Influence of age and physical fitness on miRNA-21, TGF-β and its receptors in leukocytes of healthy women

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ABSTRACT

Rationale: *The TGF-β superfamily has been shown to play an important role in a wide range of physiological as well as pathological processes including ageing, immune modulation, atherosclerosis and cancer development. The aim of the current study was to investigate (i) whether TGF-β signalling in peripheral blood mononuclear cells (PBMCs) would differ between young and old females and (ii) whether physical performance parameters of elderly women would be related to the expression of TGF-β or its receptors.*

Methods: *Sixteen healthy young (22-28 years; YF) and 90 healthy older (65-92 years; OF) females participated in the study. In addition to several components of health-related physical fitness, circulating CRP and TGF-β levels were determined together with the mRNA expression of TGF-β, TGF-βRI, TGF-βRII, and miRNA-21 (known to interfere with TGF-β signalling) in PBMCs.*

Results: *Physical fitness as determined by 6-minutes walking test (YF:median 932 (range 573-1254) m; OF:360 (114-558) m), handgrip strength (YF: 32 (24-39) kg; OF:18(10-30) kg), relative isokinetic peak torque of knee extensors (YF:1.9 (1.2-2.3) Nm/kg; OF:1.0 (0.2-1.9) Nm/kg and flexors (YF: 1.1 (0.7-1.5) Nm/kg; OF: 0.5 (0.2-1.0) Nm/kg was substantially lower in older women ($p<0.001$ for all comparisons). These changes were paralleled by an increase in hs-CRP (YF: 0.9 (0.1-4.3)mg/L; OF: 2.3 (0.3-56.7)mg/L,$p<0.001$). Serum levels of TGF-β and TGF-β mRNA levels from PBMCs did not differ between young and old women whereas, both TGF-βRI/GAPDH (YF: 4.07 (1.38-14.60); OF: 2.08 (0.14-28.81); $p=0.020$) and TGF-βRII/GAPDH levels (YF: 3.16 (1.14-10.25); OF: 1.71 (0.51-14.86); $p=0.020$) were lower with respect to old age. In elderly women, only TGF-βRI expression correlated negatively with miRNA-21 expression in PBMCs ($\rho=-0.315$; $p=0.004$). Interestingly, hs-CRP and miRNA correlated positively with handgrip strength ($\rho=0.237$ and $\rho=243$, $p<0.05$), while none of the TGF-β-related parameters were related to physical performance.*

Conclusion: *The results suggest that age affects TGF-β signalling in leukocytes by altering the expression levels of its receptors. These changes seem to occur independently of physical fitness of old women.*

Key Words: Inflamm-ageing, TGF-β Pathway, TGF-β receptors, microRNA-21, physical performance, Vienna Active Ageing Study

INTRODUCTION

Although the causes of human ageing are multifaceted, the molecular inflammation hypothesis of ageing implies that increased oxidative stress will lead to the activation of redox-sensitive transcription factors which in turn enhance the expression of pro-inflammatory genes in a variety of different cell types (8). As a consequence ageing is associated with a chronic inflammatory state, where pro-inflammatory factors such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) or C-reactive protein (CRP) are continuously present (over years) at levels higher than baseline but much lower than those found during acute inflammation (11, 64). Chronically elevated levels of IL-6 (> 2.0 ng/L) of middle-aged persons reduce the chance of successful ageing and increase the risk of future cardiovascular events or non-cardiovascular death later in life (1). Up to now it is unclear whether the main source of these pro-inflammatory factors is the chronically activated immune system (inflamm-ageing) or the senescence of cells with their senescence-associated secretory phenotype (SASP) leading to an enhanced secretion of pro-inflammatory mediators (15, 16, 18, 27).

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Besides being associated with age-related diseases such as diabetes mellitus type 2, cardiac illnesses or neurological diseases, chronic subclinical inflammation may also contribute to impaired physical function in older adults. Several large scale studies have revealed that higher levels of circulating CRP or IL-6 are associated with lower physical performance such as handgrip strength or gait speed (4, 7, 12, 51). While alterations in TNF-α, IL-6 or CRP in response to ageing, physical inactivity as well as acute and chronic exercise have been studied extensively (14, 38, 42), much less is known about the involvement of transforming growth factor-β (TGF-β) in this context.

TGF-β is known as a potent regulatory cytokine with diverse effects on haemopoietic cells. It has pivotal function in the immune system through keeping tolerance via the regulation of lymphocyte proliferation, differentiation and survival (28). Furthermore, TGF-β controls the initiation and resolution of inflammatory responses through the regulation of chemotaxis, activation and survival of lymphocytes, natural killer cells, dendritic cells, macrophages, mast cells and granulocytes (31). It has been suggested that the age-associated dysregulation of the immune system might be caused by a decreased expression and functioning of receptors or signalling rafts and/or defects in signalling pathways finally leading to altered function of immune cells and immunosenescence (25). TGF-β mediates its biological function via binding to type II and forming a complex with type I transmembrane serine/threonine kinase receptors (TGF-βRI, TGF-βRII). Ligand binding assembles a complex consisting of two type I receptor components and two type II components, whereby the type I components are phosphorylated further activating intracellular Smad proteins whose nuclear localization is required for the transcriptional regulation of target genes (35).

Interestingly, TGF-β signalling is intimately involved in biogenesis and control of microRNAs (miRNAs) as Smad proteins play a regulatory role in the processing of miRNAs in the nucleus (2, 21). MiRNAs are small non-coding RNAs that affect gene expression either by inhibiting translation of their target proteins or by degrading the respective mRNA (67). They have been shown to regulate many biological functions including ageing, immune function and the response to exercise (33, 36, 53, 63). It also has been suggested that senescent donor cells contribute to SASP by secreting not only soluble proteins but also microvesicles containing miRNAs which are taken up by recipient cells and may cause or contribute to age related pathologies like osteoporosis, atherosclerosis, Alzheimer's disease or diabetes mellitus, type 2 (62). Several miRNAs such as miRNA-155, -146a and -21 are involved in the regulation of inflammation by controlling Toll-like receptor or nuclear factor-κB (NF-κB) signalling (45, 48). MiRNA-21, despite being well established as an 'onco-miR' due to its aberrant expression in numerous cancers, seems to be of particular importance as it links inflamm-ageing to cellular senescence (44). Intracellular as well as circulating miRNA-21 levels are higher in elderly in comparison to young subjects or centenarians. This is associated with a lower TGF-βRII expression in leukocytes (46), whereby TGF-βRII is a validated target of miRNA-21 in adipocytes (24) and colon cancer cells (66). Mice lacking the TGF-βRII develop an autoimmune biliary ductular disease similar to human primary biliary cirrhosis. Using this experimental model it has been shown that the lack of TGF-β signalling leads to a down-regulation of several miRNAs in T cells but interestingly to an up-regulation of miR-21 concomitant with an increased production of TNF-α and interferon-γ (2). It is hypothesized that the up-regulation of miRNA-21 is caused by a dysregulated gene expression normally controlled by TGF-β. This would result in the activation of inflammatory pathways such as NF-κB which directly or indirectly (via induction of miRNA-21) leads to an up-regulation of inflammation. Another possibility is that a global down-regulation of miRNA expression induces multiple genes causing inflammation; this in turn could lead to elevated levels of miR-21 with a feedback up-regulation of inflammation (2).

However, TGF-β signalling seems to play an important role in relation to several diseases associated with chronic-low grade inflammation. We hypothesized that both, age and physical fitness could affect TGF-β and its receptors in peripheral blood mononuclear cells of healthy women. Therefore, the aim of the current study was to investigate (i) whether circulating TGF-β, as well as intracellular TGF-β, TGF-β receptors and miRNA-21 would differ between young and elderly women and (ii) whether these markers would be associated with parameters of physical performance.

METHODS

Subjects

Ninety elderly women (aged 65-92 years) who were recruited in 5 different senior residencies in the area of Vienna (Curatorship of Viennese Retirement Homes (KWP)) participated in the study. For the current study we used baseline characteristics of study participants intended to take part in a prospective training study. In addition 16 young women (aged 18-28 years) responded to flyers at the University of Vienna. Young as well as elderly women were sedentary (less than 1h of sports activities per week) and free of severe diseases that would contra-indicate medical training therapy or measurement of physical performance, serious cardiovascular diseases, diabetic retinopathy and regular use of cortisone-containing drugs. Written informed consent was obtained from all participants before entry into the study in accordance with the Declaration of Helsinki and after approval by the ethics committee of the City of Vienna (EK-11-151-0811).

Anthropometric Measurements

Using a commercial stadiometer (Seca, Hamburg, Germany), standing height was measured without shoes to the nearest 0.5 cm. Shoulders kept in a relaxed position and arms allowed to hang freely. Body mass was evaluated with a digital scale (BWB 700, Tanita, Amsterdam, Netherlands) to the nearest 0.1 kg with subjects lightly dressed and barefoot. Body mass index (BMI) was calculated by dividing body mass in kilograms by height in meters squared. For determining body composition (muscle and fat mass) we used bioelectric impedance analyses, due to successful validation against data obtained by magnetic resonance imaging (50). Bioelectric Impedance Analyses (BIA) were performed in the morning

after an overnight fast using a BIA Analyzer 2000-S (Data-Input GmbH, Darmstadt, Germany). Participants were asked not to perform any exercise or strenuous physical activity the day before the tests.

Determination of physical performance

To evaluate each participant's aerobic endurance a *6-minutes walking test* was conducted. Therefore, participants walked for 6 minutes as fast as possible on a 30 metre shuttle track. They were allowed to reduce their speed or to rest if the selected speed was too high to be sustained. The completed distance within 6 minutes was recorded (55).

To measure *handgrip strength* participants performed two trials of an isometric handgrip strength test (kg) using a dynamometer in a sitting position with an angle of 45° in the elbow and the lower arm on the armrest. The participant was instructed to squeeze the handle as hard as possible for 4-5 seconds and the maximum isometric contraction was recorded (SAEHAN Corporation, Masan, Korea). The two trials were separated by one minute of passive recovery (37). Out of the two trials on each arm, the best result regardless of side was used for further calculation.

The *isokinetic peak torque of knee extension and flexion* consisted of concentric isokinetic torque measurements (Lido Loredan Biomedical, Inc., Davis, USA; Range of Motion 30°-80°, speed 60°/s or 120°/s). The left leg was tested in all participants except for 3 older women with acute injuries of the left leg making it necessary to test the right leg. The best result of two trials separated by a rest period of two minutes between the attempts was documented. Absolute values were divided by body mass to obtain relative values.

Blood sampling and analyses

Routine blood analyses

Between 06:30 and 08:00 in the morning venous blood samples were taken after an overnight fast. Venous blood was collected in Z Serum Clot Activator collection tubes (Vacuette®, Greiner Bio-One GmbH, Kremsmünster, Austria) for cytokine analyses and in EDTA tubes for the determination of leukocyte subpopulation numbers. For the isolation of peripheral blood mononuclear cells (PBMCs) from whole-blood, BD Vacutainer® CPT™ Tubes containing ~130 IU Na-Heparin and 2 ml Ficoll™ (Becton, Dickenson and Company, Schwechat, Austria) were used.

After at least 30 min and at most 60 min after blood collection, the serum tubes were centrifuged (10 min, 3,000 x g). An aliquot of 1 ml was used for immediate determination of glucose, insulin and hs-CRP. The remaining serum was stored in aliquots at -80°C until further analysis. Glucose was analyzed by hexokinase method and insulin was estimated using a solid-phase, enzyme-labeled chemiluminescentimmunometric assay (IMMULITE 2000, Siemens Healthcare Diagnostics Inc., Llanberis, UK). Cholesterol, HDL cholesterol, LDL cholesterol, triglyceride and hs-CRP were routinely quantified on a Cobas 8000 (Roche Diagnostics, Vienna, Austria). Leukocytes, lymphocytes, monocytes and granulocytes were quantified by flow cytometry on a Sysmex XE-2100™ Automated Hematology System (Sysmex Austria GmbH, Vienna, Austria).

Serum levels of TGF-β

TGF-β was determined using a commercially available DuoSet development kit for performing enzyme-linked immunosorbent assays (DY240, R&D Systems; Abingdon, UK) consisting of a capture antibody (2 µg/ml of mouse anti-TGF-β1), a detection antibody (300 ng/ml of biotinylated chicken anti-human TGF-β1), and recombinant human TGF-β1 to prepare a standard curve (31-2,000pg/ml). Twenty µl of each serum sample were activated by adding 10 µl of 1N HCl, incubated at room temperature for 10 min and neutralized with 10 µl of 1.2N NaOH/0.5 M Hepes. The activated sample was diluted 20-fold with reagent diluent (0.05% Tween® 20 in PBS) and used in the assay following the instructions of the manufacturer. Spectrophotometric measurements were performedon a Victor^3 1420 Multilabel Counter (Perkin Elmer, MA, US).

Isolation of total RNA from PBMCs

PBMCs were separated from red blood cells and neutrophils by centrifugation of BD Vacutainer® CPT Tubes at 1,650 x g for 20 min at room temperature. After removing 2 ml of the plasma supernatant, the cells comprising PBMCs were resuspended in the remaining plasma and transferred to another tube. PBMCs were washed twice with PBS without Ca and Mg according to the protocol provided by the manufacturer. Finally, the pellet was carefully resuspended in 700 µl of QIAzol Lysis Reagent (Qiagen, Hilden, Germany) and stored on -80° until analysis.

Total RNA including small RNAs was isolated after thawing and incubating the samples for 5 min at room temperature using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) and following the instructions of the manufacturer. In order to prepare a miRNA-enriched fraction separated from the larger RNAs (>200nt) the RNeasy Min Elute Cleanup Kit (Qiagen, Hilden, Germany) was used. Reverse transcription was for the miRNA-enriched fraction was performed using the miScript II RT Kit (Qiagen, Hilden, Germany) while larger RNAs were reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany).

Quantitative real-time RT-PCR

TGF-β, TGF-βRI and TGF-βRI mRNA were determined usingthe respective primer assays (Hs_TGFB1_1 (QT00000728), Hs_TGFBR1_1 (QT00083412), Hs_TGFBR2_1 (QT00014350), Qiagen, Hilden, Germany) in conjunction with the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany). A standard curve was prepared by pooling equal amounts of cDNA from PBMCs of 7 young and 23 old subjects which were randomly selected from the study population. In addition, GAPDH (Hs_GAPDH_2 (QT01192646)) served as endogenous control and was used to normalize the data. Quantification was performed on an Applied Biosystems® 7500 Real-Time PCR System.

MiRNA-21 expression levels were detected using a miScript Primer Assay specific for miRNA-21 (hs_miR-21_2 (MS00009079), Qiagen, Hilden, Germany). A standard curve was prepared by using a commercially available totalRNA of peripheral blood leukocyte cells of a 24 year old female donor (Total RNA (R1234148-10), BioChain, Newark, USA). Quan-

tification was performed on an Applied Biosystems® 7500 Real-Time PCR System.

Statistical analyses

The data acquisition and data processing took place using commercial software (IBM SPSS for Windows, Version 20). Shapiro-Wilk test was used to determine if data sets were normally distributed. As most of the variables did not meet the criteria for normal distribution, Mann-Whitney U test was used in order to compare young and elderly women. Associations between variables were analysed using Spearman´s ρ correlation coefficient. Data are shown as median (minimum-maximum), statistical significance was set at $p<0.05$.

RESULTS

Subject characteristics

Old females with a median age of 84 years had a higher body mass (+23%, $p<0.001$) as well as BMI (+36%, $p<0.001$) compared to young females (median age: 25 years). Bioelectric impedance analyses revealed a higher percentage of body fat mass (+37%, $p<0.001$) and a lower muscle mass (-12%, $p<0.001$). The age-related changes in body composition were accompanied by substantial worsening of health-related parameters such as circulating glucose levels (+10%, $p<0.001$), cholesterol (+22%, $p=0.002$), LDL cholesterol (+49%, $p<0.001$), HDL cholesterol (-16%, $p=0.006$), and triglyceride (+41%, $p=0.006$) (Table 1).

Physical fitness

Aerobic fitness was determined by 6-minutes walking test (6MWT). Old females reached a significant lower distance within a time frame of 6 minutes (-61%, $p<0.001$) than younger females (Figure 1A). Strength was determined by handgrip dynamometer as well as isokinetic peak torque measurements. Isometric handgrip strength was significantly lower in old women (-43%, $p<0.001$, Figure 1B). Similarly, relative peak torque knee extension (PTE) as well as relative peak torque knee flexion (PTF) differed significantly between groups at both tested velocities (Fig. 1C-F) reflecting strength loss of M. quadriceps (-48% at 60°/s and -51% at 120°/s, $p<0.001$) and hamstrings (-53% at 60°/s as well as at 120°/s, $p<0.001$).

Inflammatory parameters

Differences in inflammatory parameters between young and old females are summarized in Table 2. The number of leukocytes in whole blood did not differ between young and old women. However, the subpopulation analysis revealed a significant higher percentage of monocytes in elderly (+24%, $p=0.007$), with no changes in lymphocytes and granulocytes. As expected, hs-CRP was significantly higher in old women in comparison to young females (+156%, $p<0.001$). Serum levels of TGF-β did not differ between young and elderly women ($p=0.290$). Similarly, TGF-β mRNA levels from PBMCs did not vary between groups ($p=0.290$). Interestingly, both TGF-βRI (-49%, $p=0.020$) and TGF-βRII mRNA levels (-46%, $p=0.020$) were lower with respect to old age.

Table 1: Subject Characteristics

Parameter	Young (n=16)	Old (n=90)	*p-value*
Age [years]	24.9 (21.7-28.4)	83.8 (65.0-92.2)	***<0.001***
Body mass [kg]	58.1 (51.0-65.2)	71.7 (46.2-112.4)	***<0.001***
Height [m]	1.65 (1.57-1.71)	1.57 (1.40-1.72)	***<0.001***
BMI [kg/m²]	21.7 (18.9-23.6)	29.6 (18.1-50.0)	***<0.001***
Body fat mass [%]	26.3 (21.2-33.2)	36.1 (14.0-50.4)	***<0.001***
Muscle mass [kg]	22.2 (20.6-23.1)	19.5 (12.9-26.4)	***0.019***
Glucose [mg/dl]	87 (67-106)	96 (79-196)	***<0.001***
Insulin [µIU/ml]	6.40 (2.50-13.50)	8.05 (1.32-41.57)	*0.068*
Cholesterol [mg/dl]	171 (136-264)	209 (144-336)	***0.002***
HDL-Cholesterol [mg/dl]	74 (54-99)	62 (33-120)	***0.006***
LDL-Cholesterol [mg/dl]	81 (47-146)	121 (43-238)	***<0.001***
Triglyceride [mg/dl]	79 (41-191)	111 (43-275)	***0.006***

Data are expressed as medians (min-max); Differences were detected using Mann-Whitney U test;
BMI (body mass index); HDL (high density lipoprotein); LDL (low density lipoprotein)

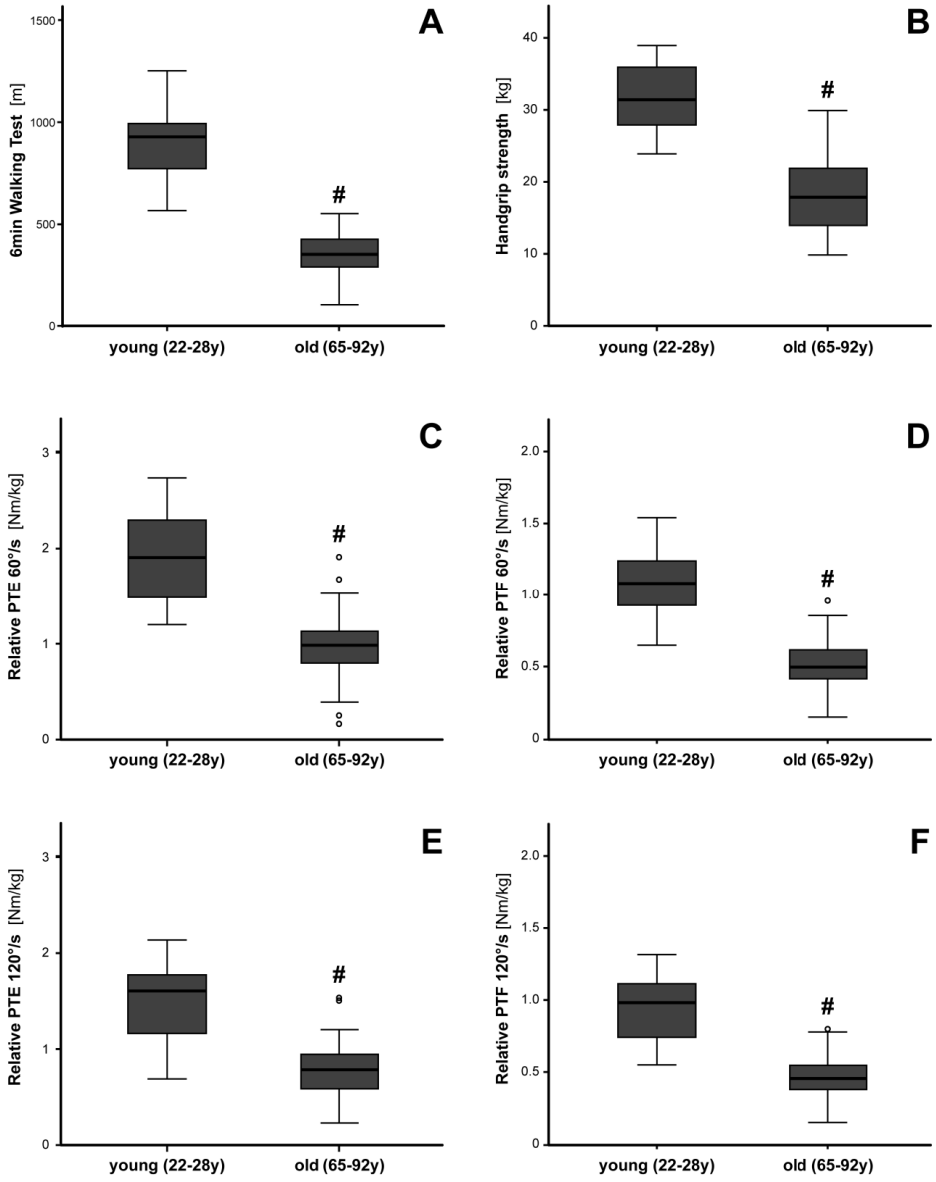

Figure 1. Age-related differences in physical fitness: (A) 6-minutes Walking Test, (B) Handgrip strength, (C) Relative Peak Torque Knee Extension (PTE) 60°/s, (D) Relative Peak Torque Knee Flexion (PTF) 60°/s, (E) Relative Peak Torque Knee Extension (PTE) 120°/s, (F) Relative Peak Torque Knee Flexion (PTF) 120°/s. # denotes a significant differences between young and old women ($p<0.001$).

To investigate whether the change in TGF-β receptor mRNA expression could be influenced by miRNA-21, its level was measured in PBMCs. While we did not detect any differences in intracellular miRNA-21 levels between young and old women ($p=0.190$), miRNA-21 in PBMCs of old women correlated negatively with TGF-βRI expression ($\rho=-0.315$; $p=0.004$) but not with TGF-βRII or TGF-β.

Correlations between fitness and inflammation in elderly women

Next we were interested whether higher fitness levels within the cohort of older women would be associated with lower pro-inflammatory states (Table 3). Indeed, hs-CRP levels and relative peak torque measurements revealed negative correlation; however, significance was reached only for relative peak torque of knee extension at 120°/s ($\rho=-0.276$; $p=0.013$). Surprisingly, hs-CRP was not associated with performance in 6MWT and even positively correlated to handgrip strength ($\rho=0.237$; $p=0.035$). Serum TGF-β, its expression level in PBMCs, and the expression of its receptors TGF-βRI and TGF-βRII were not related to any performance measure.

Body composition influences inflammatory and fitness-related parameters in elderly

As the subgroup of older women showed a substantial variation in body composition, its influence on inflammatory, fitness- and health-related variables was investigated. It has to be mentioned that age in this subgroup was even negatively associated with muscle mass ($\rho=-0.546$; $p<0.001$) but surprisingly also with BMI ($\rho=-0.219$; $p=0.039$) and body fat mass ($\rho=-0.316$; $p=0.003$).

Hs-CRP was positively correlated to BMI ($\rho=0.326$; $p=0.002$), body fat ($\rho=0.331$; $p=0.002$) and muscle mass ($\rho=0.291$; $p=0.007$), but not age. Furthermore, total leukocyte counts were positively associated with BMI ($\rho=0.342$; $p=0.001$) and body fat ($\rho=0.212$; $p=0.050$). In contrast, TGF-

Table 2: Age-related changes in immune parameters

Parameter	Young (n=16)	Old (n=90)	*p-value*
Circulating			
Leukocytes [10^9/l]	6.0 (4.6-8.0)	6.5 (3.4-13-3)	*0.208*
Lymphocytes [%]	33.8 (23.5-46.6)	31.6 (15.3-48.1)	*0.375*
Monocytes [%]	6.7 (3.7-11.5)	8.3 (1.0-14.1)	***0.007***
Granulocytes [%]	59.0 (43.8-67.2)	56.3 (38.4-75.0)	*0.584*
hs-CRP [mg/l]	0.9 (0.1-4.3)	2.3 (0.3-56.7)	***0.001***
TGF-β [pg/ml]	33,321 (23,793-42,543)	34,851 (16,667-73,681)	*0.290*
PBMCs (intracellular)			
TGF-β / GAPDH [-]	0.65 (0.30-1.59)	0.53 (0.06-3.36)	*0.387*
TGF-βRI / GAPDH [-]	4.07 (1.38-14.60)	2.08 (0.14-28.81)	***0.022***
TGF-βRII / GAPDH [-]	3.16 (1.14-10.25)	1.71 (0.51-14.86)	***0.022***
hsa-miRNA-21[copies /pg RNA]	1,821 (380-3,824)	2,452 (57-5,481)	*0.190*

Data are expressed as medians (min-max); Differences were detected using Mann-Whitney U test; hs-CRP (high sensitive C-reactive protein), PBMC (peripheral blood mononuclear cells), TGF-β (transforming growth factor-β), TGF-βRI (transforming growth factor-β receptor type I), TGF-βRII (transforming growth factor-β receptor type II), GAPDH (glyceraldehyde-3-phosphate dehydrogenase); hsa-miRNA-21 (human microRNA-21)

Table 3: Correlation between fitness and immune parameters in old women

	6MWT	Handgrip	PTE_{rel}60°/s	PTF_{rel}60°/s	PTE_{rel}120°/s	PTF_{rel}120°/s
Leukocytes	-0.100	-0.102	-0.017	0.062	-0.020	-0.039
hs-CRP	0.049	**0.237***	-0.206	-0.127	**-0.276***	-0.175
TGF-β (circulating)	-0.086	-0.082	-0.060	-0.019	-0.046	-0.120
TGF-β / GAPDH (intracellular)	-0.117	-0.043	0.033	-0.101	0.138	0.033
TGF-βRI / GAPDH (intracellular)	-0.070	-0.092	-0.124	-0.180	-0.013	-0.089
TGF-βRII / GAPDH (intracellular)	-0.175	-0.045	-0.002	-0.116	0.092	0.038
hsa-miRNA-21 (intracellular)	-0.011	**0.243***	0.101	0.034	0.075	0.015

Data indicate Spearman-Rho correlation coefficients. * $p < 0.05$, $n \geq 80$; 6MWT (6 Minutes Walking Test), PTE_{rel} (relative peak torque of knee extension), PTF_{rel} (relative peak torque of knee flexion), hs-CRP (high sensitive C-reactive protein), TGF-β (transforming growth factor-β), TGF-βRI (transforming growth factor-β receptor type I), TGF-βRII (transforming growth factor-β receptor type II), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), hsa-miRNA-21 (human microRNA-21)

βRII mRNA was negatively associated with body fat (ρ=-0.263, p=0.018) but not with BMI (ρ=-0.185; p=0.091). None of the other inflammatory variables correlated with body composition.

With respect to fitness parameters BMI and body fat correlated negatively with relative peak torque of knee extension at 120°/s (BMI: ρ=-0.282; p=0.011, body fat: ρ=-0.237; p=0.038) and partly with relative peak torque of knee flexion

at 120°/s (BMI: ρ=-0.292; p=0.009, body fat: ρ=-0.209; p=0.068). BMI and body mass were not associated with handgrip strength or 6MWT. Muscle mass correlated positively with handgrip strength (ρ=0.662; p<0.001), but not with other performance parameters.

DISCUSSION

The aim of the current study was to investigate the expression of TGF-β, its receptors and its potential modulator miRNA-21 in PBMCs in the context of age and fitness status. Young and old females differed substantially in fitness as measured by 6MWT, handgrip strength, isokinetic peak torque of knee extensors and flexors as well as serum hs-CRP levels. While serum levels of TGF-β as well as TGF-β and miRNA-21 expression levels in PBMCs did not differ between young and old females, TGF-βRI and TGFβ-RII mRNA were significantly lower in the elderly. However, within the cohort of elderly women neither TGF-β nor its receptors were associated with performance characteristics.

Initially, TGF-β was purified from human platelets (3). In mammals three different isoforms (TGF-β1, -β2, -β3) have been described, whereby TGF-β1 is the predominant form in immune cells. TGF-β is synthesized and secreted by most cell types as an inactive precursor complex, termed latent TGF-β, where TGF-β is bound non-covalently to the latency associated peptide (LAP). To be activated TGF-β has to be cleaved from the LAP using one of physiological mechanisms such as proteolytic cleavage by plasmin, cathepsin, and other enzymes, oxidation by free radicals or the interaction with thrombospondin (41). Circulating TGF-β has been detected in a variety of studies with plasma values ranging from below 0.1 ng/ml up to more than 25 ng/ml. Of course the study population differed between these studies ranging from healthy individuals of mixed gender and age-groups to different patient groups. However, methodological issues concerning plasma processing and assay system seem to be the most critical factors in determining TGF-β levels (20). In this respect it has been shown that platelet degranulation, haemolysis of erythrocytes and contamination with leukocytes can lead to an over-estimation of TGF-β protein levels in plasma, however different activation protocols to dissociate TGF-β from its complexes are in use. For this purpose, we and many other groups used acidification of serum samples prior to assessing TGF-β by ELISA (20). Furthermore, both serum from young and old females were treated in the same way, therefore minimizing the risk of bias within the study.

Regarding lifestyle-related diseases TGF-β seems to play conflicting roles as shown for cancer, where it can either act as tumour suppressor by inhibiting cell proliferation and inflammation in the early stage of cancer development or as tumour promoter by inducing metastasis or angiogenesis in later stages of the disease (52). Similarly, higher levels of TGF-β are measured in hypertensive humans (57), but increased serum levels of TGF-β may also protect patients with coronary artery disease against cardiovascular events and coronary interventions (59). With respect to age decreased levels have been reported for adults (21-67 years) in comparison to children (1-14 years) (43). Another study has revealed that TGF-β levels are higher in males than in females, but decrease with age and increase with obesity in both genders (32). However, in centenarians, serum TGF-β concentration seems to be higher than in younger adults, suggesting that high concentrations of TGF-β might be beneficial during extreme old age (6). These data are in conflict with our results as we did not detect any differences in TGF-β between young and old women. However, the broad range of age as well as other lifestyle related factors such as obesity or diabetes which were characteristic for our study could have influenced the results.

Conflicting data have also been reported with regard to TGF-β and acute exercise, whereby intensity and type of exercise seem to play an important role for data interpretation. While a graded cycling exercise to exhaustion of about 18 min duration (10) and 1 h of treadmill running at about 70-80% of VO_{2max} (22) are able to increase the concentration of circulating TGF-β, 1 h of cycling exercise at ~70% of VO_{2max} does not alter circulating TGF-β (17). However, salivary TGF-β is increased as late as 24 h after a moderate exercise bout (49). Long-term training for 6 weeks in healthy students resulted in a biphasic response of TGF-β with increased levels after 2 weeks of training and lower levels at the end of the training period (23). This is in contrast to another study in diabetic patients showing that 8 weeks of strength and aerobic training results in increased TGF-β levels which are accompanied by lower hs-CRP levels (61). Furthermore, 6 months of exercise (2.5 h per week) were able to increase TGF-β production of unstimulated as well as phytohaemaglutinin-stimulated peripheral blood mononuclear cells of persons at risk of developing ischemic heart disease (54). Taken together, it seems that chronic exercise lowers TGF-β in young and healthy persons but it might up-regulate TGF-β in patients suffering from lifestyle-related diseases.

In addition to circulating levels of TGF-β we were especially interested in expression of TGF-β and its receptors in PBMCs of young and old women. While intracellular TGF-β mRNA was not different between these two groups, lowered TGF-βRI and TGF-βRII have been detected in elderly. These results are partly in accordance with a previous study in young (20-30 years), old (75-85 years) and very old (>98 years) subjects, where leukocyte TGF-βRII mRNA were lowest in the 75-85 year old group in comparison to both, the young and the centenarians. In contrast to our study the decrease in TGF-βRII expression is accompanied by an increase in miRNA-21 levels. However, neither intracellular nor circulating TGF-β nor physical performance has been assessed in this study (46). The importance of signalling via TGF-βRII has been shown in an animal model where the induction of a TGF-βRII gene disruption results in a lethal inflammatory disease (29). On the one hand TGF-βRII is important in T-cell mediated immunity (30) but on the other hand macrophages lacking TGF-βRII have defects in expression of a set of genes that form the hallmark of the M2 polarizing program in macrophages which is important to induce the anti-inflammatory effects of M2 macrophages such as phagocytosis of apoptotic cells, resolution of inflammation and tissue repair (19, 34, 40).

Data support the hypothesis that exercise can reduce low grade inflammation in elderly (61) and provide long-term benefits with regard to cardiovascular, cognitive, psychosocial and other aspects in elderly (11). Hs-CRP has been shown to be consistently higher in elderly (26, 60), a fact that was confirmed in the current study. Moreover, hs-CRP correlated positively with BMI and body fat but negatively with relative peak torque measurements. This partly confirms several studies which revealed associations between a higher inflammatory state and lower physical performance (47, 56). Besides originating in the liver, the acute phase protein hs-CRP is produced and released from adipose tissue thereby linking obesity to a chronic inflammatory state (13, 65).

Although we detected a negative correlation between hs-CRP and isokinetic knee extension strength which is in line with many studies linking chronic inflammation to low physical performance in elderly (7, 9, 12, 58), this picture was not consistent for other performance parameters such as aerobic fitness or strength. The reason for this finding could be a complex interaction between several factors which has been suggested by Morrisette-Thomas et al. who applied principal component analysis in order to understand why inflamm-ageing does not simply reflect increases in pro-inflammatory markers (39). One especially interesting aspect in our study was that general fitness status of elderly women was not related to TGF-β, TGF-βRI or TGF-βRII expression in PBMCs. Furthermore, higher hs-CRP and miRNA-21 levels in older women were even associated with a higher handgrip strength. According to our hypothesis we would have expected higher levels of the inflammatory miRNA-21 in subjects with low physical performance as suggested by Bye et al. who showed that miRNA-21 was increased in male participants with low aerobic capacity as assessed by VO2max (5). However, these studies are sparely comparable as miRNA-21 was detected in serum, participants were male and younger and there might be a difference between performance indicators for strength or endurance. Furthermore, it would have been interesting to measure general physical activity levels by an objective method such as accelerometry as both current physical activity practice and performance are associated with inflammatory biomarkers (12).

In summary, it has been shown that TGF-β is involved in a variety of physiological as well as pathological process exerting positive but in some cases also negative effects on health. We have demonstrated that in older women TGF-β signalling in PBMCs might be impaired as reflected by a lower gene expression of its receptors TGF-βRI and TGF-βRII independent of physical fitness. However, further studies are needed to test whether a reduced expression of the TGF-β receptors indeed would reduce TGF-β signalling in PBMCs in order to get mechanistic insight as well as to reveal its functional consequences, more precisely.

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