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Biological Markers Changes at the Very Early Stage of Ageing (60-65 Years). Is there a Gender-Related Effect?

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Abstract

Ageing has been associated with progressive molecular and structural changes causing loss of function in several organs. There is a general hypothesis that very old individuals suffer profound modifications where different domains (immune, metabolic and cognitive) lose the tight functional interconnection present in younger bodies. However, it is not clear how this interconnection is affected at the very early stage of ageing and whether gender plays a role. Therefore, our aim was to evaluate some biological markers in non-institutionalized "healthy individuals" at the very early stage of ageing (60 to 65 years old, female and male). Blood was collected and serum creatinine, albumin and glucose were measured. In addition, we evaluated these individuals for lymphocytes phenotype (T CD4⁺, T CD8⁺, CD19⁺) by flow cytometry in peripheral mononuclear blood cells. It was observed that at the early stage of ageing male present higher creatinine and albumin serum levels compared to female. In addition, male had higher percentages of effector memory CD4⁺ and CD8⁺ T cells and lower percentages of naive CD8⁺ T cells. No differences were observed for B cells. These findings suggest that metabolic functions and immune system are compromised in male individuals at the very early stage of ageing. Functional studies in lymphocytes and oxidative stress in erythrocytes are required to confirm our hypothesis and in case of positive association gender differences should be considered for vaccination strategies and development of new therapies.

Keywords: Ageing; Sex-differences; Immunosenescence; Lymphocyte phenotyping.

Introduction

The natural/continuous process of ageing is associated with several changes in the organism. It is difficult to precisely identify the beginning of ageing process and in developing countries individuals are designed as "elderly" after 60 years old whereas in developed countries "elderly" are individuals over 65 years old.

Healthy ageing seems to be associated with the low burden of chronic diseases and mainly with the maintenance of a functional immune system [1,2]. Some authors have proposed that metabolism dysfunctions (i.e. kidney deteriorating function) are associated with changes in the immune system [3-5].

Most of the studies in elderly have been performed in blood cells and all of the evaluated components present changes during the ageing process. The oxidative stress has been shown to play a pivotal role in ageing by causing oxidative damage to erythrocytes as both its rate increases in elderly and the efficiency of antioxidative and repair mechanisms decreases. In agreement Kosenko et al. [6] observed that individuals elderly healthy (76.8 ± 3 years old) or with Alzheimer's disease (75 ± 2.6 years old) presented in red blood cells significant decreased activity of glutathione peroxidase (GPx), superoxide dismutase (SOD), and glutathione S-transferase (GLT) in comparison with young adults (33.3 ± 3.33 years old). Ageing has also been characterized by a process named "immunosenescence" with increased incidence and severity of infections, decline in vaccine efficacy, and autoimmunity in older adults affecting both innate and adaptive

immune responses. It has been reported that changes occur during ageing in T lymphocytes with decrease of naive cells along with the accumulation of highly differentiated T cells [7-9]. B lymphocytes in elderly individuals present reduced antibody specificity, affinity, and isotype switch [10-14]. These changes have been associated with reduced protection against new infectious agents and inefficient response to vaccines.

The decrease of naive along with the accumulation of highly differentiated T cells have been explained by the decrease in thymic output [7] and higher susceptibility of naive T cells to apoptosis after antigen activation [8]. CMV infection and malignancies have also been associated with the increase of effector memory T cells [9,15,16]. In addition, it has been reported that the age-related decrease of naive T cells and increase of T effector memory (TEM) in peripheral blood also occurs in bone marrow. These findings suggest that the increase of homeostatic cytokines (i.e. IL-15) during ageing favors the survival of an expansion of memory instead of naive cells [17].

Another important event related to changes in immune system during ageing is the declined function of hematopoietic stem cells which is characterized by impaired lymphopoiesis and enhanced myelopoiesis [18-22]. This event could favor the expansion of myeloid-derived suppressor cells (MDSCs) which have been shown to be increased in ageing individuals. MDSCs have been associated with inflammatory conditions such as infections and cancer and could contribute for the suppressive state observed in ageing individuals [23-25].

As cited above most of the studies, including those from our group, focus in the impact of age on T and B cells (adaptive immunity) but

recently neutrophils, monocytes (innate immunity), and erythrocytes have received more attention from immune gerontologists as these cells also suffers functional and phenotypical alterations with age and could thus contribute for the increase of chronic diseases and loss of the immune system efficacy. Moreover, a variety of factors play a role in the ageing process and recently it has been proposed that gender affects how ageing develops since infections, chronic diseases, and death are more frequent in elderly male individuals [26-31, reviewed in 32]. However, few studies have evaluated whether gender is related to changes observed in metabolism and immune system during the ageing process. Therefore, in this study we investigated whether there are changes in some parameters of metabolism and in lymphocytes subtypes at the early stage of ageing (60-65 years) and how these alterations occurs in female and male individuals. If we can know in advance what the crucial targets of ageing are it is possible to intervene with improved protocols in vaccination and other therapies and thus prevent more prominent changes during the process.

Materials and Methods

After obtaining the written informed consent from the ageing healthy individuals (23 women and 19 men, 60-65 years old), 3ml of heparinized peripheral blood was collected as approved by the Ethics Committee of the UNIFESP Protocol number 10904/2012.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient (Amersham Biosciences, Uppsala, Sweden) centrifugation. Cells were viable frozen in RPMI, fetal bovine calf serum, and dimethylsulphoxide -DMSO- (all products from Sigma, St Louis, MO, USA), and stored (-80°C) until cell phenotyping by flow cytometry analysis.

Cell phenotyping

Frozen PBMCs were thaw and RPMI was added. Cells were centrifuged and the pellet was diluted, counted, and volume adjusted

(1×10^6 /ml). Cells were surface stained with monoclonal antibodies CD3 APC, CD4 PerCP Cy5.5, CD8 APC Cy7, CD19 PE Cy7, CD27 FITC, and CD45RA PE (eBioscience, CA, USA) for 30 min at 4°C, washed in staining buffer (PBS with 1% BSA), and centrifuged. Live cells (based on forward and side scatter) were acquired on FACS Canto II using DIVA software (Becton Dickinson, USA). Further analyses of FACS data were performed using FLOWJO 9.3 software (Tree Star, USA).

Clinical parameters

The measurement of clinical parameters (creatinine, albumin, and glucose) was performed in blood using an automatic analyzer Dimension RXL (Siemens Laboratory Diagnostics, Tarrytown, NY, USA).

Statistical analysis

Mann-Whitney test was used for statistical analysis and values of $*p \leq 0.05$ were considered significant. All statistical analyses were performed with the aid of GraphPad PRISM software (Graphpad, La Jolla, USA).

Results

In Figure 1A it is possible to observe that at the very early period of ageing (60-65 years) male presented a higher mean serum creatinine (female=0.82 mg/dl versus male=1.02 mg/dl) than female. Male also presented higher mean serum albumin (female=3.71g/dl versus male=3.99 g/dl) compared to female (Figure 1B) whereas mean serum glucose (female=103.2mg/dl versus male=90.9 mg/dl) was similar when male and female were compared (Figure 1C).

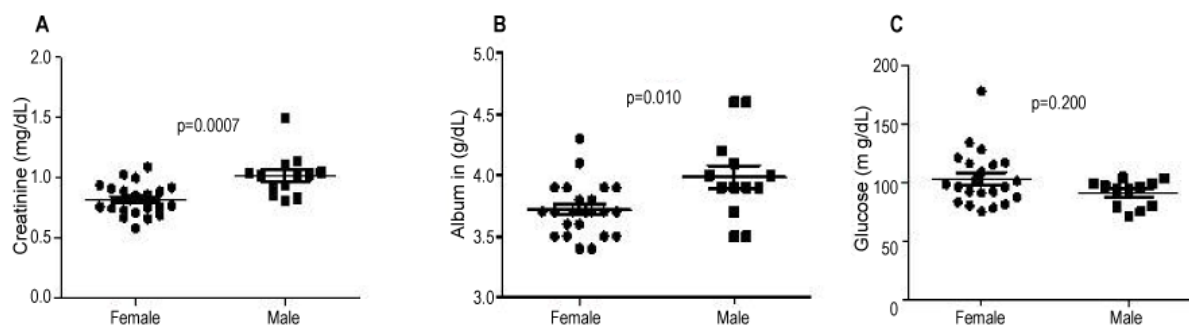


Figure 1: Blood levels of creatinine, albumin, and glucose from individuals of 60-65 years old (female versus male).

Changes in the percentage of T (CD4⁺, CD8⁺) and B lymphocytes and in CD4/CD8 ratio have been shown during the ageing process. In this study the evaluation of mean CD4⁺ (female=47.4% versus male=43.3%), CD8⁺ (female and male=23.2%), CD19⁺ (female=11.9% versus male=11.2%) and the ratio CD4⁺/CD8⁺ (female=2.28 versus male=2.13) showed no statistical difference when female and male at the early stage of ageing (60-65 years) were compared (Figure 2).

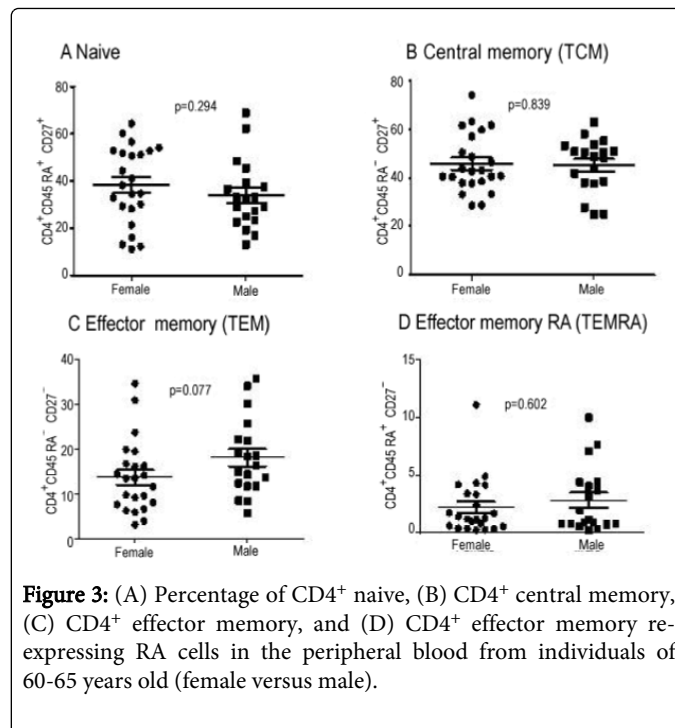
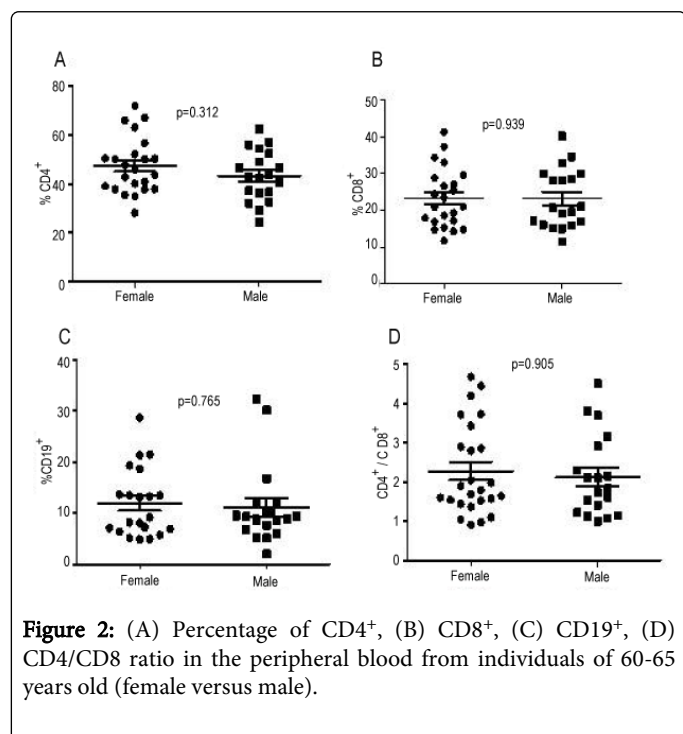
Decrease of naive cells and increase of a memory phenotype is reported in elderly individuals and it has been associated with

impaired immune response to new pathogens and vaccination. Therefore, our next step was to investigate whether lymphocytes at different stages of maturation (naive, memory, and effector) show percentage changes in female and male individuals at the early stage of ageing (60-65 years). We performed the phenotype of blood circulating cells by flow cytometry using as markers CD45RA⁺ and CD27⁺. The presence of these two markers in T lymphocytes is related with naive function whereas the decrease or loss (CD45RA^{neg}CD27⁺,

CD45RA⁺CD27^{neg}, and CD45RA^{neg}CD27^{neg}) is related to memory and effector functions [33].

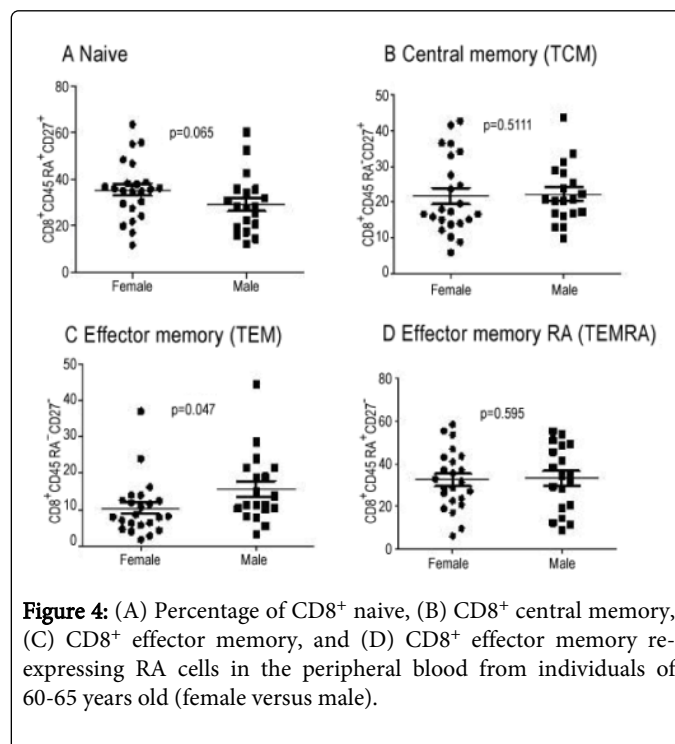
Figure 3C shows that at the early stage of ageing (60-65 years) there was a trend towards higher mean percentage of CD4⁺ T lymphocyte effector memory (TEM) phenotype in male individuals (female=13.6% versus male=18.1%, p=0.077) whereas other phenotypes (naive, TCM, and TEMRA) presented similar mean percentages when female and male individuals were compared. CD8⁺ T cells showed a trend towards higher mean expression of naive (Figure 4A) phenotype in female (female=35.5% versus male=29.2%, p=0.065) whereas effector memory (TEM female=10.4% versus male=15.6%, p=0.047) phenotype was significantly higher in male individuals (Figure 4C). CD8⁺ central memory (TCM) and effector re-expressing RA (TEMRA) T cells were expressed in similar percentages when female and male were compared (Figures 4B and 4D respectively).

Ageing has been associated with alterations in peripheral B cell developmental system leading thus to the imbalance in naive and memory populations and decreased response to new extracellular pathogens. In B cells (CD19⁺) the expression of CD27 is a marker of primed memory cells (CD19⁺CD27⁺) and its engagement promotes the differentiation of memory B cells into plasma cells. Our study showed that both naive and memory phenotype was expressed at similar mean percentages when female and male individuals at the early stage of ageing (60-65 years) were compared (Figure 5A and 5B). We observed a mean percentage of naive B cells over 60% (CD19⁺CD27⁻; female=60.8% versus male=62.7%) whereas memory B cells were under 40% (CD19⁺CD27⁺; female=37.0% versus male=34.7%).



Discussion

Recent findings point to the importance of the immune system integrity for a healthy ageing. Therefore, it is crucial to understand how changes occur in the immune system during the ageing process and the associated events.



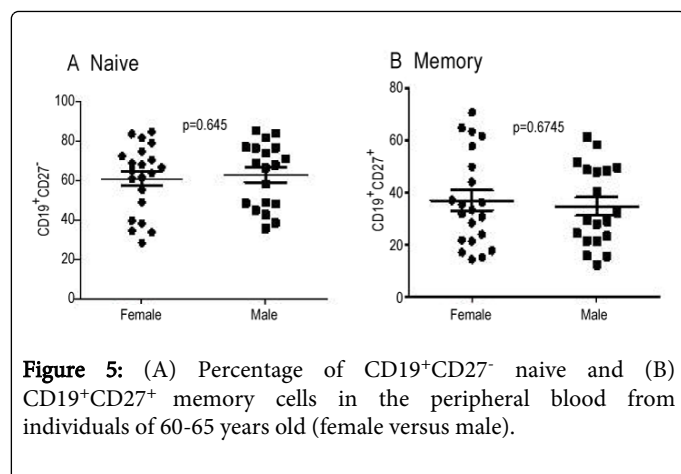


Figure 5: (A) Percentage of CD19⁺CD27⁻ naive and (B) CD19⁺CD27⁺ memory cells in the peripheral blood from individuals of 60-65 years old (female versus male).

Bucci et al [34] followed centenarians for five years and found that individuals who reached longer survival presented similar serum creatinine compared to those who lived less (1.03 ± 0.37 and 0.99 ± 0.3 mg/dL respectively). Instead, longer survival was related to CD4⁺ and CD8⁺ higher percentage of naive cells, higher ratio activated/memory and effector/memory phenotype suggesting that in very old individuals the immune system is more susceptible to changes than renal function and could have a significant impact on survival. In our study, at the early stage of ageing (60-65 years), male individuals presented significantly higher mean serum creatinine (female=0.82 mg/dl versus male=1.02 mg/dL) than female along with lower percentage of naive CD8⁺ T cells (female=35.5% versus male=29.2%) and higher percentage of T effector memory CD4⁺ (female=13.6% versus male=18.1%) and CD8⁺ (female=10.4% versus male=15.6%) suggesting more changes in the immune system of the male gender.

Weinberger et al [35] found that CD4⁺ T lymphocytes are less susceptible to age-related phenotypic and functional changes than CD8⁺ T cells. In agreement we found for CD4⁺ T cells that only the effector memory (TEM) phenotype showed a trend towards higher mean percentage in male than in female individuals ($p=0.077$). For CD8⁺ T cells we observed changes in two subtypes leading to a lower percentage of naive and a higher percentage of TEM in male compared to female individuals.

In healthy adult donors Hamann et al [36] observed for CD8⁺ T cells $55 \pm 17\%$ of naive, $25 \pm 11\%$ of TCM, $4 \pm 3\%$ of TEM, and $13 \pm 13\%$ of TEMRA. Comparing with Hamann's data our results showed for both gender at the early stage of ageing (60-65 years) a lower mean percentage of naive (female=35.5% versus male=29.2%) and a higher mean percentage of TEM (female=10.4% versus male=15.6%) and TEMRA (female=32.4% versus male=33.1%) CD8⁺ cells. Although our data are in line with other authors' findings confirming that the ageing process is characterized by accumulation of senescent CD8⁺ T cells which were in our study more prominent in male individuals, functional evaluation and other senescence markers are crucial to confirm our hypothesis. Henson et al. [37] found that CD8⁺ TEMRA (median 22%) from healthy young individuals presented low proliferative activity and senescence features (KLRG1, CD57, γ H2AX) but were still capable of cytotoxic activity and secretion of TNF- α and IFN- γ after activation. Moreover, in senescent CD8⁺ T cells anaerobic glycolysis was preferentially used to generate energy, these cells produced more reactive oxygen species (ROS), had more mitochondrial dysfunction and lowest telomerase activity after stimulus. The group [38] also evaluated elderly individuals (65-82

years old) and found that senescence features (KLRG1, CD57, γ H2AX) were significantly higher expressed in ageing individuals compared to young adults. In addition, in elderly individuals the proliferative capability of naive and EMRA CD8⁺ T cells was lower and telomerase activity decreased in all subtypes of CD8⁺ T cells. Intriguingly, naive and EMRA CD8⁺ T cells from elderly were capable of significant higher production of TNF- α and IFN- γ and naive cells from elderly expressed higher percentage of perforin and granzyme in opposite to EMRA CD8⁺ T cells. They also found that targeting (inhibition) of both the p38 MAPK (senescence) and PD-1 (exhaustion) pathways increases their proliferative responses to polyclonal and antigen-specific stimuli while maintained the effector capacity which could be an important strategy during vaccination.

Ageing also affects B cells (humoral response) and in addition to the decreased percentage of the naive phenotype it has been reported that elderly subjects present reduced antibody specificity, affinity and isotype switch which contribute for the impairment in the protection against infections and poor response to vaccines [39]. Bancos & Phipps [40] observed a mean percentage of 40% for naive and 60% for memory B cells in the peripheral blood of elderly subjects. In opposite, we observed a mean percentage of naive B cells over 60% (female=60.8% versus male=62.7%) whereas memory B cells were under 40% (female=37.0% versus male=34.7%). This difference could be due to our studied population that was at the early stage of ageing (60-65, $n=23$ female and 19 male) whereas [40] evaluated individuals were older (60 to 72 years old; $n=6$ female and 3 male). We did not find any differences in the percentages of naive and memory B cells when female and male were compared. Other changes instead of number could occur in B cells at the early stage of ageing such as inadequate function, insufficient help from T cells with consequences for antibody production, specificity, affinity and isotype [41].

Several studies have shown that male suffer higher morbidity and mortality compared to female due to infections [26-30]. Also, a more efficient immune response is obtained in female after vaccinations [31]. Considering influenza vaccine one study showed that female receiving half dose developed similar antibodies in relation to male receiving full dose [42]. A Japanese study showed age-related changes in lymphocyte subsets suggesting a faster progression of immunosenescence in men with a more pronounced decline of T cells including naive CD4⁺ and CD8⁺CD28⁺, B cells, T-cell proliferative capacity, and IL-6 secretion, and a weaker increase in CD4⁺ memory T cells and NK cells [43]. In agreement we found that male individuals at the very early stage (60-65 years) are more negatively affected by changes in T cells.

In conclusion, in our studied population the very early stage of ageing (60-65 years) was characterized by more prominent changes in male individuals such as higher mean creatinine levels in serum and increase of memory in detriment of naive T cells in peripheral blood. Functional studies in lymphocytes and oxidative stress in erythrocytes are required to confirm our hypothesis and in case of positive association gender differences should be considered for vaccination strategies and development of new therapies.

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