

# The Running Practice Modulates Inflammatory Markers and not Alters Global DNA Methylation in Elderly Men

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## Abstract

**Objectives:** The current study aimed to evaluate 1) the impact of the regular running practice 2) as well the acute effect of running race on inflammatory markers and DNA methylation status in peripheral blood of health elderly men.

**Methods:** Fifteen male volunteers aged 60 years and older were recruited. They were allocated in two groups: runners (RUN, n=8) and sedentary (SED, n=7), considering if they were sedentary or amateur street runners. All participants, SED and RUN groups, were submitted to a basal blood collection. After 5 days, two blood samples were collected in the RUN group: 30 min before and immediately after a 5-10km outdoor running performance. The blood samples (15mL) were collected for the epigenetic and inflammatory measurements, which were done using specific kits, according to the manufacturer's instructions.

**Results:** In the basal period, no significant differences were observed on DNA global methylation status between the SED and RUN groups. However, the SED group showed increased IL-4 and IL-6 levels when compared to the RUN individuals (p= 0,040 and 0,035, respectively). The IL-6 and IL-4 levels were significantly higher in the EXE group after the running performance compared to the before running period (p=0,004 and 0,013 respectively), while DNA methylation status remained unaltered.

**Conclusion:** Our data suggest that DNA methylation status might not be consider a potential biomarker related to the healthy elderly runners phenotype. Furthermore, the inflammatory response may indicate a possible regulatory effect provided by the endurance exercise in the elderly group because IL-4 and IL-6 are synergic cytokines directing an immune response.

**Keywords:** Exercise; Running; Aging; Epigenetic; Inflammation;

## Introduction

Aging has become increasingly in the world population and the projection for 2050 is that people over 60 years old be around 2 billion [1]. To clarify specific mechanisms of the biological aging process and the elaboration of combined strategies for the management of elderly health are urgent in order to guarantee the quality of life of this population. Emerging experimental

and clinical evidences suggest that the imbalance of epigenetic machinery plays a pivotal role in both normal aging process as well in age-related diseases [2,3]. Epigenetic process result in heritable phenotypical alterations in the expression of specific genes without alter the primary DNA sequence. The hallmarks of epigenetic gene regulation include DNA methylation and histone acetylation levels modifications [4].

DNA methylation is a process catalyzed by DNA methyl transferases (DNMTs) enzymes and is usually associated with transcriptional repression and gene silencing [5]. Histone acetylation status is regulated by histone acetyl transferases (HATs) and histone deacetylases (HDACs). HATs produce a larger chromatin structure, characteristic that allows transcriptional activation, whereas HDACs exert the opposite effect [6].

Nowadays, it is observed increased research on both pharmacological and non-pharmacological tools to prevent and minimize the physiological age modifications and diseases. In this context, exercise has been pointed out as an interesting strategy promoting several benefits in the elderly individuals [7]. In this context, a recent study conducted by Zhang and colleagues (2015) showed that DNA methylation levels are modulated in elderly people previously sedentary submitted to an interval walking training protocol. Furthermore, this response was related to an improvement on inflammatory status, suggesting that exercise might induce beneficial effects on health by engaging epigenetic signals [8].

Among the modalities of physical exercise, recreational running is markedly growing as an option, even among the aged population. The beneficial impact of running practice on human physiological systems it is well described [9]. It was recently reported the impact of the running practice on epigenetic modulation in middle-aged individuals [10]. However, this response in elderly individuals has been poorly investigated. Furthermore, although many studies have shown that exercise can induce epigenetic changes in peripheral blood of different populations [11,12] it is important to note that most

of these investigations are conducted with previously sedentary individuals, while findings with well-trained populations are scarce.

Importantly, age-related changes of immune system contribute to increased ratios of infection diseases, poor vaccine response and cardio metabolic diseases which are linked with the chronic low grade inflammation observed in elderly subjects [13]. This process is namely immuno senescence and was mediated by change in both cellular adaptive and innate immune system, as well as in pro and anti-inflammatory cytokine levels such as interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, and Tumour Necrosis Factor-Alpha (TNF- $\alpha$ ) [14]. Cytokines produced by immune cells play a critical role in the development of host immunity against infection. It has been established that Interferon-gamma (INF- $\gamma$ ) represents a type 1 (T1) cytokines, which are involved in cellular immune response [15,16]. In contrast, interleukin-4 (IL-4) participate of type 2 (T2) immune response against extra-cellular/humoral pathogens. In addition, T1 and T2 cytokines responses are mutually inhibitory [17]. Thus, the INF- $\gamma$ /IL-4 ratio has been used as an indicator of immune polarization [18]. Currently, it is well established that an acute bout of strenuous endurance exercise inhibits the responses of T1 cytokines due to actions of glucocorticoids and changes in intracellular transcription factors in immune cells [19,20]. In addition, these changes can contribute to anti-inflammatory effects of exercise in active exercisers.

Finally, it is known that long-term endurance exercise training also modulates inflammatory markers, specifically, is able to induce a reduction in serum interleukin 6 (IL-6) and interleukin 4 (IL-4) levels [21]. In addition, cardio respiratory fitness is inversely associated with several markers of inflammation, such as IL-6, demonstrating that exercise training might plays a pivotal key for the control of low-grade inflammation [22]. Data from epidemiological studies demonstrated an association between the levels of physical activity, lower levels of bacterial and viral infections, as well as distinct immune profile in elderly subjects characterized by low levels of pro inflammatory mediators [23,24]. Nevertheless, to date, no study evaluated the impact of acute outdoor endurance training on cytokine markers in well-trained elderly individuals.

In view of these considerations, the aim of the present study was to compare the basal levels of inflammatory parameters (IL-4 and IL-6) and global DNA methylation status in the peripheral blood from elderly healthy amateur runners (RUN group) with elderly sedentary individuals (SED group). In order to investigate the acute outcomes of exercise in trained individuals, we also analyzed the effect of a running race (5-10 km) on these markers in the EXE group.

## Material and Methods

### Ethical Procedures

The study was approved by the Ethics Committee of Methodist University Center IPA (protocol number 1190948) and all participants provided informed written consent prior to participation.

### Subjects

Participants off both groups (RUN and SED) were recruited through an advertisement on the Social Network Sites. They were allocated in two groups: runners (RUN, n=8) and sedentary (SED, n=7), taking into account the question that they were sedentary or amateur street runners. As criteria to compose RUN group, volunteers should be regular amateurs runners, practicing running bouts three times a week for at least two years. The amount of physical activity in the RUN group was accessed by a self-report questionnaire of training characteristics. To be included in SED group, individuals should not have engaged in regular practice of physical exercise in the last six months prior to data collection.

Exclusion criteria for both groups were the presence of chronic diseases, the use of drug-containing HDAC inhibitors and smoking. The participants were oriented not to use anti-inflammatory and antibiotic medicines 72h before all blood collections. All volunteers should be physically independent and do not have cognitive limitations that could prevent the understanding of tests.

### Experimental Trials

In this comparative, controlled, cross sectional study, volunteers went to the Laboratory of Exercise Physiology at the Centro Universitário Metodista-IPA after a twelve-hour of fasting period and blood samples (15 ml) were obtained from an antecubital vein (basal period) for measurement of inflammatory and epigenetic measurements. The body composition and the body mass index (BMI) in both group were also assessed.

### Global DNA Methylation Levels Analysis

Global DNA methylation levels in the plasma were quantified using Methyl Flash™ Methylated DNA Quantification Kit (Epigentek, Catalog number P-1034, NY, USA) according to the manufacturer's information. Capture antibody in this kit binds to 5-methylcytosine and it was measured the total DNA methylation level as a percentage of total DNA present in each plasma sample. A standard curve with positive and negative controls was run with the samples. Thus, 100 ng of DNA (1-8 ng/ $\mu$ l) was bound to the plate at 37°C for 90 min. The methylated fraction of DNA was detected using capture and detection antibodies. The relative optical density (OD) units were quantified by reading the absorbance in a FLUOstar Optima micro plate reader (Labtech). Methylated DNA amount was proportional to the OD measured. Absolute amount of methylated DNA was quantified from a standard curve, and the slope of the standard curve was used to calculate the percentage of methylated DNA (5-mC%) in the plasma samples.

### Cytokines Measurement

The levels of IL-4 and IL-6 in the plasma were determined by Enzyme-Linked Immunosorbent Assay (ELISA) protocols according the manufacturer's recommendations. IL-4 and IL-6 were analyzed using ELISA Development Kit of Peprotech Inc, New Jersey, USA.

### Statistical analysis

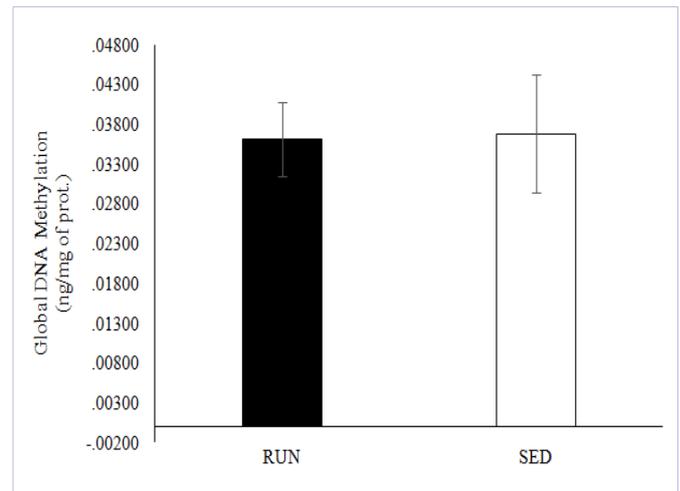
Data normality was verified by the Shapiro–Wilk test. To assess the differences in normally distributed and non-parametric variables, the t test and the Mann–Whitney test were used. Characteristics of volunteers was presented as media ± standard derivation or absolute frequency (%). To compare anthropometric parameters, DNA methylation levels and inflammatory markers between the RUN and SED groups, we utilized an unpaired t test. Pearson correlation was used to identify the evaluated correlations. PASW Statistics 20.0 for Windows (SPSS) was used to perform Statistical analyses. The significance level was set to 5% (p<0,05).

### Results

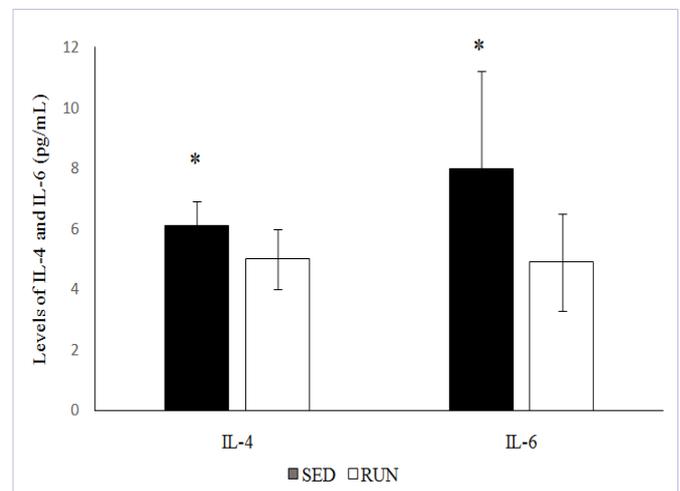
Participants characteristics (SED and RUN groups) are highlighted in Table 1 & Table 2 illustrates the training characteristics of the RUN group.

In the basal period, DNA methylation levels did not differ between groups, as highlighted in Figure 1. Regarding the inflammatory markers, the SED group showed increased values of IL-4 (p= 0.040) and IL-6 (p= 0.035) when compared to RUN group (Figure 2).

In the race day, the RUN group was encouraged to exercise to their limit. The DNA methylation levels remained unaltered comparing the before and after the outdoor running performance (p=0.081, Figure 3). However, as showed in Figure 4, higher IL-6 levels were observed after the running compared to the before period (p<0.05).



**Figure 1:** Basal global DNA methylation levels of running (RUN) and sedentary (SED) groups. Values are expressed as mean ± SD



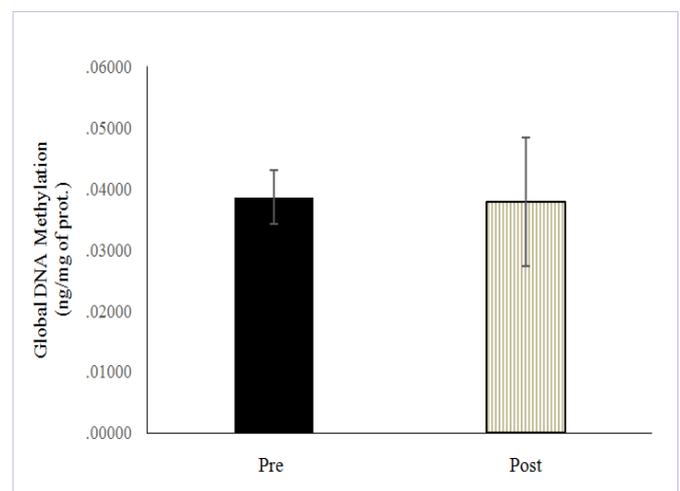
**Figure 2:** Basal plasmatic levels of IL-4 and IL-6 of sedentary (SED) and running (RUN) groups. Values are expressed as mean ± SD.\* Denotes statistical difference when compared with pre period (p<0.05)

**Table 1:** General characteristics of the volunteers

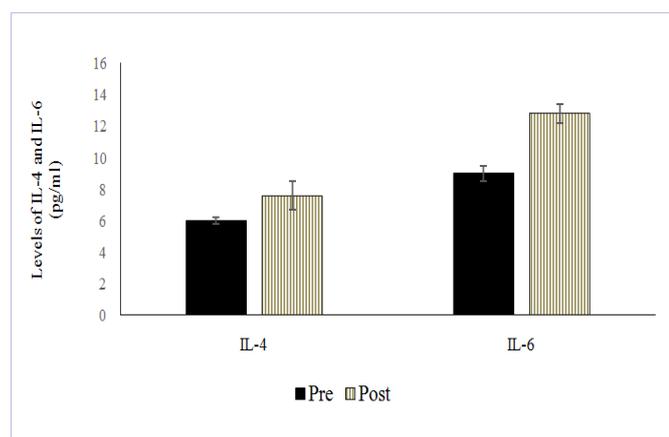
Variables	SED (n=7)	RUN (n=8)
Age (years)	64.8 ± 4.2	64.2 ± 3.9
Weight (Kg)	73.1 ± 5.78	71.1 ± 5.79
Height (cm)	172 ± 0.03	174 ± 0.04
BMI (kg/m <sup>2</sup> )	24.5 ± 2.30	23.3 ± 1.34

**Table 2:** Data related to training characteristics of the Running group (n=8)

Variables	Frequency	
Running Experience (years)	4-Feb	50%
	20-Oct	25%
	>20	25%
Frequency (sessions/week)	3-Feb	75%
	6-Apr	25%
Session duration (minutes)	60	75%
	>60	25%



**Figure 3:** Global DNA methylation of running group (RUN) in response to acute exercise performance. Values are expressed as mean ± SD.



**Figure 4:** Plasmatic levels of IL-4 and IL-6 of running group (RUN) in response to acute physical exercise performance. Values are expressed as mean  $\pm$  SD.\* Denotes statistical difference when compared with pre period ( $p < 0.05$ ).

## Discussion

To our knowledge, this is the first study that investigated the effects of acute and chronic physical exercise exposure, focused on the running practice on DNA methylation status of well-trained elderly subjects.

No significant differences were observed on DNA methylation levels between the SED and SED individuals at baseline. Conversely, our group recently reported that sedentary elderly individuals showed higher levels of global histone H4 acetylation when compared to the runners ones [25]. Taken together, these findings suggest that global histone H4 acetylation, and not DNA methylation status, may be consider an important epigenetic mark linked to endurance phenotype in well-trained-elderly individuals.

The current study also demonstrated that global DNA methylation plasma levels remained unaltered in the RUN group after the running race compared to the before period, evidencing that this epigenetic marker is not acutely altered in response to running in this population. Our data corroborate those obtained by Robson-Ansley and colleagues [26] reporting that a single bout of a prolonged and strenuous exercise protocol (running in a treadmill) was not able to alter DNA methylation levels in peripheral blood of well-trained young-aged men. In addition, global histone H4 acetylation levels also are not altered in response to a street running race in both middle-aged and elderly well-trained individuals [25,10]. Taken together, these results led us to hypothesize a possible epigenome adaptation in response to chronic exercise exposure in both young and aged-well trained individuals.

In contrast, a remarkable reduction on DNMT3B concentrations, an indicative of DNA hypomethylation status, was observed in peripheral blood of active young-aged males following a 120-min of treadmill running [6]. In addition, Zhang and colleagues showed a significant increase on DNA methylation levels in elderly sedentary individuals submitted to a walking interval training protocol during six months when

compared to control ones [8]. Significant changes on global DNA methylation levels was also demonstrated in preciously sedentary COPD individuals submitted to a single exercise session [27]. Furthermore, sperm DNA was also demethylated in response a 3 months of exercise training in healthy young men. When combining these findings, it appears entirely suppose that the physical conditioning (sedentary versus trained) are a pivotal factor related to exercise modulation on DNA methylation levels. Specifically, that this epigenetic mark is remarkably responsive to short and long-term exercise interventions in previously sedentary people.

Among the benefits provided by the endurance exercise, increased anti-inflammatory cytokines in parallel to lack of changes in pro inflammatory mediators, representing lower T1/T2 cytokine ratio, are directly to the intensity of the exercise [28]. The results of our study demonstrated that an acute bout of outdoor 5-10km endurance challenge induced an increase in T2 inflammation, as demonstrated by higher IL-4 levels after the workout. These effects are attributed, at least in part, to the elevations of miokine levels, glycoproteins like cytokines that are express by the muscle tissue during and after physical stress [29].

Muscle contraction stimulates the secretion of IL-6 with a variety of functions, especially the modulation of metabolism and the induction of an anti-inflammatory environment through the stimulation of anti-inflammatory cytokines [30]. Our data demonstrated a significant increase in systemic IL-6 immediately after bout in elderly runners. Despite the fact that IL-6 is recognized as a pro-inflammatory cytokine, acute exercise can induce an increase in IL-6 secretion from muscle cells with anti-inflammatory effects such as reduction of nuclear-kappa beta factor expression in mononuclear cells, a master regulator of pro inflammatory transcription of DNA, and stimulation of T2 cytokines [29]. Marathons and ultra-endurance exercise can induce an elevation in order to 100x of IL-6 levels in serum of athletes after the bout [31,32]. The increases in serum concentrations of IL-6 observed in our study was more attenuated, however, we cannot rule out that these differences in IL-6 after the running may be related to the duration of the race.

There has been consistent evidence that acute high intensity endurance exercise induce an elevation in IL-4 levels in both young trained or untrained individuals. In addition, cytokines regarding T1 inflammation, like  $\text{INF-}\gamma$ , are suppressed many hours after bout [19,33]. The suppression may last for more than 24 hours after bout [34]. Moreover, the changes in the cytokines produced by T1 and T2 cells are similar to falls observed in the frequency of the T cells that produce these cytokines [33]. Across qualitative analysis, Kakanis and coworkers [35] demonstrated an increase in IL-4 levels in addition to a T1/T2 shift after 2h of cycling exercise at 90% of second ventilatory threshold. Interesting, a non-significant increase in T-cells expressing IL-4 in parallel to decreased the percentage of T cells expressing  $\text{INF-}\gamma$  was observed after intensive treadmill running at 75% of  $\text{VO}_2\text{Max}$  [20,21]. Mechanistically, these changes in T1/T2 balance may be related to events associated to exercise-induced up-regulation of GATA3 and CCR4 genes, transcription factor genes related to IL-4

and T2 phenotype [19]. In addition, higher expression of IL-6 was correlated with the percentage of CD8+ T cells that producing IL-4 after exercise [33].

Hormonal changes is also postulated as exerts effects on cytokine response. Glucocorticoids (i.e. cortisol) binds in their cytoplasmic/nuclear receptors on antigen presenting cells to inhibits the expression of IL-12, a pivotal cytokine to induce T1 cells in adaptive immunity [36]. Catecholamines also suppress the production of INF- $\gamma$  and related T1 cytokines, and are able to stimulate the production of T2 cytokines [37]. It was demonstrated a negative correlation between adrenaline and the peripheral frequency of Th1 cells after 2h of 75%VO<sub>2</sub>max endurance exercise [33]. In addition, T1 cells express higher density of  $\beta$ -adrenergic receptors compared with T2 cells. Thus, the higher catecholaminergic influence of response can induce both trafficking of circulating T1 cells from the systemic circulation to inflamed tissue, which can reduce the frequency of T1 cells in peripheral circulation [38]. Taken together, these data may indicate a possible regulatory effect provided by the endurance exercise in the elderly group because IL-4 and IL-6 are synergic cytokines directing a T2-prone response [39].

## Conclusions

In summary, our data presents a first attempt to bridge the gap between exercise and the modulation of DNA methylation status in well-trained elderly individuals. The findings here obtained could suggest that this epigenetic signal might not be consider a potential biomarker related to the elderly runnings phenotype as well exerts a stable profile in response to long-term running practice. Furthermore, the inflammatory profile presented by elderly runners in response to chronic and acute physical effort may indicate a possible regulatory effect provided by the endurance exercise on aging processes.

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