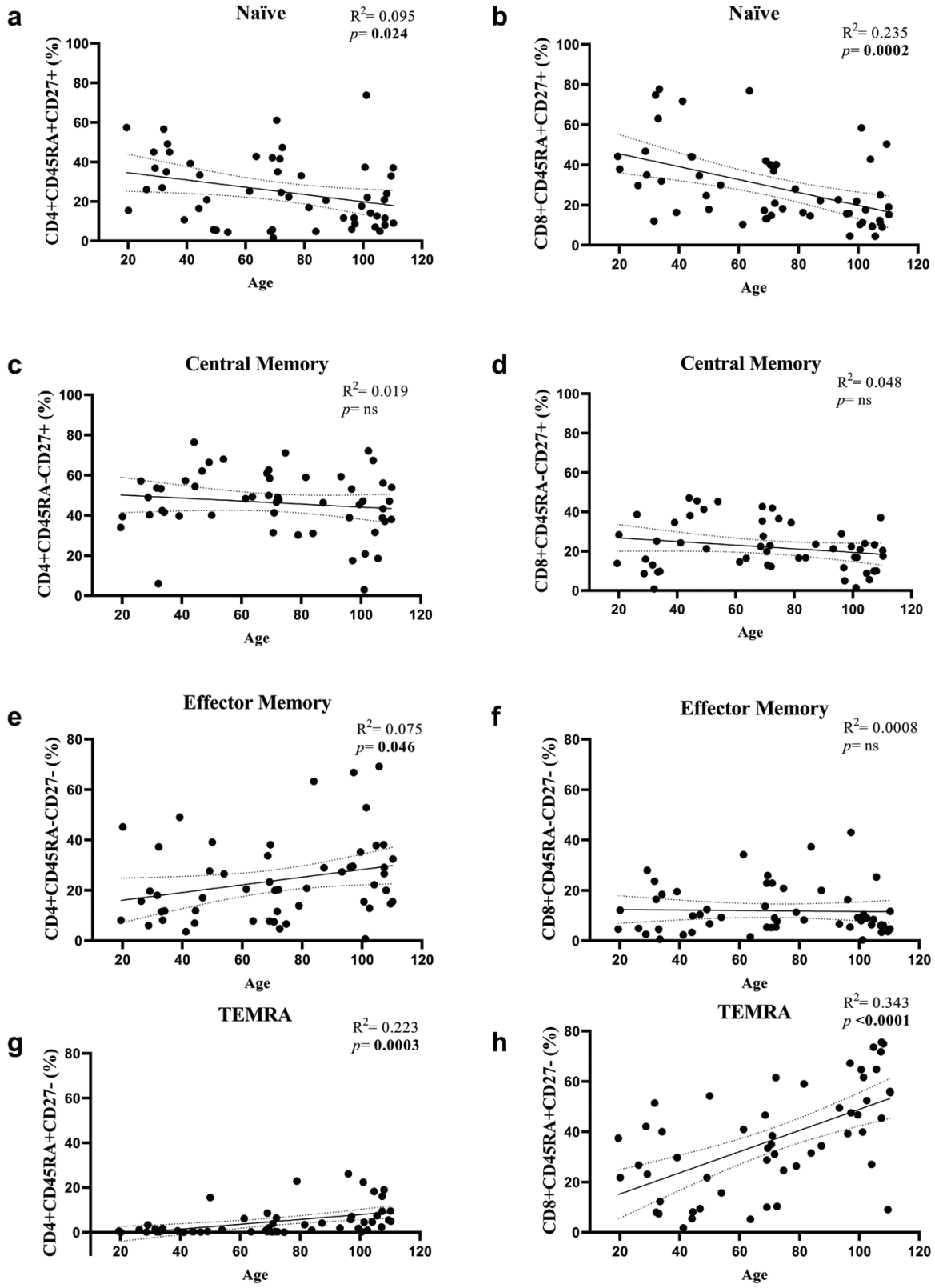


Figure 5. Age-related changes in T-cell subsets. Linear regression analysis shows the relationship between CD4 and CD8 T cell subsets and age in all individuals (n = 54). Each point represents data from an individual healthy donor. The coefficient of determination and P-values are shown on the graphs. ns = not significant; R^2 = R squared; TEMRA, terminally differentiated effector memory

been shown to be significantly associated with the presence and progression of several inflammatory diseases [33], and serum levels of CRP and IL-6, that increase in response to inflammatory *stimuli* [34, 35]. Although we observed a highly significant age-related increase in IL-6, CRP and NLR in all individuals or only in the male population, some oldest

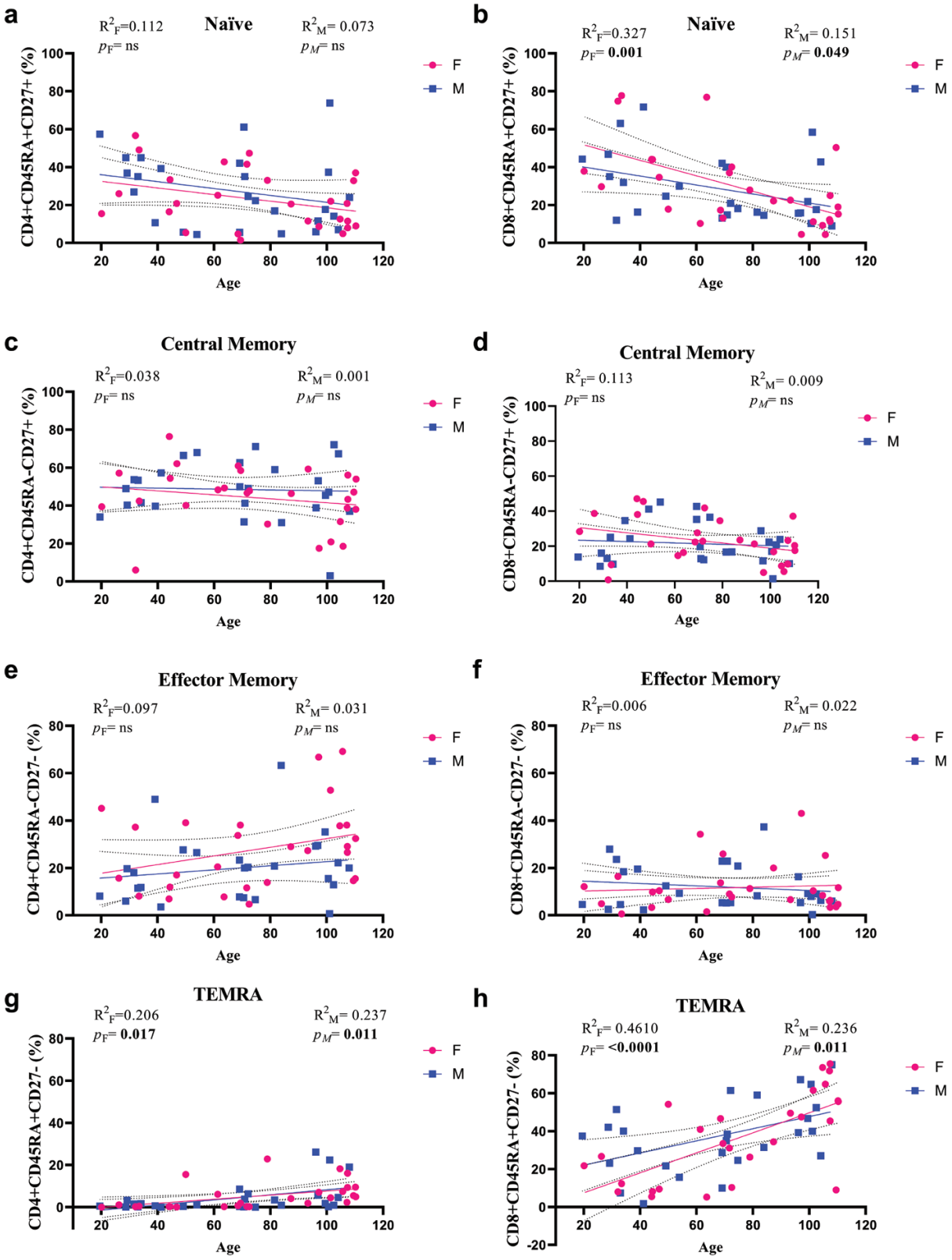
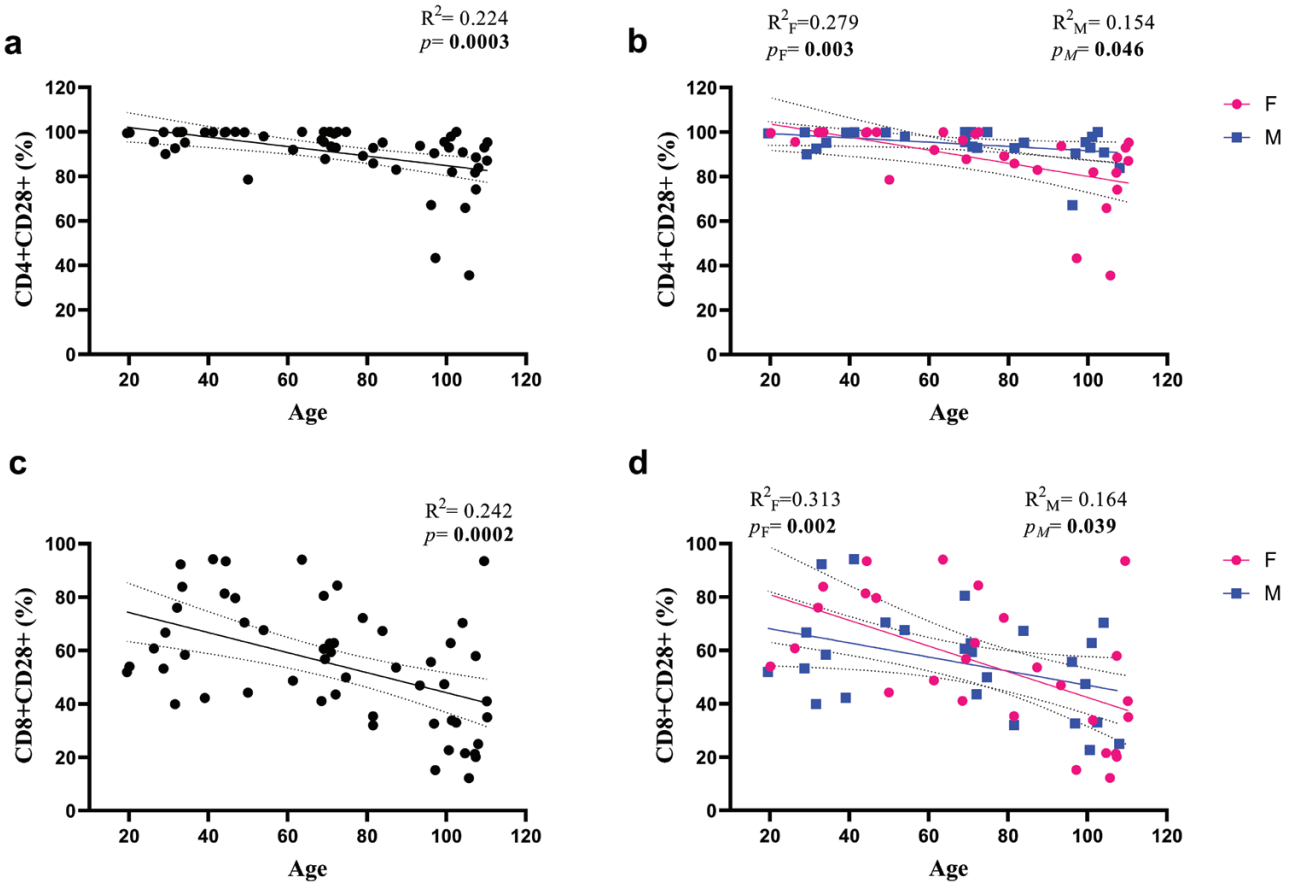


Figure 6. Age-related changes in T-cell subsets according to gender. Linear regression analysis shows the relationship between CD4 and CD8 T cell subsets and age in males (n = 26) (blue line) and females (n = 28) (pink line). Each point represents data from an individual healthy donor. The coefficient of determination and P-values are shown on the graphs. ns = not significant; R^2 = R squared; TEMRA, terminally differentiated effector memory

centenarians showed monocyte count, NLR, IL-6, and CRP levels comparable to younger subjects, while others showed the highest values.

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



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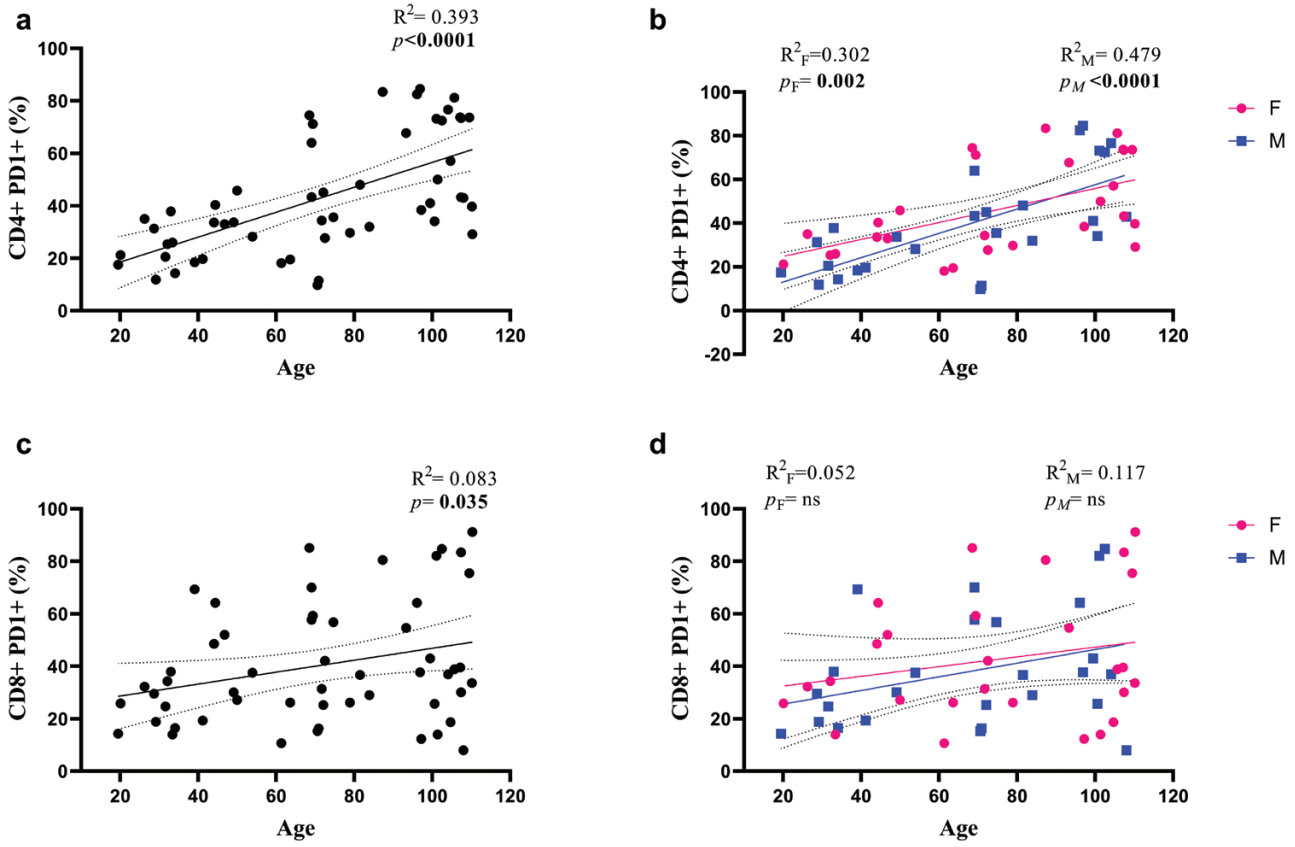
Percentages	CMV positive individuals				
	Adult (n=12)	Older (n=11)	LLI (n=11)	Oldest cent. (n=8)	*p*-value
CD4+CD28+	95.25±5.914	91.97±5.468	83.62±17.67	80±18.96	ns
CD8+CD28+	57.52±13.37	53.94±12.95	40.31±17.75	38.25±26.76	ns

Percentages	CMV negative individuals		
	Adult (n=7)	Older (n=3)	*p*-value
CD4+CD28+	99.90±0.08	100±0.00	ns
CD8+CD28+	79.01±18.67	71.57±18.86	ns

Figure 7. CD28 expression on T cells and age. Linear regression analysis shows the relationship between CD4 + CD28 + percentages (a and b), CD8 + CD28 + percentages (c and 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. (e) Age group comparison for CD4+/CD8+ CD28+ percentages in CMV positive individuals obtained by one-way ANOVA test and in CMV negative individuals obtained by *t*-test. CD4+CD28+ and CD8+CD28+ percentages are shown as mean±standard deviation per group. *P*-values >0.05 is not significant. *F* = female; LLI = long-lived individuals; *M* = male; Oldest cent. = oldest centenarian; R^2 = *R* squared

individuals since inflammation is an important malleable driver of ageing up to extreme old age [36]. However, deleterious effects of inflamm-ageing in centenarians can be counteracted by several mechanisms [37–39]

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 [3, 10, 17, 40]. In our study, 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. Linear regression analysis revealed a significant increase in proportions of TEM CD4 T cells and 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 [41, 42]. However, again we observed large inter-individual variability among the over 100.



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	CMV positive individuals				
Percentages	**Adult (n=12)**	**Older (n=11)**	**LLI (n=11)**	**Oldest cent. (n=8)**	***p*-value**
CD4+PD1+	29.65±16.66	41.65±25.18	61.73±18.55	57.13±20.27	**0.002** (Adults *vs* LLI); **0.021** (Adults *vs* Older cent.)
CD8+PD1+	32.59±20.08	37.64±23.94	43.18±25.69	50.13±29.49	ns
	CMV negative individuals				
Percentages	**Adult (n=7)**	**Older (n=3)**			***p*-value**
CD4+PD1+	25.79±7.50	35.53±7.8			ns
CD8+PD1+	33±18.02	52.20±8.75			ns

Figure 8. PD1 expression on CD4 and CD8 T cells and age. Linear regression analysis shows the relationship between percentages of CD4+ PD1+ (a and b) and CD8+PD1+ (c and 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. (e) Age group comparison for CD4+/CD8+ PD1+ percentages in CMV positive individuals obtained by one-way ANOVA test and in CMV negative individuals obtained by t-test. CD4+PD1+ and CD8+PD1+ percentages are shown as mean±standard deviation per group. P-values > 0.05 is not significant. F = female; LLI= long-lived individuals; M = male; ns = not significant; Oldest cent.= oldest centenarian; PD-1= programmed death-1; R^2 = R squared

Highly differentiated T cells typically lose expression of the costimulatory receptor CD28 molecule, needed for T cell activation and expressed in all CD4 and CD8 naïve T cells. 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 [43–45]. If CD8+CD28– can expand in the absence of CMV, the accumulation of CD4+CD28– T cells with ageing is mainly caused by chronic antigenic stimulation by this persistent infection, although they are uncommon compared to CD8+CD28– T cells [46]. CMV infection is, indeed, a major 'trigger' for the expansion of CD4+CD28– cells, increasing frequencies more than 10-fold on average (in CD8 T cells, the effect was a 2-fold increase) [47]. 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+ cells than individuals not only from the same group cohort but also from younger ones. In CMV-negative subjects, CD28 expression in CD4 T cells appears not to change with ageing while it would seem to decrease in CD8 T cells but the lack of a group of CMV-negative LLIs and oldest centenarians do not allow us to analyse the exclusive effect of age in these subjects.

We also investigated the exhausted status of T cells, using the PD-1 marker. PD-1 is involved in the regulation of CD8

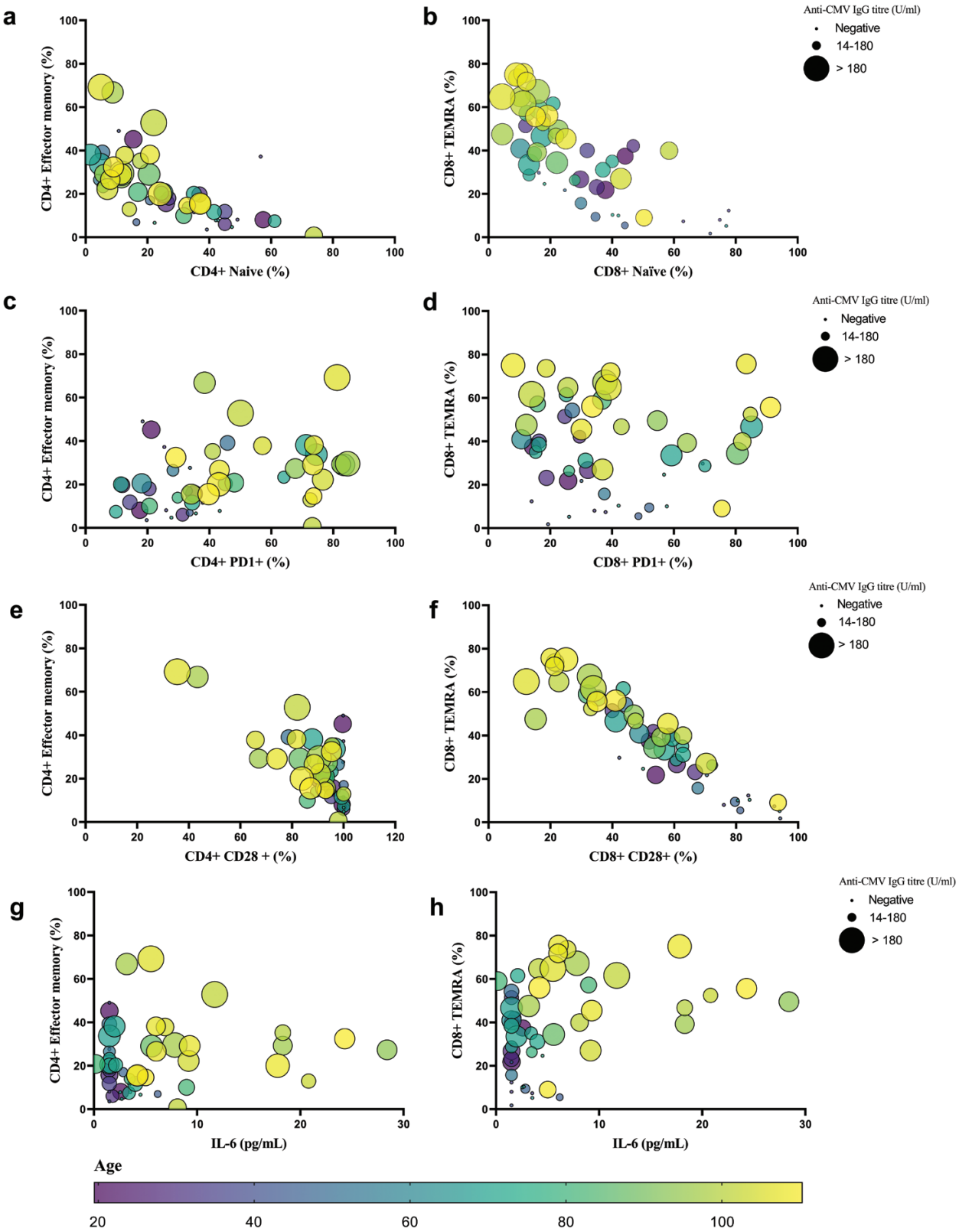

Figure 9. Bubble plot showing the relationship between age, anti-CMV IgG titre, and percentages of specific T-cell populations. The size of each symbol is proportionate to the IgG titre (U/ml), while the symbol's colour represents the individual's age on a continuous scale. Each point represents data from an individual healthy donor. %, percentages; IL-6 = interleukin-6; PD1, programmed cell death protein 1; TEMRA, terminally differentiated effector memory

T-cell exhaustion during chronic viral infection and it is also transiently expressed by activated CD8 T cells during the acute phase of viral infection [48–50]. 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.

Single-investigative descriptive analysis of the parameters under study appears to show a combined effect of CMV infection and age on decreasing percentages of CD8 naive and concomitant increase in TEMRA, in increasing PD1 expression in CD4 T cells, and in decreasing CD28 expression in CD8 combined with increasing TEMRA. Despite its age and anti-CMV IgG titre, the oldest centenarian individual showed high percentages of CD28+ and low percentages of TEMRA CD8 T cells. As above reported, it was proposed that latent CMV infection causes large clonal expansions of terminally differentiated CD28–CD4+ and 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. In contrast, increased IL-6 levels appear to correlate exclusively with increasing age. The lack of a CMV-negative centenarian control group does not allow us to be able to analyse the exclusive effect of age on these subjects.

Several studies have reported associations between CMV and all-cause mortality in some cohorts, while other studies have found little or no association between CMV and decreased life span or chronic disease. One mechanism through which CMV could impact age-related diseases could be the triggering of inflammatory responses with each episode of reactivation, but evidence linking CMV to an increase in systemic pro-inflammatory markers remains scarce and controversial [10, 51]. CMV seropositivity has also been shown to be associated with more than double the risk of hospitalization for COVID-19 [52], and CMV positivity increased the cardiovascular risk of older men [53]. An association between CMV seropositivity and cardiovascular mortality had been reported in a meta-analysis some years ago [54]. However, a recent meta-analysis that included a larger number of subjects with a wider age range, as well as more data that allowed for adjustments for more confounders, showed that CMV infection is not associated with cardiovascular and all-cause mortality in different cohorts of older people [55]

Several studies would show that CMV positivity can impair various aspects of immune function in aged models and humans, but there is no actual increase in infection mortality in CMV-infected individuals compared to controls [51]. More relevant is the evidence that CMV might provide benefits to the adult, and even older host, by augmenting immune defence against other infections [56, 57]. Then, CMV is responsible for a large subset of TEM virus-specific cells, and many of these are TEMRA. These cells are perfectly equipped to control the virus without further T-cell expansion [10, 16, 58]. There is some evidence that CMV status in older people does not seem to impact responses to vaccination [56].

Discordant results have also been obtained in studies concerning the role of CMV positivity in European longitudinal studies on the so-called Immune risk profile that evaluated factors predicting mortality and morbidity at very old age. As reviewed by Caruso et *al*. [10], in Swedish studies a risk of death in old CMV-positive people with a reversed CD4/CD8 *ratio* was observed, whereas Dutch results suggested that immunosurveillance against CMV could be crucial for remaining longevity. Moreover, a protective effect of CMV in Belgian women was observed, whereas very old CMV-negative women experienced the highest mortality rates. It is noteworthy that these populations had quite different birth dates and their life expectancies at birth were quite different [10, 59].

So, CMV seems to be compatible with long life and preserved immune function, and there is some evidence that it can help the host fight off other infections [16, 56, 60]. Support comes from data showing that fewer older centenarians, almost certainly CMV-positive, died than the rest of the older population in 2020 COVID-19 pandemic [61–63]. In agreement, three Brazilian supercentenarians recovered from COVID-19, showing robust IgG levels and neutralizing titers versus SARS-CoV-2 [64].

The previously discussed discordant results underscore that the immunosenescence process and its relevance are highly context-dependent (see also below).

Overall, our study shows that several immune parameters, especially of semi- and super-centenarians, exhibit great variability. Everybody had a different immunological history during his/her life because of the different antigenic *stimuli* encountered, called “immunobiography” [9]. In addition, socio-economic status, education, lifestyle, nutrition, physical activity, and microbiota are known to affect the immune system [10, 65–67]. Genetic control of CMV infection could also positively or negatively modulate virus reactivation [68] since persistent CMV infection requires continuous control by the host immune system which is itself altered with age [69]. All this could have different effects on different people.

Anyway, 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 might be not unfavourable for centenarians, and in particular for the oldest ones.

However, this study has two limitations, (i) the cross-sectional nature of data; and (ii) the impossibility of having adequate CMV negative controls since practically all the 90+ Sicilian population is CMV positive.

In conclusion, our results lend credence to the idea that immune ageing may be more of a differential adaptation than a general immune alteration. In this perspective, older centenarians have successfully adapted to a history of insults thus achieving exceptional longevity.

Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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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.

Conflict of Interests

The authors declare that they have no conflict of interest.

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Data Availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Author Contributions

C.C., M.E.L., and S.M. conceived and designed the study. A.A., C.C., G.A., M.E.L, and S.A. recruited and selected participants. A.C., A.M.C., C.M., R.C., M.D.S, and M.E.L performed experiments and statistical analysis. C.C., M.E.L., and S.M. wrote the report. A.N.A. and G.C. made substantial intellectual contributions to the work and commented on and revised the report. All authors read and approved the final manuscript.

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