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BRIEF REPORT

Characterization of senescence biomarkers in rheumatoid arthritis: relevance to disease progression

Laura E. Petersen[1] · Jaqueline B. Schuch[1,2] · Lucas A. de Azeredo[3] · Talita S. A. Baptista[4] · Julia G. Motta[1] · Aline D. do Prado[5] · Moisés Evandro Bauer[1,2]

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Abstract

Rheumatoid arthritis (RA) has been associated with early senescent features. However, the effects of disease progression on senescence markers are largely unknown. Here, we evaluated key senescence markers in RA, including telomere length and T cell differentiation stages as well as cytomegalovirus (CMV) serology, previously associated with premature aging. In a cross-sectional study, 44 patients with active (Ac-RA), 26 patients with controlled (Co-RA), and 30 healthy controls were recruited. Peripheral blood was collected and differentiation stages of T cells analyzed by multi-color flow cytometry. Enzyme-linked immunosorbent assays were used to evaluate the CMV serology. The telomere length was measured by multiplex quantitative PCR. Patients with Ac-RA presented lower percentage of intermediate-differentiated T cells (CD4+CD27-CD28+ and CD8+ CD27-CD28+; $p < 0.001$). All patients had a reduced proportion of cytotoxic T cells, and higher CD4/CD8 ratio compared with controls ($p < 0.001$). A lower proportion of CMV IgG+ subjects was found in the Co-RA group, ($P < 0.001$), although no differences in the CMV IgG titers were observed between groups. The groups had similar leukocyte telomere length. In addition, age was negatively correlated with CD8+CD27+CD28+ T (early-differentiated) cells ($P < 0.05$). Positive correlations between CMV IgG titers and age ($P < 0.05$) and CD4+CD27-CD28- T (late-differentiated) cells ($P < 0.01$) were observed. Furthermore, disease duration was correlated with CD4+CD27+CD28+ T cells ($r = -0.318$, $p < 0.05$) and CD4+CD27-CD28- T cells ($r = 0.308$, $p < 0.05$). Our findings indicate that CMV and age may have a similar impact on T cells in both RA patients and controls.

Key Points

- *Patients and controls were homogenous regarding CMV IgG titers and TL.*
- *A lower proportion of CMV IgG+ subjects was found in the Co-RA group.*
- *Anti-CMV levels were positively correlated with age and percentage of CD4+CD27-CD28- (late-differentiated) T cells.*

Keywords Cytomegalovirus · Differentiation of T cells · Disease activity · RA · Telomere length

Laura E. Petersen and Jaqueline B. Schuch contributed equally to this work.

✉ Moisés Evandro Bauer
mebauer@pucrs.br

[1] Laboratory of Stress Immunology, School of Sciences, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Av. Ipiranga 6681, Porto Alegre, RS 90619-900, Brazil

[2] Graduate Program in Biomedical Gerontology, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, Brazil

[3] Graduate Program in Medical and Health Sciences, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, Brazil

[4] Developmental Cognitive Neuroscience Laboratory (GNCD), Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, Brazil

[5] Rheumatology Service, Nossa Senhora da Conceição Hospital – Grupo Hospitalar Conceição (GHC), Porto Alegre, Brazil

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease associated with increased morbidity and mortality rates [1]. The disease progression has been associated with several age-related features, including accelerated aging of the immune system (immunosenescence), poor cognition, and increased prevalence of age-related pathologies [2, 3].

Previous studies have reported several immunosenescence changes in RA. These changes included accelerated thymic involution, increased clonal expansions of circulating T cells, loss of the T cell CD28 costimulatory receptor, and shortened cellular telomeres that are all key hallmarks of accelerated aging [4, 5]. In aging studies, human cytomegalovirus (CMV), an asymptomatic β-herpes virus infection, has been shown to play a role in driving the expansion of senescent T cells (CD8+CD28-) and implicated with reduced T cell receptor (TCR) repertoire, and increased plasma levels of pro-inflammatory cytokines (e.g., IL-6) [6, 7]. Increasing evidence points out these aspects may contribute to the RA pathogenesis [3, 8]. Premature aging could be an important trigger to the disease onset or associated with the disease progression, since the incidence of RA increases with age [3]. Although many hallmarks of aging have been reported in adults with RA, the role of disease progression on these markers is largely unknown.

In this study, we investigated the senescence markers in RA considering the following disease activity: controlled (Co-), active (Ac-)RA, and healthy controls. We hypothesized that Ac-RA subjects would have more senescent T cells with shorter telomeres as compared with Co-RA and healthy controls. We also speculated the CMV may have an important impact on these senescent features.

Materials and methods

Subjects

In this cross-sectional study, 102 patients were screened at the Rheumatology Unit of the São Lucas Hospital (Porto Alegre, Brazil), and 44 patients were included with Ac-RA and 26 patients with Co-RA. The diagnosis was performed accordingly to the American College of Rheumatology classification criteria by well-trained rheumatologists and Disease Activity Score (DAS)-28 criteria was used the categorized patients in Co- (DAS-28 < 3.2) and Ac-RA (DAS-28 > = 3.2). The control group ($n = 30$) included healthy individuals recruited from local community by convenience. No individual in the control group reported any health-related problem. Both patients and healthy individuals have similar age and education level. The exclusion criteria included illiteracy, daltonism, anemia, neoplasias, infections, diabetes, cardiovascular disease, dementia, brain trauma, high dose of glucocorticoids (> 10 mg/ml), and treatment with biological agents. All participants provided written informed consent before inclusion (in accordance with declaration of Helsinki), and the study was approved by the Ethical Research Committee of PUCRS.

Blood collection and immunophenotyping of peripheral blood mononuclear cells

Peripheral blood was collected and peripheral blood mononuclear cells (PBMCs) were isolated accordingly to previous work [9]. PBMCs were stained with a combination of monoclonal antibodies: antiCD3, anti-CD4, anti-CD8, anti-CD27, anti-CD28 (all from BD Biosciences, San Jose, USA) to discriminate different stages of T cell differentiation by the following criteria: CD27+CD28+ (early differentiated), CD27−CD28+ (intermediate-differentiated), and CD27−CD28− (senescent cells) [9]. The gating strategy is shown in Fig. 1. A minimum of 20,000 lymphocytes was identified by size (FSC) and granularity (SSC) and acquired using a FACS Canto II flow cytometer (BD Biosciences). Data were analyzed using the FlowJo V.10 software (Tree Star Inc., Ashland, USA).

Cytomegalovirus serology

Plasma samples were analyzed for both IgM and IgG antibodies anti-CMV using enzyme-linked immunosorbent assays (ELISAs; Euroimmun – Lübeck, Germany). Sensitivity and specificity were estimated in 100%. The cutoff of 1 OD and 22 IU/mL were considered positive (reactive) for IgM and IgG antibodies anti-CMV, respectively. Detection limit were 0.05 OD (IgM) and 0.4 IU/mL (IgG).

DNA isolation and telomere length (TL) analysis

Genomic DNA was extracted from peripheral blood using the PureLink Genomic DNA kit (Invitrogen, Carlsbad, California, USA) following the manufacturer's instructions and stored at − 20 °C. NanoDrop™ One Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to set the DNA concentration for each sample in 50 ng/μl. The TL was measured via multiplex quantitative PCR (*qPCR*) method in Rotor-Gene Q (Qiagen, Hilden, Germany) following guidelines described by Cawthon [10]. A standard curve was used to determine the ratio of the telomere (T) repeat copy number to a single-copy gene (S) copy number (i.e., the albumin gene). The T/S ratio is proportional to the average TL. The standard curve included 5-point serial dilution of reference DNA from a single person, ranging from 100 to 6.25 ng. Ct values for the amplification of the telomere

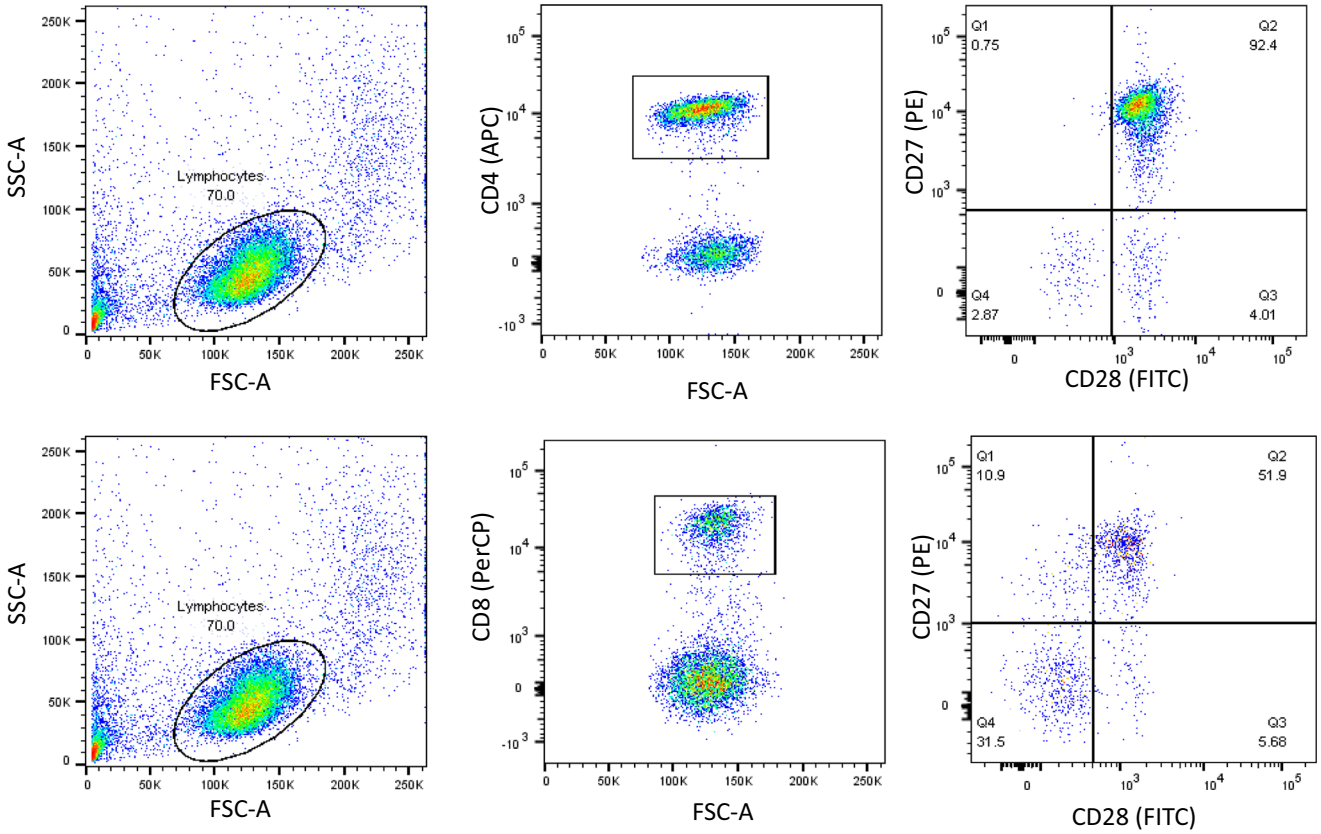

Fig. 1 The gating strategy used to discriminate different stages of T cell differentiation: CD27+CD28+ (early differentiated), CD27−CD28+ (intermediate-differentiated), and CD27−CD28− (senescent cells) [9]

template were provided at 74 °C and for albumin at 88 °C. Melting curve analysis was used to verify specificity of product synthesis.

Statistical analyses

All variables were tested for normality of distribution using Shapiro–Wilk test. The comparison of clinical and demographic data between the three groups (controls, Co-RA, and Ac-RA) was performed using chi-square (χ^2) or ANOVA. Generalized linear modeling (GzLM) was used to analyze immunosenescence markers between the three groups adjusting for potential confounders (sex). Linear or gamma distribution was selected based on the distribution outcome. Bonferroni pairwise multiple correction test was used to compare means between groups. Exploratory correlation analyses were performed to evaluate the relationship among age and senescence markers using Pearson or Spearman tests. Statistical Package for Social Sciences, SPSS Statistics V.20 software (SPSS Inc., Chicago, IL, USA) was used to conduct statistical analyses. The significance level was set at $\alpha = 0.05$. Figures were plotted in GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Demographic and clinical data are shown in Table 1. Patients with RA and controls were similar regarding age, education level, and smoking data. Immunophenotyping analyses are depicted in Table 2. A lower percentage of intermediate-differentiated T cells (CD4+CD27-CD28+ and CD8+CD27-CD28+) was found in patients with Ac-RA ($p < 0.001$). In addition, a reduced proportion of cytotoxic T cells and higher CD4/CD8 ratio was found in patients with Co- and Ac-RA ($p < 0.001$).

None of the subjects had IgM reactivity to CMV, excluding acute viral infection. Similar CMV IgG titers were observed among patients and controls (Fig. 2a). However, the Co-RA group had a lower proportion CMV IgG+ subjects (50%) as compared with healthy controls (96.7% of controls) or Ac-RA (86.5%), $\chi^2 = 19.8$, $p < 0.001$ (Fig. 2b). No differences in TL measures were detected between controls, Co- and Ac-RA groups ($p = 0.533$; Fig. 2c).

We next explored the relationships between age, clinical data, and immunosenescence markers. It was found a negative correlation between age and percentage of CD8+CD27+CD28+ T (early-differentiated) cells ($r = -0.241$, $P = 0.031$; Fig. 3a). In addition, the anti-CMV levels were positively correlated with age ($r = 0.235$, $P = 0.026$; Fig. 3b) and

Table 1 Demographic and clinical data

	Controls ($n = 30$)	Controlled-RA ($n = 26$)	Active-RA ($n = 44$)	E	*P* value
Males[a]	7 (23.3)	13 (50.0)	6 (13.7)	$\chi^2 = 11.390$	**0.003**
Age, years[b]	56.20 (9.8)	57.72 (6.7)	55.37 (11.0)	$F = 0.460$	0.632
Disease duration[c]	–	5.00 (14.0)	5.00 (8.0)	$Z = -0.146$	0.884
Schooling[b]	8.87 (3.6)	8.77 (4.2)	7.53 (3.7)	$F = 1.367$	0.260
Smoking (yes)[a]	4 (13.3)	6 (23.1)	6 (13.6)	$\chi^2 = 1.310$	0.519
DAS-28[c]	–	2.77 (0.6)	4.66 (1.5)	$Z = -6.763$	**< 0.001**
RF+[a]	–	13 (50)	21 (47.7)	$\chi^2 = 0.034$	0.854
Treatment					
Methotrexate, *n* (%)	–	23 (88.5)	34 (77.3)	$\chi^2 = 1.353$	0.245
Dosage, mg/day		18.69	20.74	$F = 1.986$	0.109
Glucocorticoids, *n* (%)		10 (38.5)	31 (70.5)	$I^2 = 6.894$	**0.009**
Dosage, mg/day		4.18	6.20	$F = 1.096$	**0.045**
Sulfasalazine, *n* (%)		0 (0)	5 (11.4)	–	–
Dosage, mg/day		–	1800.00	–	–
Leflunomide, *n* (%)		6 (23.1)	20 (45.5)	$\chi^2 = 3.505$	0.061
Dosage, mg/day		15.00	19.50	$F = 23.611$	0.101
Hydroxychloroquine, n (%)		5 (19.2)	9 (20.5)	$\chi^2 = 0.015$	0.902
Dosage, mg/day		400.00	355.55	$F = 9.600$	0.169

Significant *p* values are highlighted in bold

RA Rheumatoid arthritis, *DAS-28* Disease Activity Score, *RF* Rheumatoid factor

[a] *N* (%), [b] mean (standard deviation), or [c] median (interquartile range)

percentage of CD4+CD27-CD28- T (late-differentiated) cells ($r = 0.313$, $P = 0.008$; Fig. 3c). Considering disease duration, significant correlations were found with percentage of CD4+CD27+CD28+ T cells ($r = -0.318$, $p = 0.030$; Fig. 3d) and percentage of CD4+CD27-CD28- T cells ($r = 0.308$, $p = 0.035$; Fig. 3e). No correlations were found between the remaining clinical and TL/immune variables.

Discussion

Here, we explored for the first time the possible connection between disease severity and senescence markers in RA. The senescence markers included the analysis of PBMC telomere length (TL) and evaluation of different stages of the T cell differentiation.

Table 2 Immunophenotyping of lymphocyte subsets

Markers (%)	Cell type	Controls	Controlled-RA	Active-RA	Statistics (Wald)	*P* value
CD3+CD4+	Th	47.52 (1.2)	51.12 (2.3)	50.79 (1.3)	4.374	0.112
CD4+CD27+CD28+	Early-differentiated T cell	82.13 (1.2)	84.45 (1.4)	85.92 (0.9)	5.404	0.067
CD4+CD27-CD28+	Intermediate-differentiated T cell	7.66 (0.4)[a]	6.21 (0.7)	5.44 (0.4)[b]	15.992	**< 0.001**
CD4 + CD27-CD28-	Late-differentiated T cell	8.64 (0.8)	7.27 (1.2)	6.6 (0.8)	3.367	0.186
CD3+CD8+	Tc	25.27 (1.0)[a]	19.89 (1.8)[b]	19.76 (1.6)[b]	12.242	**0.002**
CD8+CD27+CD28+	Early-differentiated T cell	55.81 (3.1)	48.72 (4.3)	46.69 (2.7)	4.871	0.088
CD8+CD27-CD28+	Intermediate-differentiated T cell	5.94 (0.4)[a]	6.65 (0.7)[a]	4.18 (0.3)[b]	14.176	**0.001**
CD8+CD27-CD28-	Late-differentiated T cell	30.73 (3.1)	36.80 (4.6)	37.38 (2.8)	2.777	0.249
CD4/CD8	Ratio	1.96 (0.1)[a]	3.15 (0.4)[b]	2.65 (0.2)[b]	19.819	**< 0.001**

Data were analyzed by Generalized Linear Modeling test (gamma or linear distribution) adjusted for age and sex. Mean (Standard Error)

[a,b] Indicate differences between groups (Bonferroni post hoc test) and significant *p* values are highlighted in bold

Th helper T cell; *Tc* cytotoxic T cell

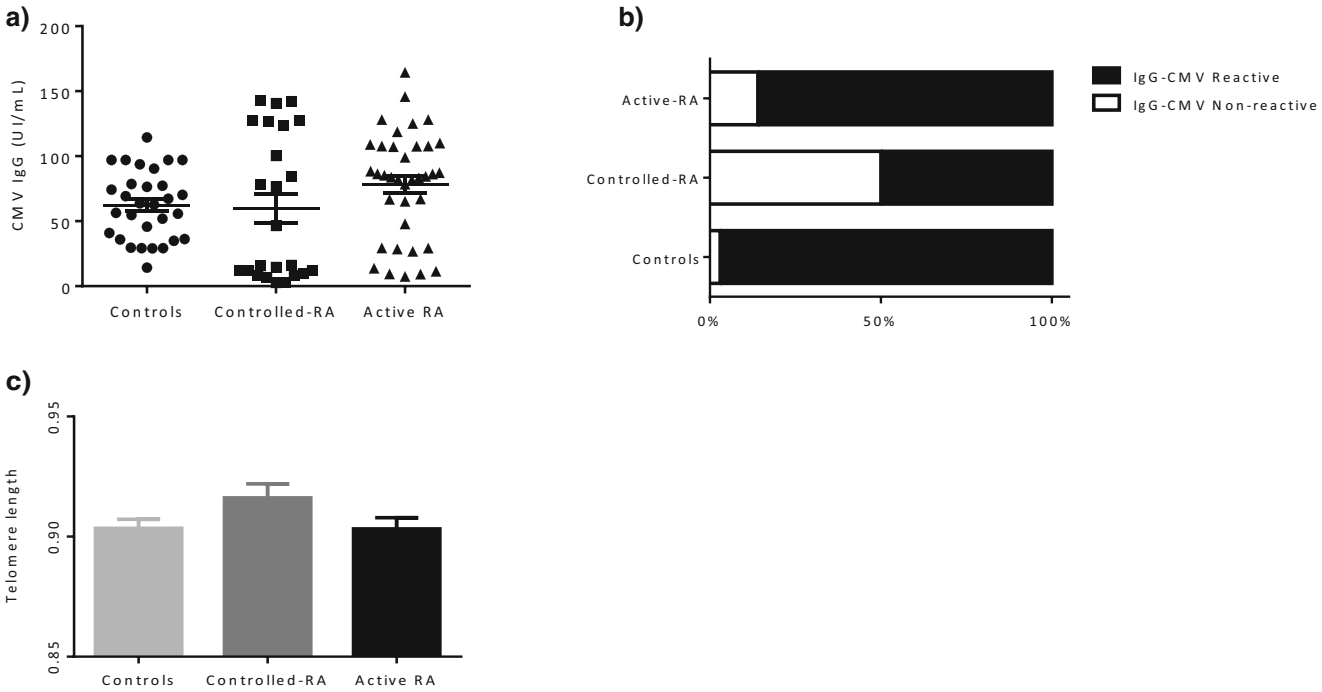

Fig. 2 Senescence markers analyzed in controls, controlled-RA and active-RA groups. **a** The IgG anti-cytomegalovirus (CMV) levels. Wald = 1.299, *P* value = 0.522, **b** percentage of individuals CMV IgG+ in each group. $\chi^2 = 19.843$, *P* value < 0.001 **c** Telomere length analysis. Wald = 1.258, *P* value = 0.533

A reduced proportion of intermediate-differentiated T cells (CD27-CD28+) and cytotoxic T cells were observed in RA patients, especially in those with active disease. Cytotoxic T cells play roles in the host defense against viruses and some bacteria, limiting such infections, and also exert cytotoxic effects on tumor cells [11]. Furthermore, the reduction of CD27-CD28+ T cells might be a compensatory modification associated with the decrease of CD8+ T cells, since those cells present proinflammatory and cytotoxic profile. In this sense, the reduction of these cells could increase susceptibility to infections and cancer, and functional studies should be performed to further evaluate this link.

The healthy immunosenescence involves thymic involution and remodeling changes in peripheral lymphocytes, characterized by depletion of naïve T cells, increase of late-differentiated and memory T cells, and reduction in the diversity of the TCR (T cell receptors) [12]. During aging and chronic viral infections, the T cell costimulatory molecule CD28 is lost, interfering in cellular signaling and functional aspects of these cells [13]. The late-differentiated T cells increase the production of pro-inflammatory cytokines and cytotoxicity through the expression of perforin and granzymes [3]. Over the years, the expansion of CD28- T cells has been reported in RA [14–17]. Here, no differences between groups were observed for this cellular subset. However, patients with longer disease duration presented lower percentage de CD28+ T cells and expansion of CD28- T cells. The CMV serology was investigated here, as previous aging studies indicated that CMV is associated with accelerated immunosenescence—of note in T cells [6]. Increasing evidence has indicated that expansions of CD28- T cells occur more often in individuals infected by CMV. In fact, in RA and other chronic inflammatory diseases, this specific expansion was driven by the presence of CMV infection [18–21]. Concurring with the lack of changes in late-differentiated T cells (CD27-CD28-), the RA groups and controls had similar CMV IgG titers. In spite of no clinical association, the CMV IgG levels were positively correlated to age and CD4+ CD27-CD28- T (late-differentiated) cells. The CMV infection may interfere with the cellular aging process, promoting chronic replicative stress, inducing the IFN-α expression by dendritic cells and by promoting telomere shortening [6]. Our data considering the correlations among IgG anti-CMV, age, and early and late-differentiated T cells might support this hypothesis, independently of the clinical status. In other words, the CMV and age may have a similar impact on T cell senescence program between RA patients and healthy controls. This should be further confirmed by longitudinal studies.

In addition, telomere shortening is considered a hallmark of aging and biological clock in humans. Telomeres are DNA-protein complexes, characterized by TTAGGG-rich repeats at the ends of chromosomes. Shortening of the TL occurs during cell division and reflects the replicative history of cells [22]. A meta-

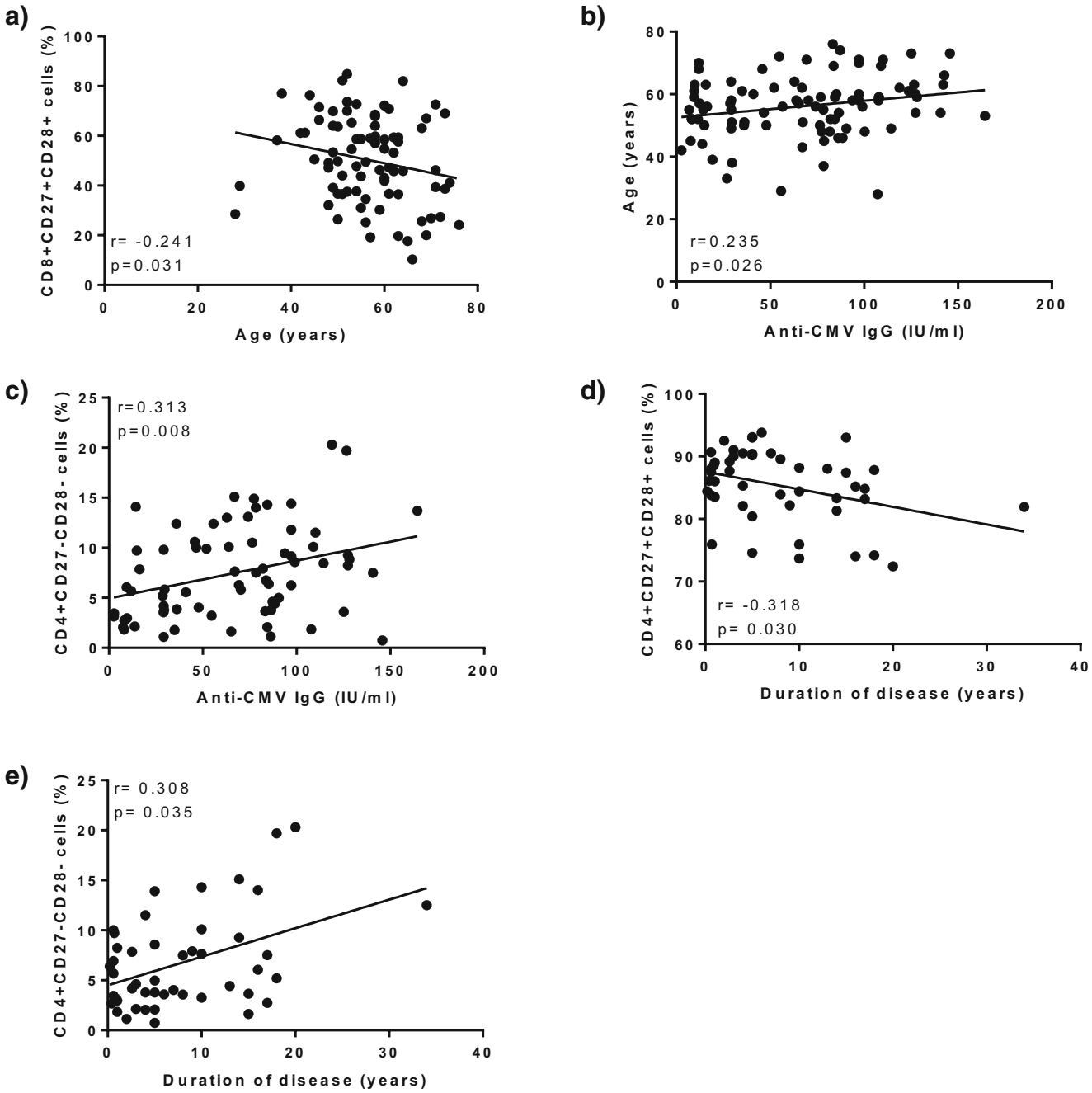

Fig. 3 Correlations of clinical and laboratories data in RA patients and control

analysis indicated that shortened TL was associated with RA [5], although some studies did not find such association [5, 23, 24], in accordance with our findings. The clinical status was not associated with TL in our study.

Our findings should be considered in the perspective of some limitations. As mentioned above, senescence markers are influenced by many characteristics. The relative sample size and low statistical power could prevent us to explore other potential associations. For instance, CMV serology can be considered an important factor for immunosenescence, and deeper analyses comparing CMV seropositive and seronegative individuals could clarify the possible role of this marker in the RA. However, the limited sample size prevented us from finding such potential associations. In addition, the studied clinical groups were quite homogenous regarding the immune variables and we speculate that accelerated senescence may be a feature of patients with more aggressive disease progression. This should be explored by future studies. Finally, it should be mentioned that no information was gathered here concerning the involvement of extra-articular manifestations or acute phase proteins.

In conclusion, we report changes in the percentage of intermediate-differentiated T cells (CD27-CD28+) and cytotoxic T cells in Ac-RA compared with Co-RA and controls. In addition, anti-CMV levels were positively correlated with age and percentage of CD4+CD27-CD28- T (late-differentiated cells).

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Compliance with ethical standards

The institutional review board approved this study and all patients provided written informed consent for participation.

Disclosures None.

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