

STUDY PROTOCOL

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Interaction between cytokine gene polymorphisms and the effect of physical exercise on clinical and inflammatory parameters in older women: study protocol for a randomized controlled trial

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Abstract

Background: Aging is associated with chronic low-grade inflammatory activity with an elevation of cytokine levels. An association between regular physical activity and reduction of blood levels of anti-inflammatory cytokines is demonstrated in the literature pointing to an anti-inflammatory effect related to exercise. However, there is no consensus regarding which type of exercise and which parameters are the most appropriate to influence inflammatory markers. Evidence indicates that the single nucleotide polymorphism (SNP) can influence the synthesis of those cytokines affecting their production.

Methods/Design: The design of this study is a randomized controlled trial. The aim of this study is to investigate the interaction between the cytokine genes SNP and the effect of physical activity on older women. The main outcomes are: serum levels of sTNFR-1, sTNFR-2, interleukin (IL)-6, IL-10, measured by the ELISA method; genotyping of tumor necrosis factor- (TNF)-alpha (rs1800629), IL6 (rs1800795), IL10 (rs1800896) by the TaqMan Method (Applied Biosystems, Foster City, CA, USA); and physical performance assessed by Timed Up and Go and 10-Meter Walk Tests. Secondary outcomes include: Geriatric Depression Scale, Perceived Stress Scale and aerobic capacity, assessed by the six-minute walk; and lower limb muscle strength, using an isokinetic dynamometer (Biodex Medical Systems, Inc., Shirley, NY, USA). Both exercise protocols will be performed three times a week for 10 weeks, 30 sessions in total.

Discussion: Investigating the interaction between genetic factors and exercise effects of both protocols of exercise on the levels of inflammatory cytokine levels can contribute to guide clinical practice related to treatment and prevention of functional changes due to chronic inflammatory activity in older adults. This approach could develop new perspectives on preventive and treatment proposals in physical therapy and in the management of the older patient.

Trial registration: (ReBEC) RBR9v9cwf

Keywords: Polymorphism, Cytokines, Older adult, Exercise, Physical performance, BDNF

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Background

Aging is associated with a chronic low-grade inflammatory process characterized by a systemic elevation, from two to four times, of plasma levels of cytokines as interleukin (IL)-1, tumor necrosis factor alpha (TNF- α), IL-6, acute phase proteins, soluble TNF receptors (sTNFR) and IL-10, among others [1]. The balance between production and release of those cytokines has been related to the emergence or aggravation of chronic conditions related to aging, disability and increased mortality in older adults [1,2]. High levels of cytokines are associated with a reduction of physical performance and muscle function [3-7]. The underlying mechanism by which these cytokines contribute to a functional deterioration in older adults seems to be their catabolic effect, leading to a reduction in muscle mass and strength that are related to sarcopenia [1,8].

TNF- α is an early mediator of inflammation since it starts and coordinates the acute phase response and induces the production of a second wave of cytokine expression, such as IL-6, IL-8 and C-reactive protein [9]. It also stimulates the production of sTNFR that acts as its natural inhibitor; therefore, regulating its biological function. Since these receptors are more stable molecules than TNF- α in circulation, they are more reliable markers of plasma TNF- α levels, and hence of the inflammatory response [10]. A few authors argue that TNF- α is behind the age-related inflammatory changes [11,12], being associated with the development of insulin resistance and metabolic syndrome [13], and also with reduction of muscle mass and strength loss due to its catabolic action [14].

IL-6 is a cytokine that has both pro- and anti-inflammatory roles and is involved in controlling and coordinating inflammatory responses. It is produced by different cell types, which include the skeletal muscle cells [15]. On the other hand, IL-10 is an anti-inflammatory cytokine that is essential to inflammatory activity control and resolution that is triggered and sustained by other mediators [16]. The IL-10 inhibitory effect on IL-6 and TNF- α cytokines is well established in acute inflammatory processes [17], but not in chronic inflammation, such as we see during aging. The impact of physical exercises on their plasma levels is not known.

Differences seen in protein expression among people can occur as a result of functional genetic variations in the promoter area of these molecules gene [18]. The most common variations seen on human genomes are the single nucleotide polymorphism (SNP). Evidence points that SNPs, which are present in the genes of several molecules involved in inflammation, could affect their gene transcription and synthesis [18], modulating the inflammatory response severity. Some polymorphisms are associated with a greater production of inflammatory mediators.

Thus, gene variations could explain in part the variability in the production of cytokines, and the greater liability of certain people to clinical conditions that are mediated by the elevation of these markers' production, such as chronic conditions related to aging and longevity [19-21].

The expression of TNF- α , IL-6 and IL-10 is influenced by functional polymorphisms at their promoter areas. The polymorphisms in TNF- α (rs1800629), IL-6 (rs1800795) and IL-10 (rs1800896) have been associated with several acute and chronic diseases and with longevity as well [1,19,22]. For instance, Oberbach *et al.* [22] identified that changes in the IL-6 plasma levels in response to exercise were influenced by the -174 G/C polymorphism, suggesting that genetic factors related to cytokine production could be determinant to individual effects of the anti-inflammatory response promoted by exercise. However, literature presents contradictory results related to their activity and effects on plasma levels of these mediators, especially considering the older population. One possibility is that differences shown in the literature are due to interactions between lifestyle and gene factors, along with cultural and ethnic differences of the different studied samples.

Observational studies have pointed out that physically active people have lower concentrations of inflammatory cytokines, suggesting that regular exercise may alleviate the chronic inflammatory activity associated with aging [15,23,24]. Consistent evidence has demonstrated that physical activity induces an elevation of anti-inflammatory cytokine systemic levels [25-27], with the skeletal muscle tissue being indicated as an endocrine organ that produces and releases cytokines called miokines [15].

The production and release of IL-6 during physical activity seem to rely on factors such as type, intensity and duration of exercise [26]. Despite the possible benefits of physical exercise on chronic inflammatory process, information about the parameters of the ideal exercises for regulating cytokines have not been established [28-31], with research results being conflicting.

Therefore, the primary objective of this clinical trial is to investigate the existence of an interaction among TNF- α rs1800629, IL6 rs1800795, IL10 rs1800896 and gene polymorphisms with the effect of physical exercise in older women.

Methods/Design

Recruitment of participants

This study is a randomized controlled trial for which community-dwelling older women will be recruited and randomly assigned to either one of the following physical activity protocol: muscle strengthening exercises (MSE) or aerobic exercises (AE). Participants will be recruited from the community through publicity in local

newspapers and community centers for older persons. The study will be conducted at the Universidade Federal de Minas Gerais, Belo Horizonte, Brazil.

After the initial evaluation, participants will be randomized into either the MSE or AE group. Randomization will be performed by means of sealed brown envelopes without identification. The researcher responsible for the evaluations will be blinded to the participants' group.

The study was approved by the Ethics Committee of Universidade Federal de Minas Gerais (ETIC 038/2010) and all volunteers will give their written informed consent to participate, according to the principles of the Helsinki Declaration (1964).

Sample

Inclusion criteria

Community-dwelling older women who are sedentary and aged 65 years or more will be included in the study. Sedentary older women are those who have not practiced any regular physical activity (that is, at least three times a week, 40 minutes minimum) in the last three months.

Exclusion criteria

Older people with cognitive impairment detectable by the Mini Mental State Examination [32], acute phase inflammatory disease, a history of cancer, current use of immunomodulatory medications, amputation or lower limb fracture in the last six months, presence of neurological sequelae or current participation in an alternative exercise program will be excluded from the study.

Baseline assessment

A standardized questionnaire will be applied by trained researchers to collect sample characteristics, socio-demographic data and information on the clinical condition of older adults.

Primary outcome measure

1. Plasma levels of TNF- α , sTNFR-1, sTNFR-2, IL-6, IL-10: 5 ml of blood will be withdrawn from the participants and immediately centrifuged at 1,500 rpm to obtain plasma. Aliquots will be removed in a laminar flow hood and stored at -80 °C. Plasma levels of cytokines will be determined by ELISA (enzyme-linked immunosorbent assay) method with high sensitivity kits (Quantikine[®]HS, R&D Systems, Minneapolis, MN, USA). According to the manufacturer the detection level of the method was 5 pg/ml for TNFRs, 0.15 pg/ml for IL-6 e 0.75 pg/ml for IL-10.
2. *Genotyping* – Blood samples will be obtained from all individuals in ethylenediaminetetraacetic acid

(EDTA) anti-coagulant and genomic DNA will be extracted using a phenol-chloroform method from unfractionated whole blood, and stored at -20 °C. TaqMan genotyping assays will be obtained from Applied Biosystems, Inc. (Foster City, CA, USA). The assay identification code for each respective SNP is IL-10 (rs1800896), TNF (rs1800629). A customized assay will be used for the IL-6 SNP gene. All amplifications will be carried out in an ABI 7900HT thermal cycler (Applied Biosystems, Inc.) using TaqMan Genotyping Master Mix and following the manufacturer's recommended amplification conditions.

3. Physical Performance will be assessed by means of Timed Up and Go (TUG) [33] test and the 10-Meter Walk Test (10MWT) [34]. These tests will be used because they have demonstrated high reliability and are commonly used to assess function in older people. The TUG measures, in seconds, the time taken to stand up from a standard chair, walk a distance of three meters, turn, walk back to the chair and sit down. The TUG score demonstrates high inter-rater and intra-rater reliability (intraclass correlation coefficients (ICCs) 0.99 and 0.99 respectively) [35]. The 10MWT has good reliability (ICC = 0.78 e ICC = 0.93), and is a good marker for mobility and fall risk [36].

Secondary outcome measure

1. Muscle strength will be assessed using a Biodex[®] System 3 Pro isokinetic dynamometer (Biodex Medical Systems Inc., Shirley, NY, USA). This instrument has been accepted as the "gold standard" for assessment of muscular performance [37]. The muscle groups assessed will be the knee joint extensors and flexors of the dominant limb at angular speeds of 60° and 180° in concentric contractions, with 5 and 15 repetitions respectively, at intervals of 30 seconds between each velocity. All procedures will be performed according to the assessment protocol suggested by the manufacturer, such as positioning of the volunteer, calibration, correction for gravity, familiarization and strong verbal encouragement. Variables chosen for analysis are work normalized by body mass (%) at 60°, and Power at 180°.
2. Aerobic capacity, as measured by the 6-Minute Walk Test (6MWT). The 6MWT has been used in patients who have different disorders, such as neurological, cardiothoracic, infant or rheumatologic dysfunctions, and its correlation with maximum aerobic capacity is considered to be satisfactory [38,39]. Data related to systemic blood pressure,

heart rate and subjective perception of effort using the Borg scale will be recorded.

3. Geriatric Depression Scale (GDS) is a screen for the presence of depression in older people. It comprises 15 individual questions; the cut-off-points will be 5/6 (non-case/case) [40,41].
4. The level of stress will be assessed by the 14-item Perceived Stress Scale validated for Brazilian older persons. This scale assesses three factors considered to be keys in the experience of stress: how the subject evaluates his life as unpredictable, uncontrollable and overloaded [42].

The salivary cortisol level will be measured as an objective measurement of stress [42]. The salivary cortisol level will be collected with cortisol specific Salivette tubes (Sarstedt.Salivette-swab). The dosage of the salivary levels will be performed using the ELISA method (*Salimetrics*).

Intervention

The participants will be divided into two groups: strengthening exercises (SE) and aerobic exercises (AE) groups. Both groups will be submitted to a protocol lasting 10 weeks, 30 sessions in total, 3 times a week, under the direct supervision of a physical therapist.

AE protocol

This protocol will consist of aerobic activities, including a 5-minute warm-up routine, 40 minutes of aerobic exercises – walking and free weight exercises for both upper and lower limbs, and a 5-minute cool down period, as recommended by the American College of Sports Medicine [43]. Heart rate will be maintained at 60% of age-predicted maximum heart rate during both warm-up and cool-down periods, and between 65% to 80% levels during the aerobic activity.

Blood pressure and heart rate will be measured at the beginning and at the end of every session. To ensure the safety of participants and to guarantee the proper training zone, each one of them will be monitored by a cardiac monitor.

SE protocol

This program was based on a previous study (ISRCT-N62824599) [44] developed by the Pain and Inflammation in Rehabilitation and Aging Studies Laboratory (Laboratório de Dor e Inflamação em Reabilitação e Estudos do Envelhecimento - LADIRE) research group.

The session will consist of a 10-minute walk, followed by stretching the rectus femoris, psoas, hamstrings and triceps surae muscles. Strengthening exercises will be performed for the following movements: hip flexion, abduction, adduction and extension; knee flexion and

extension; mini-squat. The load, suitable for each participant, will be calculated by one repetition maximum (1RM) test. Participants will initiate the exercises at 50% of 1RM, adjusting the load after two weeks (seventh session) to 75% of 1RM. The RM will be recalculated for sessions 13 and 22, and the exercises will be performed at 75% of the newly established RM. Participants will be reassessed after 30 sessions.

Sample size

Based on the multivariate linear regression analysis that will be performed in this study to explore the SNP influence on the effects of exercise on plasma levels of cytokines and on physical performance, 140 older adults need to be included, considering the sample calculation of $(10 \times (K + 1))$, where K is the number of explanatory variables of the model. The explanatory variables are age, anthropometric measurements (Body Mass Index and Waist Circumference), physical activity level, presence of depression and stress levels (Perceived Stress Scale and salivary cortisol), TNF- α rs1800629 (AA + AG versus GG), IL6 rs1800795 (GG versus CC + GC), IL10 rs1800896 (GG versus AA + AG), genes polymorphisms.

Statistical analyses

Insight into the sample characterization will be provided using descriptive statistics, including measures of central tendency (mean and median) and variability (range and standard deviation). Violation of Hardy-Weinberg equilibrium will be tested by the chi-square test.

The Kolmogorov-Smirnov test will be used to verify the normal distribution of data. The comparison for between and within groups data will be analyzed using an analysis of variance (ANOVA). *Post hoc* testing will be undertaken with LSD tests.

The multivariate linear regression analysis will be performed to explore the interaction and the effect of polymorphisms on the variables: plasma levels of cytokines and physical performance, considering the genotype of each investigated.

Statistical analysis will be performed using the *Statistical Package for Social Sciences* (SPSS Inc., Chicago, IL, USA), version 17.0, and α level will be set at .05.

Discussion

The elevation of the plasma inflammatory cytokine levels has as its main consequences sarcopenia [6,18,19], reduction of function and independence of the older adult. We have previously demonstrated a weak to moderate negative correlation between high plasma levels of IL-6 and sTNFR-1, and reduced muscle strength and physical performance in older women [3,4].

Within this context, physical exercise has been presented as one of the most effective strategies to influence

both an improvement in physical performance and a decrease in plasma levels of inflammatory markers in the older adult [7,24,29]. However, even though physical exercise has been widely performed in clinical practice, there is no consensus on which type of exercise and what clinical parameters are the most influential on the inflammatory markers. Thus, the results of this clinical trial could contribute to the standardization and management of clinical practice related to prevention and treatment of functional changes in the older adult.

A series of evidence suggests that the SNP could affect the production of cytokines, also influencing physical, cognitive and behavioral performance, and muscle strength in the older adult [18,19,22]. We have recently investigated the effect of the -174 G/C polymorphism of the IL-6 gene on the plasma IL-6 levels in both community dwelling and institutionalized older women. Homozygotes women for the G allele showed high IL-6 levels, and an interaction between polymorphism and housing conditions (community/institution) was observed, with a higher effect of GG genotype on IL-6 levels in the institutionalized women [45].

The genes involved in the regulation of the chronic inflammatory process can contribute not only to the individual variability of cytokine production and physical performance in the older adult, but also to the response of these variables to physical exercise. Still, it is important to note that the evaluation of a single cytokine genotype without considering its interaction with other genotypes from different biological markers could lead us to misinterpretations, as their combined action could result in different effects. Therefore, analyzing and understanding the influence of genetic factors on the effects of different exercise protocols as proposed by this study may contribute to the development of new perspectives on preventive and therapeutic approaches in physical therapy and in general management of the older patient. This trial was designed to be reproducible in both research and clinical environments.

Abbreviations

10MWT: 10-Meter Walk Test; 1RM: one repetition maximum; 6MWT: 6-Minute Walk Test; AE: aerobic exercises; EDTA: ethylenediaminetetraacetic acid; ELISA: Enzyme Linked Immunosorbent Assay; GDS: Geriatric Depression Scale; IL-10: interleukin-10; IL-6: interleukin-6; IL-8: interleukin-8; MSE: muscle strengthening exercises; SNP: single nucleotide polymorphism; sTNFR: soluble TNF receptors; TNF- α : tumor necrosis factor alpha; TUG: Timed Up and Go.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DSP, LSMP, ALT and DAGP will be responsible for the conception and implementation of the study, as well as for its writing and final corrections. RCD will help in the implementation and supervision of the study. DMCA and BZQ will be responsible for supervision of the intervention. AMA and DCF will be responsible for the evaluations. DSP, ECCM and BZQ will be responsible for the molecular genetic studies. ASM and DSP will be

responsible for the immunoassays. DSP, DAGP and FJM will conduct the data analysis. All authors contributed to and approved the final manuscript.

Acknowledgments

This work was funded by Fapemig (17621/2011) CAPES, CNPq and (Brazil).

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Received: 17 January 2012 Accepted: 4 July 2012

Published: 8 August 2012

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doi:10.1186/1745-6215-13-134

Cite this article as: Pereira et al.: Interaction between cytokine gene polymorphisms and the effect of physical exercise on clinical and inflammatory parameters in older women: study protocol for a randomized controlled trial. *Trials* 2012 **13**:134.

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