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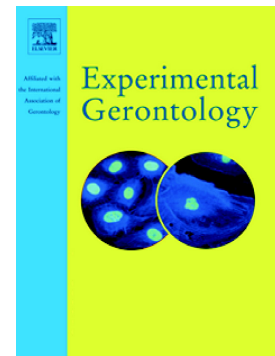
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**RELATIONSHIP BETWEEN INFLAMMATORY BIOMARKERS AND TESTOSTERONE
LEVELS IN MALE MASTER ATHLETES AND NON-ATHLETES**

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ABSTRACT

BACKGROUND: Aging is often associated with low-grade systemic inflammation and reduced anabolic hormone levels. To investigate whether lifelong exercise training can decrease the age-related low-grade inflammation and anabolic hormone levels, we examined hormonal and inflammatory parameters among highly-trained male masters athletes and age-matched non-athletes. **METHODS:** From 70 elite power and endurance master athletes – EMA (51.3 ± 3.6 yr), 32 young controls - YC (23.7 ± 3.9 yr) and 24 untrained age-matched controls - MAC (47.2 ± 8.0 yr) venous blood was drawn to measure inflammatory parameters (interleukin-6 [IL-6], tumor necrosis factor- α [TNF- α] and interleukin-10 [IL-10]) and circulating hormones (luteinizing hormone [LH], total testosterone, estradiol, sex hormone-binding globulin [SHBG] and free androgen index [FAI]). **RESULTS:** EMA showed a better anti-inflammatory status than MAC (higher IL-10 and IL-10/IL-6 ratio and lower IL-6), but a lower anti-inflammatory status than YC (higher TNF- α) ($p < 0.05$). The MAC group had lower testosterone levels compared to the YC and EMA group ($p < 0.05$), and lower estradiol levels and testosterone/LH ratio compared to YC ($p < 0.05$). In the control groups (MAC and YC), testosterone correlated negatively with age and proinflammatory parameters, and positively with anti-inflammatory parameters. **CONCLUSION:** Elite master athletics elevated levels of anti-inflammatory cytokines above that seen in non-athlete peers and mitigated the age-related reduction in testosterone levels.

Keywords: older athletes; hormonal profile; hypogonadism; master athletes; inflammation

1. Introduction

Aging is associated with systemic inflammation - reflected by an increase in inflammatory cytokines, such as tumor necrosis factor (TNF- α) and interleukin-6 (IL-6), and a reduction in anti-inflammatory cytokines, such as interleukin-10 (IL-10) - and a decline in many anabolic hormones (Bobjer et al. 2013; Maggio et al. 2006). Part of the systemic inflammation may be attributed to an age-related increase in body fat (Cesari et al. 2005; Degens 2010), promoting the conversion of testosterone via aromatase in adipose tissue into estradiol, hence reducing androgen levels (Fui et al. 2014).

However, a physically active lifestyle is an effective way to mitigate the changes (Degens et al. 2012). Thus, master athletes are aging successfully (Kusy et al. 2015; Lepers et al. 2016). Previous studies have shown the beneficial effects of lifelong exercise in master athletes on oxidative stress (Aguiar et al. 2019), inflammatory status (Aguiar et al. 2020), nitric oxide production (Sousa et al. 2019), hormonal profile (Ari et al. 2004; Hayes et al. 2013), and telomere length (Aguiar et al. 2019).

Some exercise-induced adaptations, such as the production of testosterone and cortisol, may depend on the training intensity. For instance, the testosterone concentration may decrease after exhaustive training (Daly et al. 2005) and thereby diminish its anabolic effect, and while cortisol can improve mobilization of energy substrates that will enhance performance when produced in excess, it can impair recovery from exercise (Brownlee et al. 2005; Daly et al. 2004).

Aguiar et al. compared inflammatory (TNF- α and IL-6) and anti-inflammatory (IL-10) cytokines in master athletes, middle-aged individuals, and untrained young people, and concluded that both endurance and sprint athletes have lower levels of inflammatory cytokines, as well as higher levels of anti-inflammatory cytokines (Aguiar et al. 2019). Additionally, Ari et al. (2004) observed that master athletes with a long training history (41 years on average) had higher levels of testosterone, growth hormone (GH) and insulin-like growth factor-I (IGF-1), and the testosterone level was

positively associated with training time. This is not an isolated observation as Hayes et al also found higher circulating testosterone levels in master athletes than age-matched non-athletes (Hayes et al. 2013). However, no studies have related the hormonal profile to inflammatory parameters in master athletes.

Therefore, the present study has the following aims: 1) to compare inflammatory parameters (TNF- α , IL-6 and IL-10) and hormonal profile (luteinizing hormone [LH], testosterone, estradiol, sex hormone-binding globulin [SHBG], and free androgen index [FAI]) of elite master athletes and their untrained peers; 2) to assess the association between hormonal profile and markers of inflammation. The hypothesis was that master athletes have a better inflammatory and hormonal profile than age-matched non-athletes. We also hypothesize that master athletes have a similar inflammatory and hormonal profile than healthy young non-athletes.

2. Methods

The Research Ethics Committee of the Catholic University of Brasilia (CAAE: 22997219.3.0000.0029) approved this study that followed the guidelines of the declaration of Helsinki. All subjects signed an informed consent form with all procedures and possible risks explained clearly and thoroughly.

2.1. Participants

For the present analysis, a sample of males was composed of 70 elite power (100m, 200m, 400m, 110m with barriers, among others) and endurance (5km to the marathon, and triathletes) master athletes (EMA: 51.3 ± 8.0 years) competing at regional, national and international levels, and 32 young (YC: 23.7 ± 3.9 years) and 24 middle-aged (MAC: 47.2 ± 8.0 years) non-athletes. The average number

of training years in master athletes was 24.95 ± 9.92 years. They were currently training 9.26 ± 3.69 hours per week and participated in 8.33 ± 5.45 competitions per year. The self-reported best performance of the master athletes ranged from 68% to 99% relative to the age-group world records (5-yr intervals) registered in the World Masters Athletics (WMA). All participants in our sample reached the podium at least once in the national, South American and/or world master championships.

The master athletes were recruited among the Brazilian Master Athletics Championship participants held in São Bernardo do Campo, Brazil, in 2018, Grandprix del Mercorsur held in Montevideú Uruguay, in 2019, and World Master Indoor Athletics Championships held in Torún, Poland, in 2019. The data from the non-athletes were obtained as part of a previous study at the Catholic University of Brasília (Aguilar et al. 2019; Aguilar et al. 2020; Deus et al. 2019; Rosa et al. 2020). The inclusion criteria for elite master athletes were: (a) systematic training for at least 15 years and (b) active training and participation in national and international competitions at the time of data collection. The exclusion criterion of the control group was training or participating in competitive sports. In addition, all participants should be free from a history of inflammatory diseases, a history of cardiometabolic diseases (such as type II diabetes mellitus, systemic arterial hypertension), smoking and regular use of drugs, including hormone replacement.

2.2. General procedures

All volunteers arrived at the laboratory after an 8-hour fast for a blood sample collection from an antecubital vein (~4 mL each using Vacutainer tubes with and without EDTA) for pro- and anti-inflammatory parameters, lipid profile, blood glucose, anamnesis and anthropometric measures. The body mass index (BMI) was calculated and relative body fat was estimated using the seven-fold protocol (Jackson et al. 1978). A single researcher measured all skinfolds with the Lange[®] caliper

(Cambridge Scientific Instruments). Body fat percentage using an equation described elsewhere (Siri 1961).

2.3. Lipid profile and blood glucose

Triglycerides, high-density lipoprotein (HDL-c), total cholesterol (TC) and blood glucose were assessed using commercial kits (Labtest, Minas Gerais, Brazil) according to the manufacturer's instructions. Low-density lipoprotein (LDL-c) was determined using Friedewald's formula (Friedewald et al. 1972).

2.4. Cytokines

TNF- α , IL-6 and IL-10 were analyzed in triplicate by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The detectable limit for TNF- α , IL-6 and IL-10 were 10 pg/mL, 18 pg/mL and 1.0 pg/mL, respectively. The overall inter-assay coefficients of variation for TNF- α , IL-6, and IL-10 were 10.0% and 6.5% for 8.0%, respectively. The ratios between pro- and anti-inflammatory cytokines (i.e., IL-10/IL-6 ratio) were also determined.

2.5. Hormonal parameters

An outsourced reference laboratory carried out hormonal analyses. The analyses of LH, total testosterone, estradiol and SHBG were performed with the automatic immunoassay equipment Atellica® - Siemens® using chemiluminescence (Fasano et al. 2019). A commercial Roche kit (Roche Diagnostics®, Mannheim, Germany) applicable to the Modular E170 automated platform that uses biotinylated anti-testosterone antibody and a testosterone derivative marked with a ruthenium complex was used. The separation was carried out using microparticles covered with streptavidin, captured by magnetic action, and subjected to washing. The reading was then performed by applying a voltage that

induced light by the ruthenium complex (electrochemiluminescent test). A standard solution containing only the reagent was prepared for all procedures, and a control solution containing the reagent plus a known solution. All hormonal measurements were performed in duplicate (Maes et al. 1997; Mather et al. 2015; Morimoto et al. 1997). The free androgen index (FAI) was calculated as $TT \text{ (ng/dL)} / SHBG \text{ (ng/dL)} \times 100$ (Cooper et al. 1998; Nanjee et al. 1985).

2.6. Statistical Analyses

The normality and homogeneity of the data were analyzed by the Shapiro-Wilk and Levene tests, respectively. A general linear model with one factor (one-way ANOVA) was used to compare the hormonal variables between the groups (YC, MAC and EMA). In addition, an analysis of covariance (ANCOVA) was applied to test for differences in testosterone levels between the groups adjusted by age and BMI. The Pearson's correlation coefficient was calculated to determine the association between variables. Cohen's d was used to assess the effect size of the comparisons, considering the following classification to measure the magnitude of effect size: small, $d = 0.2$ to 0.49 , moderate, $d = 0.5$ to 0.79 , and large, $d > 0.8$ (Cohen 1992). The total sample size used in this study ($n = 126$) conferred a statistical power of 99% ($1 - \beta = 0.99$) for the main analysis, a significance level of 5% ($\alpha = 0.05$), and moderate effect size ($d = 0.5$). The significance level was set at 5% ($p \leq 0.05$). All procedures were performed using Graph Pad Prism version 6.0 (Graph Pad Software, San Diego - California), Gpower[®] version 3.1 (Franz Faul, Germany) and Statistical Package for the Social Sciences - SPSS Statistics[®] version 23 (IBM Corporation, New York - United States).

3. Results

The participant characteristics are shown in Table 1. EMA and YC had lower BMI ($p < 0.001$) and body fat ($p < 0.001$), and higher lean mass ($p < 0.001$) than the MAC group. EMA also showed a

higher LDL-c ($p = 0.004$), TC ($p = 0.002$) and LDL/HDL ratio ($p = 0.012$) than MAC, and higher TC than the YC. Triglycerides, HDL-c and blood glucose did not differ between groups ($p > 0.05$). TNF- α was higher in the EMA group compared to the other two groups ($p < 0.05$). IL-6 was higher ($p < 0.05$) in the MAC group compared to YC and EMA. IL-10 was lower ($p < 0.05$) in the MAC group than the other two groups. IL-10/IL-6 ratio was higher ($p < 0.05$) in the EMA group compared to MAC and lower ($p < 0.05$) than YC. There were no significant differences between groups in FAI.

*****Table 1 about here*****

Comparisons of hormonal parameters between EMA, YC and MAC are shown in Figure 2. The MAC group (446 ± 216) had lower testosterone ($p = 0.002$) than the YC (663 ± 265) and EMA (712 ± 209) groups, which in turn, were not significantly different from each other (Fig. 1A). The testosterone analysis adjusted by age and BMI (ANCOVA) showed the same differences ($p = 0.002$). In addition, LH was not significantly different between the groups. There was no significant difference in testosterone/estradiol ratio between groups (Fig. 1C). However, the MAC group (29.5 ± 9.8) had a lower ($p < 0.05$) level of estradiol (Fig. 1D) than the YC group (38.6 ± 11.3) with no significant difference from the EMA group (34.9 ± 9.7). The SHBG level (Fig. 1E) did not differ significantly between the YC (19.1 ± 10.5) and the MAC group (27.3 ± 4.1), but it was lower than YC ($p < 0.05$) in the EMA group (31.5 ± 16.2). The MAC group (72.2 ± 70.8) had a lower ($p < 0.05$) testosterone / LH ratio (Fig. 1F), than the YC group (156.7 ± 133.3) and a moderate effect size ($d = 0.64$) compared to the EMA group (144.6 ± 142.9).

*****Figure 1 about here*****

In the non-athletes, total testosterone was negatively correlated with age ($r = -0.42$; $p = 0.003$; Fig. 2A), but the testosterone level did not differ significantly between YC and EMA (Fig. 2F). In the pooled data of the young non-athletes and elite master athletes, correlations between testosterone and TNF- α ($r = -0.05$; $p = 0.77$; Fig. 2B), IL-6 ($r = -0.04$; $p = 0.81$; Fig. 2C), IL-10 ($r = 0.01$; $p = 0.97$; Fig. 2D), and IL-10/IL-6 ratio ($r = 0.10$; $p = 0.53$; Fig. 2E) were non-significant. In the pooled data of the young and middle-aged non-athletes, correlations between testosterone and TNF- α ($r = -0.57$; $p = 0.01$; Fig. 2G), IL-6 ($r = -0.61$; $p = 0.01$; Fig. 2H), IL-10 ($r = 0.45$; $p = 0.03$; Fig. 2I), and the IL-10/IL-6 ratio ($r = 0.52$; $p = 0.01$; Fig. 2J) were significant.

Figure 2 about here

4. Discussion

The main findings of the present study were that master athletes have less inflammation and have higher testosterone levels than their non-athletic peers. The levels were similar to those of healthy young people. This indicates that regular, intense physical activity may attenuate the age-related low-grade systemic inflammation and changes in the hormonal profile.

Lifestyle factors appear to significantly impact age-related detrimental effects in male hormone production (López-Otín et al. 2013; Schumacher et al. 2008). Lower testosterone production in the middle-aged than the young non-athletes in our study may be due to a reduction in the quantity and sensitivity of Leydig cells for LH (van den Beld et al. 2018). The higher LH level in the sedentary middle age group may be a consequence of the feedback loop to the hypothalamus, where low testosterone results in an enhanced release of LH from the pituitary gland that may, however, not be adequate to fully maintain testosterone levels. Indeed, in the normal situation, this negative feedback loop between testosterone and LH is also mediated by the low-testosterone-induced release of

gonadotropin-releasing hormone (GnRH) from the hypothalamus, where it has been shown that the GnRH production by the hypothalamus is attenuated in old age (Basaria 2013; Kaufman et al. 2005; van den Beld et al. 2018). Furthermore, inflammation may impair testosterone production, as we observed a negative correlation between inflammatory cytokines and testosterone in the non-athletes.

Some studies have shown a significant inverse relationship between hormonal levels, especially testosterone, with inflammation markers (Bobjer et al. 2013; Maggio et al. 2006; Nettleship et al. 2007). Inflammation is the body's response to cell damage. One of the main characteristics of inflammation is the release of cytokines by cells of the immune system that increase vascular permeability, oxidative stress (Aguiar et al. 2020) and leukocyte chemotaxis (Vodo et al. 2013). With physical inactivity and aging, the cessation of secretion of cytokines after an insult is attenuated (Bruunsgaard et al. 2001), which contributes to a proinflammatory state, characterized by elevated levels of TNF- α and IL-6. As discussed above, the inflammatory state may inhibit testosterone production, which corresponds with our observation that in the non-athletes the correlations between testosterone and inflammatory cytokines were negative, but not so in the master athletes.

It has been reported that there is an association between hypogonadism and systemic inflammation in 33- to 41-year-old men (Bobjer et al. 2013). The hypogonadism and systemic inflammation in such young men may result from a high volume and intensity of exercise, especially in endurance sports (Hooper et al. 2018; Lane et al. 2019). Indeed, with a chronic increase in the volume/intensity of exercise, there is an increased production of cortisol, which has a negative impact on LH levels, inhibiting testosterone secretion in the testes through the high production of hydrocortisone (Cumming et al. 1983). The resulting reduction in testosterone levels may contribute to low-grade systemic inflammation as it has been reported that testosterone has an anti-inflammatory role in physically active individuals (Bianchi 2019). In addition, testosterone is converted to estrogen and

binds to the estrogen receptor, which protects against the accumulation of body fat and inflammation (Davis et al. 2013)

Another possible explanation for such a correlation is that the age-related reduction in physical activity (Ingram 2000) that over time leads to an excessive accumulation of body fat, increasing the level of circulating proinflammatory cytokines (Aguiar et al. 2020), as we saw in the middle-aged compared to the young non-athletes. Chronic inflammation induces a higher expression of aromatase in adipose tissue, an enzyme that converts testosterone to estradiol (Cochino et al. 2013; Fui et al. 2014; Kershaw et al. 2004). This process may further increase the production of inflammatory cytokines, such as TNF- α and IL-6, reducing testosterone levels and inhibiting LH function, especially in obese individuals (Ruige et al. 2012), generating a vicious cycle of hormonal and inflammatory damage. It is, therefore, possible that the inflammatory state and lower testosterone are due to a combination of high levels of adiposity (% body fat) caused by low physical activity levels.

Regular exercise has been reported to reverse the deleterious effects of aging and three months of moderate run training (60min/day 6x/week) resulted in a 14.5% increase in dihydrotestosterone in 40- to 75-year-old men that remained 8.6% above baseline even eight months after training cessation (McTiernan 2008). Also testosterone and SHBG levels may be elevated by regular exercise, as seen in 60- to 70-year-old endurance athletes who had higher SHBG levels than their minimally trained peers (~ 2.5h/week) (Cooper et al. 1998), similar to the observations in our study. This is significant, as SHBG concentrations are inversely proportional to insulin resistance (Feng et al. 2019), and illustrates the important role of regular exercise for the prevention of insulin resistance and diabetes in old age, combined with our observation that master athletes had higher testosterone than age-matched non-athletes, regular exercise will likely help maintain testosterone during aging.

Master athletes show that a healthy lifestyle associated with a good body composition can preserve hormonal levels and demonstrate better anti-inflammatory parameters (Aguiar et al. 2020).

Physical exercise in advanced age still stimulates Leydig cells to increase testosterone production (Tremellen et al. 2018). In addition, master athletes have higher IL-10 compared to their sedentary peers (Aguiar et al. 2020; Minuzzi et al. 2017) that mediate a greater production of T cells, thus enhancing their immune tolerance and hormone production (Minuzzi et al. 2017). Testosterone can also preserve telomere length (Simoes et al. 2017), nitric oxide bioavailability (Sousa et al. 2019), and levels of irisin and klotho (Rosa et al. 2020), as seen previously in master athletes.

5. Limitations

The study has some limitations. One is that no objective measures of aerobic and neuromuscular physical fitness were collected (i.e. $\text{VO}_{2\text{máx}}$). Free testosterone was also not directly assessed, but we did calculate the free androgen index as a reasonable alternative. In addition, the training phase or diet of each athlete was not considered, which may have an impact on the hormonal and inflammatory status of the athletes.

6. Conclusions

In conclusion, elite master athletes mitigate the age-related decrease in testosterone levels and diminishes the low-grade systemic inflammation compared to non-athlete peers. These results indicate that lifelong athletic training and a healthy lifestyle have rejuvenating effects on many biological parameters that culminate in a better quality of life in old age.

Conflict of interest statement

The authors declare no conflict of interest.

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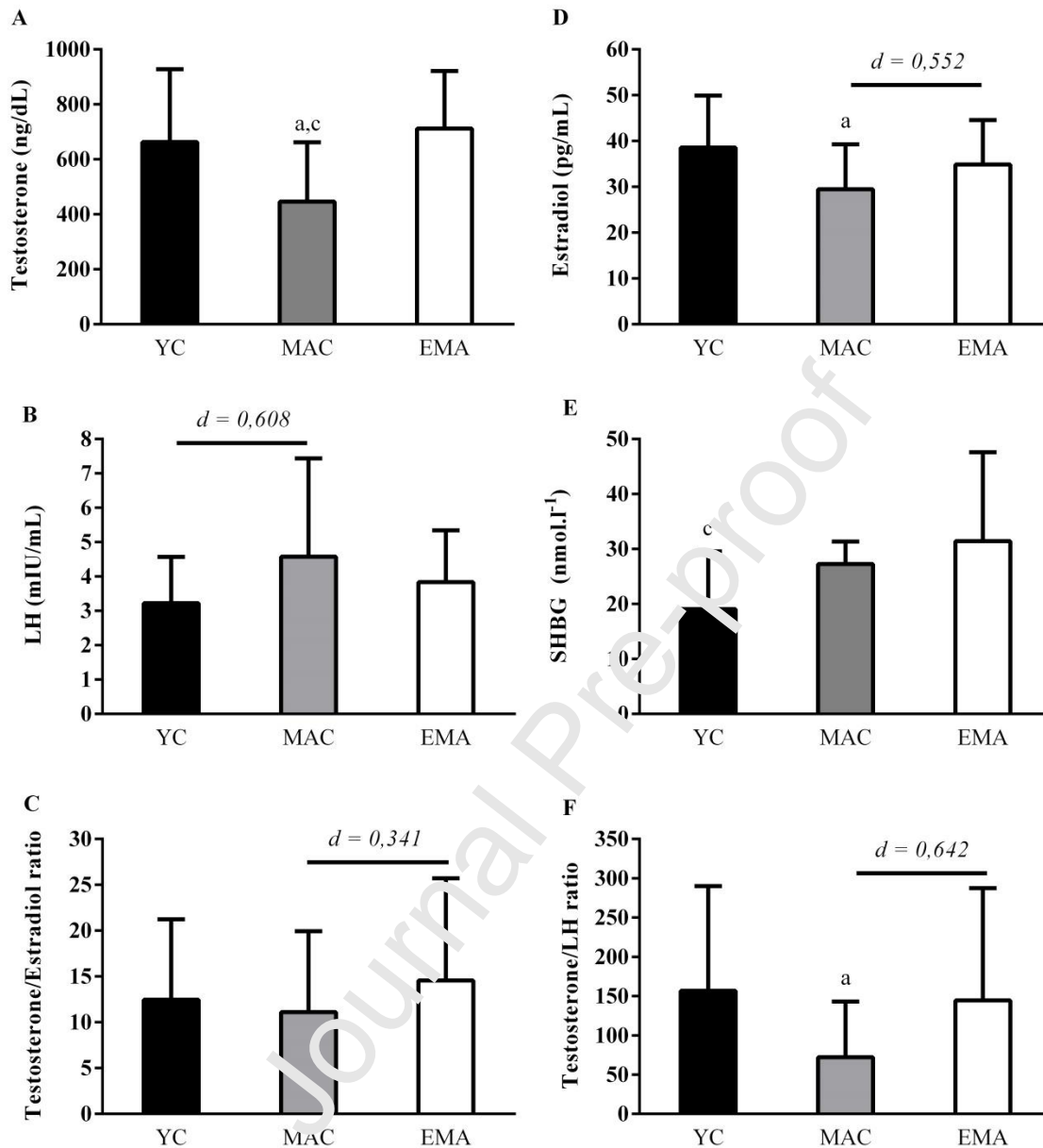
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Figure

1. Comparison of the hormonal profile between young, middle-aged controls and elite master athletes. Figure A (YC = 27; MAC = 21; EMA = 56); Figure B (YC = 24; MAC = 16; EMA = 55); Figure C (YC = 32; MAC = 24; EMA = 70); Figure D (YC = 24; MAC = 19; EMA = 56); Figure E (YC = 31; MAC = 24; EMA = 69); Figure F (YC = 31; MAC = 24; EMA = 69) a - significant difference from young group; b - significant difference from middle age group; c – significant difference from elite master athletes.

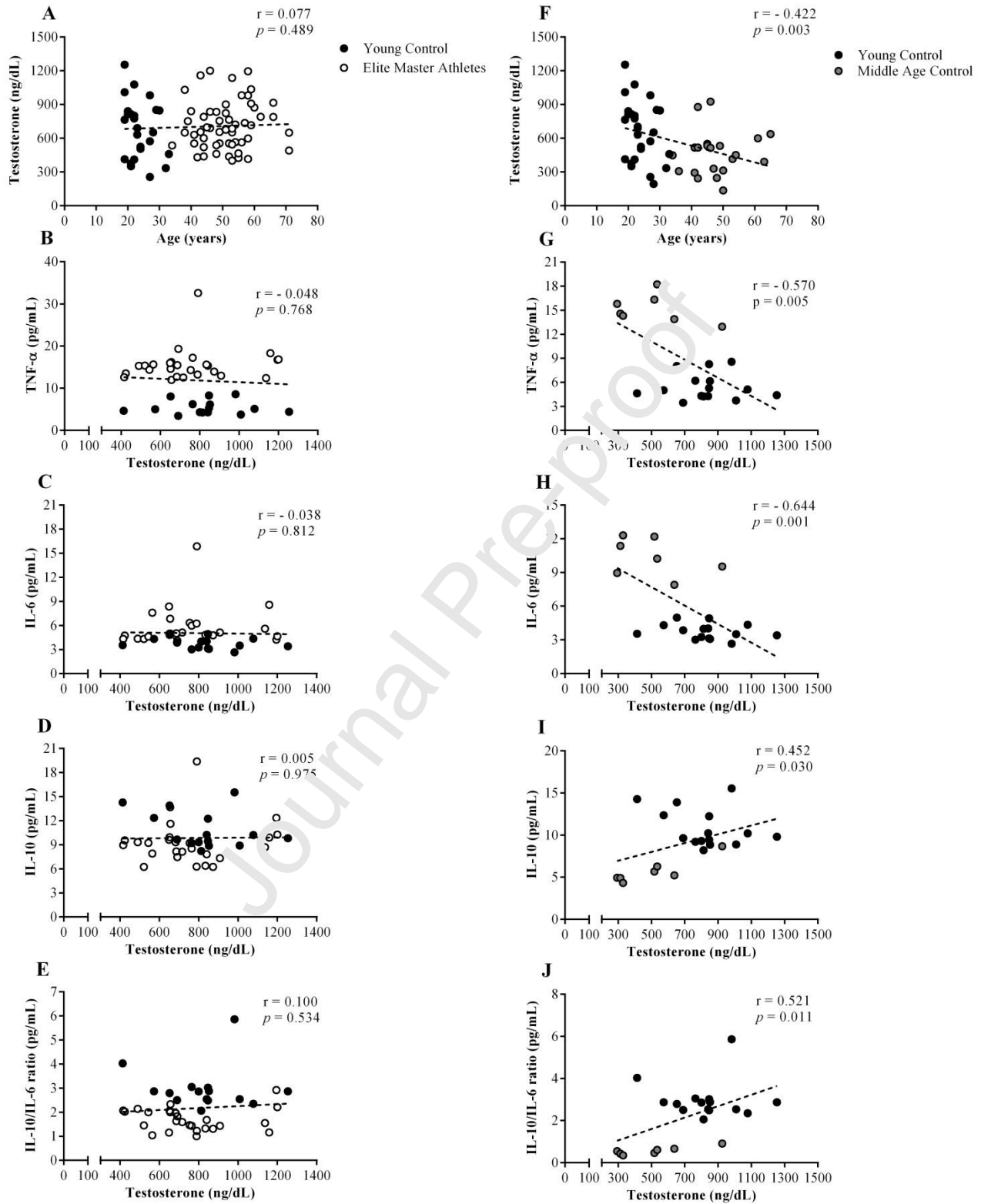


Figure 2. Correlation between testosterone, inflammatory parameters and age for young control and master athletes (left panel) and untrained participants (right panel). Figures A (n = 81), B, C, D and E (n = 41); Figures F (n = 48), G, H, I and J (n = 22).

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Author statement

Lucas P Barbosa: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing. **Samuel S Aguiar:** Methodology, Formal analysis, Investigation, Writing - Review & Editing. **Patrick A Santos:** Investigation, Writing - Review & Editing. **Thiago S Rosa:** Investigation, Writing - Review & Editing. **Larissa A Maciel:** Methodology, Formal analysis, Writing - Review & Editing. **Lysleine A Deus:** Writing - Review & Editing. **Rodrigo V P Neves:** Writing - Review & Editing. **Patricio L A Leite:** Investigation, Writing - Review & Editing. **Sara D Gutierrez:** Investigation & Editing. **Caio V Sousa:** Writing - Review & Editing. **Marko T Korhonen:** Writing - Review & Editing. **Hans D:** Writing - Review & Editing. **Herbert G Simões:** Supervision, Project administration, Funding acquisition, Writing - Review & Editing.

Table 1. Description of body composition and biochemical parameters.

	Young	Middle-aged	Elite Master
	Control	Control	Athletes
	(n = 32)	(n = 24)	(n = 70)
Age (yrs)	23.7 ± 3.9	47.2 ± 8.0 ^a	51.3 ± 8.0 ^a
BMI (kg·m⁻²)	24.3 ± 2.7	27.2 ± 2.9	23.5 ± 3.7 ^{a,b}
Body fat (%)	14.6 ± 4.9	22.1 ± 4.2	13.4 ± 4.4 ^{a,b}
Lean mass (%)	85.4 ± 4.9	79.2 ± 6.7	87.0 ± 5.0 ^{a,b}
Triglycerides (mg·dL⁻¹)	76.6 ± 29.4	114.9 ± 50.3	89.7 ± 40.3
HDL-c (mg·dL⁻¹)	69.3 ± 25.1	90.3 ± 32.2	71.51 ± 32.5
LDL-c (mg·dL⁻¹)	74.0 ± 33.3	35.8 ± 40.7	104.2 ± 52.2 ^b
TC (mg·dL⁻¹)	161 ± 32	138 ± 36	199 ± 45 ^{a,b}
LDL/HDL ratio (mg·dL⁻¹)	1.29 ± 0.75	0.37 ± 0.79	2.22 ± 1.78 ^b
Blood glucose (mg·dL⁻¹)	84.6 ± 5.8	86.5 ± 11.5	86.2 ± 7.1
TNF-α (pg/mL)	5.37 ± 1.57	13.83 ± 3.56 ^a	15.31 ± 3.88 ^a
IL-6 (pg/mL)	3.65 ± 0.69	10.08 ± 1.44	5.73 ± 2.30 ^{a,b}
IL-10 (pg/mL)	10.70 ± 2.14	5.54 ± 1.34	9.21 ± 2.65 ^{a,b}
IL-10/IL-6 ratio (pg/mL)	3.01 ± 0.85	0.55 ± 0.16	1.69 ± 0.47 ^{a,b}
Free Androgen Index	128.87	79.18	79.44
(ng/dL)			

TNF-α (YC = 17; MAC = 10; EMA = 29); IL-6 (YC = 17; MAC = 10; EMA = 29); IL - 10 (YC = 17; MAC = 10; EMA = 29); IL-10/IL-6 ratio (YC = 17; MAC = 10; EMA = 29). ^a - different from young group; ^b - different from middle aged group; BMI - body mass index; HDL - high-density lipoprotein; LDL - low-density lipoprotein; TC - total cholesterol.

HIGHLIGHTS

- Physical exercise mitigates the effects of biological aging.
- Healthy lifestyle mitigates the reduction of androgen hormones.
- High levels of testosterone indicate higher anti-inflammatory cytokines.

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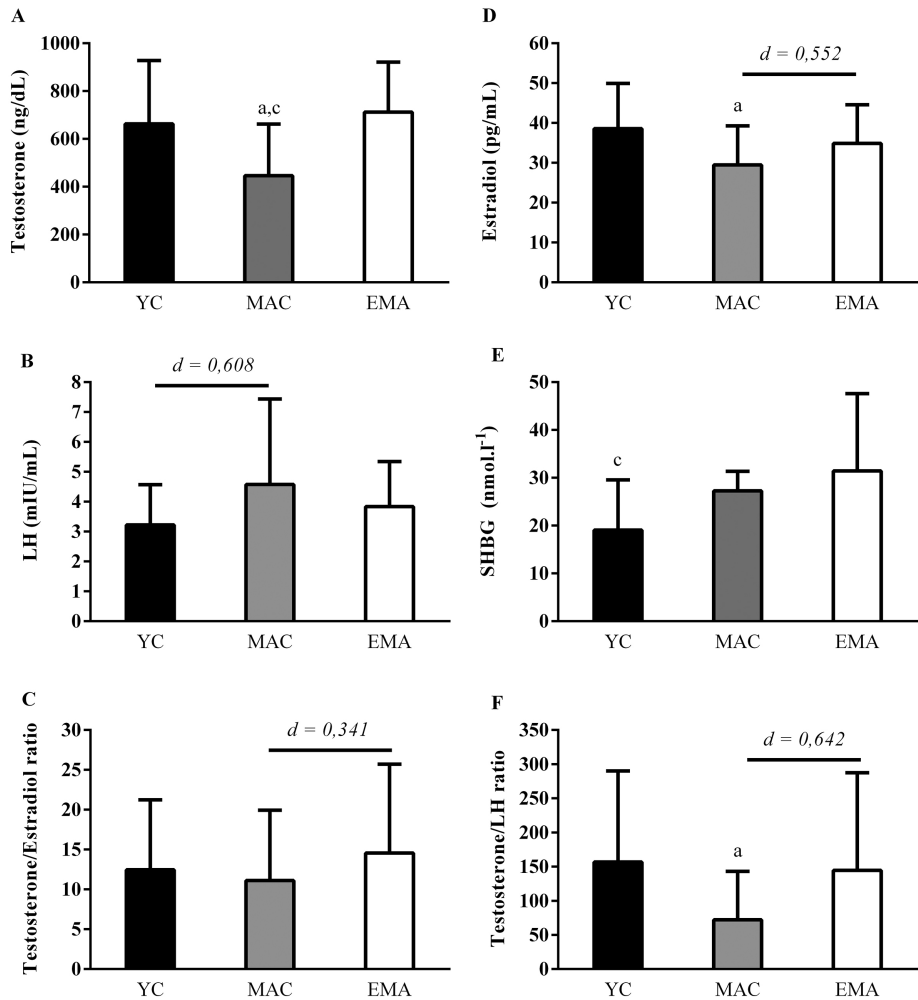


Figure 1

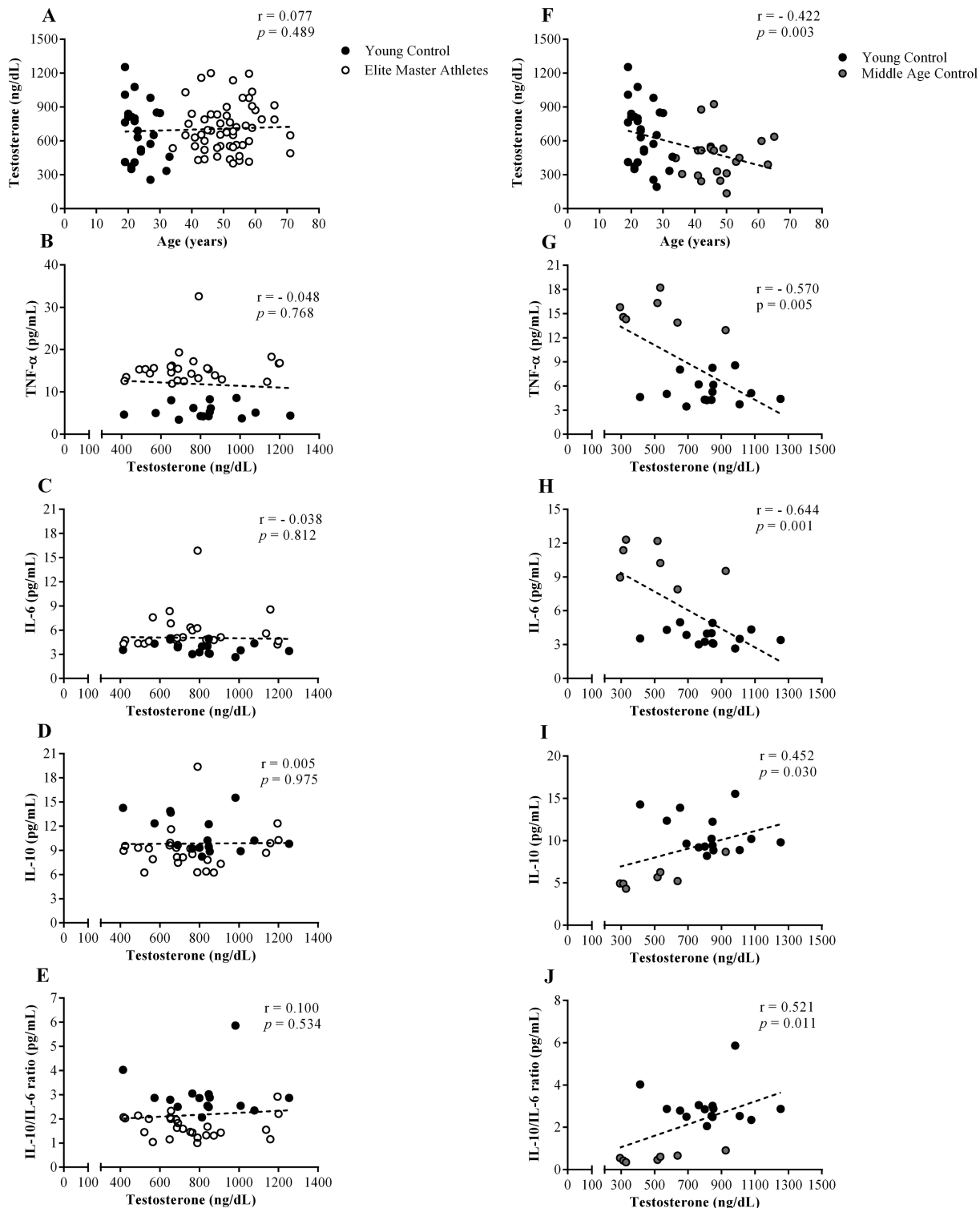


Figure 2