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DISSERTATION / DOCTORAL THESIS

Titel der Dissertation /Title of the Doctoral Thesis

Active Ageing – Influence of age and physical performance on transforming growth factor-β signalling

verfasst von / submitted by

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Human passions have mysterious ways, in children as well as grown-ups. Those affected by them can't explain them, and those who haven't known them have no understanding of them at all.

Michael Ende – the neverending story -

I. ABSTRACT

Chronic low-grade inflammation is an undesirable side effect of ageing associated with inactivity and obesity. Transforming growth factor-β (TGF-β) is a multimodal protein with various cellular functions ranging from tissue remodelling to regulation of immune functions. According to recent literature, physical performance, especially aerobic exercise exerts beneficial effects to low-grade inflammation whereas the influence of resistance exercise training remains unclear.

This thesis is based on three papers out of the Vienna Active Ageing Study. Therefor, the story of this thesis is based on standard values in TGF-β signalling and the difference in young and old women, while in the second step the influence of 6 months of progressive resistance training was assessed on physical performance parameters and TGF-β signalling.

More precisely, the aim of study I was to investigate whether TGF-β signalling in peripheral blood mononuclear cells (PBMCs) would differ between young and old females and additionally whether physical performance parameters of older women would be related to the expression of the signaling pathway. While serum levels of TGF-β and TGF-β levels from PBMCs did not differ between young and old women, both TGF-β receptor I (TGF-βRI) and TGF-βRII levels were lower in older participants. Interestingly, high-sensitivity C-reactive protein (hs-CRP) and microRNA-21 (miRNA-21) correlated positively with handgrip strength, while none of the TGF-β-related parameters were related to physical performance.

Study II was focused on the effects of elastic band resistance training on muscular strength in older adults living in retirement care facilities. Therefore, participants were randomly assigned to one of three intervention groups: resistance exercise training (RT), RT in combination with nutrient supplementation (RTS), or cognitive training group (CT). Six months of a low intensity resistance exercise using elastic bands and own bodyweight is beneficial in improving functional performance of institutionalised older people while multinutrient supplementation did not lead to any additional benefits to the effects of RT in improving muscular performance.

Further, study III concerns the effect of the three different interventions on systemic inflammation and TGF-β signalling in PBMCs. The improvements in physical performance

were paralleled by a reduction in TGF-βRI expression in PBMCs, while circulating inflammatory markers were unaffected. Additional protein and vitamin supplementation did not provoke further effects. Interestingly, muscular endurance of upper and lower body as well as aerobic performance at baseline were negatively associated with changes in circulating TGF-β during the first three months of intervention. Another interesting outcome of the study was that drop-outs were characterised not only by lower physical performance but also by higher TGF-β and TGF-βRI expression, while miRNA-21 expression was decreased in PBMCs.

Conclusively, strength training is beneficial even in the oldest old and potentially influences TGF-β signalling through increased receptor expression. Furthermore, adherence to the study was significantly related to alterations in the TGF-β pathway at baseline.

II. KURZFASSUNG

Der Zustand eines chronisch erhöhten Entzündungslevels kennzeichnet menschliches Altern und steht in Zusammenhang zu Inaktivität und Übergewicht. Der transformierende Wachstumsfaktor-β (TGF-β) ist ein multi-funktionelles Protein, mit zahlreichen Funktionen auf Zellebene, welches gewebsmodellierende bis hin zu immunregulierende Aufgaben, erfüllt. Aktuelle Studien zeigen, dass der körperliche Zustand insbesondere die Ausdauer betreffend, die altersbedingte chronische Inflammation positiv beeinflussen kann, wobei dieser Effekt durch das Krafttraining bisher noch nicht eindeutig belegt werden konnte.

Diese Arbeit beruht auf drei Publikationen innerhalb der Vienna Active Ageing Study. Die Geschichte hinter dieser Arbeit beschäftigt sich zuerst mit Normwerten von Parametern des TGF-β Signalweges und die Unterschiede zwischen jungen und alten Frauen, wobei im zweiten Schritt der Einfluss von progressiven Krafttraining bei alten Menschen auf ihr körperliche Leistungsfähigkeit und wiederum den TGF-β Signalweg, untersuchte wurde.

Genauer gesagt, war das Ziel der 1.Stude, den Unterschied des TGF-β Signalweg von peripheren mononukleären Blutzellen (PBMCs) zwischen jungen und alten Frauen zu erheben. Zusätzlich wurde der Einfluss von sportlicher Leistung auf TGF-β und den Rezeptoren erhoben. Die Ergebnisse zeigten, dass sich TGF-β im Serum und TGF-β in PBMCs zwischen alten und jungen Frauen nicht unterschieden haben, während geringere Konzentrationen sowohl im TGF-β Rezeptor I (TGFβ-RI) als auch im TGF-βRII in der älteren Population festgestellt wurden. Interessanterweise korrelierten hs-CRP und microRNA-21 (miRNA-21) positiv mit der Handgriffkraft. Darüber hinaus wurden keine signifikanten Korrelationen zu körperlicher Leistung gefunden.

Studie 2 beschäftigte sich mit dem Effekt von Krafttraining mit elastischen Bändern auf verschieden Kraftparameter bei älteren Personen, die in Pensionisten-Wohnhäusern wohnen. Dazu wurden die ProbandInnen randomisiert einer von folgenden drei Gruppen zugeteilt: Krafttraining (RT), RT in Kombination mit einem eiweißreichen Nahrungssupplement (RTS), oder kognitives Training (CT). Herausgefunden wurde, dass sechs Monate progressives niedrig intensives Krafttraining die körperliche Leistungsfähigkeit verbessert, wobei eine zusätzliche Nahrungsergänzung zu keinen größeren Effekt führt, als das Training alleine.

In der 3. Publikation wurde der Effekt der verschiedenen Interventionen auf die chronisch erhöhte Inflammation und den TGF-β Signalweg in PBMCs untersucht. Daraus resultierte, dass die Verbesserungen in der körperlichen Leistung mit einer Reduktion des TGF-βRI in PBMCs einhergingen, wobei die Inflammationsmarker unverändert blieben. Eine zusätzliche Nahrungsergänzung hat auch hier zu keiner Veränderung geführt. Interessanterweise zeigte die Kraftausdauer der oberen und unteren Extremitäten sowie die globale Ausdauer eine negative Korrelation mit den Veränderungen im TGF-β. Ein weiteres interessantes Ergebnis liefert die Analyse der ausgeschiedenen Personen, die nicht nur durch eine geringere physische Leistungsfähigkeit charakterisiert waren, sondern auch durch eine erhöhte TGF-β und TGF-βRI Ausschüttung, sowie eine verringerte miRNA-21 Expression in PBMCs.

Zusammenfassend kann man sagen, dass sich Krafttraining auch im hohen Alter positiv auf die körperliche Leistungsfähigkeit auswirkt, und möglicherweise durch die Erhöhung des Rezeptor 1 einen Einfluss auf den TGF-β Signalweg hat. Zusätzlich hat sich gezeigt, dass ein frühzeitiges Ausscheiden aus der Studie mit einer verstärkten Expression im TGF-β Signalwerg bei den Ausgangswerten einhergeht.

III. LIST OF PUBLICATIONS

The present doctoral thesis is based on following articles:

Article 1:

Halper, B., Hofmann, M., Oesen, S., Franzke, B., Stuparits, P., Vidotto, C., Tschan, H., Bachl, N., Strasser, E.M., Quittan, M., Wagner, K.H. & Wessner, B. *Influence of age and physical fitness on miRNA-21, TGF-β and its receptors in leukocytes of healthy women.* Exerc Immunol Rev, 21:154-63.

Article 2:

Oesen, S., Halper, B., Hofmann, M., Jandrasits, W., Franzke, B., Strasser, E.M., Graf, A., Tschan, H., Bachl, N., Quittan, M., Wagner, K.H. & Wessner, B. *Effects of elastic band resistance training and nutritional supplementation on physical performance of institutionalised elderly - A randomized controlled trial.* Experimental Gerontology. 2015

Article 3:

Schober-Halper, B., Hofmann, M., Oesen, S., Franzke, B., Wolf, T., Strasser, E.M., Bachl, N., Quittan, M., Wagner, K. H. & Wessner, B. *Influence of elastic band resistance training on TGF-ß pathway in leukocytes of institutionalized older people: the Vienna Active Ageing Study (VAAS).* Immunity and ageing. 2016

Further related articles of the Vienna Active Ageing Study:

Hofmann, M., Halper, B., Oesen, S., Franzke, B., Stuparits, P., Tschan, H., Bachl, N., Strasser, E.M., Quittan, M., Ploder, M., Wagner, K.H. & Wessner, B. *Serum concentrations of insulin-like growth factor-1, members of the TGF-beta superfamily and follistatin do not reflect different stages of dynapenia and sarcopenia in elderly women***.** Exp Gerontol. 2015 Apr; 64:35-45

Hofmann, M., Schober-Halper, B., Oesen, S., Franzke, B., Tschan, H., Bachl, N., Strasser, E.M., Quittan, M., Wagner, K.H. & Wessner B. *Effects of elastic band resistance training and nutritional supplementation on muscle quality and circulating muscle growth and degradation factors of institutionalized elderly women: the Vienna Active Ageing Study (VAAS).* Eur J Appl Physiol. 2016 Mar 1.

Franzke, B., Halper, B., Hofmann, M., Oesen, S., Peherstorfer, H., Krejci, K., Koller, B., Geider, K., Baierl, A., Tosevska, A., Strasser, EM., Wessner, B., Wagner, KH. Vienna Active Ageing Study Group. *The influence of age and aerobic fitness on chromosomal damage in Austrian institutionalised elderly.* Mutagenesis. 2014 Nov; 29(6):441-5

Franzke, B., Halper, B., Hofmann, M., Oesen, S., Jandrasits, W., Baierl, A., Tosevska, A., Strasser, E.M., Wessner, B. & Wagner, K.H. Vienna Active Ageing Study Group. *The impact of six months strength training, nutritional supplementation or cognitive training on DNA damage in institutionalised elderly.* Mutagenesis. 2015 Jan; 30(1):147-53

Franzke, B., Halper, B., Hofmann, M., Oesen, S., Pierson, B., Cremer, A., Bacher, E., Fuchs, B., Baierl, A., Tosevska, A., Strasser, E.M., Wessner, B., Wagner, K.H., Vienna Active Ageing Study Group (VAAS). *The effect of six months of elastic band resistance training, nutritional supplementation or cognitive training on chromosomal damage in institutionalized elderly***.** Exp Gerontol. 2015. May; 6: 16-22

Grimpampi, E., Oesen, S., Halper, B., Hofmann, M., Wessner, B. & Mazzà, C. *Reliability of gait variability assessment in older individuals during a six-minute walk test.* J Biomech. 2015 Nov 26; 48(15):4185-9

IV. TABLE OF CONTENT

V. ABBREVIATIONS

BMP	Bone morphogenetic proteins
CT	Cognitive Training
hs-CRP	high-sensitivity C-reactive protein
IL	Interleukin
miRNA	MicroRNA
NK	Natural Killer
nt	Nucleotides
RISC	RNA-induced silencing complexes
RNA	Ribonucleic Acid
R-SMAD	receptor-regulated SMAD
RT	Resistance Training
RTS	Resistance Training + Supplement
SBE	SMAD-binding element
TGF-β	Transforming Growth Factor-beta
TGF-βRI	Transforming Growth Factor-beta Receptor I
TGF-βRI	Transforming Growth Factor-beta Receptor II
TNF-α	Tumor Necrosis Factor-α
VAAS	Vienna Active Ageing Study

1 Introduction

1.1 Aim of the doctoral research

The Research Platform Active Ageing was approved in early 2011 and conducted as a multicentre study by the Department of Nutritional Sciences, Centre for Sport Science and University Sports (Department of Sports and Exercise Physiology) and the Karl Landsteiner Institute for Remobilization and Functional Health. The aim of the study was to capture the effect of training with and without additional dietary supplementation on muscle strength, -function and -mass, immune system, molecular muscle-specific markers, DNA-damage and oxidative stress parameters in old institutionalised subjects.

The focus of this doctoral research was the influence of age and physical performance on immunological parameters in old persons. Therefore the first scientific task was to get an overview of the differences between young and old subjects. Those immunological alterations were measured in the circulating and intracellular transforming growth factor-β (TGF-β) and its related parameters like TGF-β Receptor I and II (TGF-βRI/II) as well as microRNA-21 (miRNA-21) in peripheral blood mononuclear cells (PBMCs) beside other circulating inflammatory parameters.

The second part of the doctoral research was the major part of the Vienna Active Ageing Study (VAAS), broach the issue of the influence of a six month progressive resistance training with elastic bands compared to training accompanied by nutritional supplementation and cognitive training in institutionalised old persons.

The third and main part of this thesis is focused on adaptions of circulating as well as intracellular TGF-β signalling and inflammatory parameters after the six months of intervention.

1.2.1 Ageing and the inflammatory adaptions

Active and healthy ageing remains one of the main goals of modern society as the occurrence and management of age-associated diseases such as metabolic disorders, cardiovascular

diseases, cancer, and cognitive impairments represent a huge burden to the individuals, their families and the national health care systems in general[1].

Ageing is characterized by a complex remodeling of thousands of biological variables necessary for maximizing the feasibility of survival, based on the product of genetic, environmental and stochastic factors[2]. During the last decade, several declarations and hypothesis of ageing occurred, whereby the inflammation hypothesis of ageing attracted most attention. The ageing process is accompanied by a chronic low-grade inflammation in older subjects compared to younger[3]. This chronic low-grade inflammation is also termed as "inflammageing" by Franceschi[4]. The process is described as a global reduction in the capacity to cope with a variety of stressors and a concomitant progressive increase in pro-inflammatory status[4]. Thereby the activation of redox-sensitive transcription factors by age-related oxidative stress causes the up-regulation of pro-inflammatory gene expression in a variety of different cell types, resulting in a low-grade chronic systemic pro-inflammatory state[5]. More precisely, this state is characterized by enhanced levels of pro-inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α). Those cytokines have been shown to rise with age and be involved in the pathogenesis of most age-associated diseases[6,7]. Evidence is based on vitro and in vivo studies, showing that IL-6 and TNF-α can inhibit protein synthesis[8]. C-reactive protein (CRP), an acute phase protein produced by the liver in response to IL-6, is also a useful marker of inflammageing and moreover a predictor of risk for cardiovascular and other diseases[9].

Those inflammatory imbalances are even associated with frailty and the development and progression of severe age-related conditions like cardiovascular diseases and type 2 diabetes mellitus. Moreover, this ongoing phenomena is a highly significant risk factor for both, morbidity and mortality in older people, as most if not all age-related diseases share an inflammatory pathogenesis[10] (Figure 1).

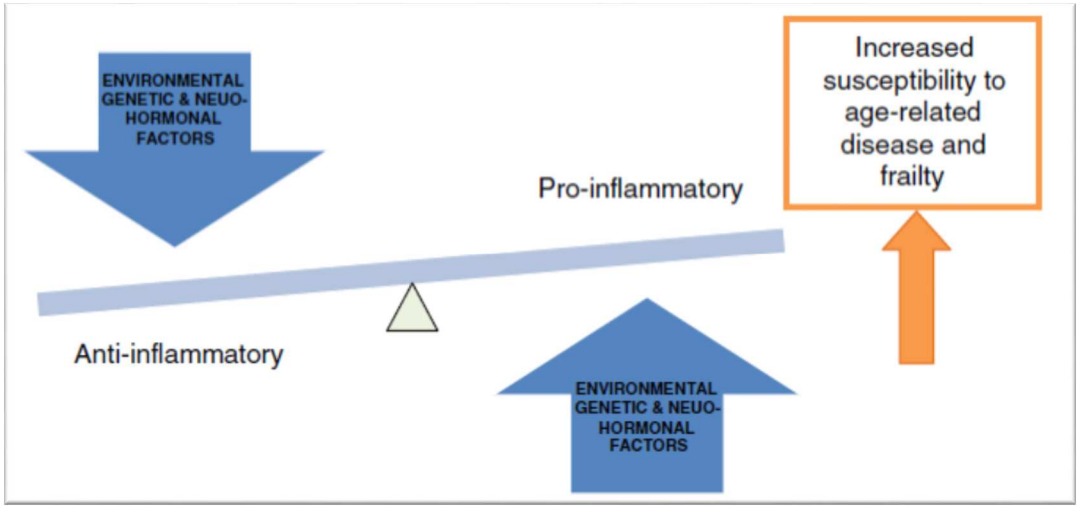

Figure 1: Relationship between pro- and anti-inflammation in ageing (Baylis et al. 2013)[11]

Conclusively, chronic inflammation in older persons can contribute to loss of muscle mass and strength and the combination likely leads to a perpetual cycle of poor function capacity, low physical fitness and persistent inflammation among older subjects[3].

The parallel and related ongoing biological process occurring in age concerns the progressive deterioration of adaptive and innate immune responses, as a result of age-dependent decreases in immunological competence, is also known as 'immunosenescence`[12, 13]. In this scenario, the ageing process is related to changes in the numbers of innate immune cells, which can be explained in some cases by the redistribution of cell subsets[14]. Generally a reduction of the natural killer (NK) cells mediated cytotoxicity and disturbances in macrophage-derived cytokine release, lead to increased prevalence of infections[15]. In general, ageing is associated to decreased main functions of innate immune cells, caused by an altered signal transduction pathways and changes in the expression of various innate immune cell receptors. As a consequence of these changes, the ability to collaborate in the initiation of the adaptive immune response can be impaired[14]. Panda et al. (2009) also note that normal human ageing affects several aspects of the innate immune response, which leads to a reduced ability to provide the prompt response to viral and bacterial pathogens and also to influence and integrate with the adaptive immune response[16]. Pawelec (2007) declares that immunosenescence contributes to heightened susceptibility of older subjects to infectious diseases. However, the defense against pathogens is impaired mainly because of alterations in adaptive immunity mediated by B and T cells[17]. Interestingly, Spielmann et al.

(2014) reported that relations between obesity and T cell differentiation are associated to immunosenescence in adolescents[18].

Nevertheless, the precise etiology of inflammageing and its potential causal role in contributing to adverse health outcomes remain largely unknown[19].

1.2.2 Transforming Growth Factor-β

Pioneers in the field of molecular biology in 1980s were used to research a hormone with one main function and this function only. The multifunctional nature of transforming growth factor-β (TGF-β) does not fit with this paradigm. The effects of TGF-β were different or even opposite, depending on the cell type and the conditions[20].

The effects of TGF-β are mediated by three ligands, namely TGF-β1, TGF-β2 and TGF-β3 through TGF-β type I and type II receptors[21]. Among these three isoforms, TGF-β1 is predominantly expressed in the immune system by different lineages of leukocytes and stromal cells[22]. Unlike most cytokines, TGF-β is synthesized as an inactive large precursor molecule containing a self-inhibiting propeptide in addition to the active form of TGF-β. Proteolytic processing results in a heterodimer containing an active TGF-β in association with either a latency-associated protein or a latent TGF-β binding protein, which keeps TGF-β inactive[23, 24]. Under physiologic conditions, active TGF-β bound, or an intracellular soluble form is liberated from these proteins following additional stimuli from acidic pH, integrins, thrombospedin-1 and proteases, enabling it to exert its function by binding to its receptor[24, 25].

TGF-β is known as a potent regulatory cytokine with diverse effects on haemopoietic cells and a pivotal function in the immune system through keeping tolerance via the regulation of lymphocyte proliferation, differentiation and survival[26]. Moreover, TGF-β controls the initiation and resolution of inflammatory responses through the regulation of chemotaxis, activation and survival of lymphocytes, NK cells, dendritic cells, macrophages, mast cells and granulocytes[27]. Hs-CRP has been shown to directly stimulate TGF-β and induce other genes that contribute to collagen deposition[28].

As an example of this dual function, under the influence of the TGF-β cytokine TH17 as well as Treg cells can develop from naïve CD4+ T lymphocyte precursors[29, 30]. The presence of TGF-β leads to a differentiation of naïve T cells into Treg cells that express the transcription factor forkhead box p3[31]. In contrast together with IL-6, which is produced by the activated innate immune system in case of infection or inflammation[32], TGF-β induces the differentiation of naïve T cells into TH17 cells that express the transcription factors Retinoic acid receptor γ (RORγ) and RORα while IL-6 inhibits the Treg cell differentiation that is induced by TGF-β[32]. Summarised, TGF-beta inhibits the development of immunopathology to self or nonharmful antigens without compromising immune responses to pathogens[27].

In tumour progression, TGF-β has a decisive role through suppressing inflammation, which can drive tumorigenesis and also affect the recognition and destruction of tumour cells through the regulation of immune cell function[33]. Therefore, high levels of TGF-β are produced by many types of tumours including melanomas and cancers of the breast, colon, esophagus, stomach, liver, lung, pancreas, prostate, and hematologic malignancies[34, 35]. Interestingly, in early stages of tumourigenesis, TGF-β seems to act as a tumor suppressor[36]. Conclusively, TGF-β has either a tumor-suppressing or tumor-promoting function depending on cellular context[33].

1.2.3 TGF-β Superfamily

The TGF-β superfamily contains more than 30 structurally related polypeptide growth factors including TGF-βs (1-3), activins (A,B), inhibins (A,B), bone morphogenetic proteins (BMP) (1-20), growth differentiation factors including myostatin, nodal, leftys (1,2), and Müllerianinhibiting substances[20, 37, 38, 39]. TGF-β superfamily members are expressed in most cell types and play essential roles in differentiation and tissue morphogenesis[21].

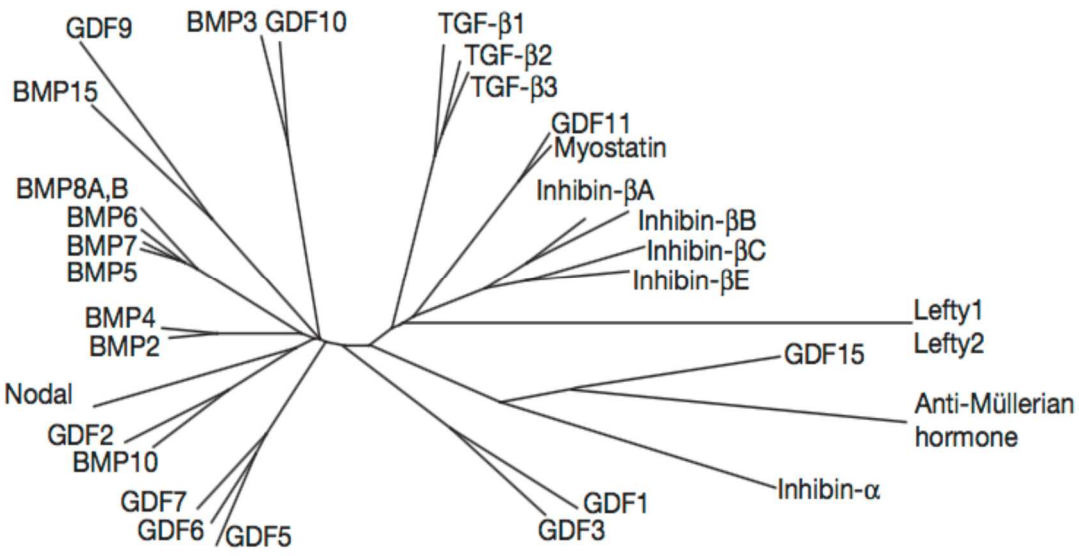

Figure 2. TGF-β superfamily members depicted in form of a phylogenetic tree (Shi et al., 2011)[40]

The TGF-β receptor system, its activation mechanism and SMAD proteins, which function both as substrates for TGF-βRs and signal transducters, came to light in quick succession[20]. Upon phosphorylation by the receptors, SMAD complexes translocate into the nucleus, where they cooperate with sequence-specific transcription factors to regulate gene expression. The vertebrate genome encodes many ligands, fewer type II and type I receptors, and only a few SMADs (Figure 3). In contrast to the perceived simplicity of the signal transduction mechanism with SMAD, the cellular responses to TGF-beta ligands are complex and context dependent[21]. The TGF-βRII is the specific receptor for TGF-β ligands, produced as latent high molecular weight complexes, with high affinity once activated by proteolytic cleavage or structural modification of the latent TGF-β complexes[21]. In total, five type I (activin-like receptor kinase family) and seven type II receptors have been fully identified[41].

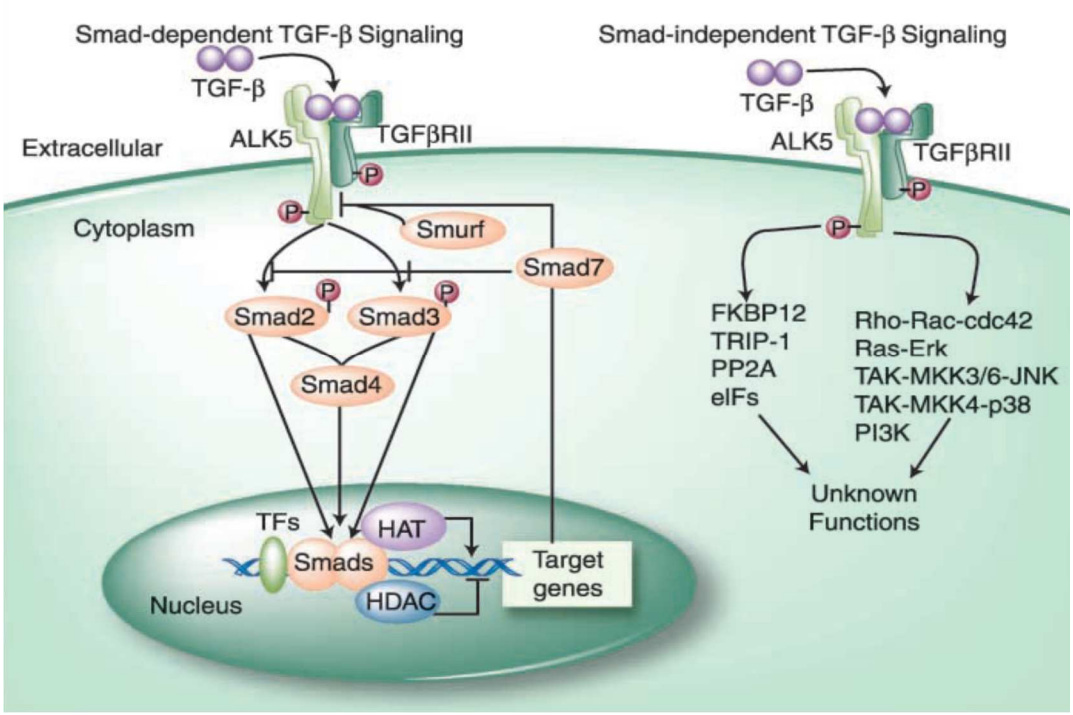

Figure 3: Smad-dependent and Smad-independent signaling following TGF-β binding to its receptor (Chang et al. 2002)[41]

The members of TGF-β superfamily normally function to regulate embryonic development and cellular homeostasis, including regulation of proliferation, differentiation, apoptosis, and extracellular matrix remodeling in a cell and context specific manner[39]. Furthermore, alterations in TGF-β superfamily pathway, including either germ-line or somatic mutations or alterations in the expression of members of these signaling pathway often leads to human disease, including developmental disorders, vascular diseases, and cancer[42].

Not only the pivotal protein TGF-β, is associated to cancer but also the TGF-βRs. Both the TGF-βRs type I and II as well as SMAD proteins are mutated in several cancer diseases while, TGF-βRII inactivating mutations are frequently found in colon cancers associated with microsatellite instability[43, 44]. More detailed, TGF-β RII and SMAD4 are commonly inactivated through mutation and loss of heterozygosity in several types of carcinoma[43].

1.2.4 miRNAs

There are three main types of the ‘classic’ ribonucleic acid (RNA), namely the messenger RNA (mRNA), the transfer RNA (tRNA) and the ribosomal RNA (rRNA). mRNAs are translated into proteins while tRNA and rRNA have housekeeping roles during this translation process[45]. However, small RNAs like microRNAs (miRNAs) are not translated into proteins. Lin-4 and let-7 were the first two miRNAs discovered and identified in the 1990s, in worm Caenorhabditis elegans[45].

MiRNAs are small non-coding RNAs, about 22 nucleotides (nt) long, with an important role as regulators of post-transcriptional gene expression by inhibiting translation of their target proteins or by degrading the respective mRNA[46, 47]. They bind to the 3′UTR of the target mRNA, and the specificity of the binding is dictated mainly by the seed region of the miRNA (from 2 to 8 nt). Hundreds of target genes, often belonging to the same pathway, are regulated by the same miRNA. To increase the complexity of this post-transcriptional regulation several miRNAs can target the same transcript[48].

It has become evident that miRNAs might regulate thousands of human genes by either mRNA degradation or suppression of mRNA translation[49]. Recently it has been estimated that 84–89% of miRNA-induced suppression of gene expression is the result of degradation of target mRNAs[50]. Currently, close to 1,900 human miRNA precursors are listed in the miRBase registry, which give rise to 2,588 mature miRNAs[51, 52].

In detail, the miRNA-biogenesis starts with transcription of miRNA genes by RNA polymerase II or III to generate primary miRNA transcripts (pri-miRNA) in the nucleus[53, 54, 55] (Figure 4). In the next step the primiRNA transcripts are processed by the microprocessor complexes Drosha-DGCR8, resulting in about 70 nt long pre-miRNAs[55, 56, 57]. The hairpin formed pre-miRNAs are exported from the nucleus through Exportin 5[58]. Then pre-miRNAs are processed into about 22 nt long miRNA duplexes (miRNA/ miRNA*) by RNAse III Dicer[56]. It continues with miRNA biogenesis the functional strands of the miRNA duplexes are bound by Argonaute proteins to form RNA-induced silencing complexes (RISC), whereas the other strands of the duplexes are degraded[57]. The single strands of mature miRNA guide RISC to its target mRNAs to downregulate gene expression by suppressing mRNA translation or mRNA degradation[59].

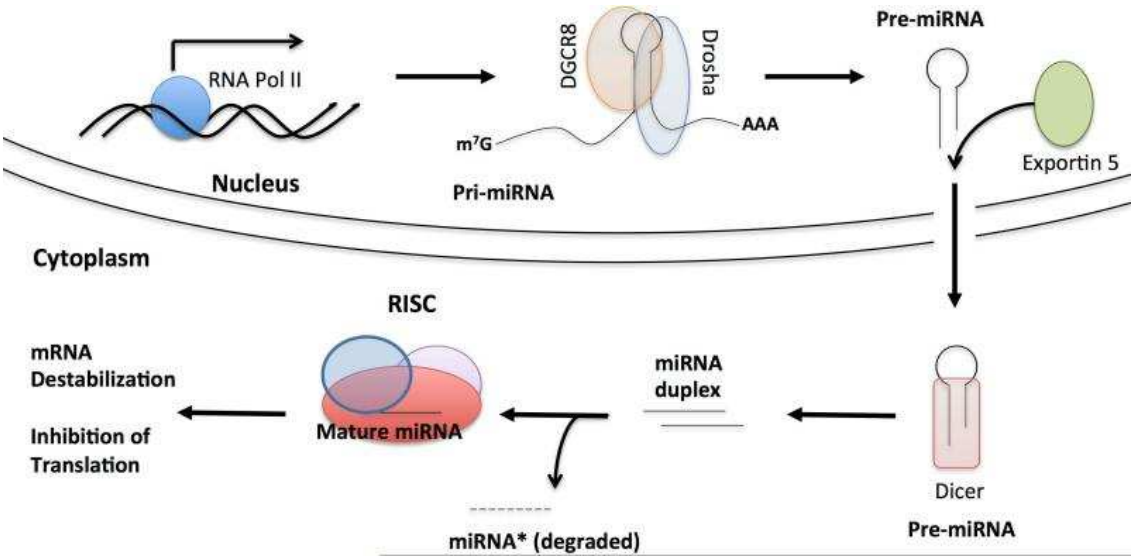

Figure 4: Biogenesis of miRNAs (Blahna & Hata 2012)[60]

MiRNA regulation itself can be influenced by SMADs. This regulation can be classified into two different mechanisms[61]. The first type is described through transcriptional regulation of miRNAs by SMADs largely which resembles the canonical TGF-β signaling pathway. The binding of a TGF-β ligand results in phosphorylation of a receptor-regulated SMAD (R-SMAD) and the formation of an R-SMAD / Co-SMAD heterodimer, afterwards the complex is translocated to the nucleus and binds to the SMAD-binding element (SBE) to regulate the transcription of miRNA genes either positively or negatively, to undergo regular miRNA processing[61]. The second mechanism is the posttranscriptional regulation of miRNA biogenesis that acts on pri-miRNAs in the cell nucleus[61, 62]. The phosphorylation of an R-SMAD induces the import into the nucleus where it recognizes and binds an SBE-like sequence that is located in the stem section of the pri-miRNA. Next the R-SMAD recruits the microprocessor complex Drosha-DGCR8 to the pri-miRNA to stimulate processing of the pri-miRNA into pre-miRNA[61, 62]. Interestingly, TGF-β and BMP modulate the fast posttranscriptional induction of miRNA-21 and miRNA-199a in human pulmonary smooth muscle cells[63] (Figure 5). Further research revealed even more miRNAs, including miRNA-105, miRNA-215, miRNA-421, miRNA-509 are regulated post-transcriptionally by BMP and TGF-β[62].

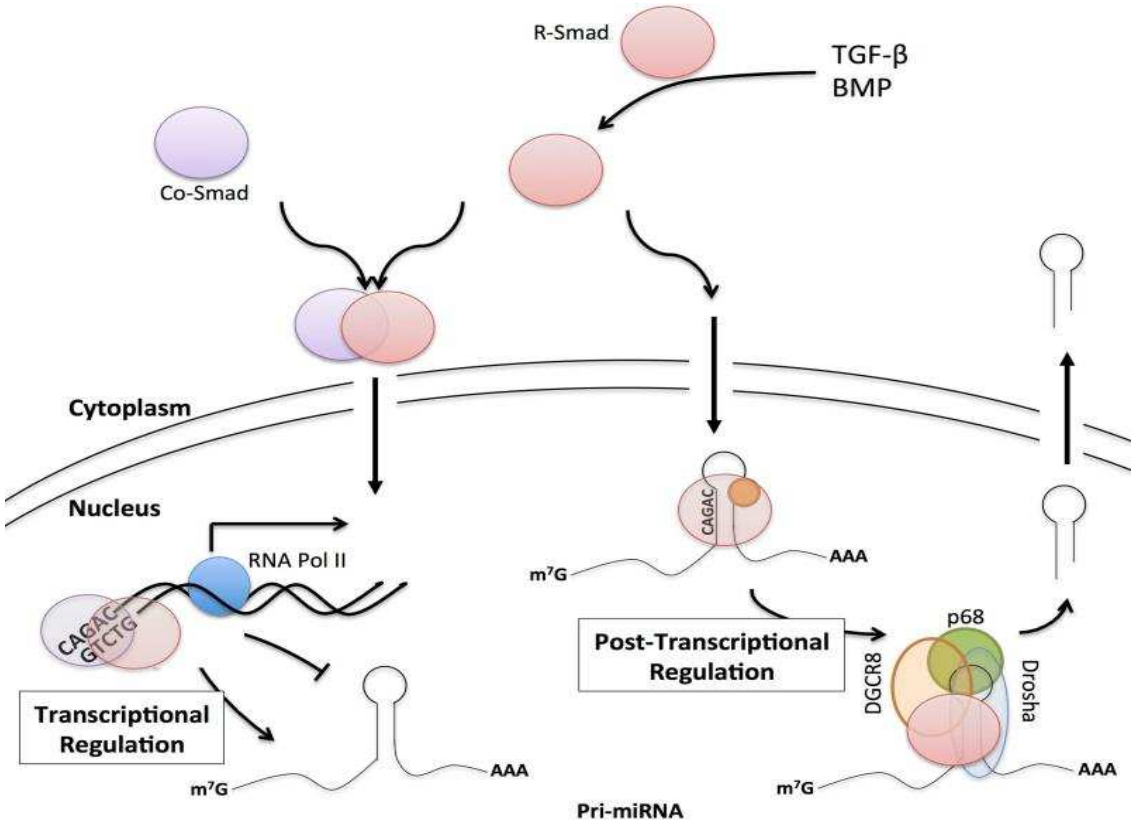

Figure 5: Regulation of miRNA expression by SMADs (Blahna & Hata 2012)[60]

In addition, miRNAs can target SMAD proteins[64]. Most SMAD proteins are targeted from even one or more miRNAs[60]. Marquez et al. (2010) showed that miRNA-21 down-regulates SMAD7, which is a negative regulator of TGF-β signalling[65]. However, miRNA-21 is a validated target of both TGF-βRI and TGF-βRII[64] (Figure 6). Moreover, an overexpression of miRNA-21 is associated with elevated levels of pro-inflammatory cytokines in a dominant-negative TGF-βRII mouse model[66].

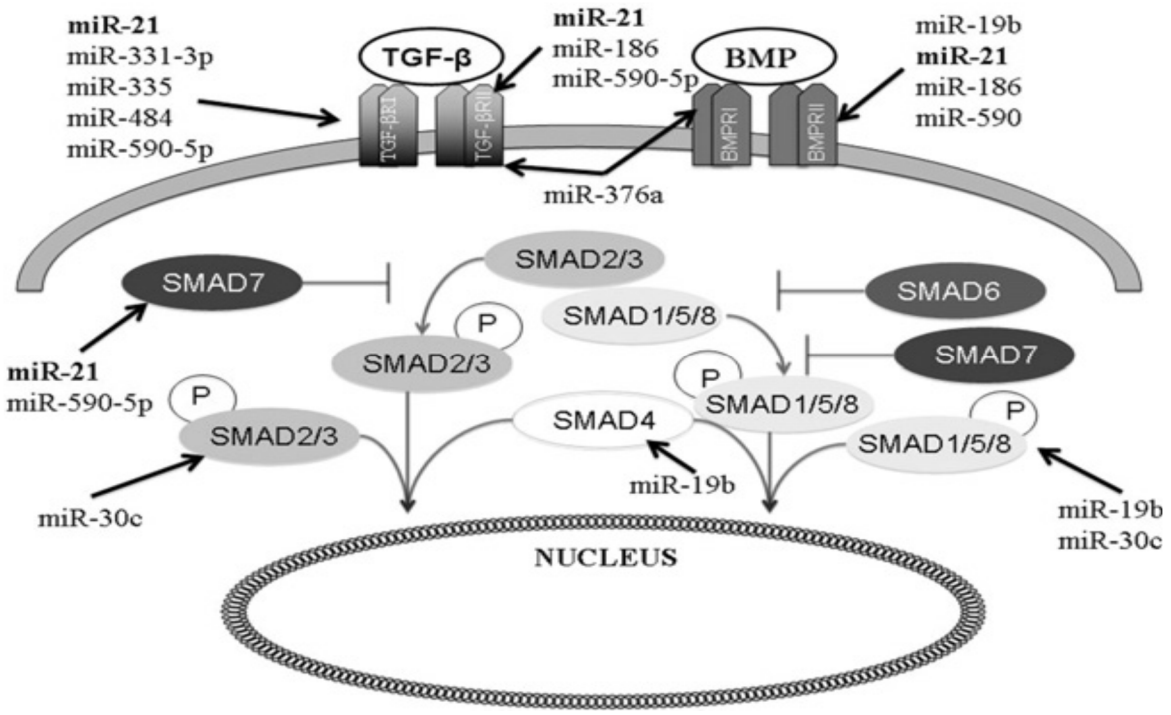

Figure 6: The TGF-beta signaling pathway[64]

It seems therefore reasonable to expect that miRNA-21 have been shown to regulate many biological functions including ageing and seems to be a key regulator of immune cell function and development as well as disease pathogenesis[67-69,71]. Therefore, the function of miRNA-21 is focused and more precisely described in this doctoral thesis.

Regarding cancer, the identification of several targets of miRNAs which are actually classical oncogenes or tumour suppressors has led to the widely accepted idea that miRNAs play pivotal roles in cancer initiation, progression and metastasis[70, 71]. MiRna-21 was first noted as an apoptotic suppressor in various cell lines but it plays also a crucial role in a plethora of biological functions and diseases including cardiovascular diseases and inflammation[72, 73]. Asthma is a chronic inflammatory disease characterized by inflammation of the airways, tissue remodelling and decreased respiratory function. MiRNAs may play an important role in asthma as it is characterized by marked changes in gene and protein expression in the lung[74]. More precisely, IL-12p35 expression is downregulated during asthma, which is correlated with increased expression of miRNA-21[75].

Furthermore, miRNA-21 was induced in 8 of 10 inherited muscular diseases[76]. Soares et al. (2014) have shown, that gain and loss of function experiments in vivo revealed that miRNA-206 and miRNA-21 were sufficient and required for atrophy programmes[72].

Summarised, recent data have demonstrated that also the expression of miRNAs in PBMCs changes with ageing, suggesting that miRNAs and their predicted targets may be potential diagnostic indicators of aging and/or age related diseases[77].

1.2.5 Influence of physical fitness on immunological parameters

The loss of muscle mass and function, the so called sarcopenia, is a consequence of human ageing. The increasing prevalence of sarcopenia and critical illness in older subjects has resulted in a deadly intersection of disease processes[78]. The lethality of this combination appears to be the result of altered muscle metabolism, decreased mitochondrial energetics needed to survive critical illness, and a chronically activated catabolic state likely mediated by TNF-α[78].

Although exercise programs can prevent or ameliorate this ageing process, the effectiveness of exercise interventions to build muscle and effect metabolic improvements is less efficient in older individuals compared to young[79] (Lutz, 2012, 22935594). Those differences in trainability occur due to multiple cellular and biochemical changes like inflammatory processes, as mentioned before.

The second point is the gain of adipose tissue as a serious issue in ageing and a growing health concern in all ages. Importantly, the combination of obesity and sarcopenia carries a high health risk[79]. Lutz et al. propose that the link between body composition (the increased ratio of fat to muscle) and immunity in ageing causes in an increased expression of adipokines and decreased expression of myokines that affect immune system[79]. Or rather, myokines have a pivotal role in the protection against diseases associated with low-grade inflammation, hyperlipidemia, cardiovascular diseases, type 2 diabetes, insulin resistance and cancer and they are involved in the mediation of health-beneficial effects of exercise[80]. Myokines are secreted by contracting skeletal muscles into the circulation, while some directly act on the muscle itself[81, 82]. Furthermore, myokines are able to create a systemic anti-inflammatory environment and provoke specific endocrine effects on visceral fat[80].

With respect to exercise, one well researched myokine is IL-6. This IL-6 is expressed by type 1 and type 2 muscle fibres and acts directly in the muscle and peripherally in different organs via a hormone like way[83]. In inactive muscle, the cytokine encoding gene is silent, but it is activated rapidly when muscle starts to contract[83]. In general, IL-6 is the first myokine / cytokine released into circulation during exercise and it can increase up to a 100-fold concentration[83].

Pederson et al. (2004) have shown that skeletal muscle maintains its function as an endocrine organ in age, although even a normal capability of IL-6 cytokine production and release is preserved in healthy old compared to younger adults[84]. The increase in IL-6 induced by exercise depends on several facts like exercise duration, intensity, recruited muscle mass and the individual endurance capacity[85]. However, chronic exercise leads to reduced level of IL-6 as shown by Lima et al. (2015), examining 10 weeks of aerobic exercise[86].

In general, IL-6 derived from muscles, seems to inhibit low-grade production of TNF-α, thus TNF-α induces insulin resistance[83]. Pederson (2011) described that the reaction to exercise is characterized by a distinct increase in IL-6, followed by IL-1ra, TNF-R and IL-10[87] as similarly shown by Gleeson (2006) (Figure 7).

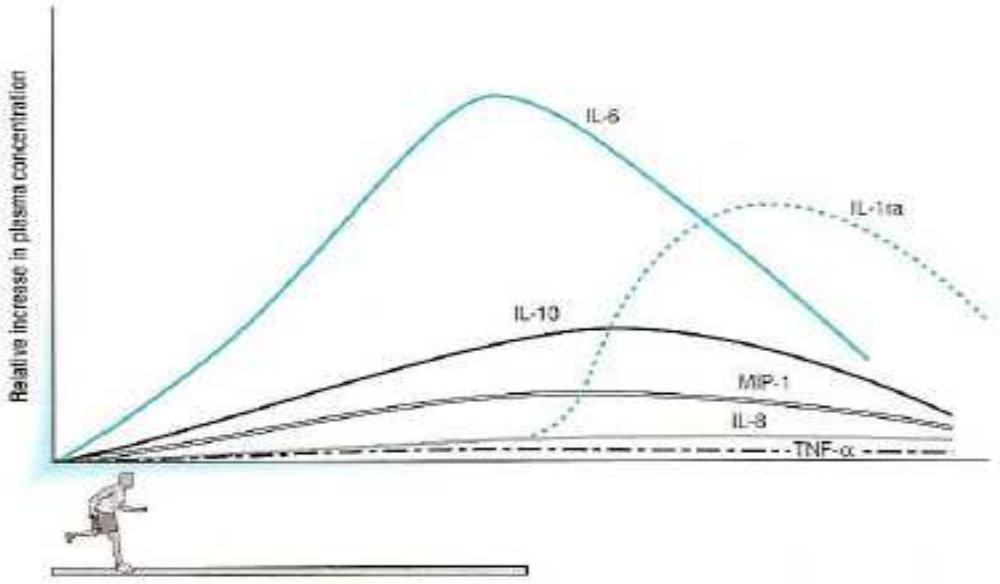

Figure 7: Changes in cytokine levels after intense exercise (Gleeson, M. (2006)[88].

In higher aged women, a recent study has shown that strength training alone did not change any inflammatory parameters while strength training combined with fish oil supplementation enhanced the production of several cytokines (interferon-γ, IL-2, IL-6 and IL-10)[89]. Touvra et al. (2011) have shown that strength training combined with aerobic exercise training increased circulating TGF-β and reduced hs-CRP without altering the levels of IL-6, IL-10, interferon-γ and TNF-α in older patients with type II diabetes[90].

Besides myokines and cytokines, many other inflammatory processes occur during and after exercise. Also in miRNAs a lot of changes occur due to exercise training. Acute as well as chronic endurance exercise can provoke rapid miRNA adjustments[91]. Russel et al. (2013) have shown that an acute bout of endurance exercise at a moderate intensity leads to a decrease in miRNA-9, -23a, -23b and -31 while miRNA-1, -133a, -133-b and -181a were increased 3 hours post exercise. Interestingly, 10 days short-term training resulted in enhanced level of miRNA-1 and -29b, whereas miRNA-31 remained on a decreased level similarly to acute exercise bout[92]. Another acute and brief exercise examined a 2 min bout of cycling for 10 times interspersed by a 1-minute rest which leaded to significantly altered numbers of miRNAs in monocytes in 12 healthy, male participants[93]. Nielsen et al. (2014) observed that acute aerobic exercise and endurance training showed a significant decrease in the circulating miRNA-146a, but no effect on miRNA-21 level directly after the exercise while a decrease in miRNA-21 was found after the whole intervention period[91]. In circulating miRNAs, Baggish et al. (2011) have shown that after an exhaustive cycling exercise miRNA-146a and miRNA-21, were significantly up-regulated directly after acute exercise but decreased after one hour of rest. This test was repeated after 90 days of rowing intervention with the result that only miRNA-146a displayed a further significantly increased plasma concentration immediately following while both, levels of miRNA-146a and miRNA-21 were still enhanced at rest in comparison to each individual´s resting baseline[94].

Focusing strength training, Davidsen et al. (2011) examined the differences of 56 young, healthy men, divided into ‘high responders’ and ‘low responders’, after performing a 12-week resistance training program 5 days per week. Interestingly, miRNA-451 was up-regulated while miRNA-29a, miRNA-26a and miRNA-378 were down-regulated in muscle of low responders, although no changes in high responders were found[95].

Although studies concerning strength training and miRNAs are rare, current studies indicate that miRNA-21 is associated to muscular function and seems to be up-regulated in ageing[64, 72, 76]. Therefore, miRNA-21 is focused in this investigation.

The positive effects of resistance training on age-related loss of muscle mass and function have been shown repeatedly[96]. Recently TGF-β pathway was converged as a modifier for muscular dystrophy besides regulating inflammatory processes[26, 97], but data investigating the effects of resistance training on TGF-β signalling are scarce. Thus, exercise training supplemented by dietary intervention is not yet researched concerning TGF-beta pathway.

2 Methods

Shortly, the Vienna Active Ageing Study (VAAS) was conducted in a randomized, controlled, observer-blind design. The participants were randomly divided into three parallel groups – resistance training (RT), RT combined with nutritional supplement (RTS), cognitive training (CT) – and matched for gender. Blood samples were collected and physical performance tests were executed before, after three months and after six months of intervention. The aim of the primary VAAS was to assess and compare the effects of either a resistance training intervention, a resistance training and nutritional supplementation intervention or a cognitive training intervention on institutionalized older people.

This thesis is focused on the TGF-β signalling changes and the inflammatory effects resulting from the intervention. To have an idea of standard values and to judge changes in consequence of the intervention, firstly we compared TGF-β signalling parameters of old subjects with younger participants. Furthermore association of TGF-β pathway to physical performance in older participants at baseline was examined.

Second paper deals with the main outcome of six months of intervention on physical parameters, thus the base of the VAAS.

The third publication focusses the TGF-β signalling and its changes after three and six months of intervention.

In accordance to different aims in those three papers, study population, measured parameters and statistical analysis differ. Therefore, detailed methods are pointed out in current paper.

3 Papers

3.1 Publication I

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

Halper B, Hofmann M, Oesen S, Franzke B, Stuparits P, Vidotto C, Tschan H, Bachl N, Strasser EM, Quittan M, Wagner KH & Wessner B.

Exercise Immunology Review. 2015; 21:154-63

Participation:

Halper Barbara: Recruiting, sample collection, blood analyses, literature research, writing the paper

Hofmann Marlene: Sample collection, collection of anthropometric data, support in laboratory analyses, editing the paper

Oesen Stefan: Recruiting, collection of physical fitness parameter, editing the paper

Franzke Bernhard: Support in recruiting and sample collection, editing the paper

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)

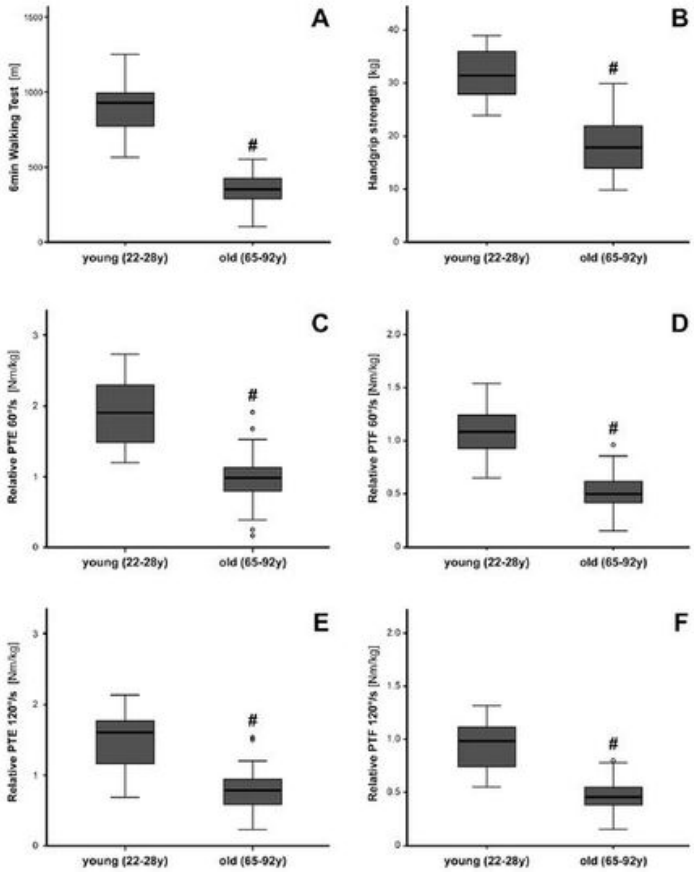

Figure 1. Age-related differences in physical fitness: (A) 6-minutes Walking Test, (B) Handgrip strength, (C) Relative Peak Torque Knee Extension (PTE) 60°/s, (D) Relative Peak Torque Knee Flexion (PTF) 60°/s, (E) Relative Peak Torque Knee Extension (PTE) 120°/s, (F) Relative Peak Torque Knee Flexion (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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3.2 Publication II

Effects of elastic band resistance training and nutritional supplementation on physical performance of institutionalized elderly — A randomized controlled trial

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Participation:

Oesen Stefan: Recruiting, collection of physical fitness parameter, writing the paper

Halper Barbara: Recruiting, literature research, editing the paper

Hofmann Marlene: Collection of anthropometric data, editing the paper

Franzke Bernhard: Support in recruiting, editing the paper

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Effects of elastic band resistance training and nutritional supplementation on physical performance of institutionalised elderly — A randomized controlled trial

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ABSTRACT

Objectives: To evaluate the effects of elastic band resistance training in combination with nutrient supplementation on muscular strength and the ability to perform mobility-related activities of daily living in older adults living in retirement care facilities.
Design: Randomized controlled trial, with a 6-month intervention period.
Setting: A retirement care facility, Vienna, Austria.
Participants: One hundred and seventeen older adults (14 males (12%) and 103 females (88%)), aged 65 to 97 years (mean age: 82.8 ± 6.0), having a mini-mental state examination score ≥23 and no chronic diseases posing a medical contraindication to training therapy.
Intervention: Participants were randomly assigned, but stratified by sex, to one of three intervention groups: supervised resistance exercise training (RT), RT in combination with nutrient supplementation (RTS), or cognitive training group (CT). All interventions were performed two times a week for 6 months. RT was designed to train all major muscle groups using elastic bands. The nutrient supplement (rich in proteins, vitamin D, B2, B12) was distributed every morning as well as after each RT session.
Measurements: A battery of motor ability tests and functional test were performed prior to as well as following 3 months and finally after 6 months of intervention. These tests included isokinetic torque measurements of the knee extensors and flexors in concentric mode at 60 and 120°/s, isometric handgrip strength, senior arm-lifting test, chair stand test, maximum walking speed and a 6-minute walking test (6MWT).
Results: A repeated-measures ANOVA analysis revealed significant improvements in physical function of lower ($p = 0.002$) and upper extremities ($p = 0.006$) for RT and/or RTS in comparison to CT. For isokinetic measurements, 6MWT, and gait speed time effects ($p < 0.05$) were detected without any group × time interaction effects. Dropouts showed lower performance in chair stand test ($p = 0.012$), 6MWT ($p = 0.003$), and gait speed ($p = 0.013$) at baseline than that of the finishers of the study.
Conclusion: Six months of a low intensity resistance exercise using elastic bands and own body weight is safe and beneficial in improving functional performance of institutionalised older people. Multinutrient supplementation did not offer additional benefits to the effects of RT in improving muscular performance.

Abbreviations: ACSM, American College of Sports Medicine; AJLL, artificial joints of lower limbs; CT, cognitive training group; H, hamstrings; MMST, Mini Mental State Test; MNA, Mini Nutritional Assessment; PT, peak torque; RT, resistance training group; Q, quadriceps; RTS, resistance training plus nutritional supplement group; WA, walking aids; 6MWT, 6-minute walking test.

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1. Introduction

Since the mid-twentieth century, the proportion of older persons in the total population has been rising continuously. Based on a recent projection of the United Nations globally the number of persons aged 60 years and over will more than double from 841 million people in 2013 to more than 2 billion in 2050, and by 2050 the share in older persons aged over 80 is expected to be more than three times than that of the present (United Nations, 2013). With increasing age progressive physiological changes occur that lead to a decrease in the function of various organ systems including the muscular system. Ageing-induced losses of skeletal muscle mass, strength, power and function typically occur from midlife onwards and are well documented (McGregor et al., 2014; von Haehling et al., 2010). The preservation of muscle strength and lean muscle mass are key components to perform activities of daily living and to maintain a satisfactory health-related quality of life (Geirsdottir et al., 2012).

Lifestyle choices such as regular physical exercise (both aerobic as well as strength exercises) and a balanced diet containing a sufficient amount of essential amino acids are generally regarded to be effective measures capable of preventing or counteracting age-related muscular decline (Pasiakos et al., 2015). Evidence suggests that intense resistance exercise interventions using free weights and exercise machines may reverse declines in muscle strength, lean muscle mass and functional capacity of older individuals (Romero-Arenas et al., 2013; Silva et al., 2014; Van Roie et al., 2013). The other side of the coin is that resistance exercise training applying high intensity modes and/or maximum strength assessments may pose a higher risk of injury or overuse especially to older people (Liu and Latham, 2010). Unlike traditional resistance exercise, elastic tubing provides adjustable resistance (the level of resistance varies according to rate and maximal stretch of the material such as greater stretch produces greater resistance) allowing a smooth progression (Martins et al., 2013). Not least because of this issue, elastic band exercise is frequently used for therapeutic training. It has been documented as a safe and effective strategy to enhance muscular performance finally improving the ability to perform functional tasks of older individuals (Colado and Triplett, 2008). Additional advantage of exercise training with elastic resistance bands includes high acceptance in older adults, its simplicity, versatility, portability, minimal space requirements and the comparatively low costs (Fahlman et al., 2011).

When resistance training is combined with protein supplementation, muscle protein synthesis is stimulated to an extent where the net protein balance (muscle protein synthesis minus muscle protein breakdown) becomes more positive than that with feeding alone (Phillips et al., 2005). However, there is evidence that the aged muscle is less responsive to the anabolic stimuli of amino acids and exercise, a state that is termed 'anabolic resistance' (Cuthbertson et al., 2005; Rennie, 2009). With respect to protein quality and quantity it has been hypothesized that protein supplements for the elderly should be rich in essential amino acids such as leucine in order to stimulate muscle protein synthesis in a way similar to that of younger adults (Yang et al., 2012). Besides proteins, vitamin D has been suggested to influence muscular performance, especially in subjects with low or deficient vitamin D levels (Halfon et al., 2015). Hence, a combination of resistance training with protein and vitamin D supplementation has been shown to be superior in preserving muscle mass during a weight loss programme in elderly than resistance training alone (Verreijen et al., 2015).

With these aspects in mind, the current study aimed to compare three different supervised training interventions (cognitive training, elastic band resistance training, and elastic band resistance training plus nutritional supplementation) on muscular strength and the ability to perform functional tests related to activities of daily living in older adults living in retirement homes.

2. Methods

2.1. Participants

Of 230 older men and women who were approached at 5 different retirement care facilities located in Vienna, 117 persons (103 females and 14 men, respectively) met the inclusion criteria and volunteered to participate in the study. Participants were required to be over the age of 65 years and physically cleared for medical stability for study participation. The exclusion criteria referred to recommendations of the American Heart Association (Williams et al., 2007) and included cognitive impairment (mini-mental state examination scores being <23) (Folstein et al., 1975), chronic diseases posing a contraindication to training therapy, serious cardiovascular diseases, diabetic retinopathy, and regular use of cortisone-containing drugs. In addition, subjects did not perform any regular resistance training. Before being included in the study, all subjects were comprehensively informed about the purpose, procedures, benefits and risks of and discomfort that might result from study participation. Written informed consent was obtained from all participants. The present study was conducted in accordance to the Austrian laws (including Doctors Act, CISA, Data Protection Act), the Declaration of Helsinki (as revised in Edinburgh 2000), and in analogous accordance with the ICH-GCP guidelines. The study has been approved by the ethics committee of the City of Vienna (EK-11-151-0811) and registered at ClinicalTrials.gov, NCT01775111.

2.2. Experimental design

To examine the effects of 6 months of supervised, progressive resistance exercise training with and without nutrient supplementation, participants of this controlled study were randomly assigned to one of three intervention groups. These intervention groups either performed resistance exercise training (RT), or the same resistance training protocol in combination with nutrient supplementation (RTS), or cognitive training (CT), the latter serving as the control group. Exercise intervention started immediately following baseline pre-testing which included anthropometric measurements, isokinetic torque measurements of knee extensors- and flexors in concentric mode, measurement of isometric handgrip strength of the dominant hand, and a battery of functional tests including upper body lift and reach test, chair stand test, maximum walking speed, and the 6 minute walking test (6MWT). In order to compare physiological adaptations, tests were repeated following 3 and 6 months of intervention, respectively. The tests were supervised by the same investigators. For each measurement time point the assessments were performed on two different days between 9:00 and 11:00 using identical equipment, the same subject positioning as well as standardized instructions. In addition, the order of testing remained the same on both testing days (day 1: Gait speed, chair stand test, 6MWT; day 2: Functional reach test, handgrip strength, isokinetic peak torque measurement (PT 60°/s Q., PT 60°/s H., PT 120°/s Q., PT 120°/s H.), arm-lifting test).

2.3. Resistance exercise intervention

The individualized exercise intervention using elastic band exercise as resistance (Thera-Band®, The Hygenic Corporation, Akron, OH, USA) was designed to train all major muscle groups (training volume and intensity were progressively increased) and was performed two times per week on non-consecutive days (separated by at least 48 h). Exercise training took place in small groups of not more than 10 participants, guided and closely supervised by appropriately trained and experienced sport scientists to ensure safety and compliance. Resistance exercise training was based on guidelines provided by the American College of Sports Medicine (ACSM) for older adults (Nelson et al., 2007). Each exercise session consisted of a general warm-up of 10 min, followed by a resistance training session (35–40 min) incorporating one to two

exercises (in a slow controlled manner) for each of the six muscle groups (legs, back, abdomen, chest, shoulder and arms), and was completed by a cool-down routine. Following an adaptation phase of 4 weeks using low external resistance (yellow Thera-Band®, 1 set of 15 repetitions per exercise with a higher resistance only if the subject was obviously unchallenged) exercise intensity was progressively increased by adapting the resistance of the elastic band (based on the Thera-Band® force-elongation table) (Page and Ellenbecker, 2011) from yellow to red and further to black. Additionally, the exercise volume was enhanced by increasing the number of sets from one to two. Rate of progression was based on individual improvements (band colour was changed if participant would have been able to perform two more repetitions in the second set and reported to be below seven on the OMNI Resistance for active muscle scale (0 extremely easy to 10 extremely hard)) (Lagally and Robertson, 2006). In the final training phase 56.9% of the participants had switched to the red elastic band while 43.1% even used the black one (specific information on the exercises are shown in Supplemental file 1).

2.4. Nutrient supplementation

In addition to resistance exercise training, subjects of RTS group received a nutrient supplement drink (FortiFit, NUTRICIA GmbH, Vienna, Austria) every morning after breakfast, as well as immediately after each training session. Each drink had a caloric value of 150 kcal and contained 20.7 g protein (3 g leucine, >10 g essential amino acids), 9.3 g carbohydrates, 3 g fat, vitamins (800 IU vitamin D, 2.9 mg vitamin B6, 3 μg vitamin B12) and minerals. A research dietician distributed the supplements and monitored adherence. Participants were instructed to maintain their regular food intake.

2.5. Cognitive training

The CT group served as sham-control to the resistance-exercise training groups mentioned above. Different from common control groups not receiving any treatment CT subjects participated in activities including cognitive tasks (memory training) and coordinative tasks (such as manual dexterity) twice weekly to provide a timely effort which was equal to those of the RT and RTS groups, respectively (Gatterer and Croy, 2004).

2.6. Anthropometric assessment

Body mass and body height were measured following standardized anthropometric procedures using a digital scale and a stadiometer during each testing session. Body mass was measured to the nearest 0.1 kg (SECA Model 877, Seca GmbH & Co. KG, Hamburg, Germany) and height was measured to the nearest 0.1 cm using a portable stadiometer (SECA Model 217, Seca GmbH & Co. KG, Hamburg, Germany). Body mass index was calculated as body mass relative to height in meters squared (kg/m^2).

2.7. Nutritional status

The Mini Nutritional Assessment (MNA) was used to assess nutritional status (Vellas et al., 1999). According to standard analyses subjects were classified as having a normal nutritional status (>24 points), as being at risk for malnutrition (17–23.5 points) or as being malnourished (<17 points).

2.8. Isokinetic dynamometry

Due to the relevance of the muscles of the lower extremities to activities of daily living, quadriceps and hamstring muscles were selected for muscle strength assessment. Isokinetic peak torque measurements of knee extensors and flexors were performed using a LIDO Multijoint II isokinetic loading dynamometer (Loredan Biomedical Inc., Sacramento, CA, USA). Participants were tested in a sitting position with their hip flexed at approximately 90° and subjects securely strapped to the seat of the chair using adjustable trunk and waist stabilisation belts. The anatomic axis of the knee rotation at the knee joint was aligned with the machine axis of rotation to insure similar movements for all participants. A thigh strap on the test leg was used to restrict any lateral movement at the knee, allowing only knee flexion and extension. Finally, a resistance pad was placed on the distal tibia, and the ankle of the leg being tested was securely strapped to the dynamometer lever arm. Limits for the knee extension/flexion movement (range of motion) was set between 30° and 80° of knee angle (with 0° being fully extended) with 80° of knee flexion as the starting position. Calibration and gravity correction procedures were performed before starting the test protocol. Following a general warm-up subjects were carefully familiarised with the equipment by performing submaximal trials of the extension–flexion movement with increasing intensity. Finally, two continuous maximal repetitions of knee extensors and knee flexors were performed concentrically at each angular velocity (60°/s and 120°/s) for peak torque recording. Between each of the recorded tests for a given angular velocity subjects rested for 2–3 min before performing the next maximum effort test. Participants were verbally encouraged to make a maximum effort throughout the whole range of motion. The highest extensor and flexor peak torques (Nm) of the two maximal trials at each angular velocity were chosen for analyses (Patterson and Spivey, 1992).

2.9. Handgrip strength

Handgrip strength of the right hand was measured to the nearest kilogram (kg) using a Jamar hand dynamometer (Sammsons Preston, Inc. Bolingbrook IL, USA). Subjects were seated with their elbow unsupported and bent at an angle of 90°. Prior data collection, the width of the dynamometer handle was adjusted to the individual hand size (with the middle phalanx resting on the inner handle) and participants performed two submaximal trials to get acquainted with the instrument and measurement procedure. Finally, participants were encouraged to perform a maximal contraction within approximately 4 to 5 s. After a rest of 60 s, participants were asked to perform a second trial. The highest score of maximum voluntary contraction was used for data analyses (Mijnarends et al., 2013).

2.10. Chair stand test

Chair rise performance is influenced by strength and power of the lower extremities. The mechanical power required to perform repetitive chair-rise movements has been shown to be an important indicator of mobility, fall risk, and functional debility in older individuals (Rikli and Jones, 2013). For this test the maximum number of completed cycles of unsupported chair rises (from a seated to a fully erected position (hip and knees straightened)) completed within 30 s was counted. A straight-back chair with a seat height of 46 cm was placed against a wall for support and safety purposes. Prior data collection and following a demonstration by the tester, participants performed a 2–3 repetition practice trial to familiarise with the technique. Participants were instructed to keep their arms crossed at the wrists and held them against the chest and place their feet flat on the floor approximately shoulder-width apart. For the test participants were encouraged to complete as many full stands as possible. The number of stands executed correctly within 30 s were counted by the tester and used for data analyses (incorrectly performed stands were not counted — the last stand of the end of the 30 s limit was counted only if the subject completed more than 50% of the upward motion).

2.11. Arm-lifting test

Functional performance for sustained upper extremity activity was assessed by performing a 30 s arm-lifting task of the dominant arm. Participants sitting on an armless straight-back chair were asked to repeatedly lift a dumbbell of 2 kg (females) and 4.5 kg (males) from waist level over a distance of 30 cm to shoulder level marked by the upper edge of a box. Following initial submaximal trials for familiarisation and a subsequent rest of 60 s, participants were encouraged to complete as many correct arm lifts as possible within a time limit of 30 s. The number of cycles correctly completed were counted and used for further analyses (King et al., 2000).

2.12. Gait speed

Physical mobility was assessed by measuring maximal walking time over a distance of 6 m on a flat surface on a hallway using an electronic photocell timing system (Brower Timing System, UT, USA). Participants were allowed to increase their speed within 2 m before the starting line and were advised not to decelerate before the finish line. Walking time was determined to the nearest 0.01 s while participants were allowed to use walking aids but must not run. Each participant had two consecutive trials separated by a rest period of 2 min. Gait speed was calculated by transforming walking time for 6 m to gait speed (m/s) (Mijnarends et al., 2013).

2.13. Six-minute walking test

As indicator of aerobic endurance the distance which could be covered within 6 min was recorded. Participants were instructed to walk alone as quickly as possible (without running) around a 30 m shuttle course as many times as possible within the time limit. Turning points were marked with cones. Participants were allowed to reduce the speed or rest if necessary and resume walking based on their performance abilities. Participants were informed about elapsed time after 3, 4, and 5 min. A measuring tape fixed parallel to the walking course allowed the tester who registered the number of shuttles to calculate the final distance, whereby the nearest meter was used for data analyses (Steffen et al., 2002).

2.14. Functional reach test

The functional reach test is a clinical assessment, testing the maximal safe standing forward reach (maintaining a base of support in the standing position) as a marker of postural stability in older adults. The test examines the distance a subject can extend the centre of gravity over the base of support, quantifying the boundaries of sway (Duncan et al., 1990). Before starting the test was described and demonstrated by the tester and participants could perform a trial to get familiarised with the movement execution. Participants were instructed to stand erect (feet shoulder length apart), next to a parallel wall without touching it, arms elevated to 90° of shoulder flexion, elbows extended and hands formed to fists. The assessor records the starting position at the third metacarpal head on a yardstick attached to the wall at acromion height. Then, without moving their feet off the ground, participants performed a hip flexion by moving their trunk forward and reaching as far as they could without taking a step. At this point, the tester again located the position of the third metacarpal head. Subsequently, the subject returned to the starting position and remained still for several seconds to clearly differentiate the end of the movement. Scores were determined by assessing the difference between the start and end position. Each participant performed the test three times with the best score (cm) used for further analyses.

2.15. Determination of physical activity

Physical activity was measured using accelerometers (GT1M, Actigraph, FL, USA) at baseline (pre-training) as well as following 6 months of intervention. Technical specifications characteristics of the Actigraph accelerometer and its validity have been published previously (Kelly et al., 2013). Participants were instructed to wear the device for 5 consecutive days including one weekend on the right hip for all waking hours, except during water-based activities. Data were considered valid if participants had activity counts for at least 10 h on 3 of these days, which is in line with minimum recommendations provided by Trost et al. (2005).

2.16. Statistical analyses

Data acquisition and data analyses were performed using commercial software with data files coded and anonymized. The number of participants chosen was based on power estimation with an alpha level set at 0.05 and a power of > 0.85. The power calculation software (G * Power3.1.0) (Faul et al., 2007) estimated the sample size to be 86 using isokinetic peak torque (concentric knee extension at an angular velocity of 60°/s.; range of motion 20°–80°) as primary endpoint. Previous studies in a similarly difficult collective show a high drop-out rate of about 35–40%. Therefore, a total of about 120 subjects were included in the study.

Repeated Measurement Analyses of Variance (ANOVA) was performed accounting for group (RT, RTS, and CT), time (pre-, mid-term, and post-intervention), and the interaction term between group and time defined as fixed factors and the test subject as random factor (Intention to treat analysis). Each of the endpoints demonstrating a significant result for time or the interaction term ("promising endpoints") in the primary model were further analysed with respect to the following influencing factors: age, gender, training attendance, mental state and residence. For the "promising endpoints" first univariate random effect mixed models were used (with subject as random factor) to study the influence of each of each of the influencing factors, on each of the promising endpoints. The primary model (accounting for group, time and interaction term) was then repeated for each of the promising endpoints, now additional accounting for the influence factors being significant in the univariate analyses (further on denoted by "final" model). Analyses were performed using Proc Mixed, SAS version 9.1 system and R release 2.15.1 (SAS Institute Cary NC, USA), respectively.

3. Results

3.1. Participant flow and baseline characteristics

Details of the participant flow are displayed in Fig. 1. Briefly, 117 elderly women and men who fulfilled the inclusion criteria were randomly assigned to RT (n = 41), RTS (n = 36), and CT (n = 40). A total of 22 participants (18.8%) withdrew their willingness to participate before or immediately following baseline tests. Twelve additional participants dropped-out during the intervention period of 6 months (CT (n = 5; 4.3%), RT (n = 3; 2.6%), RTS (n = 5; 4.3%)). None of the dropouts left the intervention study as a result of injuries or adverse responses to the study but because of other medical reasons not related to the intervention. A total of 82 (70.1%) participants (CT (n = 26; 65.0%), RT (n = 31; 75.6%), RTS (n = 5; 69.4%)) completed the study after 6 months.

Study participants did not differ in most of the baseline characteristics such as age, weight, height, body mass index, cognitive performance as measured by Mini Mental State (MMST), use of walking aids (WA) or the incidence of artificial joints of lower limbs (AJLL). Although the number of male participants was generally low (12.0%), the ratio between males and females did not differ between groups (p = 0.849). Interestingly, the Mini Nutritional Assessment (MNA) revealed significantly lower scores for the CT group as compared to both, the RT and RTS

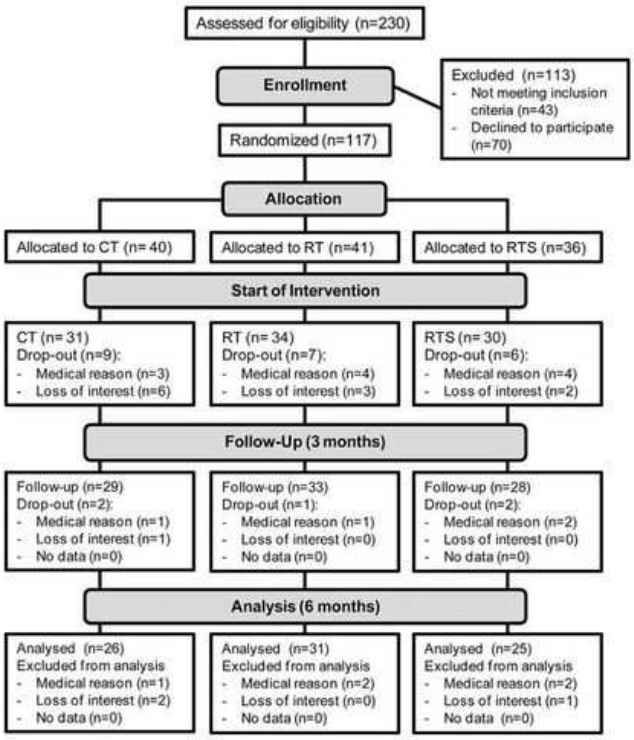

Fig. 1. Participant flow.

group (Table 1). None of the participants were classified as malnourished, 20.7% were at risk of malnutrition (32.3% of CT, 8.8% of RT, and 22.2% of RTS), whereas 79.3% showed a normal nutritional status (67.7% of CT, 91.2% of RT, and 77.8% of RTS; $p = 0.064$ for frequency distribution). When vitamin D deficiency was defined as having plasma values <25 nmol/l (Mosekilde, 2005), 20.0% of the study participants showed vitamin D deficiency (26.7% of CT, 14.3% of RT, and 20.0% of RTS; $p = 0.461$ for frequency distribution).

3.2. Muscle strength of the lower extremities (primary endpoint)

At baseline knee extensor and flexor peak torque did not differ between groups ($p > 0.05$). Peak torque significantly increased over time ($p < 0.05$) for all measurements. Post hoc analyses revealed an increase after 3 months for knee extensor peak torque at both velocities and for knee flexion at 120°/s ($p < 0.05$). After 6 months all measurements remained elevated with the exemption of knee extension at 60°/s which declined to baseline values. Interestingly, most of the elevations were reached at 3 months with only marginal changes between 3 and 6 months. No group ($p > 0.05$) or time × group interactions could be detected (Table 2).

3.3. Hand grip strength and functional performance (secondary endpoints)

Baseline measurements of handgrip strength ($p = 0.249$) and tests of functional performance such as chair stand test ($p = 0.815$), arm-lifting test ($p = 0.839$), gait speed ($p = 0.476$), 6MWT ($p = 0.911$), and functional reach test ($p = 0.142$) were similar among study groups.

With exception of handgrip strength and functional reach test, all measures of physical functioning including chair stand test, arm-lifting test, 6MWT, and gait speed significantly improved over time irrespective of group. While improvements for both, arm-lifting test ($p = 0.004$) and chair stand test ($p = 0.028$) could be detected at 3 months as well as after 6 months ($p < 0.001$), 6MWT ($p = 0.021$) and gait speed ($p = 0.004$) were increased only after 6 months.

Table 1
Baseline characteristics of participants.

	CT	RT	RTS	p-Value
Gender [females/males]	35/5	37/4	31/5	0.849
Age [years], n = 117	83.4 (±5.6)	83.0 (±5.5)	81.8 (±6.9)	0.498
Body mass [kg], n = 104	73.0 (±15.2)	72.5 (±9.7)	74.6 (±15.1)	0.802
Height [m], n = 104	1.59 (±0.08)	1.58 (±0.09)	1.58 (±0.07)	0.991
BMI [kg/m²], n = 104	28.9 (±5.0)	28.9 (±3.7)	29.8 (±6.1)	0.713
MNA [−], n = 92	24.1 (±2.9)	26.3 (±1.8)**	25.9 (±2.2)**	<0.001
MMST [−], n = 117	27.8 (±1.7)	27.1 (±2.0)	27.6 (±1.9)	0.243
WA [number (%)]	11 (32.4%)	4 (11.4%)	6 (18.8%)	0.095
AJLL [number (%)]	3 (11.1%)	3 (9.1%)	1 (3.3%)	0.516

Values are shown as mean (± standard deviation). p-Values refer to differences between groups (Chi Square Test, one-factorial ANOVA).
CT (cognitive training group); RT (resistance training group); RTS (resistance training + supplement group); MNA (Mini Nutritional Assessment); MMST (Mini Mental State Test); WA (regular use of walking aids); AJLL (artificial joints of lower limbs).
** $p < 0.01$ versus CT.

Table 2
Isokinetic peak torque measurements.

Parameter	Group	Mean (± standard deviation)			Differences to baseline		p-values		
		Baseline	3 months	6 months	Δ (pre − 3 m)	Δ (pre − 6 m)	Time	Group	Time × group
PT 60°/s Q. rel. [Nm/kg]	CT	1.03 (±0.29)	1.09 (±0.34)*	0.97 (±0.38)	0.03 (±0.16)	−0.06 (±0.21)	**0.017**	0.476	0.230
	RT	0.98 (±0.32)	1.06 (±0.29)*	1.07 (±0.26)	0.07 (±0.18)	0.06 (±0.21)			
	RTS	1.07 (±0.42)	1.14 (±0.41)*	1.13 (±0.40)	0.06 (±0.16)	0.01 (±0.18)			
PT 60°/s H. rel. [Nm/kg]	CT	0.55 (±0.17)	0.61 (±0.15)	0.56 (±0.21)*	0.03 (±0.12)	0.00 (±0.12)	<**0.001**	0.552	0.061
	RT	0.51 (±0.16)	0.54 (±0.15)	0.60 (±0.14)*	0.02 (±0.10)	0.07 (±0.12)			
	RTS	0.59 (±0.22)	0.61 (±0.18)	0.66 (±0.21)*	0.02 (±0.13)	0.06 (±0.11)			
PT 120°/s Q. rel. [Nm/kg]	CT	0.82 (±0.27)	1.00 (±0.25)***	0.84 (±0.31)*	0.14 (±0.23)	0.01 (±0.19)	<**0.001**	0.794	0.683
	RT	0.82 (±0.26)	0.95 (±0.25)***	0.89 (±0.23)*	0.13 (±0.18)	0.05 (±0.17)			
	RTS	0.84 (±0.33)	0.98 (±0.37)***	0.95 (±0.34)*	0.15 (±0.20)	0.08 (±0.18)			
PT 120°/s H. rel. [Nm/kg]	CT	0.48 (±0.16)	0.63 (±0.15)***	0.56 (±0.23)***	0.12 (±0.16)	0.07 (±0.13)	<**0.001**	0.667	0.302
	RT	0.48 (±0.14)	0.55 (±0.14)***	0.55 (±0.15)***	0.07 (±0.10)	0.05 (±0.11)			
	RTS	0.52 (±0.19)	0.60 (±0.18)***	0.60 (±0.22)***	0.07 (±0.16)	0.06 (±0.11)			

Values are shown as mean (± standard deviation). p-Values refer to main effects of time and group as well as time × group interactions (repeated measurement ANOVA). Significant effects ($p < 0.05$) are shown in bold. CT (cognitive training group); RT (resistance training group); RTS (resistance training + supplement group); PT (peak torque);Q (Quadriceps, knee extension); H. (hamstrings, knee flexion); rel. (relative to body mass).
* $p < 0.05$ and *** $p < 0.001$ vs baseline for time effects.

An additional time × group effect could be detected for chair stand test ($p = 0.002$) and arm-lifting test ($p = 0.006$), indicating that changes over time were different between groups (Table 3). For the chair stand test a significant larger increase was found for RT (+17% at 3 and 6 months; $p < 0.001$) and RTS (+9% at 3 months and +15% at 6 months; $p = 0.007$) compared to CT. Interestingly, ameliorations in the arm-lift test were detected only in RTS (+19.1% at 3 months and +56% at 6 months; $p = 0.002$). No statistical differences could be observed between CT and RT ($p = 0.312$) as well as between RT and RTS ($p = 0.056$) (Fig. 2).

In order to determine whether a change in everyday physical activity could have influenced the primary and secondary outcomes, steps per day as marker of physical activity were determined by accelerometers. Valid data could be obtained from 61.5% of the participants at baseline but only from 36.7% after 6 months. Baseline measurements revealed an average of 4382 ± 2026 steps per day with no differences between intervention groups. Interestingly, after 6 months this measure decreased to 4197 ± 1696 steps per day ($p = 0.050$), while no group or time × group interaction effects could be detected.

3.4. Confounding factors

In general, a significant influence of age (with older participants having worse performance indices) was observed in several parameters, including peak torque measurements of knee extensors (120°/s) and flexors (60°/s and 120°/s) ($p < 0.001$), the 30-second arm lifting test ($p < 0.001$), and the 6MWT ($p = 0.016$), respectively. Besides exercise performance, older participants also had poorer scores in the mini mental state examination ($p = 0.019$).

Gender differences with higher values for male participants were observed in concentric isokinetic peak torque measurements of knee extensors (120°/s) and flexors (60°/s and 120°/s) ($p < 0.001$) and

Table 3
Handgrip strength and functional performance.

Parameter	Group	Mean (± standard deviation)			Differences to baseline		p-Value		
		Baseline	3 months	6 months	Δ (pre − 3 m)	Δ (pre − 6 m)	Time	Group	Time × group
Handgrip strength [kg]	CT	17.0 (±6.6)	17.3 (±8.2)	16.6 (±7.6)	0.8 (±2.6)	−0.3 (±2.4)	0.639	0.197	0.470
	RT	19.8 (±6.3)	20.2 (±5.6)	20.4 (±5.8)	−0.1 (±2.8)	0.4 (±2.7)			
	RTS	18.8 (±7.3)	19.2 (±7.0)	19.8 (±6.6)	0.2 (±2.5)	0.3 (±2.5)			
Arm-lifting test [repetitions]	CT	24 (±10)	23 (±9)**	23 (±10)***	2 (±9)	3 (±7)	<**0.001**	0.159	**0.006**
	RT	24 (±10)	28 (±10)**	30 (±12)***	2 (±4)	6 (±6)			
	RTS	22 (±11)	27 (±11)**	34 (±10)***	4 (±5)**	10 (±8)**			
Chair stand test [repetitions]	CT	11 (±5)	11 (±5)*	11 (±5)***	−1 (±2)	0 (±2)	<**0.001**	0.076	**0.002**
	RT	11 (±4)	14 (±4)*	15 (±4)***	2 (±3)***	2 (±3)***			
	RTS	12 (±5)	13 (±6)*	15 (±7)***	1 (±3)**	2 (±)**			
6MWT [m]	CT	369 (±111)	380 (±119)	389 (±113)*	−5 (±51)	9 (±59)	**0.006**	0.825	0.469
	RT	367 (±92)	394 (±89)	422 (±91)*	19 (±43)	23 (±45)			
	RTS	359 (±101)	393 (±158)	396 (±143)*	29 (±101)	29 (±98)			
Gait speed [m/s]	CT	1.48 (±0.57)	1.62 (±0.75)	1.62 (±0.67)**	0.05 (±0.36)	0.08 (±0.30)	**0.010**	0.800	0.296
	RT	1.49 (±0.39)	1.60 (±0.47)	1.71 (±0.52)**	0.11 (±0.31)	0.19 (±0.41)			
	RTS	1.50 (±0.70)	1.50 (±0.68)	1.62 (±0.83)**	−0.08 (±0.40)	0.00 (±0.46)			
Functional reach test [cm]	CT	27.3 (±5.3)	27.9 (±6.0)	27.5 (±6.5)	0.6 (±4.7)	0.8 (±5.9)	0.371	0.081	0.453
	RT	29.6 (±6.4)	29.9 (±7.9)	29.7 (±7.1)	0.3 (±6.1)	0.0 (±7.2)			
	RTS	30.4 (±7.1)	29.9 (±5.6)	32.6 (±6.8)	−0.5 (±6.3)	2.4 (±6.9)			
Physical activity [steps/d]	CT	4865 (±1576)	–	4713 (±1473)*	–	−152 (±1169)	**0.050**	0.818	0.380
	RT	4723 (±2271)	–	3988 (±1745)*	–	−735 (±1339)			
	RTS	4487 (±2236)	–	4194 (±1813)*	–	−293 (±862)			

Values are shown as mean (± standard deviation). p-Values refer to main effects of time and group as well as time × interaction effects (repeated measurement ANOVA). Significant values ($p < 0.05$) are shown in bold.
CT (cognitive training group); RT (resistance training group); RTS (resistance training + supplement group); 6MWT (Six-Minute Walking Test).
* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs baseline. ## $p < 0.01$ and ## $p < 0.001$ vs CT.

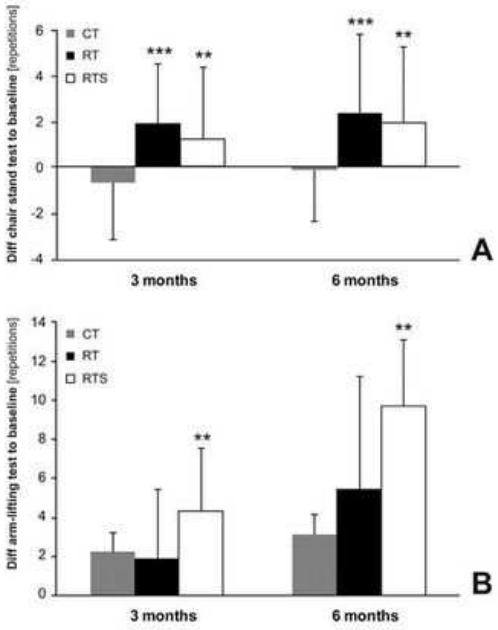

Fig. 2. Effects of resistance training ± nutritional supplement on functional performance. Values represent the difference in repetitions between baseline and 3 months or 6 months that could be performed within 30 s in the chair stand test (A) and the arm-lifting test (B); CT (control group); RT (resistance training group); RTS (resistance training + supplement group); $^{**}p < 0.01$; $^{***}p < 0.001$ vs control group.

handgrip strength ($p < 0.001$). On average, men had larger walking speeds than women ($p = 0.003$) and reached higher distances in 6MWT ($p = 0.039$).

Attendance at the training sessions as calculated by the ratio between attended sessions and offered sessions in percent was 71% (±26.5%) with no significant differences between groups ($p > 0.05$). However, participants with higher attendance at the training sessions were more likely to have superior peak torque values of knee flexors at 120°/s ($p = 0.026$), and performed better in the 30 s chair stand test ($p = 0.030$) and the 6 MWT ($p = 0.019$), respectively.

MMST was affected by age ($p = 0.019$) but MMST did not influence any of the strength or functional performance parameters.

3.5. Baseline differences between finishers and drop-outs

As outlined in the participant flow (Fig. 1), in total nearly 30% of study participants quit the study directly after the baseline measurements (18.8%), between baseline and 3 months (4.3%) or between 3 and 6 months (6.8%). Interestingly, finishers of the intervention study had significantly higher performance indices already at the baseline tests in some of the functional parameters, namely chair stand test ($p = 0.012$), 6 MWT ($p = 0.005$) and gait speed ($p = 0.013$) compared to those subjects who dropped out from the intervention (Table 4).

Table 4
Baseline differences between finishers and drop-outs.

	Finishers	Drop-outs	p-Value
Participants [number (%)]	82 (70.1%)	35 (29.9%)	
Age [years]	82.4 (±6.2)	83.8 (±5.5)	0.221
MMST [−]	27.6 (±1.9)	27.3 (±2.0)	0.397
Chair stand test [repetitions]	12.0 (±4.7)	8.9 (±4.2)	**0.012**
6MWT [m]	379 (±91)	307 (±120)	**0.005**
Gait speed [m/s]	1.55 (±0.57)	1.19 (±0.44)	**0.013**
Functional reach test [cm]	28.9 (±6.4)	29.7 (±6.1)	0.694
Arm-lifting test [repetitions]	23.5 (±10.9)	23.8 (±4.7)	0.906
Handgrip strength [kg]	18.8 (±6.9)	17.2 (±5.5)	0.421

Values as mean (± standard deviation). p-Values refer to differences between the finisher and drop-outs (Chi Square-, independent t-test). Significant values ($p < 0.05$) are shown in bold. MMST: Mini Mental State Test; 6MWT: Six-Minute Walking Test.

4. Discussion

The main finding of the study was that six months of progressive elastic resistance training can be effective to enhance parameters of physical function such as number of repetitions in the chair stand test as well as the performance in the arm-lifting test even at old age. However, this type of resistance training was not able to enhance isokinetic muscle torque of knee extensors and flexors, defined as primary outcome. This is different to many other resistance exercise studies with older adults using high-intensity programmes with free weights or weight machines for several weeks. There is evidence that training loads need to be high and of sufficient long duration if strength and muscle mass are to increase (Raymond et al., 2013; Silva et al., 2014; Steib et al., 2010). Intensities of 60–85% of the individual maximum voluntary strength have been recommended to increase muscle strength by increasing muscle mass and even higher intensities are required to improve the rate of force development in the elderly (Mayer et al., 2011). The study participants were instructed to choose the resistance (and colour) of the elastic band in a way that they should be able to perform two sets of exercises with a maximum of 15 repetitions per set and with a resting period of 30 to 60 s. Several equations have been developed to predict the one repetition maximum strength based on submaximal tests. Even if the accuracy of these equations is linked to specific exercises or populations, 15 repetitions as used in current study correspond to approximately 65% of the one repetition maximum (American College of Sports Medicine, 2009; Baechle and Earle, 2008). Therefore, the intensity chosen in our study can be defined as moderate (Steib et al., 2010). Although the participants were carefully supervised and encouraged, it cannot be excluded that some participants worked at a lower intensity potentially explaining the lack in strength gains. It might be of advantage in future studies to monitor elastic band exercises using stretch-sensors which have been shown to measure total and single repetition time-under-tension reliably (Skovdal Rathleff et al., 2013).

While higher training intensities seem to be superior to low or moderate intensities for improving maximal strength, low and moderate intensity programmes are frequently used especially for elderly subjects with functional limitations and with the presence of several comorbidities such as cardiovascular diseases (Liu and Latham, 2010; Pollock et al., 2000; Sousa et al., 2014). This is also the case for our study population which consisted of people who cannot be considered as thoroughly healthy as we included subjects with hypertension, hyperlipidemia, diabetes, osteoporosis, non-acute cardiac disesases and functional impairments (use of walking aids, dynapenia and sarcopenia) (Hofmann et al., 2015). One interesting outcome of our study has been that functional performance as assessed by chair stand and arm-lifting tests could be enhanced without improvements in muscle mass or strength. This is in line with the evidence demonstrating that high-intensity resistance training results in higher strength gains compared to low-intensity exercise while high intensities are not necessarily required to improve physical function (Raymond et al., 2013).

Specificity of the tests is an important issue when evaluating the effects of a training programme. We have used isokinetic dynamometry as the primary outcome as this method is considered to be the gold standard for assessing muscular strength (Gleeson and Mercer, 1996). Similar to other studies (Ostchega et al., 2004; Skelton et al., 1994) we observed good correlations of peak torques at both velocities with

handgrip strength ($r = 0.65–0.70$, $p < 0.001$), gait speed (0.53–0.54, $p < 0.001$), 6MWT (0.50–0.54, $p < 0.001$), and arm-lifting test ($r = 0.45–0.54$, $p < 0.001$). The correlations to chair stand test were somewhat lower (0.37–0.39, $p < 0.001$). In this study, two different angular velocities (60°/s, 120°/s) were used which can be considered as low to moderate also reflecting the training velocity of the elastic band resistance exercise training. We abstained from using higher velocities for assessment as institutionalised elderly might have been unable to reach the target velocity as shown previously (Lanza et al., 2003). Training with elastic bands includes concentric as well as eccentric components whereby the latter seem to be especially suitable for elderly because of the 'high-force–low-cost' manner of eccentric exercises (LaStayo et al., 2003; Skovdal Rathleff et al., 2013). Unfortunately eccentric muscle strength was not assessed in the current study making it impossible to judge the improvements in eccentric strength.

It has to be mentioned that the study population consisted of much more females (88%) than males (12%). While the proportion of males for community-dwelling men in Vienna is 34% at an age of 85 years (Statistik Austria, 2015), the proportion of males in the retirement homes is 19% in the age group of 80 to 89 years old (Bader et al., 2013) making our study population representative in terms of gender distribution of institutionalized elderly in Austria. However, with this small number of males it was not possible to conduct separate analyses for males and females. It is known that ageing affects muscle protein metabolism differently in men and women as the basal rate of muscle protein synthesis is approximately 30% higher in women than that in men, potentially explaining the slower loss of muscle mass in ageing females. On the other hand, exercise training doubles the basal muscle protein synthesis in men, whereas it is increased only by ~40% in females (Smith et al., 2008). However, it is interesting that trainability assessed by strength gains seem to be similar in older men and women (Lemmer et al., 2000; Peterson et al., 2010) making it unlikely (but not impossible) that larger or different improvements could have been observed in males.

One frequent limitation in many training intervention studies is the lack of a social activity for the control group that is of equal length and frequency to the exercising group. Therefore, we decided to control for social interactions by implementing a cognitive training for the control group. Interestingly, improvements over time were detected for many physical performance parameters such as isokinetic measurements, gait speed, and aerobic capacity as assessed by 6MWT regardless of groups indicating that also participants receiving only cognitive training improved in these parameters. In order to prove whether participants were changing their physical activity behaviour, we assessed steps per day at the beginning and at the end of the study as a measure for overall physical activity. Our data revealed that steps per day were generally low (only 10% met the recommendation of 7000 steps per day; Tudor-Locke et al., 2011) and decreased rather than increased in all groups. Therefore, it is unlikely that an increase in physical activity could have accounted for the observed improvements in performance of all groups. Future in-depth analyses will be conducted to assess whether time spent in different intensity zones or sedentary changed during the study period.

It has been shown that aerobic and/or strength training are able to improve cognitive function in patients with dementia (Bossers et al., 2015) and healthy older people (Nouchi et al., 2014). In addition, it is well known that difficulties with performance of functional activities may result from physical impairments but also from a cognitive decline (Fong et al., 2015). The question remains whether cognitive training interventions on the other hand could exert positive effects on physical performance. Usually, cognitive interventions have been developed to enhance memory and speed of processing, with the goal of maximizing current function and reducing the risk of cognitive decline in elderly populations (Acevedo and Loewenstein, 2007). However, a recent study has indicated that cognitive training might improve balance and gait in older adults known to have a history of falls (Smith-Ray et al., 2014) and longitudinal data from a large intervention trial have revealed that cognitive training ameliorates everyday functional outcomes in older adults demonstrating some transfer effects (Willis et al., 2006). Therefore, we cannot comprehensively exclude that cognitive training itself, although thought of as a control intervention, contributed to the ameliorations in gait speed and 6MWT.

Interestingly, protein supplementation was shown to be ineffective in this setting as we could not detect any differences between the trained only (RT) and the additionally supplemented group (RTS). We have assessed nutritional status by MNA and detected slightly lower scores in the cognitive group as compared to the other two intervention groups, whereby these scores did not change over time. In total, about 80% of the participants had a normal nutritional status. In previous publications we have reported that the nutritional supplementation resulted in an increased uptake of vitamin D and folic acid but not in protein being between 0.8 and 1.0 g/kg/day in the RTS group (Franzke et al., 2015a). Furthermore, plasma levels of vitamin B12 and folic acid in erythrocytes were enhanced due to supplementation (Franzke et al., 2015b). Therefore, we can confirm that the supplement was regularly consumed by the participants. There is an ongoing discussion about the optimal intake of proteins, especially in an elderly population as current recommendations for older adults range from 0.8 g/kg/day to 1.5 g/kg/day (Volkert and Sieber, 2011; Wolfe et al., 2008). It has been shown that both, protein quality and timing are important for efficiently enhancing muscle protein synthesis. In this study, we have used a protein supplement (20.7 g per serving) that contains whey protein enriched in leucine. Whey protein stimulates postprandial muscle protein accretion more effectively than do casein and casein hydrolysate due to its faster digestion and absorption kinetics and higher leucine content (Pennings et al., 2011). Increasing the proportion of leucine in a mixture of essential amino acids can reverse an attenuated response of muscle protein synthesis in the elderly (Katsanos et al., 2006). With respect to timing, the ingestion of dietary protein directly after resistance exercise increases post-exercise protein synthesis rates in young (Tipton et al., 2001) and old adults (Bukhari et al., 2015). In addition there is some evidence that providing enough protein to maximally stimulate muscle protein synthesis at each meal (breakfast, lunch, and dinner) induced the highest muscle protein synthesis in comparison to a skewed pattern with lower protein intakes for breakfast and lunch (Mamerow et al., 2014). Therefore, we assume that the pattern chosen in the current study with supplementation at breakfast and directly after the training sessions should be sufficient in order to additionally enhance muscle protein synthesis rate. However, the amount of protein could have been slightly too small as a recent dose–response study in elderly has shown that as little as 10 g of whey protein is able to prevent the significant decline in intramuscular branched chain amino acid levels, but only larger doses of whey (30 and 40 g) increase leucine level in skeletal muscle post-resistance exercise (D'Souza et al., 2014). However, even if a higher muscle protein synthesis can be achieved with protein supplementation post-exercise, this does not necessarily translate into improved muscle strength (Finger et al., 2015).

One of the most important secondary findings of our study is that those participants who quit the study before the final measurements were in a worse physical condition at baseline as measured by chair stand test, 6MWT and gait speed than those finalising the study after 6 months. We did not observe any side effects associated with the resistance exercise training or the nutritional supplementation. Reasons for dropouts mostly have been medical issues not related to the intervention but making a further participation difficult. This might be different to many other studies in older adults, pre-selecting a rather healthy and fit population of older adults but clinically relevant as a certain level of physical fitness seems to be needed before starting a group training programme.

In conclusion, six months of a moderate/low intensity resistance exercise using elastic bands and the own body weight has been shown to be beneficial in improving functional performance of institutionalised

older people with a mean age close to or even above the average life expectancy. Multinutrient supplementation did not offer additional benefits to the effects of RT in improving muscular performance.

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Appendix A. Supplementary data

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3.3 Publication III (Manuscript)

Elastic band resistance training influences transforming growth factor-ß receptor I expression in peripheral mononuclear cells of institutionalised older adults: the Vienna Active Ageing Study (VAAS)

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Elastic band resistance training influences transforming growth factor-ß receptor I expression in peripheral mononuclear cells of institutionalised older adults: the Vienna Active Ageing Study (VAAS)

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Abstract

Background: Ageing, inactivity and obesity are associated with chronic low-grade inflammation contributing to a variety of lifestyle-related diseases. Transforming growth factor-β (TGF-β) is a multimodal protein with various cellular functions ranging from tissue remodelling to the regulation of inflammation and immune functions. While it is generally accepted that aerobic exercise exerts beneficial effects on several aspects of immune functions, even in older adults, the effect of resistance training remains unclear. The aim of this study was to investigate whether long-lasting progressive resistance training (6 months) with or without nutritional supplementation (protein and vitamins) would influence circulating C-reactive protein and TGF-β levels as well as TGF-β signalling in peripheral mononuclear cells (PBMCs) of institutionalised adults with a median age of 84.5 (65.0–97.4) years.

Results: Elastic band resistance training significantly improved performance as shown by the arm-lifting test ($p = 0.007$), chair stand test ($p = 0.001$) and 6-min walking test ($p = 0.026$). These results were paralleled by a reduction in TGF-β receptor I (TGF-βRI) expression in PBMCs ($p = 0.006$), while circulating inflammatory markers were unaffected. Protein and vitamin supplementation did not provoke any additional effects. Interestingly, muscular endurance of upper and lower body and aerobic performance at baseline were negatively associated with changes in circulating TGF-β at the early phase of the study. Furthermore, drop-outs of the study were characterised not only by lower physical performance but also higher TGF-β and TGF-βRI expression, and lower miRNA-21 expression.

Conclusions: Progressive resistance training with elastic bands did not influence chronic low-grade inflammation but potentially affected TGF-β signalling in PBMCs through altered TGF-βRI expression. There appears to be an association between physical performance and

TGF-β expression in PBMCs of older adults, in which the exact mechanisms need to be clarified.

Key Words: Vienna Active Ageing Study (VAAS), TGF-β pathway, microRNA, chronic inflammation, inflammageing, strength training

Background

Population ageing resulting from a higher life expectancy concomitant with a decline in fertility will lead to a global increase in the proportion of people over the age of 60 years from 12% in 2015 to 22% by 2050 [1]. With higher ages, the prevalence of cardiovascular, metabolic and neurodegenerative disorders and cancer is enhanced. It has been shown that many of these conditions are related to compromised homeostasis between proper activation of the immune system and its resolution, leading to a limited response to pathogens and vaccines on the one hand and a chronic inflammatory state on the other hand [2, 3].

Transforming growth factor-β (TGF-β) is a multifunctional protein involved in the regulation of cell proliferation, extracellular matrix production, inflammation and immune functions [4]. Higher circulating levels of TGF-β are related to obesity with impaired insulin sensitivity [5], cardiovascular diseases among individuals with higher C-reactive protein (CRP) levels [6], type II diabetes [7, 8], and a higher age [9]. TGF-β interacts with several cell types by binding to membrane serine/threonine kinase receptors, TGF-β receptor I (TGF-βRI; also known as ALK5) and TGF-βRII, initiating diverse cellular responses [10]. An approach to modulate TGF-β signalling is to alter the expression of its receptors. Recently, it has become evident that microRNAs (miRNAs) might regulate thousands of human genes by either mRNA degradation or suppression of mRNA translation [11]. A recent investigation estimated that 84–89% of miRNA-induced suppression of gene expression is the result of degradation of target mRNAs [12]. Currently, close to 1,900 human miRNA precursors are listed in the miRBase registry, which give rise to 2,588 mature miRNAs [13, 14]. One of these miRNAs, miRNA-21, plays a crucial role in a plethora of biological functions and diseases including the development of cancer, cardiovascular diseases and inflammation [15]. Interestingly, miRNA-21 is a validated target of both TGF-βRI and TGF-βRII [16], and

overexpression of miRNA-21 is associated with elevated levels of proinflammatory cytokines in a dominant-negative TGF-βRII mouse model [17].

Regular physical activity offers protection against, and may be a useful treatment for, a wide variety of chronic diseases associated with low-grade inflammation [18]. In particular, endurance exercise training combined with dietary weight loss strategies have been shown to decrease the chronic inflammatory state associated with obesity and old age [19, 20]. Resistance training prevents or counteracts age-related loss of muscle mass and functions [21, 22], but there is an ongoing discussion whether this type of exercise alters the chronic inflammatory state associated with ageing [23-25].

In the Vienna Active Ageing Study (VAAS), we previously showed that resistance training using elastic bands for 6 months leads to an increase in functional performance of the lower and upper extremities in older people Furthermore, we detected higher CRP levels and lower TGF-βRI and TGF-βRII expression in peripheral blood mononuclear cells (PBMCs) of older women compared with younger women [26]. Therefore, the aim of this sub-study of the VAAS was to investigate whether progressive resistance training alone or in combination with a nutritional supplement containing protein and vitamins influenced expression of TGF-β, its receptors, and/or miRNA-21 in older institutionalised people.

Results

Subject characteristics

A total of 230 potential participants were screened for eligibility, and 117 participants (89 women and 12 men) agreed to participate in the VAAS. The participants were randomised into intervention groups: cognitive training (CT), resistance training (RT), and resistance training plus nutritional supplementation (RTS) (Figure 1). Blood samples were available from 95 participants (84 women and 11 men). To prevent any potential interference by acute

inflammatory processes, we excluded all subjects with CRP levels above 10 mg/L at any time point as suggested previously [27, 28]. Finally, data from 88 participants (77 women and 11 men) with a median age of 84.5 (65.2-97.4) years and a median body mass index (BMI) of 28.9 (18.1-50.0) kg/m² were included in the current study. No differences between study groups with respect to age, gender and anthropometric data were detected at baseline (Table 1).

Table 1. Baseline characteristics

	All	CT	RT	RTS	*P-Value*
Subjects [number]	88	30	32	28	
Gender [female/male]	77/11	25/5	28/4	24/2	0.602
Age [years]	84.5 (65.0-97.4)	84.5 (69.4-97.4)	84.4 (71.7-93.2)	84.3 (65.0-92.2)	0.864
BMI [kg/m²]	28.9 (18.1-50.0)	29.8 (18.1-36.9)	28.2 (22.7-40.2)	27.9 (22.9-50.0)	0.741
Leukocyte subpopulations and circulating biomarkers					
Leukocytes [x10⁹ cells /L]	6.6 (3.3-13.0)	7.4 (4.9-10.3)*	5.8 (3.1-9.8)	6.9 (4.4-13.3)*	**0.002**
Lymphocytes [cells/µl]	1990 (930-4930)	1985 (910-3510)	1835 (830-3330)	2200 (1460-4700)	0.085
Neutrophils [cells/µl]	3695 (1530-9860)	4414 (2410-6630)*	2931 (1530-6800)	3889 (2370-9860)	**<0.001**
Eosinophils [cells/µl]	200 (10-740)	225 (36-620)	160 (10-740)	220 (10-470)	0.135
Basophils [cells/µl]	40 (10-111)	45 (10-111)	35 (10-190)	35 (10-90)	0.397
Monocytes [cells/µl]	560 (250-910)	587 (72-950)	520 (260-860)	545 (320-960)	0.189
hs-CRP [mg/L]	1.95 (0.3-7.9)	2.1 (0.5-7.9)	1.9 (0.3-7.6)	1.9 (0.6-6.7)	0.918
TGF-β [µg/L]	33.3 (16.7-73.7)	38.2 (18.7-73.7)	32.8 (16.7-55.4)	32.1 (21.0-51.2)	0.083
PBMC gene expression					
TGF-β/GAPDH [-]	0.85 (0.06-3.36)	0.48 (0.23-2.66)	0.60 (0.21-2.52)	0.58 (0.06-3.36)	0.725
TGF-βRI/GAPDH [-]	2.05 (0.14-28.82)	1.99 (0.14-22.39)	2.04 (0.69-19.25)	2.11 (0.29-28.81)	0.990
TGF-βRII/GAPDH [-]	1.67 (0.51-15.85)	1.54 (0.66-7.79)	1.60 (0.66-14.85)	1.79 (0.51-10.80)	0.714
miR-21 [copies/pg]	2400 (57-4720)	2920 (343-4500)	2120 (57-4720)	2350 (550-4590)	0.765

Data are expressed as medians (min–max); Group differences were detected using X^2 or Kruskal–Wallis tests and if significant, followed by Bonferroni-corrected post-hoc analyses (* $p<0.05$ vs. RT)

Abbreviations: CT, cognitive training; RT, resistance training; RTS, resistance training plus supplement; PBMC, peripheral blood mononuclear cell; hs-CRP, high sensitive C-reactive protein; TGF-β, transforming growth factor-β, TGF-βR, TGF-β receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; miR-21, microRNA-21

Resistance training improves physical performance of older subjects

We have previously shown that resistance training intervention specifically improved muscular endurance (chair stand and arm-lifting tests), while isometric strength (handgrip test) and aerobic capacity (6-min walking test; 6MWT) increased similarly in all intervention groups [22]. Because the current study differs from the original with respect to the study

population, the data were re-analysed. At baseline, no differences in any of the physical fitness parameters were observed between intervention groups ($p > 0.05$). Similar to the previous results, chair stand performance was increased in the RT group (3 months: +18%, $p = 0.041$; 6 months: +27%, $p = 0.001$) and RTS group (6 months: +15%, $p = 0.017$). Arm-lifting performance was enhanced in RT and RTS groups only after 6 months (RT: +24%, $p = 0.007$; RTS +61%, $p = 0.007$). While handgrip strength was unaltered over time in all intervention groups (CT: $p = 0.454$; RT: $p = 0.238$, RTS: $p = 0.810$), aerobic performance during the 6MWT was slightly improved in the RT group after 6 months (+9%, $p = 0.026$) but not in the CT or RTS groups (CT: $p = 0.959$, RTS: $p = 0.080$) (data not shown).

Effects of interventions on leukocyte subpopulations and circulating inflammatory markers

At baseline, leukocyte numbers differed significantly between intervention groups ($p = 0.002$). Post-hoc analyses revealed that subjects from the RT group had lower leukocyte numbers than those from the CT group (+28%, $p = 0.002$) and the RTS group (+19%, $p = 0.039$), which was owing to lower numbers of neutrophils (CT: +51%, $p < 0.001$; RTS: +33%, $p = 0.092$). All other parameters did not differ between groups at baseline (Table 1).

Except for adaptation of the leukocyte numbers in the RT group (overall: $p = 0.001$; 3 months: $p = 0.166$; 6 months: $p = 0.001$; +5%) caused by an increase in neutrophils (overall: $p = 0.011$; 3 months: $p = 0.166$; 6 months: $p = 0.010$ +20%), we did not detect any other alterations in leukocyte, lymphocyte, monocyte or granulocyte counts. The significant overall effect on basophil granulocytes ($p = 0.011$) in the CT group could not be confirmed after post-hoc analysis (3 months: $p = 0.066$, 6 months: $p = 0.399$). Similarly, the circulating levels of TGF-β and hs-CRP remained unchanged (Table 2).

Table 2. Intervention effects on leukocyte subpopulations and circulating inflammatory markers

	Baseline (n=88)	3 months (n=71)	6 months (n=64)	*P-Value*
Leukocytes [x10^9 cells/L]				
CT	7.4 (4.9-10.3)	7.3 (4.1-11.9)	7.1 (5.4-9.4)	0.440
RT	5.8 (3.1-9.8)	6.1 (3.2-8.9)	6.0 (3.3-9.8)*	**0.001**
RTS	6.9 (4.4-13.3)	7.0 (4.5-11.7)	7.1 (4.7-13.0)	0.586
Lymphocytes [cells/µl]				
CT	1985 (910-3510)	2190 (890-3980)	2250 (930-3540)	0.387
RT	1835 (830-3330)	1780 (912-3190)	1720 (1010-3430)	0.651
RTS	2200 (1460-4700)	2340 (1570-4680)	2190 (1640-4930)	0.810
Monocytes [cells/µl]				
CT	587 (72-950)	600 (300-790)	590 (400-910)	0.534
RT	520 (260-860)	475 (250-960)	510 (250-880)	0.104
RTS	545 (320-960)	490 (300-840)	545 (330-900)	0.404
Neutrophils [cells/µl]				
CT	4413 (2410-6630)	4030 (1990-7420)	3910 (3110-5880)	0.247
RT	2931 (1530-6800)	3425 (1720-5880)	3520 (1800-7330)*	**0.011**
RTS	3889 (2370-9860)	3805 (1720-8730)	4290 (1664-8680)	0.368
Basophils [cells/µl]				
CT	45 (10-111)	30 (10-90)	30 (20-100)	**0.027**
RT	35 (10-190)	30 (10-100)	30 (10-80)	0.878
RTS	35 (10-90)	35 (10-90)	40 (10-80)	0.344
Eosinophils [cells/µl]				
CT	225 (36-620)	180 (80-570)	220 (90-330)	0.911
RT	160 (10-740)	180 (40-530)	200 (80-570)	0.381
RTS	220 (10-470)	205 (20-630)	260 (90-650)	0.137
hs-CRP [mg/L]				
CT	2.1 (0.5-7.9)	2.4 (0.2-9.2)	1.8 (0.3-8.5)	0.424
RT	1.9 (0.3-7.6)	2.2 (0.5-7.7)	2.0 (0.6-9.6)	0.645
RTS	1.9 (0.6-6.7)	2.2 (0.6-8.5)	1.6 (0.4-5.5)	0.135
TGF-β [µg/L]				
CT	38.2 (18.7-73.7)	39.7 (18.4-67.8)	40.8 (21.9-67.9)	0.953
RT	32.8 (16.7-55.4)	34.7 (18.3-59.7)	34.6 (12.7-57.3)	0.075
RTS	32.1 (21.0-51.2)	38.0 (22.9-50.8)	35.5 (22.1-52.1)	0.431

Data are expressed as medians (min–max); Differences were detected using the Friedman test and if significant, followed by Bonferroni-corrected post-hoc analyses (* $p<0.05$ vs. baseline)
Abbreviations: CT, cognitive training; RT, resistance training; RTS, resistance training plus supplement; hs-CRP, high sensitive C-reactive protein; TGF-β, transforming growth factor-β

Influence of interventions on intracellular TGF-β, its receptors, and miRNA-21 expression

The expression of TGF-β, TGF-βRI, TGF-βRII and miRNA-21 in PBMCs did not differ between groups at baseline. Resistance training led to a significant decrease in TGF-βRI levels ($p = 0.006$). Post-hoc analyses revealed that the initial decrease at 3 months (−27%, p =

0.015) was reversed at 6 months (p = 0.117). Interestingly, this decrease could not be confirmed in the RTS group, although the median level decreased similarly to the RT group. Intracellular TGF-β, TGF-βRII, and miRNA-21 expression was not influenced by any of the interventions (Table 3).

Table 3. Intervention effects on TGF-β signalling

	Baseline (n=88)	3 months (n=71)	6 months (n=64)	*P-Value*
TGF-β/GAPDH [-]				
CT	0.48 (0.23-2.66)	0.48 (0.27-4.10)	0.43 (0.25-1.93)	0.694
RT	0.60 (0.21-2.52)	0.51 (0.16-2.63)	0.52 (0.23-2.30)	0.568
RTS	0.58 (0.60-3.36)	0.47 (0.15-2.65)	0.56 (0.90-2.92)	0.549
TGF-βRI/GAPDH [-]				
CT	1.99 (0.14-22.39)	1.76 (0.46-29.89)	1.94 (0.11-9.24)	0.664
RT	2.04 (0.69-19.25)	1.69 (0.34-9.21)*	1.77 (0.54-15.91)	**0.006**
RTS	2.11 (0.29-28.81)	1.65 (0.28-15.06)	2.43 (0.52-15.04)	0.949
TGF-βRII/GAPDH [-]				
CT	1.54 (0.66-7.79)	1.37 (0.68-9.17)	1.43 (0.67-6.32)	0.873
RT	1.60 (0.66-14.85)	1.59 (0.57-13.50)	1.67 (0.62-8.79)	0.296
RTS	1.79 (0.50-10.80)	1.73 (0.44-5.25)	2.08 (0.69-6.67)	0.212
miR-21 [copies/pg RNA]				
CT	2920 (343-4500)	2840 (276-5120)	2460 (1050-4700)	0.861
RT	2120 (57-4720)	2275 (555-5600)	2540 (330-5020)	0.368
RTS	2350 (550-4590)	2535 (353-4780)	2810 (1600-4630)	0.854

Data are expressed as medians (min–max); Differences were detected using the Friedman test and if significant, followed by Bonferroni-corrected post-hoc analyses (* p<0.05 vs. baseline).
Abbreviations: CT, cognitive training; RT, resistance training; RTS, resistance training plus supplement; TGF-β, transforming growth factor-β, TGF-βR, TGF-β receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; miR-21, microRNA-21.

Because resistance training only led to marginal changes in the TGF-β signalling pathway, we examined whether different components of physical fitness (strength, muscular endurance and aerobic performance) at baseline were influenced by the TGF-β signalling response (difference between 3 or 6 months and baseline values). While isometric handgrip strength was not associated with alterations in TGF-β-related parameters, muscular endurance and aerobic performance at baseline were negatively associated with changes in circulating TGF-β levels after 3 months (chair rise test: $\rho = -0.349$, $p = 0.003$, arm-lifting test: $\rho = -0.352$, $p =$

0.024; 6MWT: ρ = −0.308, p = 0.009), but chair stand performance was positively associated with changes in TGF-βRII expression (ρ = 0.254, p = 0.033).

Drop-out analysis

During 6 months of intervention, 18 women and 3 men (27%) left the study because of several reasons (loss of interest or acute medical reasons such as joint pain or cardiological issues). The drop-out rate did not differ between groups. In addition, age, sex, and existing co-morbidities in general were not related to adherence to the study. Only the number of participants diagnosed for hyperlipidaemia was higher in finishers compared with the drop-outs (p = 0.011). Baseline performance appeared to be related to study adherence because drop-outs had lower aerobic (6MWT: −19%, p = 0.020) and chair stand (−13%, p = 0.015) performance than finishers of the study. Strength (p = 0.608) and muscular endurance (p = 0.924) of the upper extremities were not different between finishers and drop-outs. The observed differences also became evident in some of the TGF-β-related parameters because TGF-β (+106%, p = 0.016,) and TGF-βRI (+237%, p < 0.001) expression was higher in drop-outs, while miRNA-21 expression was lower (−65%, p < 0.001) (Table 4).

Table 4. Baseline differences between drop-outs and finishers

	Total (n=88)	Drop-out (n=24)	Non-Drop out (n=67)	*p-Value*
Gender [female/male]	77/11	7/1	70/10	0.980
Age [years]	84.5 (65.0-97.4)	84.8 (69.4-92.2)	84.2 (65.0-97.4)	0.502
BMI [kg/m²]	28.9 (18.1-50.0)	28.3 (18.1-43.7)	29.6 (23.4-50.0)	0.138
hs-CRP [mg/L]	1.9 (0.3-7.9)	2.0 (0.8-7.9)	1.9 (0.3-7.6)	0.413
TGF-β signalling				
TGF-β [µg/L]	33.3 (16.7-73.7)	39.3 (22.5-58.0)	32.8 (16.7-73.7)	0.178
TGF-β/GAPDH [-]	0.53 (0.06-3.36)	1.07 (0.21-3.36)	0.52 (0.06-2.66)	**0.016**
TGF-β RI/GAPDH [-]	2.05 (0.14-28.81)	6.41 (1.31-28.81)	1.90 (0.14-22.39	**<0.001**
TGF-β RII/GAPDH [-]	1.67 (0.51-14.85)	3.00 (0.91-10.80)	1.59 (0.51-14.85)	0.099
miR-21 [copies/pg RNA]	2400 (57-5720)	979 (498-3640)	2815 (57-4720)	**<0.001**
Co-morbidities				
Hyperlipidemia (n)	35	6	29	**0.011**
Hypertension (n)	81	28	53	0.700
Osteoporosis (n)	42	16	26	0.429

Cardiac diseases (n)	33	13	20	0.398
History of cancer (n)	14	5	9	0.862
Diabetes Type II (n)	17	6	11	0.877
Obesity (n)	32	10	22	0.222

Data for continuous variables are shown as medians (min–max). For co-morbidities, the respective numbers of affected participants were determined at the medical entrance examination. Obesity was defined as a BMI of >30 kg/m². Differences between groups were determined by Mann–Whitney U or X^2 tests.

Discussion

The aim of the current study was to investigate the effects of long-lasting (6 months) progressive resistance training alone and in combination with a nutritional supplement enriched with protein and vitamins on systemic inflammation and TGF-β signalling in PBMCs of institutionalised, but independent older people. Circulating TGF-β and hs-CRP levels as well as intracellular TGF-β gene expression were not influenced by elastic band resistance training. Interestingly, TGF-βRI, but not TGF-βRII, gene expression was reduced in the RT group after 3 months and returned to baseline levels thereafter.

It is well known that regular endurance training reduces the chronic proinflammatory state caused by ageing and physical inactivity [29, 30]. Adipose tissue appears to play a substantial role in this scenario because it is a major source of several hormones and cytokines [31].]. In particular, visceral fat depots and the macrophages within these depots release proinflammatory cytokines such as interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) and TGF-β1 [32]. The increase in energy expenditure caused by enhanced physical activity reduces body fat, thereby influencing the capacity to produce and release proinflammatory mediators [32]. Recommended strategies to lose weight and body fat include aerobic exercise training combined with caloric reduction. Although it is known that resistance training does not promote clinically significant weight loss, it influences body composition by increasing muscle mass and decreasing body fat [33, 34]. Thus, it has been hypothesised that muscle-strengthening exercises also exert anti-inflammatory effects. Based on intervention studies investigating the effects of strength training on inflammatory markers such as CRP, TNF-α

and IL-6, the data are ambiguous because some studies have revealed a positive effect [23, 35], whereas others did not observe any amelioration in the inflammatory state [36, 24, 25]. While the TGF-β superfamily has been studied extensively in the adaptation of muscles and tendons to exercise [37], investigations into the context of exercise immunology are scarce. A combined strength and endurance exercise programme increases circulating TGF-β [35], whereas strength training alone does not alter its levels [38], which support our results. The intervention programme altered functional performance but had no effects on body composition [22, 39]. Furthermore, we hypothesise that endurance training with or without a dietary weight loss strategy would be more effective to target the chronic inflammatory state. This hypothesis is supported by the fact that parameters measuring aerobic fitness and muscular endurance, but not handgrip strength, correlated with TGF-β alterations.

While circulating levels of TGF-β were unaffected, we showed for the first time that resistance training lowers the gene expression of TGF-βRI in PBMCs of older adults, potentially leading to decreased signalling through the type I receptor. This decrease occurred concomitant with an increase in neutrophil counts. Neutrophil granulocytes are the largest fraction of leukocytes and the first line of defence against pathogens. It has been shown that their numbers and function might be altered during the ageing process [40].]. In addition, both the number of leukocytes and the percentage of neutrophils have been shown to increase with progression to multi-morbidity in a sample size of more than 26,000 individuals [41]. Although neutrophils express receptors for TGF-β, its importance in regulating neutrophil function is poorly understood, except for a proposed effect on functional polarisation of tumour-associated neutrophils towards a pro-tumourigenic phenotype [42, 43]. However, most importantly, PBMCs, which were the source of TGF-βRI mRNA in our study, only include lymphocytes and monocytes, and not erythrocytes or neutrophils. Therefore, the decrease in TGF-βRI expression must arise from one of these cell types rather than

neutrophils. Future studies will need to investigate the exact contribution of the diverse subpopulations in the adaptation to resistance training.

Interestingly, the observed changes only occurred in the RT, but not RTS, group receiving the same strength training intervention. Similar to resistance training alone, some studies have revealed that enhanced protein intake leads to similar beneficial effects on muscle mass [44, 45]. However, a recent systematic review demonstrated that combining protein supplementation with resistance training is effective for eliciting gains in fat-free mass among older adults, but did not appear to further increase muscle mass or strength, which was similar to our study [46]. In addition to proteins, the supplement in this study contained several vitamins. Based on previous analyses, this supplement increases the uptake of vitamin D and folic acid as well as the plasma levels of vitamin B_{12} and folic acid in erythrocytes [47, 48]. There is an ongoing discussion whether antioxidant supplementation may even blunt an exercise-induced training effect [49]. Evidence indicates that reactive oxygen species modulate TGF-β signalling. In turn, TGF-β increases the production of reactive oxygen species and suppresses antioxidant enzymes [50]. Therefore, it cannot be excluded that rigorous scavenging of free radicals impaired the TGF-β pathway response in the RTS group.

A reduction in TGF-βRI expression can be caused by either lower production or an increase in degradation of its mRNA. Post-transcriptional degradation of mRNAs often involves miRNAs specific for the respective target gene [12]. We have investigated miRNA-21 that has been shown to suppress TGF-βRI and TGF-βRII [16]. Additionally, circulating miRNA-21 levels are increased in men with a low aerobic capacity as measured by maximal oxygen uptake [51]. However, its levels were not enhanced in RT or RTS groups, suggesting that other mechanisms and/or other miRNAs are involved in the down-regulation of TGF-βRI in PBMCs by strength training [52].

One striking secondary outcome of this study is that TGF-β, TGF-βRI and potentially TGF-βRII expression at the beginning of the study were higher in drop-outs compared with

finishers, while miRNA-21 expression was lower. Drop-outs were less physically fit than finishers. We confirmed that age, sex, body composition and the presence of several co-morbidities might not contribute to this effect, but the proportion of subjects with hyperlipidaemia was higher among finishers. A possible explanation for this fact could be the 'obesity paradox' that implies moderate obesity at a higher age reduces the mortality risk [53, 54].

Although this study provides interesting data on TGF-β-related parameters in the context of inflammaging and exercise training, we also have to highlight some of its limitations. Because the study is a secondary analysis of a previously conducted trial [22], it is obvious that the data need to be confirmed in a future prospective study. Furthermore, the number of female participants outnumbered the male participants by a significant degree. It also has to be mentioned that the proportion of community-dwelling men at an age of 85 years in Vienna is 34% [55], but the proportion of male individuals in Viennese retirement homes is 19% for this age group [56] making our study population representative in terms of sex distribution of institutionalised older individuals in Austria. Although it was not possible to perform sub-group analyses because of the low numbers of men, their exclusion led to the same conclusion, indicating that the data are reliable at least for older women.

Conclusion

Resistance training is beneficial, even in very old subjects, and potentially influences TGF-β signalling through altered receptor expression. Furthermore, adherence to the intervention was significantly related to alterations in the TGF-β pathway at baseline, but further information is needed to clearly understand the role of TGF-β in the context of inflammaging and resistance training.

Methods

Participants and study design

The study was conducted using a randomised observer-blind design with three parallel groups, RT, RTS and CT. Briefly, participants were untrained, over 65 years of age with a Mini-Mental State Examination score of ≥23 and free of any medical conditions that would impair their participation in a resistance training study [57].]. From a total of 117 participants in the VAAS, only those with available blood samples and a hs-CRP level below 10 mg/L at any time point were included in the current study (n=88). The present study was conducted in accordance with Austrian laws, including the Doctors Act, the Data Protection Act and the Declaration of Helsinki as revised in Edinburgh (2000), and in analogous accordance with International Conference on Harmonization - Good Clinical Practice Guidelines. Written informed consent was obtained from all participants. A detailed description of the study design has been published previously [22]. This study was approved by the ethics committee of the City of Vienna (EK-11-151-0811) and is registered at ClinicalTrials.gov, NCT01775111.

Interventions

Interventions were conducted for 6 months, and measurements were obtained at baseline, after 3 and 6 months. Twice a week, RT and RTS groups performed supervised progressive resistance training without using any equipment other than elastic bands and their own body weight. Additionally to the exercises, participants from the RTS group were encouraged to drink a supplement that was distributed every morning as well as directly after the resistance training (in total nine times per week). One portion consisted of 20.7 g protein (55 En%; 19.7 g whey protein, 3 g leucine and 10 g essential amino acids), 9.4 g carbohydrates (25 En%; 0.8 BE), 3.0 g fat (18 En%), 1.2 g fibre (2 En%), various vitamins such as 800 IU (20 µg) vitamin

D, minerals and trace elements (FortiFit; Nutricia GmbH, Vienna, Austria). Total energy per drink was 150 kcal. Intake of the nutritional supplement was controlled at breakfast as well as after each training session. The CT group served as a control group based on performing supervised cognitive tasks in a sitting position, which was performed twice a week to maintain all social interactions at a comparable level between groups [58].

Determination of body composition, physical fitness and health

Standing height was assessed using a commercial stadiometer (Seca, Hamburg, Germany), 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. BMI was calculated by dividing the body mass in kilograms by height in meters squared. To measure strength of the upper extremities, participants performed two trials of an isometric handgrip strength test (kg) using a dynamometer (JAMAR compatible handgrip dynamometer adapted to handle various hand sizes) in a sitting position with an angle of 90° in the elbow [59]. Functional performance of the lower extremities was measured by a chair stand test, and that of the upper extremities by an arm-lifting test as described previously [60, 22]. Aerobic capacity was assessed by the 6MWT conducted on a 30-m shuttle track [61]. Global cognitive function was determined by the Mini-Mental State Examination [62]. Co-morbidities (hyperlipidaemia, hypertension, osteoporosis, cardiac diseases, history of cancer and diabetes mellitus) were determined at the medical entrance examination. Adiposity was defined by a BMI of >30 kg/m^2 according to the World Health Organization criteria.

Blood sampling and analyses

After overnight fasting, venous blood samples were obtained in a resting state between 06:30 and 08:00 using Z serum Clot Activator collection tubes (Vacuette®; Greiner Bio-One GmbH, Kremsmünster, Austria) to analyse circulating TGF-β and hs-CRP, EDTA tubes (Vacuette®;

Greiner Bio-One GmbH) to determine leukocyte subpopulations and BD Vacutainer® CPT Tubes (Becton, Dickenson and Company, Schwechat, Austria) containing ~130 IU Na-Heparin and 2 mL Ficoll™ to isolate PBMCs.

Hs-CRP was routinely quantified on a Cobas 8000 (Roche Diagnostics, Vienna, Austria). Leukocyte subpopulations were determined by routine flow cytometric analyses. TGF-β was analysed using a commercially available DuoSet development kit to perform an enzyme-linked immunosorbent assay (DY240; R&D Systems, Abingdon, UK) following the manufacturer's instructions including plasma activation by acidification (pH 2) by adding hydrochloric acid (final concentration: 0.1 mmol/L) and neutralisation with sodium hydroxide (final concentration: 0.12 mmol/L) before measurement.

PBMCs were separated from red blood cells and neutrophils using BD Vacutainer® CPT Tubes (Becton Dickinson Austria, Vienna, Austria). The obtained pellet was carefully resuspended in 700 µL QIAzol Lysis Reagent (Qiagen, Hilden, Germany) and stored at −80°C until analysis.

Total RNA including small RNAs was isolated using a miRNeasy Mini Kit (Qiagen, Hilden, Germany) following the supplier's protocol. To prepare a miRNA-enriched fraction separated from the larger RNAs (>200 nt), we used an RNeasy MinElute Cleanup Kit (Qiagen). Reverse transcription of the miRNA-enriched fraction was performed using a miScript II RT Kit (Qiagen), whereas larger RNAs were reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen).

TGF-β, TGF-βRI and TGF-βRII mRNA levels were determined using the respective primer pairs [Hs_TGFB1_1 (QT00000728), Hs_TGFBR1_1 (QT00083412) and Hs_TGFBR2_1 (QT00014350); Qiagen] in conjunction with a QuantiTect SYBR Green PCR kit (Qiagen). In addition, glyceraldehyde-3-phosphate dehydrogenase [Hs_GAPDH_2 (QT01192646)] served as the endogenous control to normalise 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]. Quantification was performed on the Applied Biosystems® 7500 Real-Time PCR System.

Statistical analyses

Data acquisition and processing were performed using commercial software (IBM SPSS 20). The Shapiro–Wilk test was used to test for a normal distribution. Because most of the variables were not distributed normally, the non-parametric Mann-Whitney U-test or the Kruskal-Wallis test were used to compare two or more independent groups, whereas the Friedman test was applied to detect changes over time in different intervention groups. To avoid bias by multiple testing, the results were Bonferroni-corrected for post-hoc analyses. Correlations between variables were identified by Spearman's rank correlation coefficient. Data are shown as the median (minimum–maximum). Statistical significance was set at $p < 0.05$.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BSH, MH, SO, BF and TW were responsible for data acquisition, data analysis and interpretation of the data. BSH drafted the manuscript. EMS and MQ were responsible for all medical aspects in the study. BW and KHW were involved in the conception and design of the study. BW supervised all aspects of laboratory analyses. All authors were involved in revising the manuscript and approved the final version.

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4 Summary and Discussion

The aim of the current thesis was to research the influence of progressive resistance training on immunological -, especially TGF-β related parameters, in older persons. Therefore, in the first step, we investigated the expression of TGF-β, its receptors and its potential modulator miRNA-21 in PBMCs in the context of age and fitness status. In the second study, six months of elastic band resistance training was conducted to assess a broad range of functional and isometric strength tests as well as age adjusted endurance tests. The last and for this thesis the most important step was, to investigate the effect of the strength training intervention with and without supplement and control on the expression of TGF-β and the related parameters.

4.1 Young vs. old in TGF-β and its Receptors

The first scientific task was to get an overview on the differences between young and elderly persons measured. Those immunological alterations were measured in the circulating and intracellular TGF-β and its related parameters, namely TGF-βRI/II as well as miRNA-21 beside circulating inflammatory parameters.

Therefore, circulating as well as intracellular TGF-β was determined together with its receptors, miRNA-21, hs-CRP and several tests associated with physical fitness on sixteen healthy young (22-28 years) and 90 healthy older (65-92 years) participants. Young and old females differed substantially in fitness as measured by 6-minutes walking test, 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 elderly. However, neither TGF-β nor its receptors were associated with performance characteristics in elderly women.

Initially, TGF-β was purified from human platelets[98]. Circulating TGF-β has been detected in a variety of studies with plasma values ranging from below 0.1ng/ml up to more than 25ng/ml[99]. Of course the study population differed between these studies ranging from healthy individuals of both 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[100]. In this respect it has been shown that platelet degranulation, hemolysis 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. Therefore, we and many other groups used acidification of serum samples prior to assessing TGF-β by ELISA[100]. For this purpose, 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 act on the one hand as tumour suppressor by inhibiting cell proliferation and inflammation in the early stage of cancer development and on the other hand as tumour promotor by inducing metastasis or angiogenesis in later stages of the disease[101]. TGF-β modulates even collagen biosynthesis and releases collagen byproducts such as procollagen type III N-terminal propeptide. These factors are related to several cardio vascular risk factors including age, obesity and hypertension. Moreover, individuals with higher levels of either TGF-β or procollagen type III N-terminal propeptide were more likely to be black, less physically active and have higher concentration of hs-CRP[102]. Similarly, higher levels of TGF-β are measured in hypertensive humans, whereas increased serum levels of TGF-β may also protect patients with coronary artery disease against cardiovascular events and coronary interventions[103, 104].

With respect to age decreased levels have been reported for adults (21-67 years) compared to children (1-14 years)[105]. More detailed, it has been revealed that TGF-β levels are higher in males than in females, but decrease with age and increase with obesity in both genders[106]. Interestingly, in centenarians, serum TGF-β concentrations seem to be higher than in younger adults, suggesting that high concentrations of TGF-β might be beneficial during extreme old age[107]. These results 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.

Additionally to circulating levels of TGF-β we were focusing expression levels of TGF-β and its receptors in PBMCs of young and old women. While intracellular TGF-β was not different between these two groups, lowered TGF-βRI and TGF-βRII expressions have been

detected in older participants. These results are partly in accordance with a recent 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[108]. However, neither intracellular nor circulating TGF-β nor physical performance has been assessed in this study. 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[109]. On the one hand TGF-βRII is important in T-cell mediated immunity while 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[110, 111, 112, 113]. Recent studies support the hypothesis that exercise training can reduce low grade inflammation in older subjects and provide long-term benefits with regard to cardiovascular, cognitive, psychosocial and other aspects in older subjects[90, 114]. In accordance to our study, hs-CRP has been shown to be consistently higher in older subjects[115, 116].

4.2 Associations between TGF-β and physical fitness in older women

Another interesting point is the association of TGF-β signalling to physical fitness of older participants at baseline. Interestingly, hs-CRP correlated positively with BMI and body fat but negatively with relative peak torque measurements. This fact confirms at least partly with several studies which revealed associations between a higher inflammatory state with lower physical performance[117, 118]. Moreover, hs-CRP correlated positively with BMI and body fat but negatively with relative peak torque measurements. 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[119, 120]. Although, we detected a negative correlation between hs-CRP and isokinetic knee extension strength, which is in line with diverse studies linking chronic inflammation to low physical performance in older participants[121, 122, 123, 124], this finding was not consistent for other performance parameters such as aerobic fitness. The reason for this result 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 inflammageing does not simply reflect increases in pro-inflammatory markers[125]. Interestingly, in our study the general fitness status of older 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. 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[114]. However, these studies are sparely comparable as miRNA-21 was detected in serum, participants were younger men and there might be a difference between performance indicators for strength and endurance. Moreover, it would have been interesting to measure general physical activity levels as both current physical activity practice and performance are associated with inflammatory biomarkers[123].

In summary, it has been shown that TGF-β is involved in a variety of physiological as well as pathological processes 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 I and II independent of physical fitness[1].

Before summarising and discussing the results of six months of progressive elastic resistance training on TGF-β pathway related parameters, the findings of the primary Vienna Active Ageing Study are in focus to get an idea of the physical effect of the intervention before pay attention to the physiological, or more precisely the immunological.

4.3 Effect of intervention on physical parameters

The second question in this thesis was the effect of six months of progressive elastic resistance training on parameters of physical function such as number of repetitions in the chair stand test as well as the performance in the arm-lifting test even at old age. However, this type of progressive resistance training was not able to enhance isokinetic muscle torque of knee extensors and flexors, defined as primary outcome. This is not in accordance to many other resistance exercise studies also focusing older subjects using high-intensity

programmes with free weights or weight machines for several weeks. There is evidence that training loads need to be high and of adequate long duration if strength and muscle mass are to increase[126, 127, 128]. Intensities of 60–85% of the individual maximum voluntary strength have been approved to increase muscle strength by increasing muscle mass and even higher intensities are required to enhance the rate of force development in older subjects[129]. The study participants were instructed to choose the resistance (and colour) of the elastic band in a way that they should be able to complete two sets of exercises with a maximum of 15 repetitions per set and with a resting period of 30 to 60 seconds. Several equations have been developed to predict the one repetition maximum strength based on submaximal tests. Even if the correctness of the equations is linked to specific exercises or population groups, 15 repetitions as used in the current study correspond to approximately 65% of the one repetition maximum[130]. Therefore, we determined a moderate intensity in our study. Although the participants were carefully supervised and encouraged, it cannot be excluded that some participants worked at a lower intensity which potentially explains the lack in strength gains. It might be of advantage in further studies to monitor elastic band exercises using stretch-sensors which have been shown to measure total and single repetition time-under-tension reliably[131]. While higher training intensities seem to be more effective compared to low or moderate intensities for improving maximal strength, low and moderate intensity programmes are frequently used especially for older people with functional limitations and with the presence of several comorbidities such as cardiovascular diseases[132, 133, 134]. This is also the case for the current study population which consisted of people who cannot be considered as thoroughly healthy as we included subjects with hypertension, hyperlipidemia, diabetes, osteoporosis, non-acute cardiac diseases and functional impairments (use of walking aids, dynapenia and sarcopenia)[135]. Interestingly, we examined that functional performance as assessed by chair stand and arm lifting tests could be enhanced without improvements in muscle mass or strength. This result is accordance to Raymond et al. (2013) demonstrating that high intensity resistance training results in higher strength improvements than low-intensity exercise while high intensities are not necessarily required to improve physical function[126].

Specificity of the tests is an important issue when evaluating the effects of an exercise programme. In the current study, we used isokinetic dynamometry as the primary outcome as this method is the gold standard for testing muscular strength[136]. In accordance to other studies[137, 138] we observed significant correlations of peak torques at both velocities with

handgrip strength, gait speed, 6-minutes walking test, and arm-lifting test. The correlations to chair stand test were somewhat lower. In this study, two different angular velocities (60°/s, 120°/s) were used which can be considered as low to moderate also reflecting the performed training velocity of the elastic band resistance training. We desist from using higher velocities for assessment as institutionalised older subjects might have been unable to reach the target velocity as shown previously[139]. Training with elastic bands includes concentric as well as eccentric components whereby the latter seem to be especially suitable for older subjects because due to 'high-force–low-cost' manner of eccentric exercises[131, 140]. Unfortunately, eccentric muscle strength was not assessed in the current study. It has to be mentioned that the study population consisted of much more women (88%) than men (12%). While the proportion of males for community-dwelling men in Vienna is 34% at an age of 85 years[141], the proportion of males in the retirement homes is 19% in the age group of 80 to 89 years old[142] making our study population representative in terms of gender distribution of institutionalised older people in Austria. However, with this small number of men it was not possible to conduct separate analyses for men and women. It is known that ageing affects muscle protein metabolism differently in men and women as the basal rate of muscle protein synthesis is approximately 30% higher in women than that in men, this fact potentially explains the slower loss of muscle mass in ageing women. On the other hand, exercise training doubles the basal muscle protein synthesis in men, whereas it is increased only by ~40% in females[143]. Interestingly, trainability assessed by strength gains seem to be similar in older men and women[144, 145] making it unlikely (but not impossible) that larger or different improvements could have been observed in males.

One limitation frequently occurring in training intervention studies is the lack of a social activity for the control group with an equal length and frequency to the exercising group. Therefore, we decided to control for social interactions by implementing a cognitive training for the control group. Interestingly, for many physical performance parameters including isokinetic measurements, gait speed, and aerobic capacity as assessed by 6-minutes walking test, improvements over time were detected regardless of groups indicating that also participants receiving only cognitive training improved in these parameters. In order to prove whether participants were changing their physical activity behavior, we assessed steps per day at the beginning and at the end of the study to measure an overall physical activity. Our data revealed that steps per day were generally low, more precisely only 10% fulfilled 7000

steps per day recommended by Tudor-Locke et al. (2010) and decreased rather than increased in all groups[146]. Therefore, it is unlikely that an increase in physical activity could have accounted for the observed improvements in performance independent of allocated group. Future in-depth analyses will be conducted to assess whether time spent in different intensity zones or sedentary changed during the study period. Recent studies have shown that aerobic and/or strength training are able to improve cognitive function in patients with dementia and healthy older people[147, 148]. Additionally, it is known that difficulties with performance of functional activities may result from physical impairments but also from a cognitive decline[149]. The question still remains is whether cognitive training interventions exert positive effects on physical performance. Usually, cognitive interventions have been developed to enhance memory and speed of processing, with the goal of maximizing function and reducing the risk of cognitive decline in older people[150]. However, a recent study has indicated that cognitive training might improve balance and even gait in older subjects known to have a history of falls[151]. Moreover, longitudinal data from a large intervention trial have revealed that cognitive training ameliorates everyday functional outcomes in older adults demonstrating some transfer effects[152]. Therefore, we cannot comprehensively exclude the cognitive training effect, although thought of as a control intervention, contributed to the ameliorations in gait speed and 6-minutes walking test.

In conclusion, focusing the functional outcomes, six months of a moderate/low intensity resistance exercise using elastic bands and the own body weight has been shown to be beneficial in improving functional performance of institutionalized older subjects.

4.4 Effect of intervention on TGF-β, its receptors and miRNA-21

Last but not least, the main question of the current doctoral thesis was to investigate the effects of this long-lasting progressive resistance training alone and in combination with a nutritional supplement on systemic inflammation and TGF-β signalling in PBMCs of institutionalised older people. Circulating TGF-β and hs-CRP levels as well as intracellular TGF-β gene expression were not influenced by elastic band resistance training. Interestingly, TGF-βRI, but not TGF-βRII, gene expression was reduced in the RT group after 3 months and returned to baseline levels thereafter.

Although we examined in the first paper, that none of the inflammatory parameters were related to physical performance state, the chronic pro-inflammatory state caused by ageing and physical inactivity can be reduced through regular endurance training[1, 153, 154]. Adipose tissue seems to have a substantial role in this complex because it is a major source of several hormones and cytokines[155]. More precisely, visceral fat depots and the macrophages within these depots release pro-inflammatory cytokines such as IL-6, TNF-α and TGF-β[156]. The increase in energy expenditure caused by enhanced physical activity reduces body fat, thereby influencing the capacity to produce and release pro-inflammatory mediators[156]. Recommended strategies to lose weight and body fat include aerobic exercise training combined with caloric reduction. Although it is known that resistance training does not promote clinically significant weight loss, it influences body composition by enhancing muscle mass and decreasing body fat[157, 158]. Moreover, it has been hypothesised that muscle-strengthening exercises also exert anti-inflammatory effects. Based on intervention studies investigating the effects of strength training on inflammatory markers such as hs-CRP, TNF-α and IL-6, the data are ambiguous because some studies have revealed a positive effect[90, 159], whereas others did not observe any inflammatory adaptions[160, 161, 162].

While the TGF-β superfamily has been studied extensively in the adaptation of muscles and tendons to exercise, investigations with respect to the context of exercise immunology are rare[163]. Conflicting data have also been reported with respect 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 and 1 h of treadmill running at about 70-80% of VO2max are able to increase the concentration of circulating TGF-β, 1 h of cycling exercise at ~70% of VO2max does not lead to alterations in circulating TGF-β[164, 165, 166]. Interestingly, Rosa et al. (2014) have shown that salivary TGF-β is increased as late as 24 h after a moderate exercise bout[167]. Interestingly, 6 weeks of training 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[168] which 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[90]. Furthermore, 6 months of exercise (2.5 h per week) were able to increase TGF-β production of unstimulated as well as phytohaemaglutinin-stimulated PBMCs of persons at risk of developing ischemic heart disease[169]. While a combined strength and endurance exercise

programme increases circulating TGF-β, strength training alone does not alter its levels, which support our results[90, 170]. Furthermore, we hypothesise that endurance training with or without a dietary weight loss strategy would be more effective to target the chronic inflammatory state. This hypothesis is supported by the fact that parameters measuring aerobic fitness and muscular endurance, but not handgrip strength, correlated with TGF-β alterations.

While circulating levels of TGF-β were unaffected, we showed for the first time that resistance training lowers the gene expression of TGF-βRI in PBMCs of older adults, potentially leading to decreased signalling through the type I receptor. This decrease of TGF-βRI occurred concomitant with an increase in neutrophil counts. Neutrophil granulocytes are the largest fraction of leukocytes and the first line of defense against pathogens and it has been shown that their numbers and function might be altered during the ageing process[171]. Furthermore, both the number of leukocytes and the percentage of neutrophils have been shown to increase with progression to multi-morbidity in a sample size of more than 26,000 individuals[172]. Although neutrophils express receptors for TGF-β, its importance in regulating neutrophil function is still less researched, except for a proposed effect on functional polarisation of tumour-associated neutrophils towards a pro-tumourigenic phenotype[173, 174]. However, PBMCs, which were the source of TGF-βRI in our study, only include lymphocytes and monocytes, and not erythrocytes or neutrophils, therefore the decrease in TGF-βRI expression must arise from one of these cell types. Further studies will need to investigate the exact contribution of the diverse subpopulations in the adaptation to resistance exercise training.

Interestingly, protein supplementation was shown to be ineffective in this setting as we could not detect any differences between RT group and the additionally supplemented RTS group. However, we have assessed nutritional status by MNA and detected slightly lower scores in the cognitive group as compared to the other two intervention groups, whereby these scores did not change over time. In total, about 80% of the participants had a normal nutritional status. We already reported that the nutritional supplementation resulted in an increased uptake of vitamin D and folic acid but not in protein being between 0.8 and 1.0 g/kg/day in the RTS group[175]. Moreover, plasma levels of vitamin B12 and folic acid in erythrocytes were enhanced in RTS group[176]. Therefore, we can confirm that the supplement was regularly consumed by the participants allocated to RTS group. However, there is an ongoing discussion about the optimal intake of proteins, especially in an older population as current

recommendations for high aged population range from 0.8 g/kg/day to 1.5 g/kg/day[177, 178]. For efficiently enhancing muscle protein synthesis, both protein quality and timing are important. In this study, we have used a protein supplement (20.7 g per serving) containing whey protein enriched in leucine. Whey protein stimulates postprandial muscle protein accretion more effectively than casein and casein hydrolysate because of faster digestion and absorption kinetics and higher leucine content[179]. Increasing the proportion of leucine in a mixture of essential amino acids can reverse an attenuated response of muscle protein synthesis in older subjects[180]. With respect to timing, the ingestion of dietary protein directly after resistance training increases post-exercise protein synthesis rates in young as well as in old adults[181, 182]. Additionally, there is some evidence that providing enough protein to maximally stimulate muscle protein synthesis at each meal (breakfast, lunch, and dinner) induced the highest muscle protein synthesis in comparison to a skewed pattern with lower protein intakes for breakfast and lunch[183]. Therefore, we suggest that the chosen pattern in the current study with supplementation at breakfast and directly after the exercise training should be sufficient in order to enhance muscle protein synthesis rate. However, the amount of protein could have been slightly too small as a recent dose–response study in older people has shown that as little as 10 g of whey protein is able to prevent the significant decline in intramuscular branched chain amino acid levels, but only larger doses of whey (30 and 40 g) increase leucine level in skeletal muscle post-resistance exercise training[184]. However, even if a higher muscle protein synthesis can be achieved with post-exercise protein supplementation, it does not necessarily result in improved muscle strength[185]. Similar to resistance training alone, some studies have revealed that increased protein intake leads to similar beneficial effects on muscle mass[186, 187]. Recently a systematic review demonstrated, that combining protein supplementation with resistance training is effective for eliciting gains in fat-free mass among older subjects, but did not lead to further increase in muscle mass or strength, which was similar to our results[185]. However, there is an ongoing discussion whether antioxidant supplementation may even blunt an exercise-induced training effect[188]. Evidence indicates that reactive oxygen species modulate TGF-β signalling. More detailed, TGF-β increases the production of reactive oxygen species and suppresses antioxidant enzymes[189]. Therefore, it cannot be excluded that rigorous scavenging of free radicals impaired the TGF-β pathway response in the RTS group.

4.5 Drop-out analyses

Apart of the main outcomes, one of the most important and interesting findings of our study is, that those participants who quit the study even before the final measurements were in a worse physical condition at baseline, as measured by chair stand test, 6-minutes walking test and gait speed compared to those finishing the study. Furthermore TGF-β, TGF-βRI and potentially TGF-βRII expression were higher, while miRNA-21 expression was lower in drop-outs compared to finishers. We did not observe any side effects associated with the resistance exercise training or the nutritional supplementation. Furthermore, we confirmed that age, sex, body composition and the presence of several co-morbidities might not contribute to this effect, but the proportion of subjects with hyperlipidaemia was higher among finishers. A possible explanation for this fact could be the 'obesity paradox' that implies moderate obesity at a higher age reduces the mortality risk[190, 191]. According to other studies, investigations focusing older participants have to calculate with drop-out rates up to 37%[192], however in our study it was less than 30%. Zech et al. (2012) have shown, that drop-out rate could be related to allocation to an intervention group[193], which was not the case in our study. Dropout reasons mostly have been medical issues not related to the intervention. This might be different to many other studies in older participants, pre-selecting a rather healthy and fit population but clinically relevant as a certain level of physical fitness seems to be needed before starting a group training programme.

Resistance training is beneficial, even in very old subjects, and potentially influences TGF-β signalling through altered receptor expression. Furthermore, adherence to the intervention was significantly related to alterations in the TGF-β pathway at baseline, but further information is needed to clearly understand the role of TGF-β in the context of inflammageing and resistance training.

5 Conclusion

Chronic low-grade inflammation is an undesirable side effect of ageing. Transforming growth factor-β (TGF-β) seems to play a role in the inflammageing process as a multi-functional regulatory protein. It has been shown that TGF-β is involved in a variety of physiological as well as pathological processes exerting positive but in some cases also negative effects on health. Furthermore microRNA´s are involved of regulating ageing. According to current literature, physical performance is able to diminish this inflammageing process.

Therefore, this doctoral thesis is based on 3 publications within the Vienna Active Ageing Study to examine the association of physical performance on TGF-β signalling. We have shown that in older women TGF-β signalling in PBMCs might be impaired as reflected by a lower gene expression of its type I and type II receptors, though this decreases were independent of physical fitness.

Further, six months of a moderate intensity resistance exercise using elastic bands and the own body weight has been shown to be beneficial in improving functional performance of institutionalised older subjects with a mean age close to or even above the average life expectancy. Additional supplementation did not offer additional benefits to the effects of strength training alone, in improving muscular performance.

Interestingly, resistance training potentially influences TGF-β signalling through altered receptor expression. Furthermore, adherence to the intervention was significantly related to alterations in physical performance and the TGF-β pathway at baseline.

However, further information is needed to clearly understand the role of TGF-β in the context of inflammageing and resistance training.

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9 Appendix

VAAS strength exercise training programme

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Curriculum Vitae

The VAAS training program

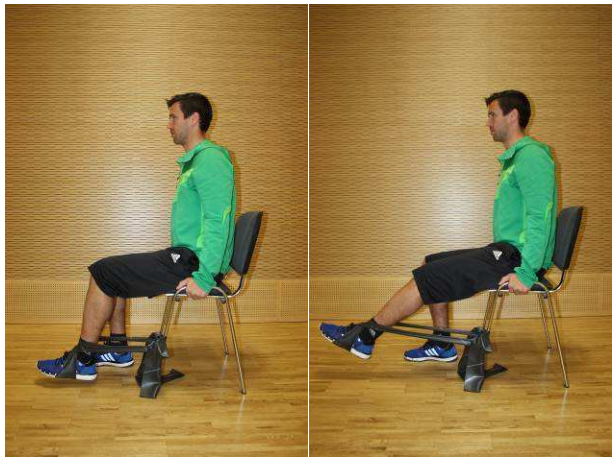
General Information
Warm up: Before every session a warm up period of 10 minutes was performed. It consisted of balance as well as low-intensity endurance exercises such as walking.
Strength training (30-40min): The execution of the exercises (using elastic bands or the own body weight) was supervised and the exercise order remained the same in every session. The participants were instructed to perform all exercises in a slow and controlled manner without sudden jerk acceleration. The exercises should be executed with the full range of motion (without pain). Participants were encouraged to breathe evenly while performing the exercises, whereby exhalation should occur during the more strenuous phase of the repetition.
Cool down: After the strength training session a cool down routine was applied for about 10 minutes consisting of relaxation exercises.
Progression: The program started with an initial adaptation phase of 4 weeks using the Thera-Band® (The Hygenic Corporation, Akron, OH) with the lowest resistance (yellow). Participants were instructed to choose the stretch of the elastic band so that a maximum of 15 repetitions could be performed once (weeks 1-4). From the 5th week onwards, progression was ensured by first increasing the number of sets to two. Then the elastic band was changed from a yellow to a red and finally to a black one (see Supplementary Table 1 for the resistance of the respective Thera-Band® in dependency of stretch - 100% stretch means doubling the length of the Thera-Band®).
Supplementary Table 1: Elastic resistance pull force chart

		Resistance in Kilograms		
% stretch	**-fold stretch**	Yellow	Red	Black
25%	**1.25**	0.6	0.9	2.1
50%	**1.50**	0.8	1.1	2.8
75%	**1.75**	1.0	1.4	3.5
100%	**2.00**	1.3	1.7	4.1
125%	**2.25**	1.5	1.9	4.8
150%	**2.50**	1.7	2.2	5.5
175%	**2.75**	2.0	2.4	6.1
200%	**3.00**	2.2	2.7	6.8

Data calculated from linear regression formulas according to Page et al., 2000. J Orthop Sports Phys Ther. 30(1),A47-48.

Materials: Thera-Band® in different colors, stable chair with a sitting height of 45 cm.

Lower extremities	
M. gluteus maximus, medius, minimus, M. quadriceps femoris, hamstrings	**<u>Exercise 1: Squat</u>** *Starting position: sitting on a chair* o Raise from the chair (with or without help of the arms) o Sit down *Possibilities to increase intensity* o Squat, but without sitting down on the chair o Increasing range of motion by performing deep squats without the chair  **<u>Exercise 2: Leg extension</u>** *Starting position: sitting on a chair, elastic band is attached to one leg of the chair and the ankle of the involved leg* o Unilateral knee extension

	o Return to starting position *Possibilities to increase intensity* o Higher initial stretch of the elastic band o Higher resistance of elastic band by changing the color 

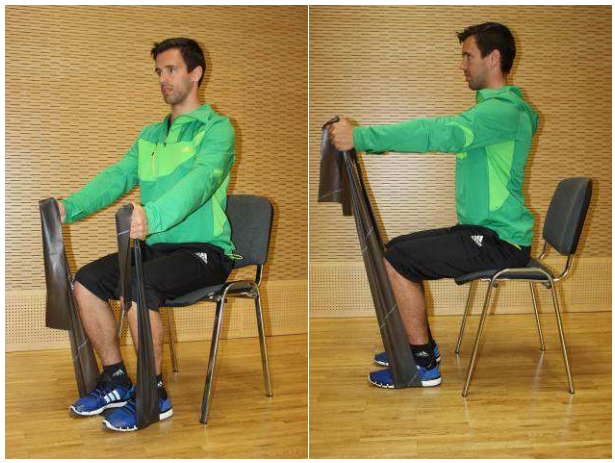
Lower extremities	
M. gluteus maximus, medius, minimus, M. quadriceps femoris, hamstrings	**<u>Exercise 3: Hip extension</u>** *Starting position: one leg stand, holding on a chair; a loop is created with the elastic band enclosing the chair and one leg at the ankle level* o Move extended leg backwards (with a leg curl at the end of the range of motion to additionally imply the hamstrings) o Return to starting position *Possibilities to increase intensity* o Higher initial stretch of the elastic band o Higher resistance of elastic band by changing the color  **<u>Exercise 4: Calf lift</u>** *Starting position: standing upright, holding on a chair*

	o Moving the heels up and down – heels should not touch the ground during the execution of the exercise *Possibility to increase intensity* o perform exercise in the one leg stand 

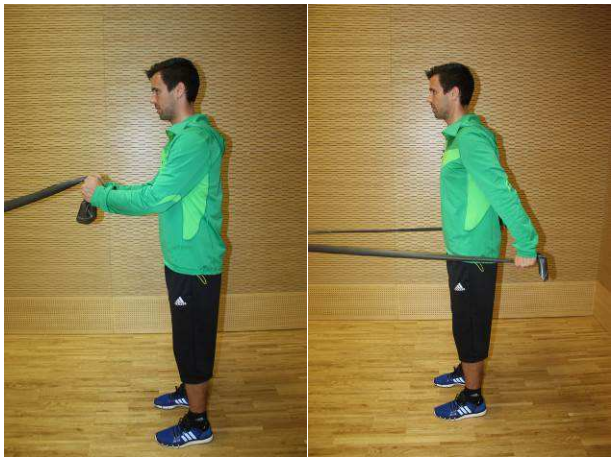
Back	
M. rhomboideus, M. trapezius M. transversus	**<u>Exercise 5: Standing row</u>** *Starting position: standing in an upright position facing wall bars (or another safe fixing point); the elastic band is fixed a bit higher than hip level and the ends of the band are wrapped around the each hand* o Pulling the arms back and bending the elbows o Squeezing shoulder blades together o Return to starting position *Possibilities to increase intensity* o Higher initial stretch of the elastic band o Higher resistance of elastic band by changing the color 

Abdomen	
M. rectus abdominis	**<u>Exercise 6:</u>** *Starting position: sitting on a chair, legs are lifted from the ground, hands are used to assure a safe position* o Hip extension and flexion, stabilisation of the abdominal muscles *Possibility to increase intensity* o The higher the legs are during the exercise the more intense; 

Chest	
M. pectoralis major	**<u>Exercise 7: Chest press</u>** *Starting position: sitting on a chair; elastic band is fixed around the upper body, each hand is grapping one end of the band* o Pushing the arms forward while bending and straightening the arms o Elbows should remain at shoulder level *Possibilities to increase intensity* o Higher initial stretch of the elastic band o Higher resistance of elastic band by changing the color 

Shoulder	
M. deltoideus	**<u>Exercise 8: Front raise</u>** *Starting position: sitting on a chair, the elastic band is fixed under both feet, one end is kept in each hand* o Moving the arms straightened arms up and down *Possibilities to increase intensity* o Higher initial stretch of the elastic band o Higher resistance of elastic band by changing the color 

Arms	
M. biceps brachii, M. triceps brachii	**<u>Exercise 9: Elbow flexion (biceps)</u>** *Starting position: sitting on a chair, the elastic band is fixed under one foot, both ends are kept one hand;* o Performing a biceps curl while resting the elbow on the knee o Return to starting position *Possibilities to increase intensity* o Higher initial stretch of the elastic band o Higher resistance of elastic band by changing the color  **<u>Exercise 10: Elbow extension (triceps)</u>**

	Starting position: standing in an upright position facing wall bars (or another fixing point), the elastic band is fixed a bit higher than hip level and the ends of the band are wrapped around the each hand o Extension and flexion of the elbow o Hint: the extended arms move further to the rear to give an extra tension on the latissimus *Possibilities to increase intensity* o Higher initial stretch of the elastic band o Higher resistance of elastic band by changing the color 

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Barbara Schober-Halper

Curriculum Vitae

Personal data

born	October, 12th 1984
nationality	Austria
family status	married, son born on Dec 18th, 2014
address	Dornburggasse 26, 7400 Oberwart
phone	+43-699-10981290
mail	barbara.halper@univie.ac.at

Education

since 10/2010	Doctoral studies Sport Science, University of Vienna, Austria *Thesis: Influence of progressive resistance training on TGFβ signalling in institutionalised older people.*
2010	Nordic Walking Instructor certificate
2009	Medical-Taping certificate
2008 – 2010	Master´s degree Sport Science, University of Vienna, Austria *Thesis: Effects of sports massage on inflammatory parameters after high intensity interval training.*
2008	Sports massage therapist
2007	Massage therapist
2005 – 2008	Bachelor´s degree Sports Sciences, University of Vienna, Austria
2004	Fitness trainer certificate
1999 – 2004	Grammar school (for athletes) Oberschützen, Austria
1995 – 1999	Secondary school (sports) Oberwart, Austria
1991 – 1995	Elementary school Oberdorf, Austria

Contributions at scientific conferences

06/2014	Invited Speaker GSAAM, Munich (Germany): "Influence of exercise on immune system"
11/2013	Oral Presentation 2. Wiener Muskeltag, Vienna (Austria): "Influence of strength training in immune system in elderly"
09/2013	Oral Presentation ISEI in Newcastle (Australia): "Age-related differences of microRNA-21 in leukocytes and its association with physical performance."
04/2013	Oral Presentation Active Ageing Conference in Vienna: "The influence of progressive resistance training on immunological parameters in institutionalised elderly."
07/2011	Poster presentation ECSS in Liverpool (England): "*Effects of sports massage on inflammatory and muscle damage markers after high intensity interval paper.*"
07/2011	Poster presentation ISEI in Oxford (England): "*Effects of sports massage on inflammatory and muscle damage markers after high intensity interval paper.*"

Scientific stay abroad

09/10 2013	Queensland University of Technology, Institute of Health and Biomedical Innovation, Brisbane (Australia)

Grants and awards

09/2013	Travel Award for Oral Presentaion on ISEI symposia in Newcastle (Australia)
09/2012	Young Investigator Price for Oral Presentation on Austrian Sports Sciences Society Congress (ÖSG)
2011	Grant of the University of Vienna („StudFG SS 2010") for finishing Master Thesis

Teaching at academic standard

04/2013	Guest Lecturer in Internship of European Master, Vienna (Austria): "Fitness Assessment"
2012-2013	Lecturer on University of Vienna (Austria): "Functional Training"
11/2011	`Teach the Teacher`- Guest lecturer in Pristina (Albania): *Active Ageing*

Professional activity

since 12/2014	Maternity leave
since 06/2014	Managing director of Kraftkollektiv KG
03/2014 – 10/2014	Research assistant at Austrian Institute of sports medicine (ÖISM)
since 06/2011	Research assistant at the Research Platform Active Ageing
2010-2012	Sports massage therapist at Werner-Schlager-Academy
2010	Trainer in Project „Gesundes Dorf"; Fonds Gesundes Österreich
since 2009	Lecturer of Functional Training in Sports Massage School, VÖSM&ÖGS, Vienna
04/2008 – 01/2010	Vitalcoach Elixia Fitness Club Millennium, Vienna
06/2006 – 01/2008	Personal trainer, Massage Insitute Matoga, Vienna
09/2005 – 05/2006	Wellness trainer, Vitalsport Therapy Centre, Vienna

Barbara Schober-Halper

Oberwart (Austria), 24th of March 2016