Improvement in the anti-inflammatory profile with lifelong physical exercise is related to clock genes expression in effector-memory CD4+ T cells in master athletes.

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ABSTRACT

Purpose: *Ageing is associated with alterations in the immune system as well as with alterations of the circadian rhythm. Immune cells show rhythmicity in execution of their tasks. Chronic inflammation (inflammaging), which is observed in the elderly, is mitigated by lifelong exercise. The aimed this study was to determine the acute effect of a maximal exercise test on clock genes, regulatory proteins and cytokine expression, and evaluate the effect of lifelong exercise on the expression of clock genes in subpopulations of effector-memory (EM) CD4+ and CD8+T cells and the association of these processes with the inflammatory profile. Therefore, this study aimed to investigate the expression of clock genes in subpopulations of effector memory (EM) CD4+ and CD8+ T cells in master athletes and healthy controls and further associate them with systemic inflammatory responses to acute exercise.*

Methods: *The study population comprised national and international master athletes (n = 18) involved in three sports (athletics, swimming and judo). The control group (n = 8) comprised untrained healthy volunteers who had not participated in any regular and competitive physical exercise in the past 20 years. Anthropometric measurements and blood samples were taken before (Pre), 10 min after (Post) and 1 h after (1 h Post) a maximal cycle ergometer test for the determination of maximum oxygen consumption ($VO_{2\,max}$). The subpopulations of EM CD4+ and CD8+ T cells were purified using fluorescence-activated cell sorting. RNA extraction of clock genes (CLOCK, BMAL1, PER1, PER2, CRY1, CRY2, REV-ERBα, REV-ERBβ, RORa, RORb and RORc) in EM CD4+ and EM CD8+ T cells as well as regulatory proteins (IL-4, IFN-γ, Tbx21, PD-1, Ki67, NF-kB, p53 and p21) in EM CD4+ T cells was performed. The serum concentration of cytokines (IL-8, IL-10, IL-12p70 and IL-17A) was measured.*

Results: *The master athletes showed better physiological parameters than the untrained healthy controls (P<0.05). The levels of cytokines increased in master athletes at Post compared with those at Pre. The IL-8 level was higher at 1 h Post, whereas the IL-10 and IL-12p70 levels returned to baseline. There was no change in IL-17A levels (P< 0.05). The clock genes were modulated differently in CD4+ T cells after an acute session of exercise in a training status-dependent manner.*

Conclusion: *The synchronization of clock genes, immune function and ageing presents new dimensions with interesting challenges. Lifelong athletes showed modified expression patterns of clock genes and cytokine production associated with the physical fitness level. Moreover, the acute bout of exercise altered the clock machinery mainly in CD4+ T cells; however, the clock gene expressions induced by acute exercise were different between the master athletes and control group.*

Keywords:
Circadian Rhythm, Cytokines, Ageing, Lymphocytes and Exercise Immunology

INTRODUCTION

The decline in numerous physiological processes due to ageing, widely reported in previous studies, results in increased vulnerability to infectious and inflammatory diseases(1). Ageing also induces perturbations of the circadian rhythm (2), including disruption of the sleep/wake cycle, body temperature, food intake and metabolism, secretion of hormones, glucose homeostasis, cell cycle regulation and immune response(3). The circadian rhythm is controlled by a central (suprachiasmatic nucleus) and peripheral clocks (organs and tissues)(3). At the cellular level, the circadian rhythm is controlled by a set of genes known as clock genes, which are transcriptional regulators that maintain rhythmic expression of their target genes over approximately 24-h cycles(4).

As a master regulator, CLOCK (circadian locomotor output cycles kaput) is a transcription factor that forms heterodimers with BMAL1 (brain and muscle Arnt-like protein), forming a transcriptional activator complex that stimulates the transcription of the Period (PER1, PER2 and PER3) and Cryptochrome (CRY1 and CRY2) genes (negative feedback) in various tissues, cells and subsets of immune cells. In turn, the cytoplasmic PERs and CRYs proteins migrate to the nucleus after dimerization to inhibit the transcription of CLOCK-BMAL1. The regulation of the circadian cycle occurs with the degradation of PER/CRY complexes in the proteasome, which

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withdraws the inhibition of CLOCK and BMAL1, as well as the restart of this feedback loop (5–7). Another regulatory mechanism is induced by the CLOCK/BMAL1 heterodimeric complex that activates the transcription of orphan nuclear receptors (REV-ERB and ROR) (8–10). REV-ERB (α and β) and ROR (a, b and c) subsequently compete for ROREs (retinoic acid-related orphan receptor response elements), present in the BMAL1 promoter. ROR activates BMAL1 transcription (10–12), whereas REV-ERB suppresses it (8,12). Therefore, BMAL1 circadian oscillations are both positively and negatively regulated by RORs and REV-ERBs.

Organisms present either a constant circadian rhythm or an endogenous rhythm. The environment operates only as a synchronizing agent (Zeitgeber)(13). Some environmental conditions adjust the oscillators that generate circadian rhythm, resulting in a continuous cycle. These external factors also serve as indicators of time and they are thus referred to as Zeitgeber (synchronizers). Daily light which restarts the central clock is the most important Zeitgeber. Therefore, the central clock in collaboration with other peripheral clocks, ensures the maintenance of the circadian cycle and can be modified by external factors such as light, social activities, diet (14,15) and physical exercise (16–18).

The rhythmic expression of clock genes has been previously described in innate and adaptive immune cells, including macrophages (19–21), microglia (22), monocytes (23), mast cells (24,25), dendritic cells (DCs) (21), B cells (21), natural killer cells (26), neutrophils (27), eosinophils (24) and CD4+ and CD8+ T cells (28,29). Furthermore, the disruption of clock gene signalling induces chronic inflammation or increases the severity of symptoms in the elderly (30).

Apart from circadian rhythm, clock genes are implicated in inflammatory response through activation of NF-κB signalling in response to various immunomodulators that are regulated by CLOCK, which is capable of regulating NF-κB-mediated transcription in the absence of BMAL1. Moreover, BMAL1 counteracts the CLOCK-mediated activation of NF-κB responsive genes. To substantiate this, Spengler et al. demonstrated the anti-inflammatory role of BMAL-1, and the activation of pro-inflammatory cytokines by CLOCK (31).

Interestingly, Nobis et al. (2019) demonstrated that clock genes in CD8+T cells modulate the response to vaccination by programming the transcriptional activities of these cells, making them more prone to efficient activation and proliferation depending on the time of day. However, deletion of BMAL1 in DCs or CD8 lymphocytes abrogated the circadian rhythm response to vaccination (32).

It is established that the ability to generate and maintain memory T cells is critical for lifelong immune health. Upon antigen encounter (after infection or vaccination), the number of antigen-specific T cells increase by several magnitudes and they differentiate into effector cells. While most of these effector cells die after the peak of their response, a subset of the subpopulation survives as memory cells that are long-lived and thereby providing protection against reinfection (33). However, the generation and maintenance of these memory T cells are compromised with ageing (33,34).

Chronic exercise may alter the profile and expression of the circadian rhythm clock genes (17). Programmed exercise has been demonstrated to alter behavioural rhythms in mammals kept in constant darkness. Previous studies highlighted that voluntary exercise influences how quick an animal synchronises a new phase of a light/dark cycle (35,36).

Physical inactivity, sedentary lifestyle and poor nutrition induce a disruption of the circadian rhythm, promoting an increase in the levels of pro-inflammatory factors and leading to chronic low-grade inflammation (37,38). Physical exercise is known to decrease TNF-α expression levels, reduce pro-inflammatory adipokine levels, and lower the expression of Toll-like receptors on monocytes and macrophages, and increase the circulating levels of anti-inflammatory cytokines in healthy populations (38–41).

Evidence has shown that the practice of lifelong regular exercise may decelerate the ageing process (42–48). Cellular senescence is a physiological process that occurs throughout life and is characterized by shortening of telomeres, damage to nuclear and mitochondrial DNA and, on immune cells, it induces the secretion of pro-inflammatory cytokines (49).

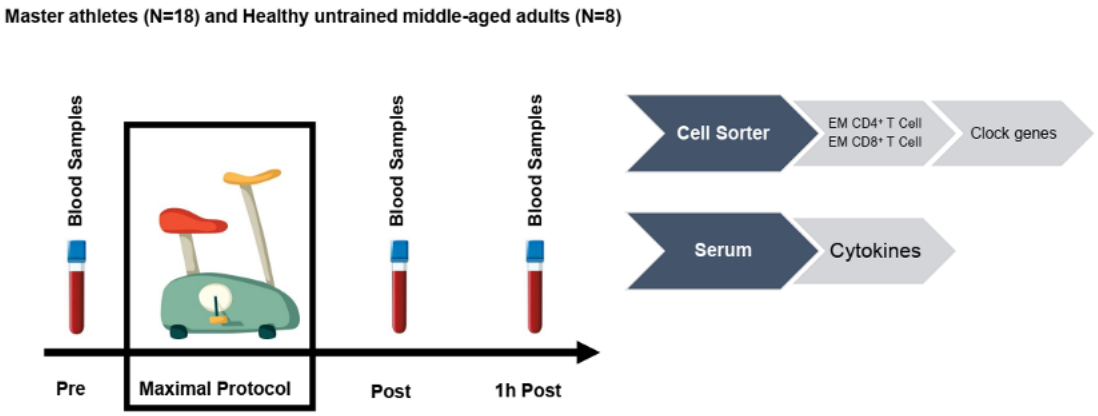
In terms of the immune system, lifelong athletes with consistent and regular years (≥20 years) of exercise practice (42,43,50,51) show reduced senescent T cell numbers and increased plasma levels of anti-inflammatory cytokines, with IL-10 levels similar to those of young adults (42–44).

A recent study demonstrated that sleep deprivation induces a robust modification in the transcriptome of cytokine and interleukin pathways as well as in clock genes (52), thereby instigating the need to clarify the inter-relationship between immunosenescence (declining of the immune system function observed during ageing) exercise and clock genes. Therefore, we hypothesised that lifelong training can be useful as a low cost intervention to improve quality of life and promote healthy ageing by reducing the number of senescent cells through regulation of the circadian rhythm. This study aimed to determine the acute effect of a maximal exercise test on clock genes, regulatory proteins and cytokine expression and evaluate the effect of lifelong exercise on the expression of clock genes in subpopulations of effector-memory (EM) CD4+ and CD8+ T cells and the association of these processes with the inflammatory profile.

METHODS

Participants

Eighteen master athletes (MA) (53.56±9.25 years) and a control group (CG) of eight untrained middle-aged adults (52.88±5.64 years) consented to participate in this study. All participants were healthy, non-smokers without any form of illness, as determined by a health and medical questionnaire. The inclusion and exclusion criteria for both groups were as previously described (42). Briefly, MA are athletes who currently participate in competitive sports and have done so for more than >20 years. We did not include athletes who trained or competed sporadically and/or older competitors who resumed training after a long period of physical inactivity. The MA group comprised athletes currently participating in national and international competitions in three sports (athletics, swimming and judo). Participants in the CG had not participated in regular physical training for the past 20 years, as determined by a questionnaire. All participants provided their written, informed consent to participate in the study, which was approved by the Ethics and Human Subjects Review Board at the Faculty of Sports Science and Physical Education, University of Coimbra.



Study Flowchart

Anthropometric measures

The stature and body mass of each participant was determined using a Harpenden stadiometer model 98,603 (Holtain Limited) and a calibrated digital scale Seca model 770 (Seca, Birmingham, UK), respectively. Measurements of weight and height were used to calculate the body mass index.

Determination of VO_{2max}

The physiological response to an incremental exercise test was determined by a maximal progressive exercise on a cycle ergometer (Lode, Groningen, Netherlands). In brief, participants began cycling with a 75 W load for 3 min (warm-up stage) followed by increments of 25 W every 3 min until volitional exhaustion. The participants cycled at a constant rate of 80 - 85 rpm.

Breath-by-breath measurements of O_2 and CO_2 were continuously recorded throughout cycling using a gas analyser with a breath by breath recording system (Quark CPET, COSMED, Rome, Italy). Participants breathed continuously through a facemask. The O_2 and CO_2 analysers were calibrated with known gases following the manufacturer's guidelines (COSMED, Rome, Italy). The adopted criterion to define if VO_{2max} was attainted was previously describe (53). Heart rate was monitored throughout the cycling by short-wave telemetry (COSMED, Rome, Italy).

Collection of blood samples

Venous blood samples (16 ml) were obtained through venepuncture in the antecubital vein and collected before exercise (Pre), 10 min after exercise (Post) and 1h after exercise (1h Post). The blood samples were collected in tubes containing EDTA and in a serum collection tube. EDTA tubes were used for flow cytometry and cell separation (cell sorting). The blood samples were centrifuged at 2000 rpm (4°C) for 10 min. Serum and plasma after the centrifugation were stored at −80°C until use.

Isolation of purified CD4+ and CD8+ T-cells

Highly purified CD4+ and CD8+ T cells were isolated by fluorescence-activated cell sorting using a FACSAria II cell sorter (BD). A five-colour combination of fluorochrome-conjugated monoclonal antibodies was used:CD3-PB (Pacific blue, clone UCHT1, Pharmingen, San Diego, CA EUA); CD4-APC (allophicocyanin, Clone 13B8.2, Beckman Coulter, Miami, FL, EUA); CD8-KO (Krome Orange, Clone 5MZ.332, Beckman Coulter, Miami, FL, USA); CD27-PECy5 (phycoerythrin-cyanine 5, clone R.8.01, Beckman Coulter) and CD45RA-PECy7 (phycoerythrin-cyanine 7, clone L48, BD Bioscience). This labelling enabled the identification EM CD4+ T cells (CD3+CD4+ CD27-CD45RA) and EM CD8+ T cells (CD3+CD8+CD27-CD45RA-). Furthermore, mRNA was extracted from EM CD4+ and EM CD8+ T cells.

RNA extraction of purified cells

The purified cells were transferred into a 1.5mL Eppendorf tube and centrifuged at 300g for 5 min; the pellet was resuspended in 350µL of RLT lysis buffer (Qiagen, Hilden, Germany).

The RNA was reverse transcribed into complementary DNA (cDNA) using reverse transcriptase. The iScriptTM Reverse Transcription Supermix for RT-qPCR kit (BIO-RAD) was used for reverse transcription of total mRNA.

The RT-qPCR SYBR® green method was used for cDNA synthesis. In brief, this method uses a fluorochrome SYBR® green which binds to the amplification product (that is, between the double strands of cDNA) and emits fluorescence that is proportional to the amplification product. This enables the monitoring of the kinetics and efficiency of the amplification process. Gene expression was determined using real-time PCR (54) using LightCycler® 480 apparatus (Roche Diagnostics, Rotkreuz, Switzerland) and SYBER green as a fluorescent marker. Quantification of gene expression was performed using the comparative Ct method (Ct = threshold cycle; number of cycles in which the PCR product reaches a detection threshold). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as the internal standard (55). Table 1 shows the sequences of the genes analysed.

Table 1. List of primers for the clock genes

Genes	Forward sequence	Reverse sequence
CLOCK	AGACCCTTCCTCAACACCAA	TGATGACCTTCTTTGCACCA
BMAL1-ARNTL	GCAGCTCCACTGACTACCAAG	TGTGAGCTTCCCTTGCATTT
PER1	TCTGCCGTATCAGAGGAGGT	CCCGGATCTTGGTCACATA
PER2	AGCTGCTTGGACAGCGTCATCA	CCTTCCGCTTATCACTGGACCT
CRY1	TGTTGAAGCAAGGAAGAAGC	TGCTCTGTCTCTGGACTTTAGG
CRY2	GGGGACTACATCAGGCGATAC	ATGCACTTGGCTGCCTTCT
REV-ERBα	CCCCAATGACAACAACACCT	CATAGGACATGCCAGCAGAAC
REV-ERBβ	ATGTCAGCAATGTCGCTTCA	CACGCTTAGGAATACGACCAA
RORa	AGGCTGCAAGGGCTTTTT	GCAGCGGTTTCTACTGGTTC
RORb	ATCAAAGCAAGTCCAGGGAAG	TGCAAACTCCACCACGTATT
RORc	GTCCCGAGATGCTGTCAAGT	TGGTTCCTGTTGCTGCTGTT
P21	TCCTCTTGGAGAAGATCAGCCG	TCCTCTTGGAGAAGATCAGCCG
IL-2	AGAACTCAAACCTCTGGAGGAAG	GCTGTCTCATCAGCATATTCACAC
IL-4	CCGTAACAGACATCTTTGCTGCC	GAGTGTCCTTCTCATGGTGGCT
P53	CCTCAGCATCTTATCCGAGTGG	TGGATGGTGGTACAGTCAGAGC
IFN-gamma	GAGTGTGGAGACCATCAAGGAAG	TGCTTTGCGTTGGACATTCAAGTC
TBX21	ATTGCCGTGACTGCCTACCAGA	GGAATTGACAGTTGGGTCCAGG
PD1	AAGGCGCAGATCAAAGAGAGCC	CAACCACCAGGGTTTGGAACTG
NF-kB p65	TGAACCGAAACTCTGGCAGCTG	CATCAGCTTGCGAAAAGGAGCC
Ki67 (MKI67)	GAAAGAGTGGCAACCTGCCTTC	GCACCAAGTTTTACTACATCTGCC
GAPDH	AGAAGGCTGGGGCTCATTT	GGTTCACACCCATGACGAAC

Serum cytokine levels

Quantification of the levels of cytokines including IL-10, IL-12p70, IL-17A and IL18 were performed using LEGENDplexTM multiplex kit (Human Inflammation Panel 1 Mix and Match Subpanel LEGENDplexTM - 5-plex; Biolegend) according to the manufacturer's instructions. The assays were performed in duplicates. Analysis was performed using BD FACSCalibur ™ flow cytometer. Data obtained were analysed using Legendplex V8.0 software (Biolegend) and the results were expressed as pg/mL.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.4.2 for windows (GraphPad Software, San Diego, California USA). The significance level was set at $P<0.05$. Descriptive statistics were presented as mean±standard deviation. Sequential changes in acute exercise at the different time points (Pre, Post, 1h Post) were analysed using repeated-measures analysis of variance or mixed-effects analysis followed by Tukey's post hoc test. Correlations between parameters and between changes in variables were analysed using Spearman's rank correlation. To estimate the strength of the exercise, Cohen's effect size was calculated and categorised by convention as small (d=0.2), medium (d=0.5) and large effect (d=0.8) using G*Power version 3.1.9.2.

RESULTS

The anthropometric data and physiological parameters are summarised in Table 2. As expected, MA showed better physiological parameters in response to maximal exercise than CG, as well as a higher value for the time test (min; P=0.005), maximum power produced by the test (watts; P=0.005), maximum ventilation (VE, ml/min; P=0.03), maximum oxygen consumption (VO_2max, ml/min and ml/min/Kg; p=0.003 and P=0.021, respectively) and carbon dioxide production (VCO_2 max, ml/min; P=0.006) (Table 2).

Table 2. Participants characteristics at baseline.

	Master Athletes (n = 18)	Control group (n = 8)	*p* value	Effect size (r)
	Mean (SD)	Mean (SD)		
Age (years)	53.56 (9.25)	52.88 (5.64)	0.849	0.08
Weight (kg)	75.45 (15.90)	72.03 (13.65)	0.602	0.22
Height (cm)	171.4 (5.31)	169.3 (0.07)	0.438	0.47
Body Mass Index (kg/m2)	25.62 (4.78)	24.99 (3.53)	0.743	0.14
Time of test	17.06 (5.38)	11.04 (2.08)	**0.005**	**1.29**
Power (watts)	209.7 (44.67)	156.3 (29.12)	**0.005**	**1.31**
VE (ml/min)	111.9 (28.90)	85.13 (23.10)	**0.030**	**0.98**
VO_{2max} (ml/min)	2997 (638.20)	2198 (362.4)	**0.003**	**1.40**
VO_{2max} (ml/min/Kg)	40.89 (10.79)	30.98 (5.14)	**0.021**	**1.04**
VCO_{2max} (ml/min)	3335 (632.2)	2614 (337.0)	**0.006**	**1.28**
VE/VO_{2max}	37.55 (6.19)	39.36 (6.85)	0.595	0.28
VE/VCO_{2max}	33.51 (5.25)	32.48 (6.85)	0.679	0.18
FC_{max}	160.4 (15.53)	160.1 (15.84)	0.968	0.02

Note: Unpaired t test. Data are Mean (SD). Values of significance ($p < 0.05$) are highlighted in bold. Large effect size ($r > 0.8$) are highlighted in bold.

The proportion of total lymphocytes, T cells, CD4+ and CD8+ T cells and EM CD4+ and CD8+ T cells in MA and CG in response to a maximum effort protocol is summarised in Figure 1. At baseline, there were no significant differences between the total lymphocyte population and subsets (T cells, CD4+ and CD8+ T cells and EM CD4+ and CD8+ T cells) in MA and in untrained individuals. The percentage of lymphocytes increased at Post only in MA, indicating that lymphocytosis is dependent of intensity and duration of exercise as opposed to the CG, whose exercise test duration was much lower. Subsets of EM CD4+ and EM CD8+ T cells were also mobilised, with a higher response in MA, as substantiated by a high percentage of EM CD4+ and EM CD8+ T cells at Post (P=0.000 and P=0.007, respectively), whereas only EM CD4+T cells in the CG were increased at Post(P =0.023) (Figure 1; Suppl. Table 1).

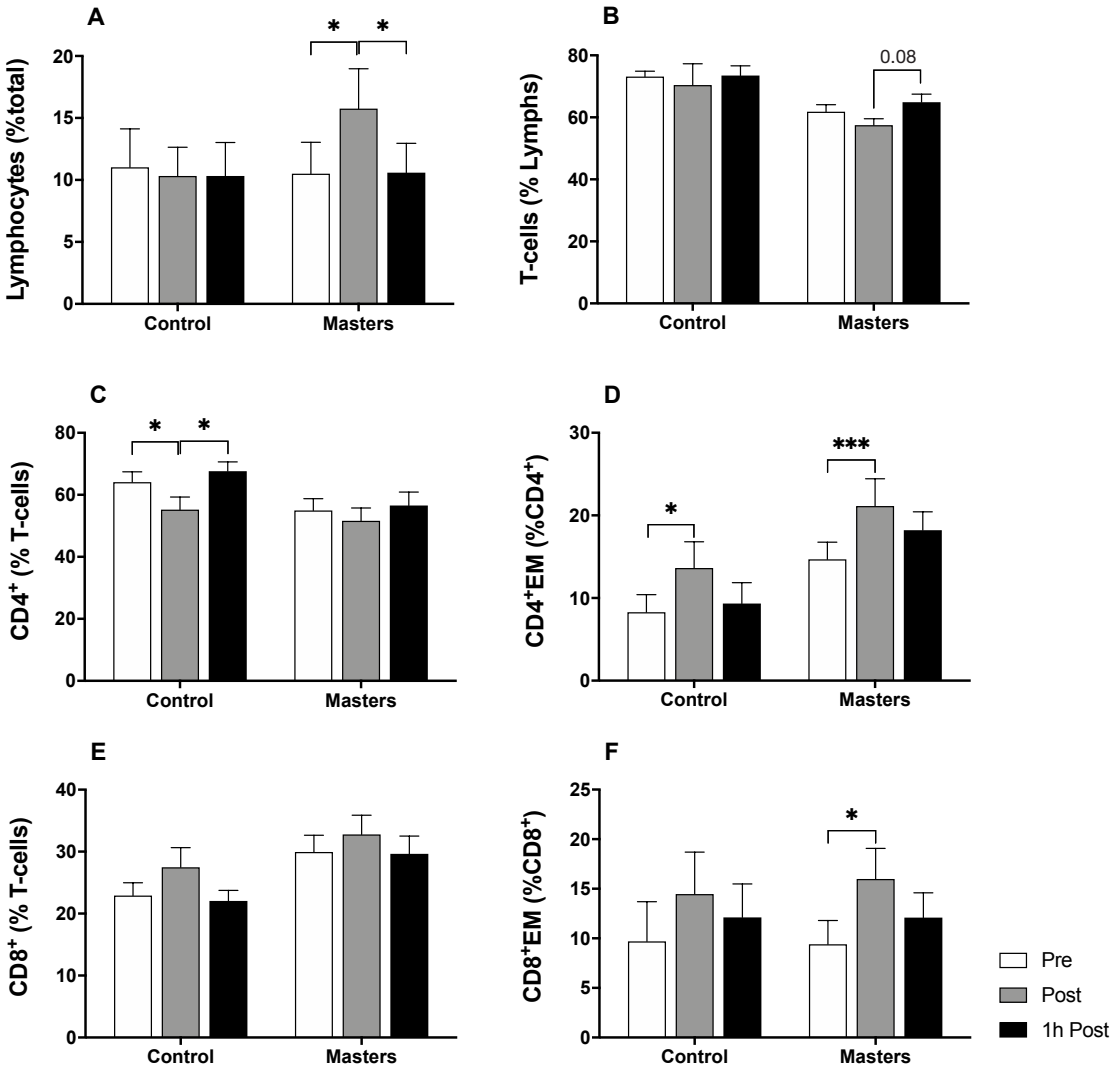


Figure 1. Proportion of total lymphocytes, T-cells, CD4+ and CD8+ T-cells, and effector-memory subpopulations in master athletes and control groups in response to a maximum effort protocol. Data represent the mean ± standard error of the mean. * $p < 0.05$; *** $p < 0.001$. Proportion of total lymphocytes (% total cells) (A), T-cells (% total lymphocytes) (B), CD4+ T-cells (% of T-cells) (C), effector-memory CD4+ T-cells (% of CD4+ T-cells) (D), CD8+ T-cells (% of T-cells) (E), effector-memory CD8+ T-cells (% of CD8+ T-cells) (F). Concentrations before (white columns = Pre), 10 min after (grey columns = Post), and 1h after (black columns = 1h Post) the exercise protocol in Control Group and Master Athletes group. Sequential changes in acute exercise at the different time points (Pre, Post, 1h Post) were analysed using repeated-measures analysis of variance or mixed-effects analysis followed by Tukey's post hoc test.

There was no effect of lifelong training on the level of pro-inflammatory cytokines (IL-12p70 and IL-17A), anti-inflammatory cytokine (IL-10) and regulatory cytokine (IL-8) at Pre (Figure 2; Suppl. Table 2). However, the levels of these cytokines were increased in MA at Post compared with that at Pre. The IL-8 level remained high at 1h Post, whereas the IL-10 and IL-12p70 levels returned to their baseline value. The IL-17A levels showed no statistical differences at 1h post compared with that at baseline and Post ($P<0.05$, Figure 2; Suppl. Table 2).

We observed a more pronounced modulation of clock genes in EM CD4+ T cells. The expression of CLOCK and BMAL1 in EM CD4+T cells was significantly increased in the CG at 1h Post compared with those at Pre and Post (Figure 3A and B; Suppl. Table 3). In the MA group, there was a significant increase in the expression of REV-ERBα and CRY1 at 1h Post compared with those at Pre and Post ($P<0.05$, Figure 3E and G; Suppl. Table 3). In EM CD8+ T cells, the expression of REV-zERBβ decreased after exercise (Figure 4H; Suppl. Table 4), with no statistical significant differences noted for other clock genes in terms of groups and moments (Figure 4; Suppl. Table 4).

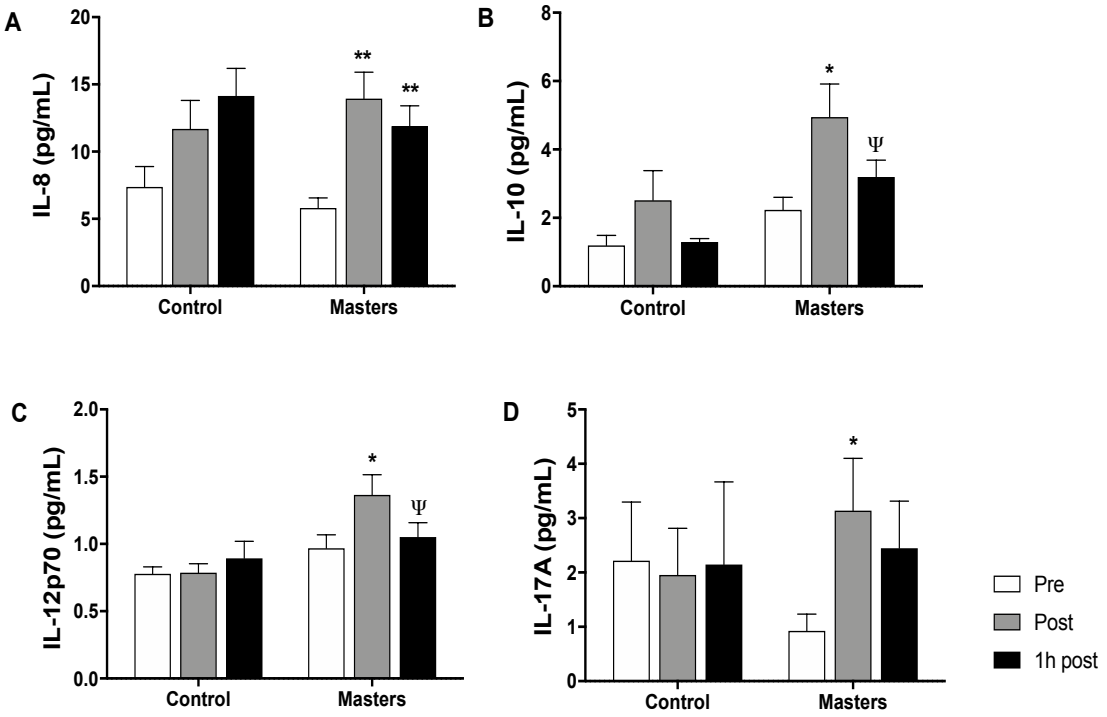


Figure 2. Differences in Cytokine levels in master athletes and control groups in response to a maximum effort protocol. Data are presented as mean with standard error of mean (SEM) bars. *$p < 0.05$ and **$p < 0.01$ compared to Pre; Ψ $p < 0.05$ compared to Post. (A) Interleukin 8; (B) Interleukin 10, (C) Interleukin 12p70; (D) Interleukin 17A. Concentrations before (white columns = Pre), 10 min after (grey columns = Post), and 1h after (black columns = 1h Post) the exercise protocol in Master Athletes group and Control Group. Sequential changes in acute exercise at the different time points (Pre, Post, 1h Post) were analysed using repeated-measures analysis of variance or mixed-effects analysis followed by Tukey's post hoc test.

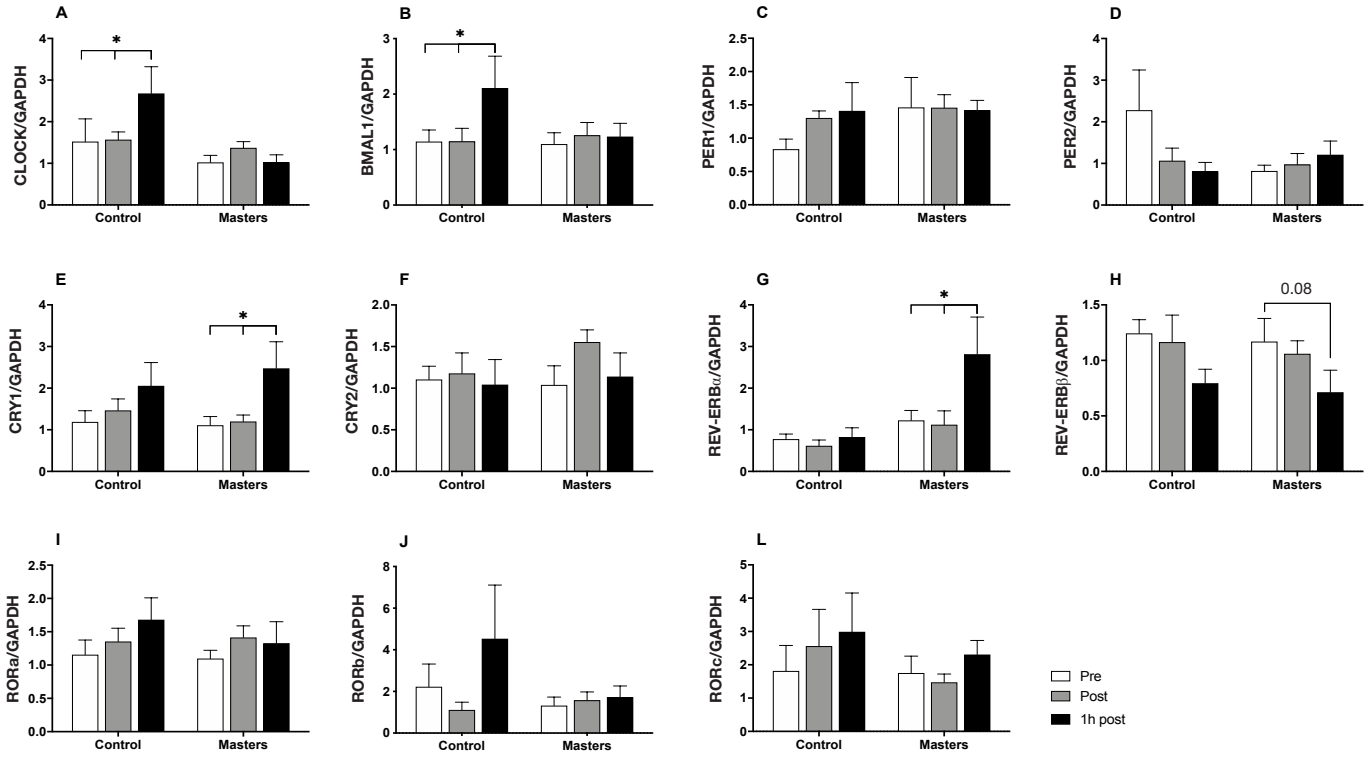


Figure 3. Differences in gene expression of clock genes in purified effector-memory CD4⁺ T-cells in master athletes and control groups in response to a maximum effort protocol. Data are presented as mean (SEM) bars. *p < 0.05 and **p < 0.01 compared to Pre; Ψ p < 0.05 compared to Post. Concentrations before (white columns = baseline), 10 min after (grey columns), and 1h after (black columns) the exercise protocol in Master Athletes group and Control Group. Sequential changes in acute exercise at the different time points (Pre, Post, 1h Post) were analysed using repeated-measures analysis of variance or mixed-effects analysis followed by Tukey's post hoc test.

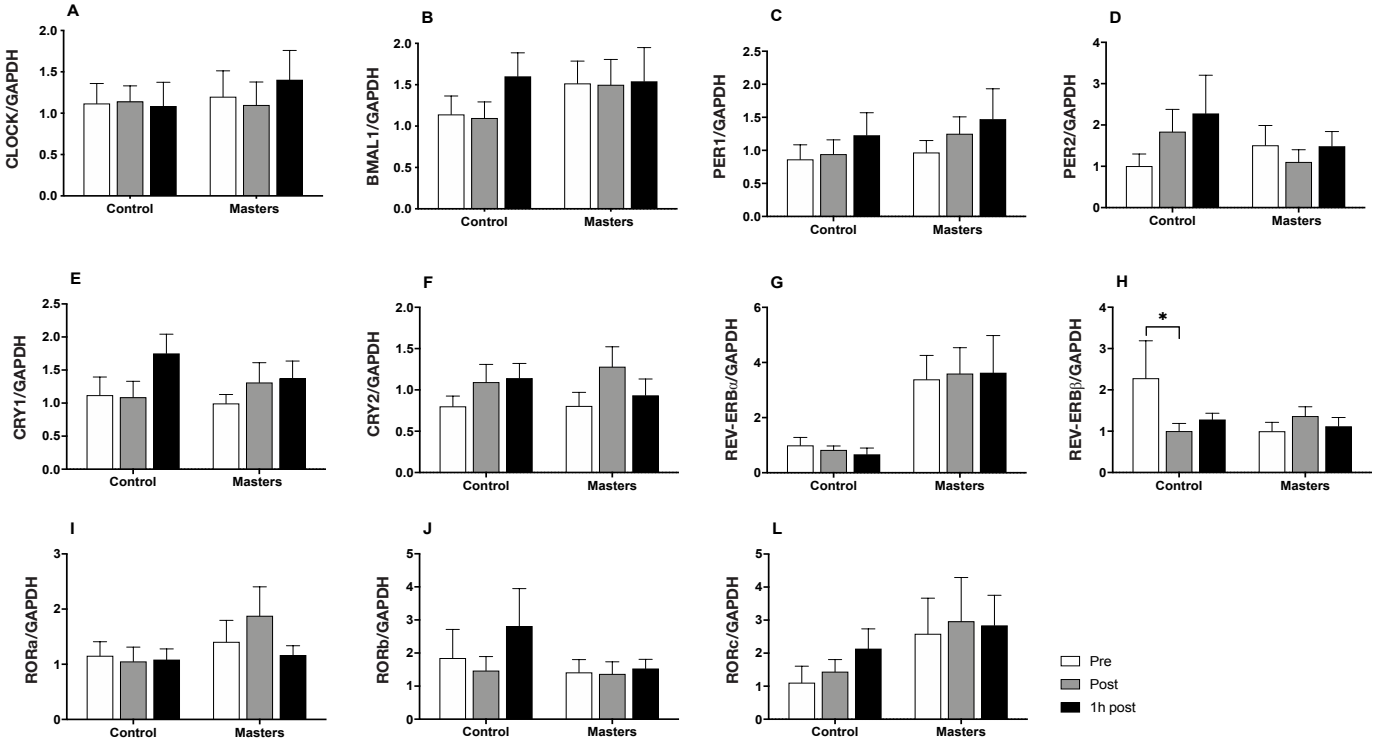


Figure 4. Differences in gene expression of clock genes in purified effector-memory CD8⁺ T-cells for master athletes and control groups in response to a maximum effort protocol. Data are presented as mean with standard error of mean (SEM) bars. *p < 0.05 and **p < 0.01 compared to Pre; Ψ p < 0.05 compared to Post. Concentrations before (white columns = baseline), 10 min after (grey columns), and 1h after (black columns) the exercise protocol in Master Athletes group and Control Group. Sequential changes in acute exercise at the different time points (Pre, Post, 1h Post) were analysed using repeated-measures analysis of variance or mixed-effects analysis followed by Tukey's post hoc test.

The expression of regulatory genes including IL-4, IFN-γ, TBX21, PD-1, Ki67, NF-kB, p53 and p21, were also analysed in the purified EM CD4+ T cells (P<0.05, Figure 5; Suppl. Table 5). In MA, only TBX21 expression increased at Post compared with that at Pre (P<0.05, Figure 5C; Suppl. Table 5).

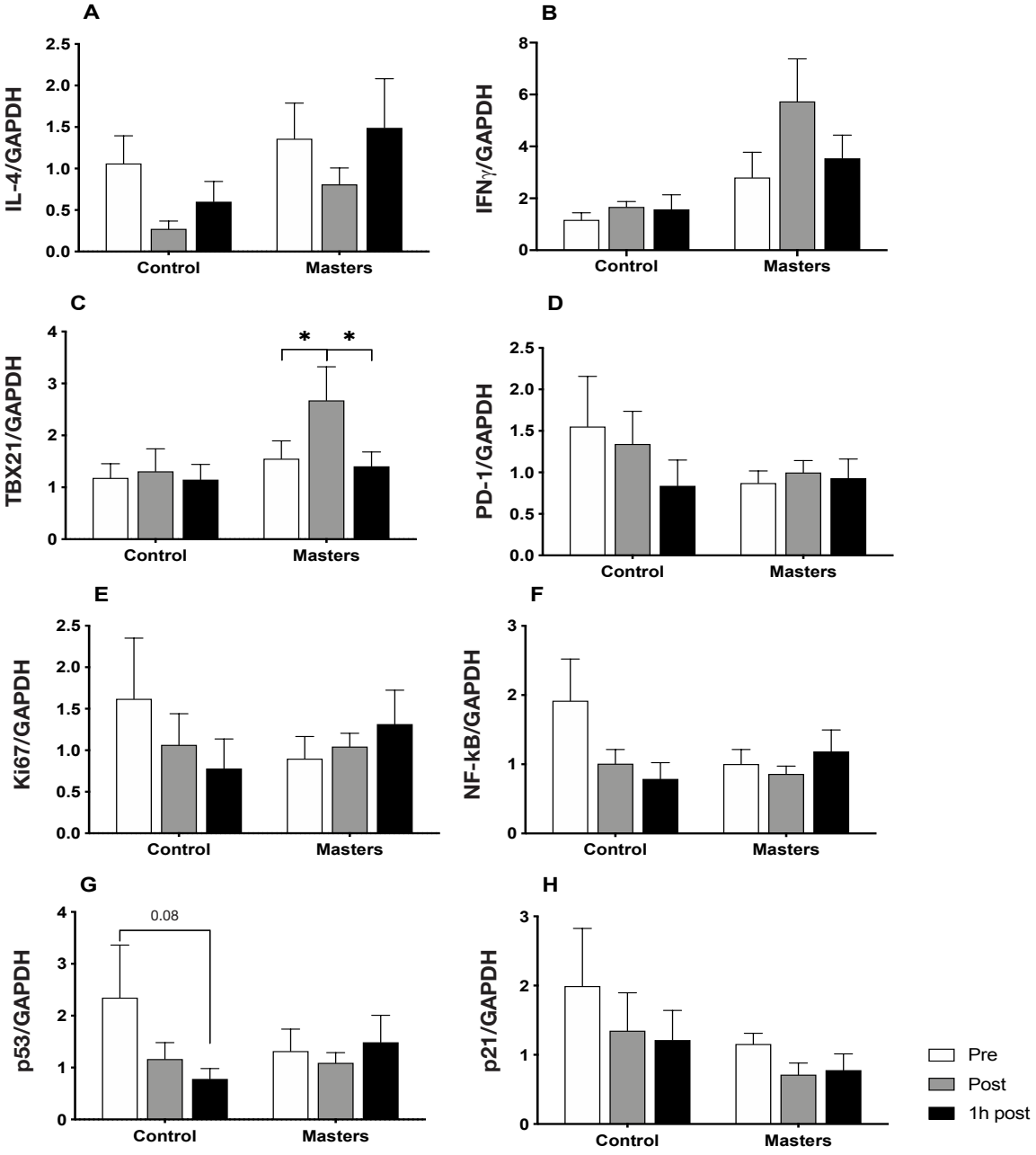


Figure 5. Differences in gene expression of regulatory proteins in purified effector-memory CD4$^+$ T-cells in master athletes and control groups in response to a maximum effort protocol. Data are presented as mean with standard error of mean (SEM) bars. *p < 0.05 and **p < 0.01 compared to Pre; Ψ p < 0.05 compared to Post. Concentrations before (white columns = baseline), 10 min after (grey columns), and 1h after (black columns) the exercise protocol in Master Athletes group and Control Group. Sequential changes in acute exercise at the different time points (Pre, Post, 1h Post) were analysed using repeated-measures analysis of variance or mixed-effects analysis followed by Tukey's post hoc test.

There was an inverse correlation between IL-8 and $VO_{2\ max}$ in MA after acute exercise (Post: r=−0.675, P=0.013; 1h Post: r=−0.774, P=0.002; Table 3). The IL-17 level was inversely correlated with test time, power and $VO_{2\ max}$ in MA at 1h Post (Pre: r=−0.701, P=0.013; Post: r=−0.762, P=0.005; 1h Post: r=−0.640, P=0.029; Table 3). There were no correlations between any cytokines and physiological parameters in the CG.

Table 3. Correlations between the cytokines and physiological parameters in master athletes and control group.

Cytokines	Physiological parameters	Master Athletes PRE		Master Athletes POST		Master Athletes 1h POST		Control group PRE		Control group POST		Control group 1h POST	
		r value	p value	r value	p value	r value	p value	r value	p value	r value	p value	r value	p value
Interleukin 8	Time of test (min)	0.168	0.598	–0.266	0.375	–484	0.095	0.485	0.355	–0.036	0.934	0.800	0.333
	Power (Watts)	0.073	0.827	–0.255	0.399	–0.511	0.077	0.470	0.344	0.079	0.876	0.632	0.500
	VO_{2max}	–0.195	0.543	–0.675	**0.013**	–0.774	**0.002**	0.371	0.497	–0.234	0.619	0.200	0.916
Interleukin 10	Time of test (min)	–0.145	0.632	–0.037	0.900	0.104	0.732	–0.436	0.327	–0.720	0.077	0.500	0.666
	Power (Watts)	–0.113	0.711	0.113	0.699	0.138	0.649	–0.519	0.233	–0.709	0.085	0.272	>0.999
	VO_{2max}	–0.329	0.271	–0.204	0.482	–0.362	0.224	–0.306	0.500	–4643	0.302	–0.632	0.500
Interleukin 12p70	Time of test (min)	0.000	>0.999	0.018	0.949	–0.060	0.837	0.449	0.333	0.102	0.450	0.458	0.450
	Power (Watts)	–0.207	0.491	0.042	0.885	–0.047	0.871	0.490	0.285	0.2874	0.600	0.750	0.300
	VO_{2max}	–0.206	0.494	0.035	0.906	–0.013	0.966	0.356	0.476	0.270	0.666	0.335	0.600
Interleukin 17	Time of test (min)	–0.440	0.151	–0.563	0.059	–0.701	**0.013**	–0.467	0.286	–1.911	0.672	–0.500	0.666
	Power (Watts)	–0.672	**0.017**	–0.576	0.053	–0.762	**0.005**	–0.563	0.209	–0.222	0.766	–0.544	0.750
	VO_{2max}	–0.626	0.033	–0.546	0.069	–0.640	**0.029**	0.259	0.590	0.753	0.105	0.632	0.500

Note: Correlations were analysed using Spearman's rank correlation.

There was a strong positive correlation between REV-ERBα expression and VO_{2max} in EMCD4+ T cells in MA after the acute exercise (Post: r=0.927, P=0.000; Table 4). In addition, REV-ERBα expression was positively correlated with test time and power in MA (Post: r=0.632, P=0.049; r=0.723, P=0.022, respectively; Table 5). Interestingly, after exercise, REV-ERBα expression in EM CD8+ T cells was negatively correlated with test time in MA and CG(Post: r=−0.701, P=0.028; r=−1.000, P=0.016; Table 3).Furthermore, the clock genes (PER1, CRY1 and CRY2)in EM CD4+ T cells at 1h Post and RORc in EM CD8+ T cells at Post were positively correlated with power (1h Post: r=0.617, P=0.035; r=0.744,

Table 4. Correlations between the Clock genes in purified effector-memory $CD4^+$ T-cells and physiological parameters in master athletes and control group.

Clock genes	Physiological parameters	Master Athletes PRE r value	PRE p value	POST r value	POST p value	1h POST r value	1h POST p value	Control group PRE r value	PRE p value	POST r value	POST p value	1h POST r value	1h POST p value
CLOCK	Time of test (min)	0.457	0.183	0.518	0.124	0.073	0.840	0.600	0.241	0.257	0.658	0.885	**0.033**
	Power (Watts)	0.685	**0.034**	0.598	0.073	0.249	0.483	0.706	0.144	0.206	0.711	0.794	0.077
	VO_{2max}	0.248	0.491	0.127	0.733	-0.478	0.166	0.771	0.102	0.142	0.802	0.314	0.563
BMAL1	Time of test (min)	0.255	0.449	-0.009	0.978	0.478	0.136	0.571	0.200	-0.285	0.556	0.071	0.906
	Power (Watts)	0.403	0.217	0.032	0.929	0.575	0.067	0.636	0.128	-0.374	0.414	-0.056	0.928
	VO_{2max}	-0.118	0.734	-0.300	0.371	0.409	0.214	0.607	0.166	-0.428	0.353	-0.321	0.497
PER1	Time of test (min)	0.449	0.143	-0.026	0.935	0.505	0.093	0.200	0.783	0.900	0.083	0.700	0.233
	Power (Watts)	0.520	0.085	0.023	0.943	0.617	**0.035**	0.410	0.500	0.820	0.133	0.564	0.366
	VO_{2max}	-0.160	0.691	-0.465	0.128	0.531	0.079	0.800	0.133	0.600	0.350	0.300	0.683
PER2	Time of test (min)	-0.012	0.973	-0.486	0.154	-0.492	0.148	0.571	0.200	0.000	>0.999	0.357	0.444
	Power (Watts)	-0.075	0.838	-0.574	0.105	-0.390	0.260	0.486	0.266	-0.093	0.852	0.187	0.700
	VO_{2max}	0.139	0.707	-0.551	0.104	-0.078	0.838	0.107	0.839	-0.214	0.661	-0.250	0.594
CRY1	Time of test (min)	0.284	0.425	0.090	0.802	0.553	0.097	0.178	0.713	-0.071	0.906	-0.214	0.661
	Power (Watts)	0.436	0.207	0.099	0.787	0.744	**0.017**	0.168	0.738	0.000	>0.999	-0.355	0.442
	VO_{2max}	0.054	0.891	-0.260	0.469	0.522	0.124	0.035	0.963	0.214	0.661	-0.428	0.353
CRY2	Time of test (min)	-0.047	0.910	-0.023	0.955	0.523	0.182	0.750	0.066	-0.428	0.353	-0.285	0.556
	Power (Watts)	0.358	0.370	0.173	0.675	0.778	**0.031**	0.673	0.114	-0.467	0.295	-0.505	0.247
	VO_{2max}	-0.166	0.703	0.095	0.840	0.381	0.359	0.142	0.782	-0.285	0.556	-0.714	0.088
REV-ERBα	Time of test (min)	0.176	0.626	0.632	**0.049**	0.376	0.283	0.500	0.4500	0.400	0.516	0.600	0.350
	Power (Watts)	0.302	0.393	0.723	**0.022**	0.478	0.162	0.223	>0.999	0.223	>0.999	0.223	>0.999
	VO_{2max}	0.187	0.607	0.927	**0.000**	0.842	**0.003**	-0.500	0.450	-0.300	0.683	-0.700	0.233
REV-ERBβ	Time of test (min)	0.803	**0.009**	0.451	0.222	0.083	0.830	-0.300	0.683	-0.102	0.900	-0.700	0.233
	Power (Watts)	0.935	**0.001**	0.623	0.081	0.356	0.342	-0.102	0.900	-0.263	0.783	-0.820	0.133
	VO_{2max}	0.466	0.212	-0.066	0.880	-0.466	0.212	0.400	0.516	-0.461	0.433	-0.900	0.083
RORa	Time of test (min)	-0.091	0.778	-0.452	0.139	-0.084	0.794	0.750	0.066	-0.428	0.353	-0.250	0.594
	Power (Watts)	0.096	0.764	-0.409	0.185	0.100	0.756	0.711	0.081	-0.430	0.347	-0.467	0.295
	VO_{2max}	-0.083	0.800	-0.587	**0.048**	0.062	0.851	0.428	0.353	-0.142	0.782	-0.714	0.088
RORb	Time of test (min)	0.203	0.525	-0.017	0.956	-0.189	0.553	0.600	0.350	0.800	0.133	0.500	0.450
	Power (Watts)	0.190	0.551	0.035	0.914	-0.326	0.297	0.368	0.533	0.579	0.333	0.368	0.533
	VO_{2max}	0.440	0.154	0.062	0.851	0.069	0.834	-0.200	0.783	-0.100	0.950	0.000	>0.999
RORc	Time of test (min)	0.134	0.697	-0.178	0.600	0.132	0.697	0.571	0.200	0.500	0.266	0.250	0.594
	Power (Watts)	0.163	0.629	-0.243	0.468	0.168	0.619	0.542	0.209	0.486	0.266	0.224	0.638
	VO_{2max}	-0.509	0.114	-0.263	0.434	-0.281	0.402	0.357	0.444	0.357	0.444	0.142	0.782

Note: Correlations were analysed using Spearman's rank correlation.

Table 5. Correlations between the Clock genes in purified effector-memory $CD8^+$ T-cells and physiological parameters in master athletes and control group.

Clock genes	Physiological parameters	Master Athletes PRE r value	PRE p value	POST r value	POST p value	1h POST r value	1h POST p value	Control group PRE r value	PRE p value	POST r value	POST p value	1h POST r value	1h POST p value
CLOCK	Time of test (min)	-0.560	0.122	-0.577	0.110	-0.585	0.103	0.428	0.353	0.071	0.906	0.714	0.088
	Power (Watts)	-0.519	0.157	-0.570	0.115	-0.604	0.090	0.318	0.495	-0.074	0.895	0.654	0.123
	VO_{2max}	-0.466	0.212	-0.566	0.120	-0.583	0.108	-0.071	0.906	-0.285	0.566	0.357	0.444
BMAL1	Time of test (min)	-0.443	0.274	-0.335	0.413	-0.323	0.434	-0.464	0.302	-0.142	0.782	0.107	0.839
	Power (Watts)	-0.414	0.319	-0.244	0.566	-0.414	0.319	-0.467	0.295	-0.018	0.990	0.018	0.990
	VO_{2max}	-0.023	0.976	-0.285	0.500	-0.452	0.267	-0.321	0.497	0.285	0.556	-0.214	0.661
PER1	Time of test (min)	-0.418	0.262	-0.276	0.470	-0.527	0.149	-0.428	0.419	0.085	0.919	0.600	0.241
	Power (Watts)	-0.290	0.445	-0.316	0.404	-0.461	0.213	-0.576	0.283	0.030	>0.999	0.394	0.466
	VO_{2max}	-0.216	0.580	-0.400	0.291	-0.416	0.269	-0.828	0.058	0.085	0.919	-0.085	0.919
PER2	Time of test (min)	-0.242	0.470	-0.018	0.959	0.191	0.570	0.085	0.919	0.028	>0.999	-0.314	0.563
	Power (Watts)	-0.264	0.429	-0.095	0.778	0.051	0.885	-0.147	0.777	-0.117	0.844	-0.382	0.477
	VO_{2max}	0.145	0.673	-0.091	0.790	0.118	0.734	-0.771	0.102	-0.428	0.419	-0.485	0.355
CRY1	Time of test (min)	-0.500	0.144	-0.469	0.172	0.024	0.952	-0.312	0.497	0.107	0.839	-0.107	0.839
	Power (Watts)	-0.417	0.229	-0.274	0.441	0.118	0.745	-0.467	0.295	0.074	0.895	-0.224	0.638
	VO_{2max}	-0.030	0.946	-0.090	0.811	-0.297	0.406	-0.642	0.138	0.071	0.906	-0.428	0.353
CRY2	Time of test (min)	-0.503	0.210	0.192	0.654	-0.457	0.255	-0.028	>0.999	0.028	>0.999	-0.142	0.802
	Power (Watts)	-0.531	0.174	0.049	0.918	-0.356	0.390	-0.235	0.677	0.000	>0.999	-0.029	0.977
	VO_{2max}	-0.239	0.564	-0.333	0.427	-0.595	0.132	-0.771	0.102	0.085	0.919	0.257	0.658
REV-ERBα	Time of test (min)	-0.597	0.073	-0.701	**0.028**	0.597	0.073	0.100	0.950	-1.000	**0.016**	-0.600	0.350
	Power (Watts)	-0.549	0.103	-0.543	0.108	0.668	**0.040**	-0.118	>0.999	-0.894	0.100	-0.335	0.600
	VO_{2max}	-0.466	0.178	-0.260	0.469	0.406	0.247	-0.100	0.950	-0.500	0.450	0.100	0.950
REV-ERBβ	Time of test (min)	-0.816	**0.010**	-0.233	0.551	-0.616	0.085	0.314	0.563	0.116	0.838	-0.428	0.419
	Power (Watts)	-0.886	**0.002**	-0.320	0.396	-0.540	0.137	0.151	0.816	-0.092	0.875	-0.637	0.183
	VO_{2max}	-0.850	**0.006**	-0.566	0.120	-0.216	0.580	-0.314	0.563	-0.579	0.233	-0.771	0.102
RORa	Time of test (min)	-0.900	**0.009**	-0.126	0.797	-0.234	0.619	-0.285	0.556	-0.035	0.963	0.000	>0.999
	Power (Watts)	-0.778	0.052	-0.111	0.840	-0.259	0.597	-0.299	0.519	0.000	>0.999	-0.149	0.761
	VO_{2max}	-0.035	0.963	-0.035	0.963	-0.285	0.556	-0.285	0.556	0.107	0.839	-0.428	0.353
RORb	Time of test (min)	-0.214	0.523	0.041	0.904	0.438	0.178	-0.464	0.302	0.071	0.906	-0.464	0.302
	Power (Watts)	-0.246	0.463	0.011	0.974	0.334	0.312	-0.523	0.223	-0.074	0.895	-0.598	0.161
	VO_{2max}	0.163	0.633	-0.177	0.599	0.018	0.967	-0.500	0.266	-0.285	0.556	-0.642	0.138
RORc	Time of test (min)	-0.383	0.346	-0.263	0.524	-0.646	0.091	-0.600	0.350	0.100	0.950	-1.000	0.333
	Power (Watts)	-0.494	0.216	-0.337	0.412	-0.783	**0.026**	-0.564	0.366	0.205	0.766	-1000	0.333
	VO_{2max}	-0.190	0.664	0.000	>0.999	-0.595	0.132	-0.700	0.233	0.700	0.233	-0.500	>0.999

Note: Correlations were analysed using Spearman's rank correlation.

There was a strong positive correlation between REV-ERBα expression and VO_{2max} in EMCD4+ T cells in MA after the acute exercise (Post: r=0.927, P=0.000; Table 4). In addition, REV-ERBα expression was positively correlated with test time and power in MA (Post: r=0.632, P=0.049; r=0.723, P=0.022, respectively; Table 5). Interestingly, after exercise, REV-ERBα expression in EM CD8+ T cells was negatively correlated with test time in MA and CG(Post: r=−0.701, P=0.028; r=−1.000, P=0.016; Table 3). Furthermore, the clock genes (PER1, CRY1 and CRY2) in EM CD4+ T cells at 1h Post and RORc in EM CD8+ T cells at Post were positively correlated with power (1h Post: r=0.617, P=0.035; r=0.744, P=0.017; r=0.778, P=0.031, respectively; Table 4; r=0.783, P=0.026; Table 5).

DISCUSSION

We demonstrated that EM T cells in MA exhibit different peripheral and cellular inflammatory responses after acute exercise compared with untrained healthy individuals. We observed increased levels of cytokines (IL-8, IL-10, IL-12p70 and IL-17A) after 10 min of acute exercise (with IL-8 and IL-10 levels remaining elevated until 1h Post) and augmented CRY1, REV-ERBα and TBX21 expression in EM CD4+ T cells in MA. Physiological parameters, including test time, power and $VO_{2\ max,}$ were negatively correlated with IL-17A levels (a pro-inflammatory cytokine) in MA at 1h Post. In untrained healthy individuals, CLOCK and BMAL1 expression was increased in EM CD4+ T cells and REV-ERBβ expression was decreased in EM CD8+ T cells after exercise.

Clock genes, inflammation and exercise

We found that the expression of CRY1 and REV-ERBα in EM CD4+ T cells was increased after 1h of acute exercise in MA. CRY1 and CRY2 are closely linked to the anti-inflammatory profile. A study using a mice model of arthritis with CRY1 and CRY2 knockout showed an increase in serum levels of IL-1β, IL-6, MMP-3 and TNF-α, with aggravated pathological changes in the arthritis score observed (56).

The kinetics of IL-17A is also very interesting because it shows an interplay with the clock family. At 10 min after ex-

haustive exercise, the circulating levels of this cytokine were increased and at the same time an elevation of TBX-21 expression was observed. Perhaps these findings may be explained by the fact that Th17 cells contain a dynamic subset of CD4 T cells that are able to develop into other subset lineages, including Th1-like Th17 cells. These cells co-express RORγt and the transcription factor TBX21 and produce IL-17 (57); however, the increase in IL-17A levels and TBX21 expression may be related to the increase in the percentage of EM $CD4^+$ cells.

On the other hand, we observed the restoration of IL-17 levels to the Pre levels 1 h after the exhaustive exercise and at this time, REV-ERBα expression was elevated 1h after the exercise in MA. REV-ERBα also has a close relationship with anti-inflammatory profile. In a study in which macrophages were stimulated with LPS and cultured with a pharmacological agonist of REV-ERBα, a decreased release of IL-6 was observed. Moreover, the deletion of REV-ERBα increased a subset of inflammatory genes in macrophages (58). In Th17 cells, a study demonstrated that REV-ERBα binds to ROR response elements to inhibit the expression of RORγt-dependent genes including IL-17A and IL-17F. Furthermore, elevated REV-ERBα expression or treatment with a synthetic REV-ERBα agonist significantly delayed and mitigated the progression of experimental autoimmune encephalomyelitis (59). Similarly, REV-ERBα negatively regulated pro-inflammatory Th17 responses in vivo (60). These results explain some of our findings, such as the negative correlation between REV-ERBα and IL-17A levels in MA. Another interesting finding was the positive correlation between REV-ERBα and the physiological parameters in MA immediately after the maximal test. Overexpression of REV-ERBα in myocytes induces an increase in the number of mitochondria and improves the respiratory capacity (61). These results have been validated in vivo via pharmacological activation of REV-ERBα as conditional loss of this gene impairs the aerobic capacity and oxidative metabolism in muscle tissue of mice through deactivation of Lkb1-Ampk-Sirt1–Ppargc-1α signalling(61). Because an imbalance between Treg and Th17 is observed in sustained inflammaging observed in senescent lymphocytes (62), the result of the anti-inflammatory regulation of clock genes in MA could be related to their better anti-inflammatory profile. Moreover, a better Treg response was observed in MA previously (44).

It is interesting to note that the clock genes altered by acute exhaustive exercise in the sedentary group were different from those in MA. In untrained healthy individuals, there was an increase in the expression of CLOCK and BMAL1 at 1h Post. A recent study revealed a change in the expression of clock genes in human leukocytes due to exercise, with BMAL1 expression increasing with exercise performed in the morning and afternoon and CRY1 expression increasing only during morning exercise (63). In healthy untrained individuals, there was a decrease in the expression of REV-ERBβ in EM CD8+ T cells. REV-ERBβ is best known for regulating genes involved in metabolism and circadian rhythm(64).

However, its effect on immune cells is still unclear, although one study showed a strong expression of REV-ERBβ in macrophages(65). In general, acute physical exercise promoted major changes in the expression levels of clock genes of EM $CD4^+$ T cells. Therefore, the effects of clock genes in lymphocytes after acute exercise and the difference between the MA and sedentary group should be better explored.

Production of cytokines

Acute exercise induces psychological and physiological stress as well as production of immunological mediators such as cytokines, chemokines and growth factors. The type, intensity and duration of these mediators depend on many factors, including pre-conditioned physical fitness, age, sex and mode of exercise (66,67).

It is interesting to note that CG did not show any difference in Pre values compared with the MA group. In our study, although CG comprised healthy volunteers, we believe that this group is not necessarily a representation of the elderly in the occidental society. However, ageing modified the number and function of circulating immune cells as well as the pattern of cytokines circulating in serum (34). Ageing, per se, induces increases in the levels of pro-inflammatory cytokines (IL-1, TNF-α, C reactive protein and IL-12)(47).

However, we observed that MA exhibit augmented cytokine levels after the maximal exercise test, but these alterations were not seen in the untrained individuals. The increase in the levels of serum cytokines during and after exercise is closely related to the intensity, type, mode and workload of the exercise (68–74). In addition, it also depends on physical fitness (75). We observed that there were no changes in the plasma IL-6 levels 10 min after a maximal exercise in both MA and age-matched untrained healthy individuals. In this study, increased serum levels of IL-10 (120% post-exercise and 48% 1h post), IL-8 (140% post-exercise and 105% 1h post), IL-12p70 (41% post-exercise and 9,4% 1h post) and IL-17A (240% post-exercise and 165% 1h post) were observed after maximal exercise in MA but not in the untrained healthy individuals (for IL-10: 110% post-exercise and 8% 1h post; for IL-8: 58% post-exercise and 91% 1h post; for IL-12p70: 1,2% post-exercise and 15% 1h post; for IL-17A: -11% post-exercise and -3% 1h post, $p>0.05$ for all). This finding may be, at least in part, due to physical fitness.

Because the production of cytokines induced by acute exercise is dependent on the duration/volume of acute exercise, Pedersen and Febbraio (2008) and Cabral-Santos et al (2019) demonstrated that the production of IL-6 and IL-10 during exercise depends on the exercise duration(74,76). In the present study, the time of maximal exercise was different between the groups: CG performed the exercise for ~11 min and MA for ~17 min (~54%). Several studies have reported that the duration of exercise exerts a major impact on the level of cytokines. Cabral-Santos et al. (2016) demonstrated that high-intensity intermittent exercise (such as 1.25km and 2.50 km treadmill runs, which have the same intensity as 1:1 min at 100% of VO_{2max} with passive recovery) effectively increased brain-derived neurotrophic factor (BNDF) and IL-6 levels immediately after exercise. Only the IL-10 response was associated with the duration of exercise, indicating the importance of exercise prescription(72). Jankord and Jemiolo (2004) demonstrated that older men that perform higher volume of lifelong exercise have higher IL-10 levels, suggesting that exercise may play a vital role in regulating the production of inflammatory markers(77).

MA exhibited a negative correlation between the levels of pro-inflammatory cytokines (IL-8 and IL-17) and physiological parameters (VO_{2max}, time of test and power) and a positive correlation between clock genes expression in EM CD4+ T cells and physiological parameters.

Pro-inflammatory cytokines (IL-17 and IL-8) have been shown to induce catabolic effects such as lipolysis and glycogenolysis (78,79). In the present study, a negative correlation between IL-8 and IL-17 levels and performance parameters was observed in MA, suggesting that lifelong exercise protects against degenerative diseases as documented in the literature(80).

Rhythmicity imposed by routine exercise, nutritional habits and sleep behaviour promote an anti-inflammatory and healthy metabolic environment. In a recent review, Parr et al. (2020), explored the deleterious effects of modern-day lifestyles on circadian biology. They suggested that the organisation of a nutritional and exercise training routine could have a potential beneficial effect on metabolic health(81).

CONCLUSION

The synchronization of clock genes, immune function and ageing presents new dimensions with interesting challenges. Acute sessions of exercise altered the clock machinery in CD-4+ T cells, but the expression of clock genes was different between MA and untrained individuals. Moreover, cytokine production was dependent on the fitness level of the elderly population. MA showed a negative correlation between inflammatory cytokine levels and physiological parameters.

LIMITATIONS

Our results do not allow the determination of a cause effect response. It is necessary to perform additional studies to clarify the relation between each clock gene and the cytokine response after exercise in the elderly.

Our sample size was limited because of the molecular approach (cell isolation by cell sorting and PCR). In addition, the nature of the control group may be a limitation in our study, because these were healthy elderly, and most of the elderly population usually as some form of disease. The lack of differences between CG and MA groups in resting conditions may be because of this.

The different modalities of exercise training could also present a limitation, because the physiological adaptations are more heterogeneous, but may also represent a strength since we showed that clock genes were modulated in lymphocytes after acute exercise independent of the exercise training modality.

STRENGTHS

This is the first work that shows a difference in clock genes expression modulated by physical fitness and acute exercise sessions in older persons. Since exercise could be a Zeitgeber it may be an excellent tool to improve the biological rhythms that are altered by ageing, and be a player in improving the inflammatory status observed during aging.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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REFERENCE

1. Clegg A, Young J, Iliffe S, Rikkert MO, Rockwood K. Frailty in elderly people. Lancet (London, England). 2013/02/08. 2013 Mar;381(9868):752–62.
2. Mattis J, Sehgal A. Circadian Rhythms, Sleep, and Disorders of Aging. Trends Endocrinol Metab. 2016 Apr;27(4):192–203.
3. Zee PC, Attarian H, Videnovic A. Circadian rhythm abnormalities. Contin (Minneap Minn) [Internet]. 2013/02/13. 2013;19(1 Sleep Disorders):132–47. Available from: http://www.ncbi.nlm.nih.gov/entrez/query. fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=23385698
4. McCarthy MJ, Welsh DK. Cellular circadian clocks in mood disorders. J Biol Rhythms. 2012 Oct;27(5):339–52.
5. Sahar S, Sassone-Corsi P. Metabolism and cancer: the circadian clock connection. Nat Rev Cancer [Internet]. 2009/11/26. 2009;9(12):886–96. Available from: http://www.ncbi.nlm.nih.gov/entrez/query. cgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19935677
6. Albrecht U, Eichele G. The mammalian circadian clock. Curr Opin Genet Dev [Internet]. 2003/06/06. 2003;13(3):271–7. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12787789
7. Robinson I, Reddy AB. Molecular mechanisms of the circadian clockwork in mammals. FEBS Lett [Internet]. 2014/06/10. 2014;588(15):2477–83. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=24911207
8. Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, et al. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell [Internet]. 2002/08/02. 2002;110(2):251–60. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12150932
9. Triqueneaux G, Thenot S, Kakizawa T, Antoch MP, Safi R, Takahashi JS, et al. The orphan receptor Rev-erbalpha gene is a target of the circadian clock pacemaker. J Mol Endocrinol [Internet]. 2004/12/14. 2004;33(3):585–608. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15591021
10. Sato TK, Panda S, Miraglia LJ, Reyes TM, Rudic RD, McNamara P, et al. A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. Neuron [Internet]. 2004/08/18. 2004;43(4):527–37. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15312651

11. Akashi M, Takumi T. The orphan nuclear receptor RORalpha regulates circadian transcription of the mammalian core-clock BMAL1. Nat Struct Mol Biol [Internet]. 2005/04/12. 2005;12(5):441–8. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15821743
12. Guillaumond F, Dardente H, Giguere V, Cermakian N. Differential control of BMAL1 circadian transcription by REV-ERB and ROR nuclear receptors. J Biol Rhythm [Internet]. 2005/11/04. 2005;20(5):391–403. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16267379
13. Aschoff J. Exogenous and endogenous components in circadian rhythms. Cold Spring Harb Symp Quant Biol [Internet]. 1960/01/01. 1960;25:11–28. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=13684695
14. Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev [Internet]. 2000/12/15. 2000;14(23):2950–61. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11114885
15. Stokkan KA, Yamazaki S, Tei H, Sakaki Y, Menaker M. Entrainment of the circadian clock in the liver by feeding. Science (80-) [Internet]. 2001/02/13. 2001;291(5503):490–3. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11161204
16. Schroeder AM, Truong D, Loh DH, Jordan MC, Roos KP, Colwell CS. Voluntary scheduled exercise alters diurnal rhythms of behaviour, physiology and gene expression in wild-type and vasoactive intestinal peptide-deficient mice. J Physiol [Internet]. 2012/09/19. 2012;590(Pt 23):6213–26. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=22988135
17. Wolff G, Esser KA. Scheduled exercise phase shifts the circadian clock in skeletal muscle. Med Sci Sport Exerc [Internet]. 2012/03/31. 2012;44(9):1663–70. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=22460470
18. Yamanaka Y, Honma S, Honma K. Scheduled exposures to a novel environment with a running-wheel differentially accelerate re-entrainment of mice peripheral clocks to new light-dark cycles. Genes Cells [Internet]. 2008/04/24. 2008;13(5):497–507. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18429821
19. Teixeira AAS, Biondo LA, Silveira LS, Lima EA, Batatinha HA, Diniz TA, et al. Doxorubicin modulated clock genes and cytokines in macrophages extracted from tumor-bearing mice. Cancer Biol Ther. 2020 Jan;1–10.
20. Keller M, Mazuch J, Abraham U, Eom GD, Herzog ED, Volk HD, et al. A circadian clock in macrophages controls inflammatory immune responses. Proc Natl Acad Sci U S A [Internet]. 2009/12/04. 2009;106(50):21407–12. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19955445
21. Silver AC, Arjona A, Hughes ME, Nitabach MN, Fikrig E. Circadian expression of clock genes in mouse macrophages, dendritic cells, and B cells. Brain Behav Immun. 2012;26(3):407–13.
22. Fonken LK, Frank MG, Kitt MM, Barrientos RM, Watkins LR, Maier SF. Microglia inflammatory responses are controlled by an intrinsic circadian clock. Brain Behav Immun. 2015 Mar;45:171–9.
23. Nguyen KD, Fentress SJ, Qiu Y, Yun K, Cox JS, Chawla A. Circadian gene BMAL1 regulates diurnal oscillations of Ly6C(hi) inflammatory monocytes. Science. 2013 Sep;341(6153):1483–8.
24. Baumann A, Gonnenwein S, Bischoff SC, Sherman H, Chapnik N, Froy O, et al. The circadian clock is functional in eosinophils and mast cells. Immunology. 2013 Dec;140(4):465–74.
25. Knudsen JG, Joensen E, Bertholdt L, Jessen H, van Hauen L, Hidalgo J, et al. Skeletal muscle IL-6 and regulation of liver metabolism during high-fat diet and exercise training. Physiol Rep [Internet]. 2016/05/18. 2016;4(9). Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=27185906
26. Arjona A, Sarkar DK. Circadian oscillations of clock genes, cytolytic factors, and cytokines in rat NK cells. J Immunol. 2005 Jun;174(12):7618–24.
27. Ella K, Csepanyi-Komi R, Kaldi K. Circadian regulation of human peripheral neutrophils. Brain Behav Immun. 2016 Oct;57:209–21.
28. Druzd D, Matveeva O, Ince L, Harrison U, He W, Schmal C, et al. Lymphocyte Circadian Clocks Control Lymph Node Trafficking and Adaptive Immune Responses. Immunity. 2017 Jan;46(1):120–32.
29. Bollinger T, Leutz A, Leliavski A, Skrum L, Kovac J, Bonacina L, et al. Circadian clocks in mouse and human CD4+ T cells. PLoS One [Internet]. 2012/01/05. 2011;6(12):e29801. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=22216357
30. Kizaki T, Sato S, Shirato K, Sakurai T, Ogasawara J, Izawa T, et al. Effect of Circadian Rhythm on Clinical and Pathophysiological Conditions and Inflammation. Crit Rev Immunol. 2015;35(4):261–75.
31. Spengler ML, Kuropatwinski KK, Comas M, Gasparian A V, Fedtsova N, Gleiberman AS, et al. Core circadian protein CLOCK is a positive regulator of NF-kappaB-mediated transcription. Proc Natl Acad Sci U S A [Internet]. 2012/08/17. 2012;109(37):E2457-65. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=22895791
32. Nobis CC, Dubeau Laramee G, Kervezee L, Maurice De Sousa D, Labrecque N, Cermakian N. The circadian clock of CD8 T cells modulates their early response to vaccination and the rhythmicity of related signaling pathways. Proc Natl Acad Sci U S A. 2019 Oct;116(40):20077–86.
33. Goronzy JJ, Weyand CM. Successful and Maladaptive T Cell Aging. Immunity. 2017 Mar;46(3):364–78.
34. Nikolich-Žugich J, Nikolich-Zugich J. The twilight of immunity: Emerging concepts in aging of the immune system review-article. Nat Immunol. 2018 Jan;19(1):10–9.

35. Edgar DM, Dement WC. Regularly scheduled voluntary exercise synchronizes the mouse circadian clock. Am J Physiol [Internet]. 1991/10/01. 1991;261(4 Pt 2):R928-33. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1928438
36. Marchant EG, Mistlberger RE. Entrainment and phase shifting of circadian rhythms in mice by forced treadmill running. Physiol Behav [Internet]. 1996/08/01. 1996;60(2):657–63. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8840932
37. Hojbjerre L, Sonne MP, Alibegovic AC, Nielsen NB, Dela F, Vaag A, et al. Impact of physical inactivity on adipose tissue low-grade inflammation in first-degree relatives of type 2 diabetic patients. Diabetes Care. 2011 Oct;34(10):2265–72.
38. de Souza Teixeira AA, Lira FS, Rosa-Neto JC. Aging with rhythmicity. Is it possible? Physical exercise as a pacemaker. Life Sci. 2020 Sep;118453.
39. Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo MA. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. Nat Rev Immunol. 2011 Aug;11(9):607–15.
40. Petersen AM, Pedersen BK. The anti-inflammatory effect of exercise. J Appl Physiol [Internet]. 2005/03/18. 2005;98(4):1154–62. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15772055
41. Teixeira AA, Lira FS, Pimentel GD, Oliveira de Souza C, Batatinha H, Biondo LA, et al. Aerobic Exercise Modulates the Free Fatty Acids and Inflammatory Response During Obesity and Cancer Cachexia. Crit Rev Eukaryot Gene Expr [Internet]. 2016/09/22. 2016;26(3):187–98. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=27650984
42. Minuzzi LG, Rama L, Chupel MU, Rosado F, Dos Santos JV, Simpson R, et al. Effects of lifelong training on senescence and mobilization of T lymphocytes in response to acute exercise. Exerc Immunol Rev. 2018;24:72–84.
43. Minuzzi LG, Chupel MU, Rama L, Rosado F, Muñoz VR, Gaspar RC, et al. Lifelong exercise practice and immunosenescence: Master athletes cytokine response to acute exercise. Cytokine. 2019 Mar;115:1–7.
44. Minuzzi LG, Rama L, Bishop NC, Rosado F, Martinho A, Paiva A, et al. Lifelong training improves anti-inflammatory environment and maintains the number of regulatory T cells in masters athletes. Eur J Appl Physiol. 2017 Jun;117(6):1131–40.
45. Mikkelsen UR, Couppé C, Karlsen A, Grosset JF, Schjerling P, Mackey AL, et al. Life-long endurance exercise in humans: circulating levels of inflammatory markers and leg muscle size. Mech Ageing Dev. 2013 Jan;134(11–12):531–40.
46. Campbell JP, Turner JE. Debunking the Myth of Exercise-Induced Immune Suppression: Redefining the Impact of Exercise on Immunological Health Across the Lifespan. Front Immunol. 2018;9:648.
47. Sellami M, Gasmi M, Denham J, Hayes LD, Stratton D, Padulo J, et al. Effects of Acute and Chronic Exercise on Immunological Parameters in the Elderly Aged: Can Physical Activity Counteract the Effects of Aging? Front Immunol. 2018 Oct;9.
48. Aguiar SS, Sousa CV, Deus LA, Rosa TS, Sales MM, Neves RVP, et al. Oxidative stress, inflammatory cytokines and body composition of master athletes: The interplay. Exp Gerontol. 2020;130:110806.
49. Ray D, Yung R. Immune senescence, epigenetics and autoimmunity. Clin Immunol. 2018 Nov;196:59–63.
50. Carrick-Ranson G, Sloane NM, Howden EJ, Bhella PS, Sarma S, Shibata S, et al. The effect of lifelong endurance exercise on cardiovascular structure and exercise function in women. J Physiol. 2020 Apr;
51. Shibata S, Fujimoto N, Hastings JL, Carrick-Ranson G, Bhella PS, Hearon CM, et al. The effect of lifelong exercise frequency on arterial stiffness. J Physiol. 2018 Jul;596(14):2783–95.
52. Foo JC, Trautmann N, Sticht C, Treutlein J, Frank J, Streit F, et al. Longitudinal transcriptome-wide gene expression analysis of sleep deprivation treatment shows involvement of circadian genes and immune pathways. Transl Psychiatry. 2019 Dec;9(1):343.
53. Howley ET, Bassett DRJ, Welch HG. Criteria for maximal oxygen uptake: review and commentary. Med Sci Sports Exerc. 1995 Sep;27(9):1292–301.
54. Higuchi R, Dollinger G, Walsh PS, Griffith R. Simultaneous amplification and detection of specific DNA sequences. Biotechnol (N Y) [Internet]. 1992/04/01. 1992;10(4):413–7. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1368485
55. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods [Internet]. 2002/02/16. 2001;25(4):402–8. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11846609
56. Hashiramoto A, Yamane T, Tsumiyama K, Yoshida K, Komai K, Yamada H, et al. Mammalian clock gene Cryptochrome regulates arthritis via proinflammatory cytokine TNF-alpha. J Immunol. 2010 Feb;184(3):1560–5.
57. Kamali AN, Noorbakhsh SM, Hamedifar H, Jadidi-Niaragh F, Yazdani R, Bautista JM, et al. A role for Th1-like Th17 cells in the pathogenesis of inflammatory and autoimmune disorders. Mol Immunol. 2019 Jan;105:107–15.
58. Gibbs JE, Blaikley J, Beesley S, Matthews L, Simpson KD, Boyce SH, et al. The nuclear receptor REV-ERBalpha mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines. Proc Natl Acad Sci U S A [Internet]. 2011/12/21. 2012;109(2):582–7. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=22184247
59. Chang C, Loo C-S, Zhao X, Solt LA, Liang Y, Bapat SP, et al. The nuclear receptor REV-ERBα modulates Th17 cell-mediated autoimmune disease. Proc Natl Acad Sci U S A. 2019 Sep;116(37):18528–36.
60. Amir M, Chaudhari S, Wang R, Campbell S, Mosure SA, Chopp LB, et al. REV-ERBα Regulates T(H)17 Cell Development and Autoimmunity. Cell Rep. 2018 Dec;25(13):3733-3749.e8.
61. Woldt E, Sebti Y, Solt LA, Duhem C, Lancel S, Eeckhoute J, et al. Rev-erb-α modulates skeletal muscle oxidative capacity by regulating mitochondrial biogenesis and autophagy. Nat Med. 2013 Aug;19(8):1039–46.
62. Schmitt V, Rink L, Uciechowski P. The Th17/Treg balance is disturbed during aging. Exp Gerontol. 2013 Dec;48(12):1379–86.
63. Tanaka Y, Ogata H, Kayaba M, Ando A, Park I, Yajima K, et al. Effect of a single bout of exercise on clock gene expression in human leukocyte. J Appl Physiol. 2020 Apr;128(4):847–54.

64. Kojetin DJ, Burris TP. REV-ERB and ROR nuclear receptors as drug targets. Nat Rev Drug Discov. 2014 Mar;13(3):197–216.
65. Lam MTY, Cho H, Lesch HP, Gosselin D, Heinz S, Tanaka-Oishi Y, et al. Rev-Erbs repress macrophage gene expression by inhibiting enhancer-directed transcription. Nature. 2013 Jun;498(7455):511–5.
66. Walsh N, Gleeson MM, Shephard R, Woods J, Bishop N, Fleshner M, et al. Position statement. Part one: Immune function and exercise. Exerc Immunol Rev. 2011;17:6–63.
67. Walsh NP, Gleeson M, Pyne DB, Nieman DC, Dhabhar FS, Shephard RJ, et al. Position statement. Part two: Maintaining immune health. Exerc Immunol Rev. 2011;17:64–6103.
68. Pedersen BK. The diseasome of physical inactivity--and the role of myokines in muscle--fat cross talk. J Physiol [Internet]. 2009/09/16. 2009;587(Pt 23):5559–68. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19752112
69. Cabral-Santos C, Gerosa-Neto J, Inoue DS, Panissa VLG, Gobbo LA, Zagatto AM, et al. Similar anti-inflammatory acute responses from moderate-intensity continu-ous and high-intensity intermittent exercise. J Sport Sci Med. 2015;
70. Gerosa-Neto J, Antunes BMM, Campos EZ, Rodrigues J, Ferrari GD, Neto JCR, et al. Impact of long-term high-intensity interval and moderate-intensity continuous training on subclinical inflammation in overweight/obese adults. J Exerc Rehabil. 2016 Dec;12(6):575–80.
71. Inoue DS, Panissa VLG, Monteiro PA, Gerosa-Neto J, Rossi FE, Antunes BMM, et al. Immunometabolic responses to concurrent training: The effects of exercise order in recreational weightlifters. J Strength Cond Res. 2016;
72. Cabral-Santos C, Castrillón CIM, Miranda RAT, Monteiro PA, Inoue DS, Campos EZ, et al. Inflammatory cytokines and BDNF response to high-intensity intermittent exercise: Effect the exercise volume. Front Physiol. 2016;
73. Lira FS, dos Santos T, Caldeira RS, Inoue DS, Panissa VLG, Cabral-Santos C, et al. Short-term high- and moderate-intensity training modifies inflammatory and metabolic factors in response to acute exercise. Front Physiol. 2017;
74. Cabral-Santos C, de Lima Junior EA, Fernandes IM da C, Pinto RZ, Rosa-Neto JC, Bishop NC, et al. Interleukin-10 responses from acute exercise in healthy subjects: A systematic review. Journal of Cellular Physiology. 2019.
75. Antunes BM, Campos EZ, dos Santos RVT, Rosa-Neto JC, Franchini E, Bishop NC, et al. Anti-inflammatory response to acute exercise is related with intensity and physical fitness. J Cell Biochem. 2019;
76. Pedersen BK, Febbraio MA. Muscle as an endocrine organ: focus on muscle-derived interleukin-6. Physiol Rev. 2008 Oct;88(4):1379–406.
77. Jankord R, Jemiolo B. Influence of physical activity on serum IL-6 and IL-10 levels in healthy older men. Med Sci Sports Exerc. 2004 Jun;36(6):960–4.
78. Shin JH, Shin DW, Noh M. Interleukin-17A inhibits adipocyte differentiation in human mesenchymal stem cells and regulates pro-inflammatory responses in adipocytes. Biochem Pharmacol. 2009 Jun;77(12):1835–44.
79. Shen H, Shi LZ. Metabolic regulation of T(H)17 cells. Mol Immunol. 2019 May;109:81–7.
80. Pedersen BK, Saltin B. Exercise as medicine - evidence for prescribing exercise as therapy in 26 different chronic diseases. Scand J Med Sci Sports. 2015 Dec;25(S3):1–72.
81. Parr EB, Heilbronn LK, Hawley JA. A Time to Eat and a Time to Exercise. Exerc Sport Sci Rev. 2020 Jan;48(1):4–10.