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Research article

Impaired Activation-Induced Telomerase Activity in PBMC of Early but not Chronic Rheumatoid Arthritis patients

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Abstract

Although telomerase activity is important in normal immune function, it is unclear whether telomerase or telomerase (dys)regulation plays a role in the pathogenic immune response in autoimmune diseases like rheumatoid arthritis (RA). In this study we evaluated the dynamics of the activation-induced human Telomerase Reverse Transcriptase (hTERT) response in RA patients and non-RA controls. The expression of the catalytic subunit of telomerase, hTERT, was measured in PBMC of RA patients and controls after *in vitro* stimulation with anti-CD3 monoclonal antibody (mAb) using real-time PCR. Anti-CD3 mAb stimulation induced activation and proliferation of the T cells in all populations studied. In early RA patients with a disease duration of less than a year, the activation-induced hTERT mRNA levels were found to be reduced as compared to healthy controls (HC). Chronic RA patients, with a disease duration of more than one year, did not show these impaired hTERT mRNA levels after stimulation with anti-CD3 mAb. Decreased hTERT mRNA levels were also found in multiple sclerosis patients and patients suffering from flu-like symptoms, indicating that these deviations are not disease-specific. The impaired activation-induced hTERT response in PBMC may be a general response of the immune cells in cases of acute or chronic immune-activation, presumably to control unwanted clonal expansions and to maintain the diversity of the TCR repertoire. Our results also indicate that clonal T cell expansions, described in RA, are probably not mediated by an elevated potency to express hTERT.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that causes irreversible destructions of tendons, cartilage and bone [1]. Despite many years of intensive investigation the etiology of this multifactorial disease has not been revealed yet. However, accumulating evidence indicates that RA is an autoimmune pathology in which T cells play a major role [2]. With increasing disease duration a number of phenotypic and functional T cell defects have been described in RA including hyporesponsiveness of T cells to stimulation, a decline in naive CD4+ T cells and a disturbance in the naive T cell receptor (TCR) repertoire indicated by a loss of TCR diversity and clonal expansion of a proportion of T cells [3-5]. The capacity of lymphocytes to clonally expand may be mediated, at least in part, through the upregulation of telomerase. Telomerase is a large ribonucleoprotein complex that synthesizes telomere repeats to maintain telomere length at a species-specific level. Telomeres shorten progressively with every cell division due to the inability of DNA-polymerase to fully replicate the extreme ends of chromosomes, the so-called end-replication problem [6,7]. This shortening of telomeres has been proposed to act as a mitotic clock that monitors cell division and provides a measure of the residual replicative capacity of cells [8,9]. Critically short telomeres may be the signal for replicative senescence and ultimately chromosomal instability in normal somatic cells [10,11]. Telomere erosion can however be prevented by upregulation or reactivation of telomerase [12,13]. The two most important subunits are an endogenous RNA subunit, human Telomerase-associated RNA (hTR), which contains a 11-base template sequence for the synthesis of telomere DNA, and the protein catalytic subunit, human Telomerase Reverse Transcriptase (hTERT) with reverse transcriptase activity [14]. Telomerase is constitutively expressed in germ line cells and in the majority of malignant tumor cells, and is repressed in most human normal somatic cells [15]. In some somatic cell populations, such as lymphocytes and hemopoietic stem cells, there is a highly regulated

transient expression of telomerase [9,16]. Telomerase activity has predominantly been studied in tumor cells, but it may also play a role in autoimmune diseases like RA. The aim of this study was to reveal the role of telomerase activity in the pathogenesis of RA. To this end the dynamics of the activation-induced hTERT response was evaluated in early and chronic RA patients and in controls consisting of healthy controls (HC), patients suffering from flu-like symptoms and multiple sclerosis (MS) patients.

2. Materials and methods

2.1. Patients and controls

The activation induced hTERT mRNA levels were studied in peripheral blood samples from 15 RA patients and 23 control subjects. All RA patients fulfilled the 1987 American College of Rheumatology criteria for the diagnosis of RA. RA patients were subdivided in early and chronic RA patients according to their disease duration. All chronic RA (cRA) patients were treated with disease-modifying anti-rheumatic drugs (DMARDs). All early RA patients (eRA) had a RA disease duration of less than a year and had not been receiving treatment prior to blood sampling. RA patients' characteristics are presented in Table 1. The Disease Activity Score (DAS) and the assessor's global assessment (AGA) score were used as an indicator for disease activity in cRA patients and early RA patients respectively. An AGA of 1 or 2 and a DAS28 lower than 2.6 were considered stable disease. The control group consisted of eight randomly selected healthy donors (4 men, 4 women, mean age = 34, range 23-50 years), eight persons affected by flu-like symptoms (3 men, 5 women, mean age = 55, range 30-66 years) and seven multiple sclerosis (MS) patients (2 men, 5 women, mean age = 45, range 36-56 years, 4 relapse-remitting (RR) and 3 chronic progressive (CP) MS patients). At the time of blood sampling, MS patients were without medication for at least three months. Infections, like flu or bronchitis, can cause acute lymphoproliferation accompanied by clonal expansions.

MS is a chronic inflammatory disease of the central nervous system (CNS) and like RA is also considered to be a T cell mediated autoimmune disease [17].

2.2. Culture conditions

PBMC were isolated using Ficoll HyPaque density gradient centrifugation (Sigma, St. Louis, MO, USA). Aliquots of $1x10^6$ freshly isolated PBMC were washed with ice cold PBS, pelleted and stored at –80° C until further analysis. PBMC were cultured *in vitro* in RPMI 1640 (Invitrogen, Merelbeke, Belgium) supplemented with 1 mM sodium pyruvate (Invitrogen), non-essential amino acids (Invitrogen) and 10% fetal bovine serum (Hyclone, Utah, USA) or autologous serum, in the presence of 5% CO_2 at 37°C. To study the kinetics of hTERT mRNA expression and telomerase bioactivity, PBMC were seeded into a 24-well plate (Greiner, Bio-One GmbH) at a concentration of $5x10^5$ cells/ml and stimulated with anti-CD3 mAb (2µg/ml) for 8 days. Each day the cells in a single well were collected and pelleted. On day 1 (after isolation), day 4 and day 8, PBMC were phenotypically characterized by flow-cytometric analysis (FACSCalibur, BD Biosciences, Erembodegem, Belgium) using anti-CD4 FITC, anti-CD4 PerCP, anti-CD8 PE, anti-CD3 PE and anti-CD25 FITC antibodies (BD Biosciences).

2.3. Quantitative analysis of the catalytic subunit hTERT

Total RNA was extracted from cell pellets with the High Pure RNA Isolation Kit according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). Expression of hTERT mRNA was measured using the LightCycler *T*elo*TAGGG* hTERT Quantification Kit (Roche Diagnostics GmbH), a real-time quantitative reverse transcriptase PCR. The housekeeping gene, porphobilinogen deaminase (PBGD), was used as a reference for relative quantification. hTERT and PBGD were amplified in two separate reactions. In brief, 1µl RNA (100-200 ng) was added to a reverse transcriptase / amplification mixture in a

total volume of 10 µl. The PCR program used consists of 10 min at 60°C for reverse transcription, 30 sec at 95°C followed by 40 cycles of denaturation (95°C), annealing (10 sec at 60°C) and enzymatic chain extension (10 sec at 72°C).

2.4. PCR-based Telomerase activity assay

Telomerase activity was measured using the *T*elo*TAGGG* Telomerase PCR ELISA^{plus} kit (Roche Diagnostics GmbH) which combines a telomere repeat amplification protocol (TRAP) assay with detection via ELISA. In brief, cells were resuspended in 200 µl lysis reagent and incubated for 30 min on ice. Two µl of the cell extract (corresponding to 2 x 10^4 cell equivalents) was added to an elongation / amplification mixture composed of 25 µl 2x reaction mixture and 5 µl of internal standard (IS) (both supplied in the kit) in a total volume of 50 µl. A combined primer elongation / amplification reaction was performed according to the following protocol: elongation at 23°C for 20 min, telomerase inactivation at 94°C for 5 min and 30 times 94°C for 30 sec, 50°C for 30 sec and 72°C for 90 sec followed by a final 10 min at 72°C. PCR products were hybridized to digoxigenin (DIG)-labeled detection probes and immobilized via the biotin label to a streptavidin-coated microtiter plate. Detection was performed with a horseradish peroxidase conjugated antibody directed against DIG (Anti-DIG-HRP) in a standard ELISA assay. A control template with a known amount of telomerase activity (provided with the kit) was used for determining the relative telomerase activity (RTA).

2.5. Statistical analyses

Spearman test was used to determine correlation between telomerase activity and hTERT mRNA expression in PBMC. RA patients and non-RA controls were compared using Mann Whitney U test. P-values < 0.05 were considered statistically significant.

3. Results

3.1. Relation between hTERT mRNA expression and telomerase activity in PBMC

The identification of differentially expressed subunits of telomerase indicated a major control point at the level of transcription. There are however other mechanisms that could play a role in the regulation of telomerase. To study the correlation between hTERT mRNA expression and telomerase activity in PBMC, we stimulated PBMC of 3 healthy controls with anti-CD3 mAb for 7 days. Each day both telomerase activity and hTERT mRNA expression were analyzed. Our data show that an increase in telomerase activity was accompanied by an increase in hTERT mRNA expression with a strong positive correlation between both parameters ($R^2 = 0.86$, $p<0.05$)) (Figure 1).

3.2. Activation-induced hTERT mRNA levels in PBMC

TCR mediated events, such as anti-CD3 mAb stimulation, can trigger telomerase expression in T lymphocytes [14]. We studied the hTERT mRNA expression profile after stimulation with anti-CD3 mAb in PBMC of 8 eRA patients, 7 cRA patients, 8 HC, 8 persons with flu-like symptoms and 7 MS patients. PBMC were stimulated with anti-CD3 mAb for 8 days and each day a proportion of the cells was pelleted. Direct *ex vivo* hTERT mRNA levels were low and in 6/7 cRA, 8/8 eRA, 3/8 HC, 8/8 flu patients and 2/7 MS patients even undetectable. After anti-CD3 mAb stimulation, hTERT mRNA levels gradually increased, peaked shortly, and slowly decreased to the basal levels thereafter (Figure 2A). The hTERT mRNA expression pattern after stimulation is significantly impaired in eRA patients as compared to HC ($p<0.01$). Remarkably, MS patients ($p<0.01$) and patients suffering from flu-like symptoms ($p<0.05$) also exhibited reduced hTERT mRNA levels (Figure 2A). In RA patients, there was no relation between disease status (active versus stable disease) and activation-induced hTERT mRNA levels (data not shown).

3.3. Maximum hTERT mRNA level after stimulation

The maximum hTERT mRNA level after anti-CD3 mAb stimulation, which was reached between day 3 and 5, was also evaluated since it may better represent the capacity of PBMC to express hTERT mRNA. The level of maximum hTERT mRNA expression was comparable in PBMC of cRA patients and HC, but was significantly lower in early RA patients compared to both HC ($p<0.01$) and cRA patients ($p<0.05$) (Figure 2B). However, this phenomenon again is not specific for early RA patients since a reduced maximal hTERT mRNA level was also found in MS patients ($p<0.01$ as compared to HC) and patients with flu-like symptoms ($p<0.05$ as compared to HC). As the average age differed between the populations studied, we tested whether there was a relation between age and the maximum level of hTERT mRNA induced by anti-CD3 mAb stimulation. We did not observe a significant correlation between the maximum hTERT mRNA level and age, neither in the HC group nor in the combined eRA, flu and MS group (Figure 2C).

3.4. Effect of anti-CD3 mAb stimulation on PBMC composition, activation and proliferation

Telomerase activity is highly regulated in T-cells. Its expression is closely linked to activation and subsequent proliferation. We therefore evaluated the T cell activation level and CD4/CD8 composition of the PBMC fraction after stimulation with anti-CD3 mAb by flow cytometry. The composition of the PBMC fraction in all groups was comparable at the three time-points studied (figure 3A and 3B). Anti-CD3 mAb stimulation was effective as it induced T cell activation (CD25 expression) in all populations studied. After 4 days of stimulation, the percentage of activated T cells was comparable in HC, eRA, cRA and MS patients and was even higher in flu patients (Figure 3C). Proliferation could be estimated by evaluating the PBGD content of the cells collected from a single well (originating from 1 million cells) as measured by real time PCR. hPBGD is a suitable housekeeping gene for the relative

quantification of a low copy gene such as hTERT. Its content will correlate well with cell numbers. Cell numbers after activation were higher in eRA, MS and flu patients and lower in cRA patients as compared to HC (Figure 3D), but the differences were not statistically significant. These data indicate that the reduced hTERT mRNA levels in eRA, MS and flu patients after stimulation are not due to a lower T cell response in these patients.

4. Discussion

Regulation of telomerase activity is a tightly controlled process in which mechanisms like telomerase gene expression (hTERT, hTR), post-translational modifications (protein phosphorylation), protein-protein interactions, and protein delocalization have been implicated [18-21]. Our data indicate that in activated T cells telomerase activity is primarily regulated at the level of transcription of the hTERT gene. These results imply that in activated T cells telomerase activity can be estimated by measuring hTERT mRNA levels.

An adequate immune response to antigen exposure requires a fast and profound (clonal) expansion of lymphocytes [14,22]. The expression of telomerase in responding T cells can act to sustain telomere length and replicative capacity during such an immune response [23]. Although telomerase activity is important in normal immune function [24], it is unclear whether telomerase or telomerase (dys)regulation plays a role in the pathogenic immune response in autoimmune diseases.

In this study we compared the dynamics of the activation-induced hTERT mRNA levels in RA patients and non-RA controls consisting of HC, people suffering from flu-like symptoms and MS patients. In the majority of both RA patients and controls we were unable to detect hTERT mRNA in freshly isolated PBMC. These data conflict with the findings of some other research groups who were able to detect telomerase activity in PBMC freshly isolated from RA patients and HC [16,22,25]. However, our results clearly illustrate that telomerase activity

is transiently upregulated after stimulation. This implicates that telomerase activity levels, measured directly *ex vivo*, are dependent on the moment and extent of the last *in vivo* activation.

In contrast to PBMC of cRA patients, PBMC of early untreated RA patients have reduced hTERT mRNA levels after *in vitro* stimulation with anti-CD3 mAb. In MS patients and patients suffering from flu-like symptoms, activation-induced hTERT mRNA levels are also reduced, which suggests that the deviations are not disease-specific. It should be noted that the average age differed between the populations studied. However, Son et al showed that age did not alter the magnitude of telomerase activity induced after *in vitro* stimulation [26]. Moreover, we did not observe a decreasing maximum level of hTERT mRNA expression with advancing age suggesting that age may not be a major confounding factor in our study. FACS analysis revealed that T cell activation and proliferation were comparable in the groups studied. This indicates that the reduced hTERT mRNA levels in eRA, MS and flu patients were not due to hyporesponsiveness of the T cells.

It has been described by Hodes et al. that the initial stimulation of T cells induces a high level of telomerase activity, but with repeated stimulation the levels of telomerase that are induced decrease progressively, resulting in telomere erosion [23]. This finding can explain the decreased telomerase induction in T cells of patients suffering from a chronic inflammatory disease like RA or MS. T cells involved in an immune reaction against a pathogen may also have experienced repeated stimulation *in vivo* at the end of the response, resulting in the decreased telomerase activity observed in the flu control group.

The inability of PBMC to express hTERT mRNA seems to be a general response of the immune cells following immune activation. It is possible that the immune system downregulates the outgrowth of potentially harmful immune cells or prevents the TCR repertoire to become severely disturbed by counteracting the fast expansion of specific T cell

subsets in cases of immune challenge. Besides activation induced cell-death (AICD), a reduced telomerase expression could be a mechanism for controlling clonal size at the end of an immune-response. Therefore the inhibition of telomerase expression in PBMC of RA patients may rather be a consequence of immune-activation than a cause of the disease. However, in contrast to early RA patients, chronic RA patients have a hTERT mRNA expression profile comparable to HC. It has been described by Koetz et al that T cells in cRA patients show an age-inappropriate telomere erosion [27]. These cells apparently have a large replicative history and a decreased capacity to proliferate. As expected, the T cell proliferative response in this patient group seemed to be impaired. It has been demonstrated that lymphocytes from RA patients have an increased sensitivity to die from AICD [28]. A fraction of the proliferative restricted cells may thus undergo apoptosis in response to stimulation. This could lead to an increase in the relative fraction of T cells expressing telomerase, resulting in the near to 'normal' hTERT mRNA levels in cRA patients. Moreover, all chronic RA patients were treated with DMARDs, which could have an influence on hTERT mRNA levels. Results obtained from this patient group may therefore not reflect the natural situation.

In conclusion, our report is the first to study the kinetics of hTERT mRNA levels in PBMC of RA patients and controls following *in vitro* activation. We have shown that PBMC, which have been challenged extensively during chronic (eRA, MS) or acute inflammation (flu), show an impaired hTERT mRNA response. This suggests a mechanism of the immune system to control unwanted clonal expansions and maintain the diversity of the TCR-repertoire. Since hTERT mRNA levels are reduced in proliferating PBMC of early RA patients, the clonal expansions described in RA are probably not mediated by an elevated potency to express hTERT mRNA.

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Table and Figure legends

Figure 1: Correlation between telomerase activity and hTERT mRNA expression in PBMC. For seven timepoints after anti-CD3 mAb stimulation, the mean RTA and hTERT mRNA expression levels in PBMC of 3 HC are plotted. Inserted graph shows the average time course of both RTA and hTERT mRNA levels in this experiment. The amount of hTERT mRNA transcripts is divided by the amount of corresponding PBGD transcripts to obtain a normalized hTERT mRNA level. A control template with a known amount of telomerase activity was used for determining the RTA. hTERT, human telomerase reverse transcriptase; PBMC, peripheral blood mononuclear cells; mAb, monoclonal antibody; RTA, relative telomerase activity; HC, healthy controls; PBGD, porphobilinogen deaminase

Figure 2: Relative hTERT mRNA levels after stimulation with anti-CD3 mAb. hTERT mRNA levels are normalized for the housekeeping gene PBGD. A) hTERT mRNA levels at 8 time-points after anti-CD3 mAb stimulation in HC (n=8), eRA patients (n=8), cRA patients (n=7), MS patients (n=7) and patients with flu-like symptoms (n=8). Mean and S.E.M. are plotted. B) Maximum hTERT mRNA levels in PBMC of HC (n=8), eRA patients (n=8), cRA patients (n=7), MS patients (n=7) and patients with flu-like symptoms (n=8) after stimulation with anti-CD3 mAb. Mean levels are depicted on horizontal bars; error bars indicate SEM. C) Maximum hTERT mRNA levels in function of the age for HC (filled squares) and MS, flu and eRA patients (open circles). * $p < 0.05$, ** $p < 0.01$

hTERT, human telomerase reverse transcriptase; mAb, monoclonal antibody; PBGD, porphobilinogen deaminase; HC, healthy controls; eRA, early RA; cRA, chronic RA; RA, rheumatoid arthritis; MS, multiple sclerosis; S.E.M., standard error of the mean

Figure 3: The composition, activation status and proliferation level of the PBMC fraction direct *ex vivo* and 4 and 8 days after stimulation with anti-CD3 mAb. Mean and S.E.M. of HC (n=8), eRA (n=8), cRA (n=7), and MS patients (n=7) and patients with flu-like symptoms (n=8) are plotted. A) Percentage of CD4+ cells. B) Percentage of CD8+ cells. C) Percentage of activated (CD25+) T cells. D) Expression of the housekeeping gene PBGD as compared to the PBGD content at day 1. *$p<0.05$; **$p<0.01$

PBMC, peripheral blood mononuclear cells; mAb, monoclonal antibody; S.E.M., standard error of the mean; HC, healthy controls; eRA, early RA; cRA, chronic RA; RA, rheumatoid arthritis; MS, multiple sclerosis; PBGD, porphobilinogen deaminase

Table1; RA patient characteristics.

	Age (yrs)	Sex	Disease activity		treatment
			DAS	AGA	
Chronic RA patients					
1	59	F	2.7		MP, MTX
2	60	M	2.1		SSZ, MTX
3	67	F	4.1		IL-1R antagonist, PSL
4	66	F	4.9		MTX
5	75	F	3.8		SSZ, PSL
6	56	F	3.6		MTX
7	51	F	3.0		MP, MTX
Early RA patients					
1	46	M		3	-
2	60	F		2	-
3	68	F		4	-
4	62	M		2	-
5	40	F		3	-
6	46	F		4	-
7	35	M		3	-
8	54	M		3	-

Abbreviations: DAS, Disease Activity Score; AGA, Assessor's Global Assessment; F, female; M, male; MP, methylprednisolon ; MTX, methotrexate; SSZ, sulfasalazin; PSL, prednisolon

Figure 1; Thewissen et al.

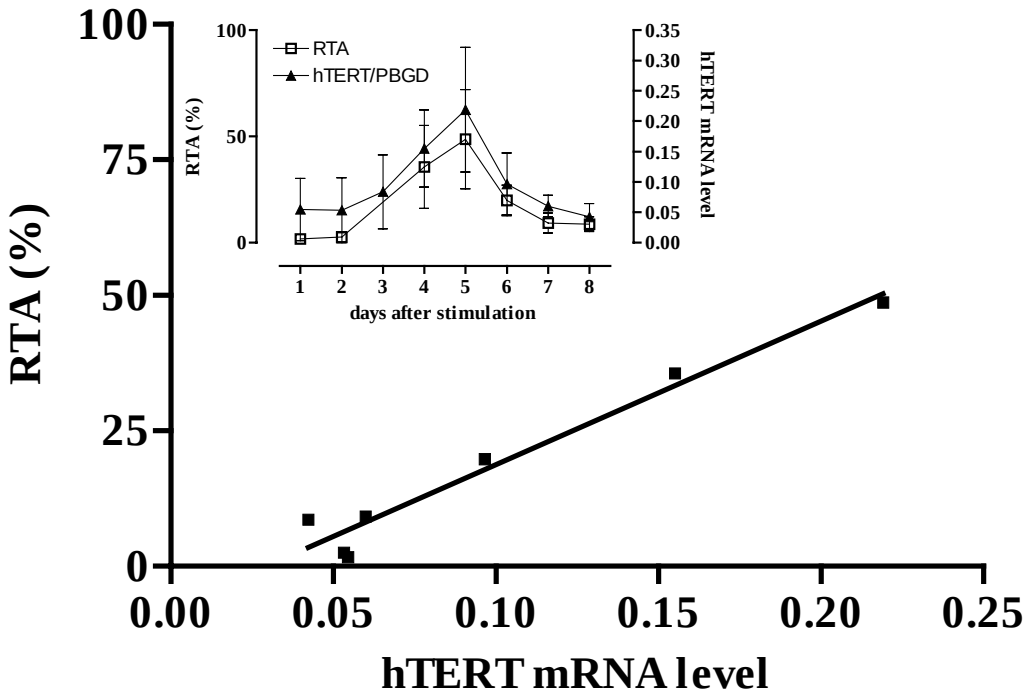

Figure 2; Thewissen et al.

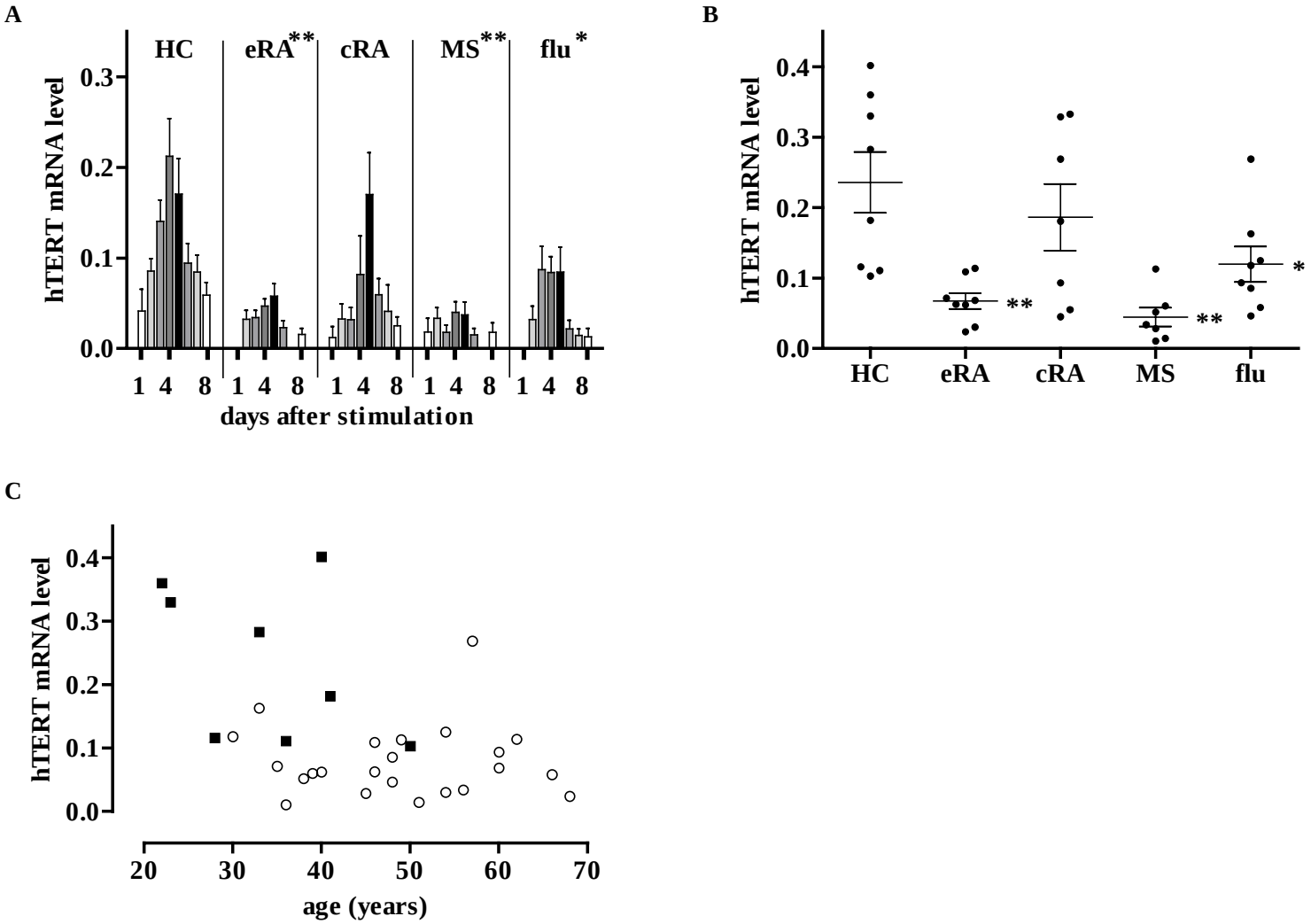

Figure 3; Thewissen et al.