Immune function, oxidative and inflammatory markers in centenarians as potential predictors of survival and indicators of recovery after hospital admission

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Abstract

Several parameters of immune function, oxidative and inflammatory stresses have been proposed as markers of health and predictors of longevity and mortality. However, it is unknown if any of these parameters can be predictive of survival in centenarians. Therefore, in a group of 27 centenarians, at the time of admission to the Clinical Hospital of Madrid, a series of immune function, antioxidant, oxidant and inflammatory parameters, were studied. Some centenarians survived and others did not, establishing two groups, "survivors" (n = 9) and "non-survivors" (n = 18). The results show that surviving centenarians display higher neutrophil chemotaxis and microbicidal capacity, NK activity, lymphoproliferation, glutathione reductase activity and basal IL-10 release. Moreover, they show lower neutrophil and lymphocyte adherence, superoxide anion and malondialdehyde concentrations and basal release of TNF-α. The ODDS RATIOS (OR) for survival for these parameters were also calculated and those with the highest ORs were the lymphoproliferative capacity and the *ex vivo* basal and stimulated release of IL-6 from mononuclear cells (OR = 136.00). Therefore, these parameters have the potential to be used in the clinical setting as predictors of survival in centenarians. In the survivors group, the same parameters were also analyzed after three months. Since survivors showed an increase in neutrophil and lymphocyte chemotaxis capacity during the recovery period, reaching similar values to those observed in healthy centenarians, these could be proposed as indicators of recovery.

Keywords: longevity, odds ratio, survival prediction

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INTRODUCTION

Centenarians have been described as the best example of successful aging, given that they largely avoided the age-related diseases responsible for the high morbidity and mortality of aging [1]. Thus, these individuals not only escape the typical infections of the elderly, but also show a peculiar resistance against most common age-related diseases through life, such as cancer, cardiovascular disease, dementia, diabetes and cataracts [2-4]. Consequently, in these individuals, morbidity is compressed toward the end of life (that is, health span approximates to life span) and then, there is a rapid onset of decline in the functional state which usually ends up in hospitalization and death. In fact, certain studies have shown high rates of disability (around 90%) among centenarians [5]. However, given that over the last decades there were not many centenarians, knowledge of evidence-based health care in centenarians is still poor [6].

Nevertheless, given that the number of centenarians is projected to rise rapidly over the next few years [7,8], there is an urgent need to identify prognosis markers of outcome in this extreme long-lived group, due to the increasing evidence suggesting the different significance of health indicators in centenarians with respect to younger seniors [9, 10]. As such, traditional metabolic indices like glucose, triglycerides, cholesterol and creatinine levels have been found to be limited as prognostic markers in centenarians [9, 11]. Moreover, whereas high systolic blood pressure in adulthood is associated with an early mortality, in centenarians it has been found to be a strong predictor of survival for at least one year [9]. In the same study, higher survival probability was associated with lower white blood counts and lower levels of inflammatory mediators in plasma, such as CRP and IL-6. Despite the fact that these studies were carried out in healthy centenarians, in which the health status was not endangered, it seems feasible that function parameters

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of immune cells as well as inflammatory mediators could have the potential to be used as predictors of survival after hospital admission.

As a matter of fact, the functional capacity of the immune system, an essential homeostatic system, has been widely proposed to be a good marker of health and predictor of longevity [12, 13] and certain immune markers have been identified to predict survival in people between 60 and 80 years of age [14-16]. In addition, among the most investigated markers as predictors of health outcomes, inflammatory markers stand out. In this context, a high pro-inflammatory state has been shown to be predictive of mortality in the elderly [17-22]. However, with respect to centenarians, the association between pro-inflammatory markers and mortality is not so clear. Some studies have found an association between pro-inflammatory cytokines such as TNF-α and dementia and mortality in centenarians [23, 24] whereas other centenarians in spite of displaying large amounts of pro-inflammatory cytokines exhibit a good clinical condition. This latter fact has led researchers to consider the idea that the observed pro-inflammatory state in centenarians could be an adaptive mechanism, and as such it should not be considered as a risk factor [25]. Additionally, oxidative stress markers have also received much attention as prognosis markers and certain redox markers have been shown to be predictive of mortality in elderly people [26-28]. However, and despite the usefulness of all the above mentioned parameters as predictors of mortality in elderly populations, their prognostic role in centenarians has scarcely been investigated.

Hence, the aim of the study was to investigate if a series of parameters of immune function, redox and inflammatory stresses in hospitalized centenarians have the potential to be used to predict survival. For this purpose, several immune function (adherence and chemotaxis of neutrophils and lymphocytes, phagocytosis and microbicidal capacity of neutrophils, Natural Killer cytotoxic activity, proliferative and cytokine release of IL-1β,

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IL-6, TNF-α and IL-10 from mononuclear cells in response to a mitogen) redox (antioxidant glutathione peroxidase and reductase activities, reduced glutathione, oxidized glutathione, intracellular anion superoxide and malondialdehyde concentrations) and inflammatory stress parameters (basal release of the above mentioned cytokines) were analyzed in a group of centenarians (N=27) at the moment of hospital admission. These parameters were afterwards related to centenarian´s survival or mortality to identify those markers with the highest predictive power. In addition, in the survivors group, the same parameters were analyzed after three months, to find out which markers could serve as indicators of recovery.

METHODS

Subjects and extraction of blood samples

Twenty-seven centenarians were recruited for this study and peripheral blood was extracted at the moment of admission to the Geriatric Service of the Hospital Clínico San Carlos in Madrid, Spain. They were divided into two groups, those who survived after admission, which will be referred to as "Survivors" (N=9) and those who did not survive, "Non-survivors" (N=18). Peripheral blood samples (10 mL) were collected in the morning, to avoid circadian changes in immune function parameters, using venipuncture and placed into sodium citrate-buffered Vacutainer tubes (BD Diagnostics – Preanalitical Systems, Madrid, Spain). Peripheral blood was also extracted from the centenarians of the survivors group after 3 months. Moreover, another group of "healthy centenarians" (N=10), who were not hospitalized during the previous year and retained certain activities of daily living (ADL) such as bathing, dental hygiene, toileting, eating, dressing, transfer and mobility, was also recruited and used as the control group. This study was performed with the informed consent of the donors and was approved by the Hospital Clínico San Carlos Ethics Committee.

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Analysis of immune function parameters

Isolation of neutrophils and lymphocytes. Both polymorphonuclear (PMNs, mainly neutrophils) and mononuclear (mainly lymphocytes) leukocytes were isolated from whole blood following a previously described method [12], using 1.119 and 1.077 density Hystopaque (Sigma-Aldrich, Spain) for neutrophil and lymphocyte separation, respectively. Collected cells (95% of viability determined using trypan blue staining) were adjusted to the corresponding final concentrations for the development of each assay.

Adherence. Briefly, 1mL of whole blood (diluted 1:1 with Hank's medium) was placed in a Pasteur pipette into which 50 mg of nylon fiber was packed to a length of 1.25 cm. After 10 minutes, the effluent had drained by gravity. The adherence index (AI) percentage was calculated as follows:

$$\mathrm{AI} = \frac{\frac{Cells}{ml} initial - \frac{Cells}{ml} effluent}{\frac{Cells}{ml} initial} \; x\; 100$$

Chemotaxis. Cell suspensions were adjusted to 0.5 x 10^6 cells (neutrophils or lymphocytes)/ml in Hank's medium and placed into a Boyden chamber. The number of cells that migrated towards formyl-Met-Leu-Phe (10^{-8} M) after 3 hours of incubation were counted and expressed as the Chemotaxis Index, as previously described [12].

Phagocytosis. Cell suspensions were adjusted to 0.5 x 10^6 neutrophils/ml in Hank´s medium and placed into migration inhibition factor (MIF) plates for 30 min at 37ºC to allow neutrophils to attach to the plastic forming a monolayer of cells. After washing, polystyrene latex beads (1.1 µm mean particle size) (LB11, Sigma-Aldrich) diluted to 1% were added to the plates. After 30 min of incubation at 37ºC, the number of beads ingested

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by 100 neutrophils was counted and expressed as the Phagocytic Index, as previously described [12].

Intracellular superoxide anion and microbicidal capacity. Cell suspensions were adjusted to 10^6 neutrophils/ml in Hank´s medium and mixed with Nitro Blue Tetrazolium (NBT: 1 mg/ ml) and with Hank's solution (basal conditions) or latex beads (stimulated conditions). After 60 min of incubation at 37 °C the intracellular reduced NBT was extracted with dioxin (Sigma-Aldrich) and absorbance was determined at 525 nm in a spectrophotometer. In addition, the percentage of superoxide anion stimulation in response to latex beads, understood as microbicidal capacity, was calculated with non-stimulated values being 100%.

Natural killer cytotoxicity. Cell suspensions were adjusted to 10^6 lymphocytes/ml in RPMI 1640 medium and placed into 96-well plates. Human K-562 lymphoma cells were added into the wells. The ratio effector/target was 10/1. These cells were cultured for 4 hours. Natural killer activity was assessed by quantifying released lactate dehydrogenase into the medium (Cytotox 96 TM Promega, Germany). The results were expressed as the percentage of tumor cells killed (% lysis), as previously described [12].

Lymphoproliferative capacity. Cell suspensions were adjusted to 0.5 x 10^6 lymphocytes/ml in RPMI 1640 supplemented with fetal bovine serum and placed into 96-well plates. The mitogen Phytohemagglutinin (PHA) (1 μg/mL per well) or complete medium were added into wells and incubated for 48h. After this incubation, supernatants were obtained for cytokine quantification. Then, ^{3}H-thymidine was added together with complete medium and incubated for 24h. ^{3}H-thymidine uptake was quantified in a beta counter both in basal and stimulated conditions and results were expressed as lymphoproliferation capacity (%), 100% being the counts per minute (cpm) in basal conditions, as previously described [12].

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Cytokine measurement. Basal and PHA-stimulated release of IL-1β, IL-6, TNF-α and IL-10 was measured simultaneously in supernatants from mononuclear cells by multiplex luminometry after 48-hours incubation (Beadlyte mouse multiplex cytokine detection system, HSTCMAG-28SK-05, Deltaclon, Spain).

Determination of redox parameters

Whole blood cells. Whole blood cells (including erythrocytes and total leukocytes) were obtained as previously described [10]. Aliquots of peripheral blood were diluted 1:1 in RPMI 1640 (Gibco, Canada) and were incubated for 4 h at 37ºC in a saturated atmosphere of humidity and CO_2. After centrifuging at 900g for 10 min, plasma was removed and the whole blood cells were stored at -80ºC until used.

Glutathione peroxidase activity. Each sample of whole blood cells was re-suspended in oxygen-free phosphate buffer (pH 7.4 50 mM). Then, it was sonicated and the supernatant (1:30) was used for the enzymatic reaction together with cumene hydroperoxide as a substrate (cumene-OOH) as previously described [10]. Oxidation of NADPH was measured at 340 nm. The results were expressed as units (U) of glutathione peroxidase activity/mg protein.

Glutathione reductase activity. The samples of whole blood cells were re-suspended in oxygen-free phosphate buffer (pH 7.4 50 mM). Then, they were sonicated and supernatants (1:5) were used for the enzymatic reaction together with GSSG 80 mM as substrate, as previously described [10]. Oxidation of NADPH was measured at 340 nm. The results were expressed as units (U) of glutathione reductase activity/mg protein.

Glutathione concentration. Whole blood cells were re-suspended in phosphate buffer (pH 8 50 mM EDTA 0.1 M). Then, they were sonicated and supernatants were used for the quantification of both reduced (GSH) and oxidized (GSSG) glutathione by the

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reaction capacity that GSSG and GSH have with o-phthalaldehyde (OPT) at pH 12 and pH 8, respectively, resulting in the formation of a fluorescent compound measured at 420 nm, as previously described [10]. Results were expressed as nmol of GSSG and GSH per milligram of protein. Moreover, the GSSG/GSH ratio was calculated for each sample.

Malondialdehyde (MDA) concentration. Quantification of malondialdehyde (MDA) was achieved using the commercial kit "Lipid peroxidation (MDA) Assay Kit" (Biovision, USA). Whole blood cells were re-suspended in 300 µl MDA lysis buffer containing 0.1mM Butylated Hydroxytoluene (BHT), sonicated and centrifuged at 13000g for 10 min. Supernatants were collected, mixed with thiobarbituric acid (TBA) and incubated in a water bath at 95°C for 60 min. Then, samples were centrifuged, supernatants collected and absorbance was measured at 532 nm, as previously described [10]. Results were expressed as nmol MDA/mg protein.

Statistical Analysis

Normality of the samples was checked by the Levene test. Differences between groups were studied using Student´s T test for independent samples. Differences in the survivor group at the moment of hospital admission and after three months were studied using Student´s T test for dependent samples. In addition, all investigated parameters were stratified into two groups to be analyzed as categorical variables. Cut-off values were chosen to maximize differences between groups. Thus, the cut-off value was established using the lowest or highest value (depending on the parameter) that differentiated the most between survivors and non survivors. Afterwards, these categorical variables were used to estimate the odds ratios (ORs), 95% confidence intervals (CIs) and Pearson Chi-Square test in logistic regression models. Two-sided $P<0.05$ was considered the minimum level of significance in all cases.

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RESULTS

The main characteristics of centenarians subdivided into “Survivors” and “Non Survivors” are reported in Table 1. There was the same proportion of men and women in each group and no differences were found between survivors and non-survivors regarding age, cause of admission or biochemical parameters.

The results regarding immune functionality are shown in Figure 1. The group of survivors showed, at the moment of admission, in comparison with the group of centenarians that died within the next three months, higher values of neutrophil chemotaxis ($P < 0.05$), Natural Killer cytotoxicity ($P < 0.001$), microbicidal capacity of neutrophils ($P < 0.001$) and percentage of lymphoproliferation in response to PHA ($P < 0.001$). Moreover, this group showed lower adherence of neutrophils ($P < 0.001$) and lymphocytes ($P < 0.01$). In addition, in response to PHA, centenarian survivors released more IL-1β ($P < 0.01$) and IL-6 ($P < 0.001$) than the non survivors. No differences were found in these parameters between those patients whose had an infection (black circles and squares) and those who did not (white circles and squares).

With respect to the redox and inflammatory state of these patients (Figure 2), no differences were found between survivors and non-survivors regarding oxidized glutathione (GSSG) (1.07±0.35; 0.96±0.23; nmol/mg protein, respectively) and reduced glutathione (GSH) (1.43±0.63; 1.66±0.74; nmol/mg protein, respectively) concentrations nor in GSSG/GSH ratios (0.74±0.42; 0.56±0.26) in whole blood. Nevertheless, the group of survivors displayed, at the moment of admission, higher glutathione reductase activity ($P < 0.01$) and lower MDA concentration in whole blood ($P < 0.05$) as well as lower neutrophil concentration of intracellular superoxide anion ($P < 0.001$), compared to non-

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survivors. With respect to the inflammatory parameters, the group of survivors displayed higher IL-6 and IL-10 and lower TNF-α release in basal conditions ($P < 0.001$) than the non-survivors.

In particular, the probability of survival within three months after hospital admission significantly increased with the increasing parameters shown in Table 2. These are: neutrophil chemotaxis (> 170 Chemotaxis Index, OR 7.00) and microbicidal activity (> 190%, OR 64.00), Natural Killer activity (> 77%, OR 34.00), PHA-stimulated lymphoproliferation (> 242%, OR 136.00), basal release of IL-6 (> 950 pg/mL, OR 136.00) and IL-10 (> 700 pg/mL, OR 59.50), PHA-stimulated release of IL-1β (> 1500 pg/mL, OR 7.00) and IL-6 (> 850 pg/mL, OR 136.00) and glutathione reductase activity (> 248 U GR/mg protein, OR 40.00). On the contrary, the probability of survival within three months after hospital admission also significantly increased with decreasing neutrophil adherence (> 33%, OR 0.017), lymphocyte adherence (> 36%, OR 0.250), basal release of TNF-α (> 202 pg/mL, OR 0.036), intracellular superoxide anion (> 43 nmol NBT/mg protein, OR 0.007) and MDA (> 3.19 nmol MDA/mg protein, OR 0.036) concentration, as indicated in Table 2.

Moreover, changes in the parameters analyzed were also investigated in the survivors group after the recovery period. It was found that after three months, survivors showed an increase in neutrophil and lymphocyte chemotaxis ($P < 0.05$; $P < 0.01$, respectively), phagocytic index ($P < 0.01$) and glutathione peroxidase activity ($P < 0.01$) as shown in Table 3, whereas all the other parameters studied remained unchanged. However, in order to ascertain the significance of these parameters as markers of recovery they were further compared with a group of healthy centenarians, who were not hospitalized during the previous year. The results show that whereas neutrophil and lymphocyte chemotaxis parameters in centenarians, after three months of recovery, reached similar values to those

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observed in healthy centenarians, the phagocytic index was still lower after the recovery period than in healthy centenarians ($P < 0.05$). Moreover, glutathione peroxidase activity was higher in centenarians after the recovery period than in healthy centenarians ($P < 0.01$).

DISCUSSION

Survival over 100 years of age used to be an extraordinary occurrence. However, over the past decades, the world population of the oldest inhabitants is growing rapidly [8, 29]. Up until 1990, there were only 100000 centenarians, which increased to nearly half a million by 2015. Moreover, the 2017 Revision of World Population Prospects from the United Nations forecasted that there will be 3.7 million centenarians worldwide by 2050. Consequently, finding prognostic markers in centenarians is an urgent need given that they will allow clinicians and clinical investigators to identify groups at lower and higher risk for mortality and consequently, to choose the most appropriate treatment strategies [30, 31].

Even though several studies have found some immune function parameters as predictors of mortality in elderly individuals [14-16, 32, 33], to the best of our knowledge, this is the first study in which immune cell function parameters have been investigated as predictive of mortality in centenarians upon hospital admission. It was demonstrated that those centenarians that survived showed a better immune function, evidenced by a lower adherence of neutrophil and lymphocytes, higher neutrophil chemotaxis and microbicidal capacity, higher Natural Killer cytotoxic activity and higher lymphoproliferation in response to the mitogen PHA at the moment of hospital admission compared to those who did not survive. With respect to the release of cytokines by mononuclear cells after

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stimulation, centenarian survivors displayed higher IL-1β and IL-6 cytokine release compared to the non-survivor group, which agrees with a previous study in which healthy centenarians were found to produce the most IL-1β and TNF-α after stimulation, compared to unhealthy ones [34]. Another study also demonstrated that 85 years-old human subjects who produce low TNF-α after a stimulus, have a more than two-fold increased mortality risk compared to peers with a higher release [35].

It is also important to recall that centenarians, in addition to displaying an excellent immune profile [12, 36, 37], also show a better redox profile than aged subjects. In a recent study, it was demonstrated that healthy centenarians exhibit a unique redox signature, characterized by low levels of oxidant parameters and very high antioxidant enzymatic activities, in comparison to individuals of other ages [10]. In the present study, those centenarians that survived also displayed lower oxidative stress levels, evidenced by a higher glutathione reductase activity and lower intracellular superoxide anion and MDA concentrations at the moment of hospital admission, in comparison to those centenarians that died. These results agree with a previous study carried out on centenarians in which those who had the best physical functional capacity also had the highest glutathione reductase activity [38].

Moreover, we attempted to establish some reference values for the investigated parameters in centenarians creating two groups, those that were above the cut-off value and those that were below. The probability of survival was then calculated using the Odds ratio (OR), and it was found that the probability of survival within three months after hospital admission significantly increased with increasing neutrophil chemotaxis, Natural Killer activity, basal release of IL-6, PHA-stimulated lymphoproliferation and release of IL-1β and IL-6 and glutathione reductase activity. However, the probability of survival increased with decreasing neutrophil adherence, basal release of TNF-α, intracellular

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superoxide anion and MDA concentrations. Interestingly, those with the highest OR were the lymphoproliferative capacity in response to PHA and the basal and PHA-stimulated release of IL-6. The proliferative capacity of lymphocytes has also been found to be predictive of mortality in elderly populations. In fact, this parameter has been used to ascertain the Immune Risk Phenotype in elderly people, which relates to mortality [16, 39]. In addition, with respect to the basal and stimulated release of IL-6, the results are surprising given that this cytokine has been generally viewed as pro-inflammatory and consequently, harmful. However, the results from the present study indicate that those centenarian who, upon hospital admission, show a higher release of IL-6 have a higher probability of survival. Thus, it could be that this cytokine may be playing an essential role regulating and orchestrating other cells. Accordingly, a recent study has unravelled a new role of IL-6 in mediating the reprogramming of cells associated with senescence [40], which could reverse the age-associated silencing of important antioxidant and anti-inflammatory genes. In addition, these results agree with a longitudinal study carried out on mice [41] in which it was found that those mice that at the very old age had a higher basal release of IL-6, were the ones that reached longevity later on.

The potential value of the parameters studied as indicators of recovery was also investigated by measuring them in the surviving centenarians after a period of three-months. It was found that most of the parameters analyzed did not change within this time frame, but neutrophil and lymphocyte chemotaxis, phagocytic capacity and glutathione peroxidase activity were found to increase in these subjects during the recovery period. In fact, neutrophil and lymphocyte chemotaxis parameters reached similar levels to those observed in healthy centenarians, and because of that, they could be used as recovery indicators. However, phagocytic capacity was still found to be lower whereas glutathione

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peroxidase was higher after the recovery period than in healthy centenarians. Therefore, it seems that these last markers require more recovery time to reach healthy values.

In summary, the results of the present study demonstrate that there is a link between a better functional capacity of immune cells and a higher chance of survival after hospital admission in centenarians. It is known that the immune system does not work alone but in close connection with the other regulatory systems, such as the nervous and the endocrine systems, forming the so-called neuro-immuno-endocrine communication, responsible for the maintenance of homeostasis of an individual [42]. Thus, a better functional capacity of immune cells reflects a better functioning of the neuro-immuno-endocrine system, as a whole, which is capable of adapting and restoring homeostasis after challenging situations, such as those causing hospital admission. Moreover, some reference cut-off values for specific markers have been provided, such as neutrophil adherence, chemotaxis and microbicidal activity, Natural Killer activity, PHA-stimulated lymphoproliferation, basal release of IL-6, TNF-α and IL-10; PHA-stimulated release of IL-1β and IL-6, glutathione reductase, intracellular superoxide anion and MDA concentrations. Thus, the combination of some of these parameters has the potential to be used in the clinical setting to evaluate the risk of mortality in centenarians. Nevertheless, it needs to be investigated if proposed reference values for the parameters studied are valid for other centenarian populations, as well as if they may need to be adjusted depending on the laboratory performance techniques. It is important to remark that these OR were obtained in a centenarian population. Thus, and although some immune function, oxidative and inflammatory parameters have been shown to be predictive of survival in the elderly, the ones reported here should also be investigated at other ages. In fact, in a previously published study from our research group carried out on mice, it was demonstrated that whereas some immune function/inflammatory and oxidative stress

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parameters are predictive of mortality across aging, some others are specific to an age group [41]. Thus, the validation of the predictive capacity of these parameters in younger populations should be performed.

An important limitation of the study is that the functional and cognitive status of hospitalized centenarians was not evaluated. In the same way, the clinical history of these patients was not taken into account. Nevertheless, these factors may have influenced the final outcome and should be included and investigated in future studies.

A strength of the study is that some of the investigated markers were found to have a high OR for survival, even though our study sample represented a very heterogeneous group of subjects, with several different causes of admission. However, given that data were analyzed retrospectively, future prospective studies are needed to confirm the predictive validity of these markers in a larger sample. Nevertheless, given that the number of extremely old individuals is rapidly increasing worldwide, the health state of centenarians as well as the identification of markers for its evaluation are important issues of public health. Accordingly, the results of the present study, although preliminary, highlight the potential usefulness of immune function and oxidative-inflammatory stress markers in predicting short-term survival after hospital admission in centenarians and provide a novel benchmark for future work aimed at establishing mortality risk indices in this population.

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Table 1. Cause of admission, biochemical and demographic characteristics of centenarians included in the study.

	Survivors	Non Survivors
N	9	18
Age	99.44 ± 0.88	100.47 ± 1.81
Men/Women (%)	33.33 %	29.41 %
Brain Ischemia (%)	66.66 %	50.00 %
Upper Respiratory Infection (%)	55.55 %	55.55 %
Urinary Infection (%)	22.22 %	33.33 %
Bone Fracture (%)	11.11 %	11.11 %
Anemia (%)	22.22 %	11.11 %
Constipation (%)	11.11 %	27.77 %
Kidney Failure (%)	22.22 %	33.33 %
Confusional State (%)	33.33 %	33.33 %
Average number causes of admission per patient	3.00	3.39
Systolic Blood Pressure (mmHg)	131.22 ± 10.42	126.61 ± 25.37
Glucose (mg/dL)	161.33 ± 92.84	134.68 ± 65.09
Creatinine (mg/dL)	1.30 ± 0.34	1.33 ± 0.66
Cholesterol (mg/dL)	150.37 ± 25.33	142.26 ± 33.11
Triglycerides (mg/dL)	72.83 ± 13.48	103.92 ± 31.28
Uric Acid (mg/dL)	8.24 ± 2.26	6.05 ± 3.02

Causes of admission are shown as percentages whereas age and biochemical data are expressed as the mean ± standard deviation of the values in each group. Because hospitalized individuals had more than one cause of admission, average number of causes per patient in each group is also shown.

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Table 2. Predictor parameters of survival within three months after hospital admission.

	Cut-off value	Odds ratio	95% Confidence Interval	P-value
Neutrophil Chemotaxis	> 170 C. Index	7.00	1.19 – 41.36	P < 0.05
Natural Killer activity	> 77 %	34.00	2.94 – 392.85	P < 0.001
Microbicidal activity	> 190 %	64.00	5.02 – 816.44	P < 0.001
PHA-lymphoproliferation	> 242 %	136.00	7.51 – 2462.77	P < 0.001
Basal release of IL-6	> 950 pg/mL	136.00	7.51 – 2462.77	P < 0.001
Basal release of IL-10	> 700 pg/mL	59.50	4.62 – 767.18	P < 0.001
PHA-induced release of IL-1β	> 1500 pg/mL	7.00	1.19 – 41.36	P < 0.05
PHA-induced release of IL-6	> 850 pg/mL	136.00	7.51 – 1462.77	P < 0.001
Glutathione reductase	> 248 U/mg protein	40.00	3.56 – 450.00	P < 0.001
Neutrophil adherence	> 33 %	0.0017	0.001 – 0.217	P < 0.001
Lymphocyte adherence	> 36 %	0.250	0.046 – 1.365	P = 0.100
Basal release of TNF-α	> 202 pg/mL	0.036	0.003 – 0.377	P < 0.001
Intracellular superoxide anion concentration	> 43 nmol NBT/mg protein	0.007	0.000 -0.133	P < 0.001
Malondialdehyde concentration	> 3.19 nmol MDA/mg protein	0.036	0.003 – 0.377	P < 0.001

Cut-off values were established using the lowest or highest value (depending on the parameter) that differentiated the most between survivors or non survivors. The Odds ratios were calculated by dividing the probability of survival by that of dying.

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Table 3. Immune and Redox parameters that changed during the 3 months-period of recovery in those centenarians that survived hospitalization. Comparison with previous data from a group of healthy centenarians.

	Survivors at the moment of admission	Survivors after 3 months period	P-value	Healthy Centenarians	P-value
Neutrophil Chemotaxis	201.22 ± 81.33	295.44 ± 57.59	P < 0.05	439.20 ± 231.79	P = 0.240
Lymphocyte Chemotaxis	258.11 ± 50.09	442.00 ± 184.01	P < 0.01	417.90 ± 148.23	P = 0.802
Phagocytic Index	194.67 ± 33.74	303.67 ± 74.43	P < 0.01	870.30 ± 288.21	P < 0.05
GPx activity	263.96 ± 112.55	487.94 ± 148.79	P < 0.01	227.74 ± 72.35	P < 0.01

Differences within individuals at the moment of admission and after 3 months were analyzed using the Student´s T test for dependent samples. The differences between the recovered and healthy centenarians were evaluated using Student´s T test for independent samples. *: P < 0.05; **: P < 0.01; ***: P < 0.001.

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FIGURE LEGENDS

Figure 1. Immune function parameters in surviving and non-surviving centenarians upon hospital admission. A) Neutrophil Adherence (%); B) Lymphocyte Adherence (%), C) Neutrophil Chemotaxis (Chemotaxis Index); D) Lymphocyte Chemotaxis (Chemotaxis Index); E) Phagocytic Index; F) Natural Killer Cytotoxicity; G) Microbicidal Activity; H) PHA-Induced Proliferation; I) PHA-stimulated IL-1β; J) PHA-stimulated IL-6; K) PHA-stimulated TNF-α; L) PHA-stimulated IL-10. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ between survivors and non survivors. Black circles and squares indicate patients suffering from infection either respiratory or urinary.

Figure 2. Redox and inflammatory parameters in surviving and non-surviving centenarians upon hospital admission. A) Glutathione Peroxidase activity; B) Glutathione Reductase activity; C) Basal Intracellular Superoxide anion and D) Malondialdehyde concentration; E) Unstimulated IL-1β; F) Unstimulated IL-6; G) Unstimulated TNF-α; H) Unstimulated IL-10. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ between survivors and non survivors. Black circles and squares indicate patients suffering from infection either respiratory or urinary.

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Figure 1

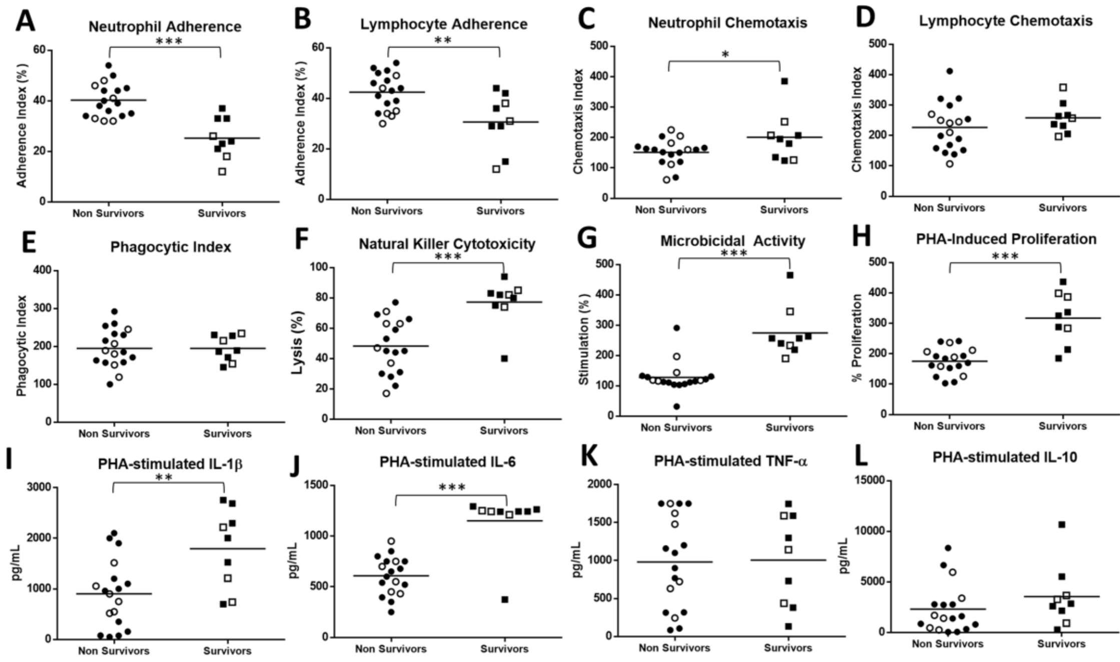

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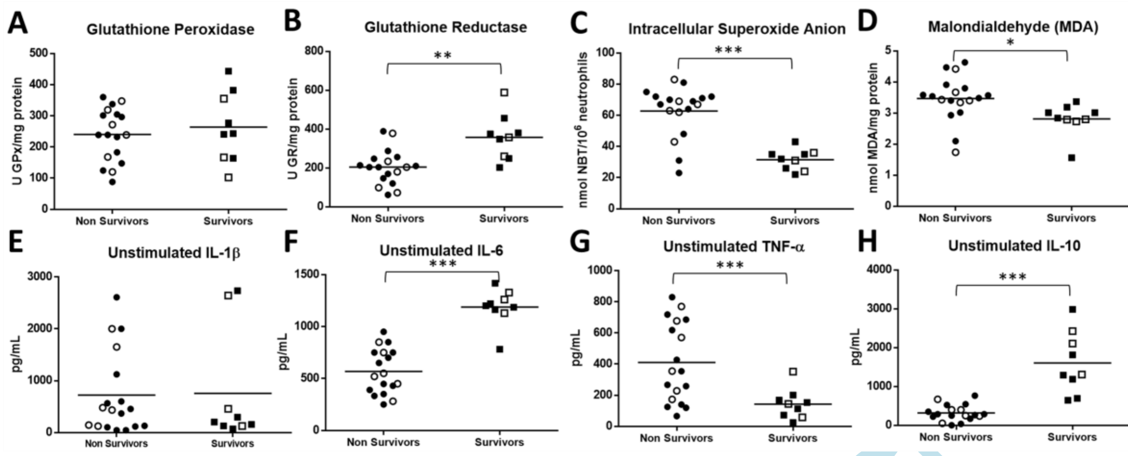
Figure 2



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