

# Serum thymosin $\alpha$ 1 levels in patients with chronic inflammatory autoimmune diseases

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## Introduction

Thymosin alpha 1 (T $\alpha$ 1) is a naturally occurring thymic peptide of 28 amino acids described by Goldstein and Coll [1]. It derives from the N-terminus tract of Prothymosin  $\alpha$  (ProT $\alpha$ ) that is cleaved by legumain, a lysosomal asparagine endopeptidase also present in mammals [2]. Both T $\alpha$ 1 and legumain share a wide distribution in different tissues, suggesting that the ProT $\alpha$  processing to yield T $\alpha$ 1 represents a generalized process in mammals [3]. Lymphoid tissues show high legumain and T $\alpha$ 1 levels, which argue for an important biological function of the peptide in this context.

## Summary

Thymosin alpha 1 (T $\alpha$ 1) is a powerful modulator of immunity and inflammation. Despite years of studies, there are a few reports evaluating serum T $\alpha$ 1 in health and disease. We studied a cohort of healthy individuals in comparison with patients affected by chronic inflammatory autoimmune diseases. Sera from 120 blood donors (healthy controls, HC), 120 patients with psoriatic arthritis (PsA), 40 with rheumatoid arthritis (RA) and 40 with systemic lupus erythematosus (SLE), attending the Transfusion Medicine or the Rheumatology Clinic at the Policlinico Tor Vergata, Rome, Italy, were tested for T $\alpha$ 1 content by means of a commercial enzyme-linked immunosorbent assay (ELISA) kit. Data were analysed in relation to demographic and clinical characteristics of patients and controls. A gender difference was found in the HC group, where females had lower serum T $\alpha$ 1 levels than males ( $P < 0.0001$ ). Patients had lower serum T $\alpha$ 1 levels than HC ( $P < 0.0001$ ), the lowest were observed in PsA group ( $P < 0.0001$  versus all the other groups). Among all patients, those who at the time of blood collection were taking disease-modifying anti-rheumatic drugs (DMARD) plus steroids had significantly higher T $\alpha$ 1 levels than those taking DMARD alone ( $P = 0.044$ ) or no treatment ( $P < 0.0001$ ), but not of those taking steroids alone ( $P = 0.280$ ). However, whichever type of treatment was taken by the patients, serum T $\alpha$ 1 was still significantly lower than in HC and there was no treatment-related difference in PsA group. Further prospective studies are necessary to confirm and deepen these observations. They might improve our understanding on the regulatory role of T $\alpha$ 1 in health and disease and increase our knowledge of the pathogenesis of chronic inflammatory autoimmune diseases.

**Keywords:** autoimmune diseases, psoriatic arthritis, rheumatoid arthritis, systemic lupus erythematosus, thymosin  $\alpha$  1

T $\alpha$ 1 plays a key role in the control of immunity, tolerance and inflammation [4,5]. It regulates immune response via a primary action on the cells of the innate immune system and thus acts as an endogenous regulator of both inflammatory and adaptive immune responses [5]. T $\alpha$ 1-induced effects are context-dependent [5]. Consistently, we showed that T $\alpha$ 1 administration increased natural killer (NK) activity in mice immunosuppressed by cancer and/or cyclophosphamide but not in normal mice [6].

T $\alpha$ 1 use in the therapy of diseases associated with immune dysfunction, namely hepatitis B virus (HBV) and hepatitis C virus (HCV), some types of cancer, severe sepsis and as an adjuvant for vaccine enhancement [7], relies

upon its ability to target different cells. Recent studies by nuclear magnetic resonance (NMR) spectroscopy reinforce this assumption and provide a model mechanism of action for which T $\alpha$ 1 undergoes a direct interaction with peculiar portions of cell membranes and then behaves as an activator of biological cascade(s) [8].

Arthritis and other rheumatic diseases share severe immune imbalances and abnormal release of mediators, resulting in damage to organs and systems. Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovitis, leading to destruction of cartilage and bone, functional limitation and disability [9]. Psoriatic arthritis (PsA) is a chronic inflammatory arthritis associated closely with psoriasis, which differs from RA in both laboratory and clinical features [10]. Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by inflammation and tissue damage due to circulating autoantibodies and immune complexes depositing in different tissues [11]. The aetiology of such diseases is still uncertain, although the role of genetic and epigenetic factors has been emphasized [12]. The pathophysiology of RA, PsA and SLE implies an intricate cytokine network participating in inflammation and in perpetuation of disease by positive feedback loops including abnormal T cell signalling and unbalanced T helper type 17 (Th17)/regulatory T cells (T<sub>regs</sub>) ratio (but may involve the overall cytokine milieu), thus promoting systemic disease [13–15].

Given the known regulatory activity of T $\alpha$ 1 on immunity and inflammation, we have analysed a large cohort of healthy individuals in comparison with patients affected by PsA, RA or SLE, looking for a possible correlation, if any, between serum T $\alpha$ 1 levels and such diseases.

## Methods

### Patients and study design

We carried out a retrospective analysis on sera from 120 patients presenting with PsA, diagnosed according to the Classification Criteria for Psoriatic Arthritis (CASPAR) [16], 40 with RA and 40 with SLE, diagnosed according to the American College of Rheumatology (ACR) revised criteria [17,18]. All of them were out-patients at the Rheumatology Clinic, Policlinico Tor Vergata, Rome, Italy. Sera from 120 consecutive blood donors of the Transfusion Medicine and Immunohaematology Section (SIMT) at the same institution served as healthy controls (HC).

The study was performed according to the Declaration of Helsinki and in accordance with the International Conference on Harmonization Good Clinical Practice Guidelines [ICH-GCP E6 (R2)]. The Ethical Committee of the University of Rome Tor Vergata approved the study protocol. All patients and controls provided written informed consent before participating in any study-related activities.

Individual medical histories, laboratory and/or clinical data at the time of blood sampling were recorded in database files and were gathered anonymously for research purpose. The clinical evaluation of patients was performed by using the Disease Activity Score (DAS) 44 and C-reactive protein levels (CRP, 0–3 mg/l) [19], or the SLE Disease Activity Index 2000 (SLEDAI-2K) [20], where appropriate.

### Laboratory assays

Peripheral blood collection for routine laboratory tests was performed in patients and controls at the time of medical examination or at blood donation, respectively. Serum aliquots, obtained from peripheral blood by standard methods, were frozen at  $-80^{\circ}\text{C}$  immediately after collection and not thawed until use for the purposes of this research.

The quantitative determination of serum T $\alpha$ 1 was performed by a competitive enzyme-linked immunosorbent assay (ELISA) using a commercial kit (T $\alpha$ 1 ELISA kit; Immundiagnostik AG, Bensheim, Germany), according to the manufacturers' instructions. Plates were read at an optical density of 450 nm on an ELISA reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA).

### Statistical analysis

Statistical analysis was performed using the statistical environment R (version 3.2.5) [21]. The Kruskal–Wallis rank sum test and the Mann–Whitney *U*-test were used for statistical comparisons among patient groups. All tests were two-sided and a *P*-value  $< 0.05$  was considered statistically significant. All *P*-values for multiple pairwise comparisons were adjusted by using Benjamini and Hochberg correction [22].

## Results

### Characteristics of the study population

Table 1 summarizes demographic and clinical characteristics of the study subjects. Overall, our study population consisted of 320 Caucasian individuals (133 males and 187 females) aged 18–72 years.

The group of patients consisted of 200 individuals (69 males and 131 females) aged 19–72 years, diagnosed clinically as RA, PsA or SLE according to the criteria specified in Methods. The HC group consisted of 120 consecutive blood donors (64 males and 56 females) aged 18–62 years. As shown in Table 1, the two major groups, namely HC and PsA, exhibited a similar gender prevalence, whereas the two smaller groups, i.e. RA and SLE, were composed mainly of female individuals, as expected based on the epidemiology of these diseases [23].

At the time of blood collection, the patients had active disease, as assessed by calculating DAS44 or SLEDAI-2K scores (Table 1), and most of them were receiving

**Table 1.** Demographic and clinical characteristics of patients and healthy controls

	HC	RA	PsA	SLE
Number	120	40	120	40
Gender female/male	56/64	33/7	62/58	36/4
Age (years)	41 (18–62)	57 (38–70)	52 (19–70)	55 (25–72)
Disease Activity Score/Index				
DAS44*	n.a.	4.7 (2.7–7.3)	4.9 (2.1–8.1)	n.a.
SLEDAI-2K <sup>†</sup>	n.a.	n.a.	n.a.	4 (0–82)
Treatments <sup>‡</sup>				
DMARD, <i>n</i> (%)	n.a.	14 (35)	40 (34)	16 (40)
CS, <i>n</i> (%)	n.a.	4 (10)	8 (7)	7 (17.5)
DMARD+CS, <i>n</i> (%)	n.a.	19 (47.5)	27 (23)	16 (40)
None, <i>n</i> (%)	n.a.	3 (7.5)	45 (37.5)	1 (2.5)

Data expressed as median (range) if not specified otherwise. HC = healthy controls; RA = rheumatoid arthritis; PsA = psoriatic arthritis; SLE = systemic lupus erythematosus. \*DAS44 (range = 0–10) is a continuous measure consisting of four variables: the Ritchie articular index (RAI), a 44 swollen joint count, erythrocyte sedimentation rate (ESR) and a general health (GH) assessment measured on a visual analogue scale. Level of disease activity: low ( $DAS \leq 2.4$ ), moderate ( $2.4 < DAS \leq 3.7$ ), high ( $DAS > 3.7$ ), remission ( $DAS < 1.6$ ) [19]. <sup>†</sup>SLEDAI-2K = SLE Disease Activity Index 2000. SLEDAI-2K  $\geq 4$  indicate active disease [20]. <sup>‡</sup>Concomitant treatments at the time of blood collection: DMARD = disease modifying anti-rheumatic drugs (methotrexate, sulphasalazine, hydroxychloroquine, azathioprine, cyclosporin; CS = corticosteroids (prednisone); none = no treatment; n.a. = not applicable.

treatment with disease-modifying anti-rheumatic drugs (DMARD) and/or corticosteroids (CS) (Table 1).

### Laboratory findings

Serum T $\alpha$ 1 levels varied considerably among different individuals in the HC group as well as in the patient group, and even within the same diagnostic group.

In the HC group, females had significantly lower serum T $\alpha$ 1 levels than males [T $\alpha$ 1 ng/ml median (interquartile range, IQR) = 28.74 (17.98–70.25) versus 78.96 (40.80–130.13), respectively;  $P < 0.0001$ , Fig. 1a].

The patients' serum T $\alpha$ 1 levels were significantly lower than in HC [18.38 (3.74–52.82) versus 53.08 (24.39–122.74), respectively;  $P < 0.0001$ , Fig. 1b]. Analysing data according to the clinical diagnosis (Fig. 1c), serum T $\alpha$ 1 was globally significantly different among the four groups ( $P < 0.0001$ ). In particular, serum T $\alpha$ 1 levels of PsA patients were dramatically lower than in HC [6.93 (2.05–21.27) versus 53.08 (24.39–122.74), respectively;  $P < 0.0001$ ], but also significantly lower than in RA [6.93 (2.05–21.27) versus 54.73 (18.76–115.95), respectively;  $P < 0.0001$ ] or SLE patients [6.93 (2.05–21.27) versus 41.37 (29.34–113.12), respectively;  $P < 0.0001$ ]. Serum T $\alpha$ 1 levels of RA or SLE patients were not significantly different from those of HC, although showing a trend towards lower values, especially in SLE.

Among all patients (Fig. 1d), serum T $\alpha$ 1 was once again globally significantly different among the treatment groups ( $P < 0.0001$ ). In particular, the patients who at the time of blood collection were taking DMARD plus CS, had higher T $\alpha$ 1 levels than those who were taking DMARD alone [29.87 (15.36–63.84) versus 10.87 (2.62–60.08), respectively;  $P = 0.044$ ] or no treatment [29.87 (15.36–63.84)

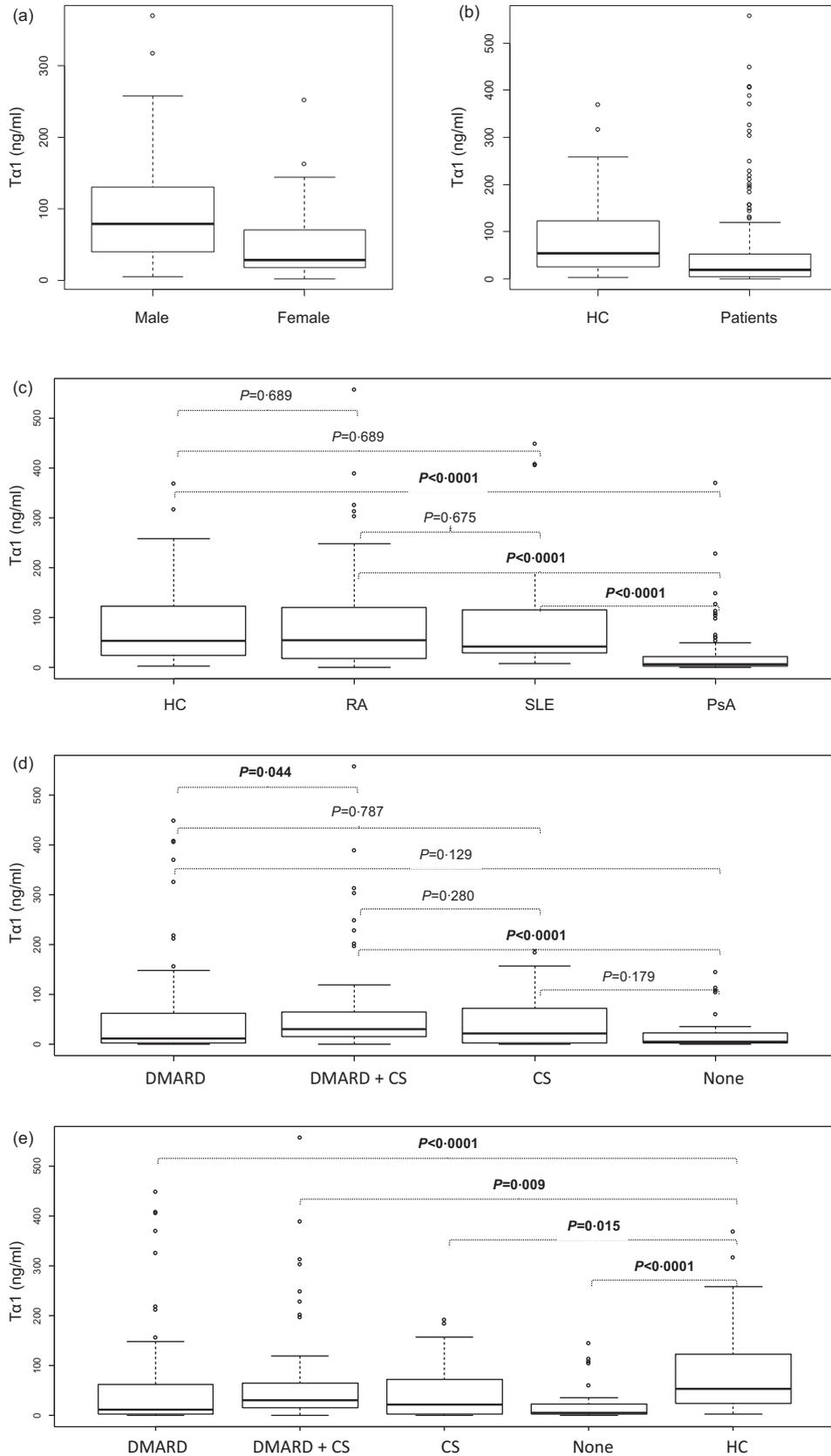
versus 4.56 (2.50–22.13), respectively;  $P < 0.0001$ ], but not of those taking steroids alone ( $P = 0.280$ ; Fig. 1d). However, serum T $\alpha$ 1 of patients taking any of the treatment types was still significantly lower than in HC (Fig. 1e), and there was no treatment-related difference in the PsA group (data not shown).

Finally, no relevant abnormalities of blood cells count, haemoglobin, cholesterol and albumin were observed (Table 2). The median ESR and CRP were high in RA patients and within the normal range for SLE or PsA patients. No significant correlation was found between T $\alpha$ 1 levels and blood cell counts or blood chemistry profile, except than a trend in ESR ( $P = 0.073$ ).

### Discussion

To the best of our knowledge, this is the first study where T $\alpha$ 1 serum levels of patients with chronic inflammatory autoimmune diseases have been analysed in relation to their demographic, clinical and laboratory characteristics and in comparison with a large cohort of healthy individuals.

The rationale of our study stems from the consideration of the potential role of malfunctioning T<sub>reg</sub> cells in chronic inflammatory immune and autoimmune diseases and from the notion that T $\alpha$ 1 is a natural circulating hormone peptide capable of influencing many components of the inflammatory/autoimmune cascade at a time [5]. Indeed, although being capable of activating adaptive and innate immunity, including dendritic cells (DC), T $\alpha$ 1 can also attenuate the immunogenic/inflammatory activity of myeloid DCs through an indoleamine 2,3-dioxygenase (IDO)-dependent pathway [24]. This finding qualifies T $\alpha$ 1 as a unique immune regulatory and pleiotropic peptide capable



**Fig. 1.** Data are shown as box-plots, where each box represents the 25th–75th percentiles. Lines inside the box represent the median value. *P*-values were calculated by Mann–Whitney *U*-test (comparisons between two groups) or Kruskal–Wallis rank sum test (comparisons between more than two groups). All *P*-values for multiple pairways comparisons were adjusted using Benjamini and Hochberg correction [22]. Box-plots represented serum thymosin alpha 1 (Tα1) levels according to gender in HC (a), to HC and patients (b), to the clinical diagnosis (c), to the treatment (d) and by comparing HC towards all treatment groups (e). HC = healthy controls; PsA = psoriatic arthritis; RA = rheumatoid arthritis; SLE = systemic lupus erythemathosus; DMARD = disease modifying anti-rheumatic drugs; CS = corticosteroids; none = no treatment.

**Table 2.** Laboratory characteristics of patients and healthy controls

Tests	HC	RA	PsA	SLE
Hb (g/dl)	14.8 (10.9–15.60)	13.0 (7.6–15.7)	13.8 (8.6–16.9)	13.3 (9.4–15.3)
WBC $\times$ 1000/ml	6.11 (3.46–9.98)	7.45 (2.99–14.3)	7.11 (0.75–16.70)	5.30 (3.40–15.2)
Lymph $\times$ 1000/ml	1.98 (1.09–4.40)	1.97 (0.93–5.43)	2.10 (1.07–3.89)	1.39 (0.61–4.10)
Neu $\times$ 1000/ml	3.31 (1.52–7.61)	4.88 (1.27–9.56)	3.99 (1.09–12.56)	2.91 (1.03–11.0)
Plt $\times$ 1000/ml	228 (139–235)	266 (206 – 569)	253 (138–471)	223 (93–384)
Albumin (g/dl)	4.60 (3.80–5.37)	4.07 (3.27–4.69)	4.29 (3.42–4.83)	4.01 (3.83–4.72)
Cholesterol (mg/dl)	184 (117–323)	205 (179–266)	206 (146–291)	208 (175–287)
CRP (mg/l)	n.a.	5.5 (0.0–58.3)	2.62 (0.0–46.0)	0.30 (0.0–19)
ESR (mm/h)	n.a.	32 (2–120)	16 (2–75)	21.5 (2–84)

Data expressed as median (range). HC = healthy controls; RA = rheumatoid arthritis; PsA = psoriatic arthritis; SLE = systemic lupus erythematosus; Hb = haemoglobin; WBC = white blood cells; Lymph = lymphocytes; Neu = neutrophils; Plt = platelets; CRP = C-reactive protein, normal values (0–3 mg/l); ESR = erythrocyte sedimentation rate, normal values (0–30 mm/h); n.a. = not applicable.

of the fine-tuning and control of the quality of immune response. The hypothetical benefits of using thymic preparations in autoimmune diseases had not already been proposed [25], but information on the circulating levels of thymic hormones in such patients was lacking.

Here we show, for the first time to our knowledge, that healthy female individuals have significantly lower T $\alpha$ 1 serum levels than the males. This evidence adds to other gender differences reported in the literature, i.e. a more intense anti-viral response observed in females which induces rapid virus clearance, but if excessively high or prolonged can result in chronic and/or inflammatory diseases [26] or a lower production of the immunosuppressive cytokine IL-10 after stimulation with selected Toll-like receptor ligands or viruses [26,27]. These findings may contribute to explaining the high prevalence of autoimmune diseases in females [23].

We then show that patients affected by chronic inflammatory autoimmune diseases show a trend towards lower T $\alpha$ 1 serum levels than in HC. In particular, PsA patients present with the lowest T $\alpha$ 1 levels, which are dramatically lower than in HC but also significantly lower than in RA and SLE patients. This finding is not surprising, as there are several differences among chronic inflammatory autoimmune diseases, not only in terms of clinical symptoms but also regarding laboratory and immunological data. As an example, despite the superficial similarity of clinical manifestations, RA and PsA show many differences at clinical, anatomical and molecular levels, conditioning different clinical response and outcome [28].

The fact that the majority of our study subjects were aged between 20 and 60 years did not allow us to find the expected correlation of T $\alpha$ 1 levels with age [29]. Similarly, the fact that our patients had active disease, and most of them were receiving treatment at the time of the blood collection, prevented us from finding significant relationships between T $\alpha$ 1 levels and disease activity scores or inflammation markers. Regarding the ESR and CRP, it is known that

normal values of both can be found occasionally in active SLE or active PsA [16,18].

In the total group of patients, the concomitant use of DMARDs and steroids had a positive impact on serum T $\alpha$ 1 levels. However, the values were always significantly lower than in HC and there were no treatment-related differences in the PsA group. In agreement with the literature, our findings thus reinforce the assumption that chronic inflammatory autoimmune diseases constitute a heterogeneous group of diseases with regard to clinical manifestations, laboratory and immunological data and therapeutic response [28]. Nevertheless, in the clinical practice they are subject to the same treatment options. Hopefully, these findings will be the object of future and larger studies, in line with recent interest in exploring the effects of various anti-rheumatic drugs, including biologicals, on immune functions in various rheumatological conditions [14].

Finally, there was no relation between T $\alpha$ 1 levels and blood cell count, haemoglobin, cholesterol and albumin, suggesting that those values should not have influenced T $\alpha$ 1 measurements. We paid attention to albumin levels, as we have reported recently that T $\alpha$ 1 uses serum albumin as a carrier [30].

The retrospective character of this research, the lack of drug-naïve patients and the quantification of T $\alpha$ 1 in a single serum sample for each individual represent important limitations of our study. Given these limitations, the present research is a preliminary exploration of the relation between serum T $\alpha$ 1 and chronic inflammatory autoimmune diseases. Our findings indicate a need of more information on the complex interplay between different cell subsets in chronic inflammatory autoimmune diseases, and also in relation to the T $\alpha$ 1 circulating levels. Indeed, the biological effects of anti-rheumatic drugs, their toxicity and the different therapeutic response, remain to be understood fully. Conversely, T $\alpha$ 1 has shown an excellent safety profile compared to other immunomodulatory agents [4,7].

Several attempts to measure serum T $\alpha$ 1 have been performed in past years [29,31,32]. Recent reports attest a renewed interest in this topic, especially in cancer, but they rely upon small numbers of subjects and the results are not univocal [33]. Interestingly in our study, performed on a large cohort of individuals, the median serum T $\alpha$ 1 level found in HC is very close to the biologically active concentration of T $\alpha$ 1, as shown in past *in-vitro* experiments [4,5,34].

Further prospective and longitudinal studies are necessary to confirm and extend our observations. They might improve our understanding of the regulatory role of T $\alpha$ 1 in health and disease and increase our knowledge on the pathogenesis of chronic inflammatory autoimmune diseases.

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### Disclosure

E. G. is a Thymosin patent holder. The other authors declare no disclosures.

### Author contributions

E. G., F. P. and R. P. conceived the study and wrote the paper; R. G., I. C. and C. B. performed the experiments and discussed the results; A. V. and D. D. C. performed statistical analyses and reviewed the paper; M. S. C., P. T. and P. C. collected and analysed the clinical data and wrote the paper; G. A. and V. C. collected and analysed blood donor data.

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