Thymic function failure and C-reactive protein levels are independent predictors of all-cause mortality in healthy elderly humans

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Abstract Relationship between thymic function and elderly survival has been suspected, despite the fact that formal proof is elusive due to technical limitations of thymic function-related markers. The newly described sj/ β -TREC ratio allows now, by overcoming these limitations, an accurate measurement of thymic output in elderly humans. Thus, the aim of this study was to determine the impact of thymic function and inflammatory markers on healthy elderly human survival. Healthy volunteers ($n=151$), aged over 65, were asked to participate (CARRERITAS cohort). Subjects were excluded if diagnosed of dementia or,

during the last 6 months, had clinical data of infection, hospital admission, antitumor therapy, or any treatment that could influence the immune status. Thymic function (sj/ β -TREC ratio), CD4:CD8 T cell ratio, C-reactive protein, interleukin-6, and neutrophilia were determined from basal samples. All basal variables and age were associated with 2-year all-cause mortality. Multivariate analysis showed that only thymic function and C-reactive protein were independently associated with time to death. In conclusion, we show, for the first time, the direct role of thymic function in human survival. C-reactive protein raise is also a marker of mortality in the healthy elderly, in a thymic-independent way.

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Introduction

Aging embraces the highly variable age-related changes in organs, tissues, and cells that confer vulnerability to stressors and disease. The immune system is not an exception, and elderly subjects show phenotypic and functional defects in both the innate and adaptive immune response. As the elderly increases, the prevalence and severity of infectious diseases disclose the outstanding importance of the immune senescence (Yosikawa 2000; Aw et al. 2007).

It is generally accepted that thymic involution, which starts just after the birth, triggers the age-associated changes of the immune system (Chiodi 1940; Henry 1967). Accordingly, among the adaptive response T cells gather most of the immune age-related defects (Linton and Dorshkind 2004; Czesnikiewicz-Guzik et al. 2008). In this line, we have previously reported that thymic function, in elderly humans, has still an impact on the rejuvenation of the peripheral naive T cell pool (Ferrando-Martínez et al. 2009). In addition, lower thymic function in elderly subjects is also associated with a higher non-antigen-driven peripheral T cell deterioration (Ferrando-Martínez et al. 2011). Overall, it is reasonable to think that thymic function could have an important role on elderly survival. On the other hand, a low-level systemic inflammatory status has been reported as characteristic of an aged immune system (DeLaRosa et al. 2006; Shaw et al. 2010). Increase of pro-inflammatory biomarkers in an age-dependent way, or *inflamm-aging*, has been proposed as a piece of evidence that the defense network, necessary in childhood and adulthood, turns out to be deleterious in the elderly (Franceschi et al. 2000).

However, despite those cellular flaws and inflammation markers that are supposed to be involved in the immune system failure, the description of a phenotype that could anticipate individuals at high risk remains elusive. In this line, the immune risk profile (IRP) describes a collection of immune-related defects that characterize the immunosenescence (Ferguson et al. 1995). The IRP is characterized by an increase in the numbers of CD3+CD8+CD28[−] cells that result in an inverted CD4:CD8 T cell ratio and is associated with persistent cytomegalovirus infection (Pawelec et al. 2005; Wikby et al. 2008). In the Swedish cohort, the inversion of the CD4:CD8 T cell ratio in peripheral blood was independently associated with mortality (Wikby et al. 1998), providing suggestion—albeit not formal proof—that loss of thymic function could be associated with aging and mortality. Therefore, notwithstanding the potential repercussion of thymic failure on human survival, lack of faithful assessment tools has hampered the verification of its biological significance. However, the sj/ β -TREC ratio (Dion et al. 2004; Ferrando-Martínez et al. 2010) allows now an accurate measurement of human thymic function from peripheral blood samples. On the other hand,

regarding the inflammation markers, a relationship between IL-6 and mortality has been described, although only in males (Baune et al. 2011). C-reactive protein raise, more if accompanied with cytomegalovirus (CMV) seropositivity, is also associated with increased human mortality rates (Simanek et al. 2011).

Altogether, the aim of this study was to determine the impact of thymic function and inflammation markers on healthy elderly mortality.

Materials and methods

Subjects During the first quarter of 2008, free-living volunteers and nursing home residents from the Aljarafe health area (Sevilla, Spain) were asked to participate in this study. Inclusion criteria included subjects aged 65 or over and self-sufficient health status. One hundred and seventy-three subjects fulfilled the criteria and were selected as potential participants of the CARRERITAS cohort. Exclusion criteria, including diagnose of dementia or any of these situations during the last 6 months: (1) clinical data of active infections, (2) hospital admission, (3) antitumor therapy, or (4) any treatment that could influence their immune status (mainly corticosteroids), were then applied. One hundred and fifty-one individuals (151 of 173, 87.3%) did not show any of these exclusion criteria and were enrolled in the CARRERITAS cohort. Demographic data and blood samples were obtained from each individual at baseline. The study was censured on January 31, 2010 (median follow-up 87 weeks [82–103]), when all participants were contacted again to assess survival rates. Time to death was calculated to determine its relationship with baseline variables.

All individuals, or their legal caregivers, were informed and consented to participate. The study was reviewed and approved by the Ethical Committee of the Virgen del Rocío University Hospital, where the research was performed.

Absolute cell counts Lymphocyte populations (CD4 and CD8 absolute numbers) were determined in fresh whole blood using the Epic XL-MCL (Beckman Coulter Inc., California) flow cytometer, according to the manufacturers' instructions. Neutrophil

numbers were obtained from a complete blood count performed in fresh whole blood using the Sysmex XE-2100 (Roche Diagnostics, IN, USA) cell counter. The inversion of the CD4:CD8 T cell ratio (Ferguson et al. 1995; Wikby et al. 1998) was selected as main indicator of the IRP. Accordingly, individuals were considered as *IRP yes* if the CD4:CD8 T cell ratio was lower than 1. Subjects were considered to present neutrophilia (*neutrophilia yes*) when their neutrophil counts exceed the reference levels established by the Hematology Service of our hospital ($\geq 7.5 \times 10^6$ cells/ml).

Herpes virus serostatus Serum samples were screened for both CMV and Epstein Barr virus (EBV) IgG antibodies using Enzyme Immunoassay Test Kits (GenWay, San Diego, CA), according to the manufacturers' instructions.

C-reactive protein quantification C-reactive protein (CRP) serum levels were quantified with the CRPLX C-Reactive Protein (Latex) kit for the Cobas C 711 automated analyzer (Roche Diagnostics) according to the manufacturers' instructions. Pathological CRP levels for adulthood, when using this kit, are established as $\text{CRP} \geq 5$ mg/l.

Interleukin-6 quantification Interleukin (IL)-6 plasma levels were performed in duplicate using a highly sensitive colorimetric enzyme-linked immunoabsorbent assay (IL-6 quantikine ELISA kit, R&D Systems, Minneapolis, MN, USA), according to the manufacturers' instructions.

sj/ β -TREC ratio quantification Thymic function was indirectly calculated in peripheral PBMC DNA using the sj/ β -TREC ratio previously described (Ferrando-Martínez et al. 2010) with minor modifications. Briefly, the six D β J β -TRECs from cluster one were amplified together in the same PCR reaction tube, while the sj-TREC was amplified in a different PCR reaction tube. Twenty-one amplification rounds were performed to guarantee an accurate quantification at the real-time PCR step. All amplicons (D β J β - and sj-TRECs) were then amplified together in a second PCR round using a LightCycler® 480 system (Roche, Mannheim, Germany). Six microliters of a 1/10 mixed dilution of the first round PCR was amplified in a 20- μ l final volume. Förster Resonance Energy Transfer specific probes for the sj-

(Franco et al. 2002) and the D β J β - (Dion et al. 2004) TRECs were previously reported.

Statistical analysis Continuous variables are expressed as median [interquartile range (IQR)] and categorical ones as percentage. Differences among categorical variables were analyzed using the chi-square test, while the Mann–Whitney *U* test was used to analyze differences between continuous variables. A survival analysis to study the relationship between time to death (dependent variable) and thymic function, IRP, age, sex, CRP, IL6, and neutrophilia status (explanatory variables) at baseline was performed. Kaplan–Meier curves were used for all time-to-event analyses, and a log rank-test bivariate analysis was performed for every variable. For the Kaplan–Meier curves, continuous variables were categorized using the median as cutoff value. A first bivariate Cox proportional-hazards regression was performed for all explanatory variables. Cox proportional-hazards multivariate regression was performed in all variables reaching a significant level ($p < 0.1$) in the bivariate analysis with the exception of the age that, as critical confounder, was included in all multivariate models disregarding previous *p* values. A *p* value < 0.05 was considered to indicate statistical significance. Hazards ratios (HR) and 95% confidence intervals (CI) were estimated for this model. COR curves were performed to determine values with increased death risk among the statistically independent variables: values were selected to maximize specificity while maintaining a good sensitivity. All statistical procedures were performed using the IBM Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) 17.0 version.

Results

Thymic function failure and IRP are both associated with mortality

One hundred and fifty-one subjects, all of them aged 65 or older (median age 79.6 years [73.5–85.8]), were included in this study. Characteristics of the cohort are shown in Table 1. In agreement with the high CMV seroprevalence existing in Spain, estimated in 93.0% in adulthood (41–60 years old) (Ory Manchon et al. 2001), 96.7% of our cohort had CMV antibodies. In addition, all CMV-negative individuals were

Table 1 Characteristics of the cohort ($n=151$)

Variable	Value	
Age (years)	Median [IQR]	79.6 [73.5–85.8]
	Min–max	65–102
sj/ β -TREC ratio	Median [IQR]	9.5 [2.6–19.3]
	Min–max	0.0–366.5
Sex (% males)		53/151 (35.1%)
IRP		12/151 (7.9%)
CMV ^a		143/148 (96.7%) ^b
EBV ^a		140/148 (94.6%) ^b
Deaths		39/151 (25.8%)

^a $n=148$ for EBV and CMV

^b Number of subjects positive for CMV or EBV IgG antibodies.

positive for EBV antibodies, indicating that our entire cohort had herpes virus persistent infection. This high prevalence did not allow comparison analyses between CMV-infected and non-infected individuals. On the other hand, IRP, defined as an inverted CD4: CD8 T cell ratio, was present in 7.9% (12 of 151) individuals of the cohort.

Thirty-nine deaths were reported (39 of 151, 25.8%) in the 2-year period. When different age strata were analyzed (Table 2), the IRP frequency ranged from 4.3% to 17.7%. Despite the oldest group (>90 years old) showed the highest percentage, in our cohort, IRP did not show a sequential increase with age. As shown in Table 2, thymic function (accurately measured with the sj/ β -TREC ratio) decreased with age while percentage of deaths increased. Not only IRP, as previously reported (Wikby et al. 2008), but also thymic function were both associated with the degree of mortality ($p=0.021$ and $p<0.001$, respectively, chi-square test). Mortality rates were similar between men and women (13 of 53, 24.5%, and 26 of 98, 26.5%, respectively, $p=0.475$).

Table 2 IRP and sj/ β -TREC ratio among the different ages

Age group	IRP	Thymic function	Deaths
65–70	1/23 (4.3%)	21.4 [7.2–58.4]	3/23 (13.0%)
71–80	5/56 (8.9%)	10.0 [2.1–19.2]	12/56 (21.4%)
81–90	3/55 (5.4%)	7.0 [3.0–16.0]	17/55 (30.9%)
>90	3/17 (17.7%)	2.1 [0.0–12.7]	7/17 (41.2%)

Relationship with mortality

Thymic function, IRP, and inflammation status are all associated with time to death

After the follow-up period (median follow-up, 87 weeks [82–103]), time to death or censor was calculated. Thymic function levels and IRP were analyzed as T cell-related variables. Neutrophilia, CRP, and IL-6 were selected as inflammation-related markers. Age and sex were included as demographic variables. A bivariate analysis showed that all tested baseline variables, except sex, were statistically associated with time to death (Table 3) in this range of ages. Higher thymic function but lower age and lower IL-6 and CRP levels were negatively associated with the risk of death. Moreover, individuals showing IRP or neutrophilia were at higher risk of death. Figure 1 shows the Kaplan–Meier survival curves for all statistically significant baseline variables. Information of the 151 subjects was successfully collected at censor date, with no loss of follow-up. The cohort was divided in two equal groups (by the median value) in continuous variables. Dichotomous variables were analyzed as follows: eight individuals showed *Neutrophilia YES* (8 of 151, 5.3%), and six out this eight died during the follow-up. On the other hand, 12 subjects showed *IRP YES* (12 of 151, 7.9%), and 7 out of this 12 died during the 2-year period.

A multivariate analysis was then performed to determine which variables were independently associated with time to death. As shown in Table 3, in our first multivariate model, lower thymic function, higher CRP levels, and presence of neutrophilia were independently associated with time to death in elderly humans (Cox proportional-hazard model). On the other hand, age, presence of IRP, and IL-6 plasmatic levels did not show a significant association in the multivariate model. We then performed a second multivariate model by adjusting by thymic function, CRP, and neutrophilia. As shown in Table 3, age was considered as a critical confounder and was also introduced in this second model, disregarding the lack of statistical significance in the first multivariate model. Thus, adjusting by age and neutrophilia status, only thymic function and CRP levels were independently associated with human survival. All co-variables of our first model were tested to analyze possible confounding factors for the neutrophilia status. Age and IL-6 plasmatic levels were the

Table 3 Association between basal variables and time to death

Variable	Bivariate analysis			Multivariate analysis ^a			Multivariate analysis ^b		
	<i>p</i>	HR	CI (95%)	<i>p</i>	HR	CI (95%)	<i>p</i>	HR	CI (95%)
sj/β-TREC ratio	<0.001	0.914	0.872–0.959	0.005	0.936	0.893–0.981	0.006	0.938	0.896–0.981
Age (years)	0.003	1.058	1.019–1.099	0.115	1.037	0.991–1.084	0.222	1.027	0.984–1.071
IRP–yes	0.005	3.230	1.422–7.334	0.499	0.648	0.184–2.279	–	–	–
IL6 (pg/ml)	0.002	1.023	1.008–1.039	0.212	0.980	0.950–1.011	–	–	–
Neutrophilia–yes	<0.001	5.739	2.525–13.041	0.032	3.891	1.125–13.466	0.123	2.227	0.804–6.165
CRP (mg/l)	<0.001	1.024	1.017–1.031	0.004	1.020	1.006–1.034	0.006	1.014	1.004–1.023
Sex–women	0.583	0.830	0.426–1.616	–	–	–	–	–	–

Bivariate and multivariate Cox proportional-hazards analysis

HR Hazard ratio, CI (95%) confidence interval

^aFirst multivariate model

^bSecond multivariate model

variables confounding the neutrophilia result in the first multivariate model (data not shown).

The C-reactive protein and thymic function index: forecast of elderly human mortality

Since thymic function and CRP levels were the only variables independently associated with time to death in our cohort, we made the attempt to define a

biomarker profile that could help to identify individuals at a higher risk to death. COR curves were performed to analyze the ability of thymic function and CRP to discriminate between survivors and non-survivors. Thymic function (COR curve area=0.745, $p<0.001$) and CRP (COR curve area=0.655, $p=0.004$) were both able to discriminate. Cutoff values were then selected from the coordinates of the curve. Thymic function value of 9.55 showed 80% of

Fig. 1 Kaplan–Meier curves for all time-to-event analyses were analyzed for all statistically significant variables from the bivariate Cox proportional-Hazards bivariate regression. All

continuous variables were dichotomized using the median as cutoff value. Log rank-test bivariate analysis was performed to assess statistical differences among the curves

specificity and 60% of sensitivity discriminating non-survivors. On the other hand, a CRP value of 14.8 mg/l showed high specificity (90%) and 41% of sensitivity in non-survivor discrimination. When Kaplan–Meier curves were performed using these cutoff values, individuals with thymic function under 9.55 or CRP over 14.8 mg/l had a statistically significant reduction in time to death (Fig. 2a and 2b). Using these intended values, we defined the C-reactive protein and thymic function (CRT) index as indicated in Table 4. A risk assessment was then performed in our cohort: higher CRT scores implied higher mortality rates, with 65.0% of mortality (13 of 20) among individuals categorized as high risk. As shown in Table 4, our index shows better positive and negative predictive values when individuals had one (intermediate) or both (high) risk factors.

We then analyzed the relationship between different risk levels described in the CRT index and time to death. As shown in Fig. 2c, time to death showed a statistically significant reduction among individuals categorized with greater risk by the CRT index. In addition, even if the multivariate analysis disregarded the neutrophilia status as an independent variable, when all three markers were combined (low thymic function, CRP ≥ 14.8 mg/l, and neutrophilia yes),

mortality rates dramatically increase up to 87.5% (seven of eight patients died during the follow-up period).

Discussion

Our results show, for the first time, that thymic function failure and C-reactive protein raise are independently associated with healthy elderly mortality. Despite the outstanding importance of the immunosenescence, both in chronological age (Aw et al. 2007; Lan et al. 2011) and the so-called premature immunosenescence (as HIV infection, Molina-Pinelo et al. 2009; Blanco et al. 2010; Appay et al. 2011), the only phenotypic characteristic that could be associated, up to date, with mortality was the CD4:CD8 T cell ratio in the very old (Ferguson et al. 1995; Wikby et al. 2008). Nevertheless, this result has been only described in the Swedish population, and it has not been validated in cohorts from other geographic regions. In this study, we confirm the increased mortality rates of individuals with an inverted T cell ratio (IRP YES in our analysis); still, we found 8% of IRP-positive subjects, a percentage lower than the 16% reported by Wikby et al. (1998). However, stricter

Fig. 2 Kaplan–Meier curves for all time-to-event analyses were analyzed for CRP and thymic function. Different groups were made with intended values after the COR analysis-defined cutoffs related with higher death risk for **a** C-reactive protein and **b** thymic function. **c** Using these values, the CRT index accurately identifies individuals at different risk levels. The number of subjects at risk are shown in basal (0 weeks) and after 6 months (24 weeks), 1 year (48 weeks), 18 months (72 weeks), and 2 years (96 weeks)

Table 4 CRT index definition and survival rates

	sj/ β -TREC ratio $\leq$ 9.55	No	Yes	No	Yes
	C-reactive protein $\geq$ 14.8 mg/l	No	No	Yes	Yes
	CRT index	Low risk	Moderate risk	High risk	
	Mortality (%)	5/68 (7.3%)	21/63 (33.3%)	13/20 (65.0%)	
	Positive predictive value ^a	7.4% (3.2–16.1)	33.3% (22.9–45.6)	65.0% (43.3–81.9)	
	Negative predictive value ^a	59.0% (48.3–69.0)	79.5% (70.0–86.7)	80.2 (72.5–86.1)	

^aPositive/negative predictive value (CI 95%)

exclusion criteria applied for the CARRERITAS cohort selection can easily explain this difference. As previously reported in the Swedish cohort, all the subjects of our cohort showing the inverted CD4:CD8 T cell ratio were also seropositive for CMV (data not shown).

CRP and IL-6 were selected as central markers of human systemic inflammation. Neutrophil increase in elderly humans has also been reported (Ferrando-Martínez et al. 2009; Strindhall et al. 2007), and recent studies suggest that neutrophil count can be affecting human atherogenesis (Drechsler et al. 2010), strongly enhancing the interest of this marker. In our first multivariate model, neutrophilia was independently associated with mortality. However, age and IL-6 levels were acting as confounding factors. Despite the fact that further analysis is needed to understand this result, we hypothesize whether IL-6 and neutrophilia could be indirect markers of tissue injury (Goldstein 2010; Baune et al. 2011) and not the primary case leading to mortality.

In our cohort, CRP raise and thymic function failure are independently associated with all-cause mortality in the healthy elderly. Relationship between CRP levels and mortality has been recently described (Simanek et al. 2011). In this study, Simanek et al. (2011) described the relationship between CMV infection and all-cause mortality within 18 years follow-up. Interestingly, the results remained unchanged after CRP adjustment. In our study, we show that, even with a 97% CMV seroprevalence, high CRP levels remain as risk factor for human mortality in a 2-year follow-up. Regarding thymic function, individuals with IRP present lower naive T cell levels as well and increased CD3+CD8+CD28–senescent T cells (Wikby et al. 2008). These data provided the suggestion, albeit not formal proof, that loss of thymic output is associated with aging and mortality. In addition, animal models showed that thymic transplant in old mice can rejuvenate several

physiological functions as well as increase mice life span (Basso et al. 2010). The description of the sj/ β -TREC ratio (Dion et al. 2004; Ferrando-Martínez et al. 2010), by overcoming the major limitations of previous thymic function-related markers (Hazenbergh et al. 2000; Harris et al. 2005), allows us for the first time to faithfully assess the potential implication of thymic function in elderly human survival. Our results show that thymic function-related deterioration of the adaptive response and systemic inflammation act independently by affecting human survival. Despite the well-described age-related atrophy (Chiodi 1940; Henry 1967), we have previously shown that thymic function in elderly humans is widely heterogenic, and the thymus that remains functional in elderly individuals has a direct role in the peripheral T cell pool maintenance (Ferrando-Martínez et al. 2009). In addition, a higher elderly thymic function correlates with younger and better-preserved peripheral naive T cell subset (Ferrando-Martínez et al. 2011). Thus, lymphoepithelial islets that can be detected in elderly individuals seem to have a satisfactory function able to still rejuvenate the peripheral T cell subset, therefore positively impacting on elderly survival.

Immunosenescence has been related with several comorbidities, such as cardiovascular risk (Cesari et al. 2003; Csiszar et al. 2003), cancer (Shurin et al. 2007; Sansoni et al. 2008), or poor response to vaccines (Hainz et al. 2005; Weinberger et al. 2008). Despite the fact that mechanisms are still partially known, T cell exhaustion and systemic inflammation are depicted as important players. In order to be stricter and have a broader sight, in this study, we have analyzed all-cause mortality instead specific comorbidities. Thus, potentially non-related deaths, such as accidental deaths, are not discarded from our database. Later studies are needed to determine the impact of thymic function failure and CRP raise in age-related particular comorbidities.

Lastly, we wondered whether the two markers determined (thymic function and CRP) could be used to identify individuals at risk in the elderly population studied. By combining these two markers, we have defined the CRT index. Mortality percentages increased and time to death decreased by augmenting the score of our index. Since all variables were only calculated on baseline, whether individuals showing a low or intermediate risk had changed their status during the follow-up by entering the high-risk status prior to death or had died without risk change should be determined. The results obtained with this CRT index, despite being interesting for gerontology practice, present some limitations that should be taken into account. First of all, the number of subjects analyzed ($n=151$) is not large enough to split the cohort in two different cohorts to make a clinical validation. Thus, larger studies with an independent cohort should be made to ratify these results. Moreover, despite the fact that CRP measurements are easily performed (in both hospitals or primary care centers), the thymic function measurement tool (the sj/ β -TREC ratio) is, up to date, a research tool. However, the simplified protocol defined by our group (Ferrando-Martínez et al. 2010) made this technique approachable by any laboratory developing usual PCR measurements and could be easily implemented to accurately monitor the adaptive immune response decline in daily gerontology practice.

All things considered, our results show the direct role of the thymus in human survival. Moreover, CRP increased levels, a conservative marker of inflammation, are also a marker of mortality in the healthy elderly, in a thymic-independent way. These results can be of great importance in the design of innovative therapies, as thymic function preservation or improvement and anti-inflammatory strategies, capable of improving elderly quality of life through immune system rejuvenation.

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