Introduction

The complement system is an innate immunity key component consisting of proteolytic cascade paths activated by pathogenic microorganisms, immune complexes and auto activation of structurally unstable C3. Corresponding to the lectin classical and alternative pathways, they lead to formation of a lytic membrane attack complex [23]. Complement plays an important role in inflammation, foreign materials opsonisation, phagocytosis facilitation and direct cytotoxic reactions, working as an antibody-dependent effector to eliminate pathogens [43]. It regulates several adaptive immune responses and is conditioned by sleep and circadian rhythms, environmental temperature and humidity, ethnicity, physical activity levels, disease, specific nutritional status and anorexia nervosa [23, 28, 34]. C3 and C4 complement components are not sensitive to acute psychological stress [35], but although depressive disorders do not affect C3, they might increase C4 serum levels [3].

It has been suggested that exigent physical conditioning elicit changes in the peripheral blood cellular and humoral components of the immune system [7]. This change is related to inflammatory and oxidative stress markers [20] with prolonged exercise and heavy training loads associated with depressed immune function [14]. In fact, well-trained individuals have lower C3 and C4 resting levels [27] and are prone to upper respiratory diseases [13]. Furthermore, nutritional status can directly affect well-trained subjects’ immune response to heavy training, because high carbohy-
drate intake during prolonged exercise limits exercise-induced im-
mune depression [14] and inadequate nutrition negatively
influences immunecompetence after heavy exertion [15]. In addi-
tion, cellular immunity responds better than humoral immunity to
nutritional supplementation, as glutamine supplementation im-
proves: (i) cellular (but not humoral) immunity functions in severe-
depressed immune system subjects and (ii) cellular immunity
(like CD4/CD8 ratio), although IgG, IgM, C3 and C4 plasma concen-
trations did not change in severe burn patients [29].

Dietary protein and specific micronutrients deficiencies have
been associated with immune dysfunction, but benefits regarding
high doses of anti-oxidant intake are not sufficiently studied. This
is very relevant once antioxidant vitamins and trace elements mod-
ulate immune cell function through regulation of redox-sensitive
transcription factors [44], although this supplement effect on im-
une humoral function is not well investigated. Since the complem-
ent system is a central mediator of inflammation [43], its im-
provement might elicit some immune surveillance against exer-
cise-induced inflammatory focus. We aimed to verify if supplemen-
tation with antioxidant vitamins, minerals and trace el-
ements can alter immune humoral function and total complement
activity after a period of heavy physical exertion. It was hypothe-
sised that supplementation induces complement system benefits
post-heavy physical training.

Material and Methods

Sample

24 male firefighters volunteered to participate and were randomly
divided into supplemented and placebo groups (concealed alloca-
tion was implemented). The inclusion criteria were that subjects
were professional firefighters; healthy (assessed through medical
tests); with no muscular, bone or articular pathologies and visual
or hearing deficits; and with a positive classification in physical con-
ditioning tests. Subjects with any incapacitating physical or organ-
ic pathology were excluded. There were no differences between
groups regarding age, anthropometrical and physical conditioning
characteristics (►Table 1), and no dropouts occurred during the
study. Experimental procedures were conducted in accordance with
Helsinki Declaration and ethical principles for medical research in-
volving human subjects [16].

Testing protocol

The current study was randomized, double-blinded and placebo-
controlled with supplemented and placebo groups receiving, over
35 consecutive days, a proprietary supplement (Ever-Fit Plus, Pris-
far® with 15 mg of beta-carotene, 200 mg of vitamin C, 136 mg of
vitamin E, 200 μg of selenium, 15 mg of zinc and 100 mg of mag-
nesium) and a placebo powder (maltodextrin with artificial flavour
and colour), respectively. The training period included 5 microcy-
cycles of 5 training units (including 30 min of military drills plus
90 min of technical skills with and without fire protective clothing)
and 2 resting days. This and the contents of the weekly physical
conditioning program are displayed in ►Fig. 1. Participants avoid-
ed physical exertion over the weekends during the study period.
Anthropometrical evaluation included stature; body mass; tricipi-
tal, bicipital, sub-scapular and supra-iliac skinfold thickness; and
fat mass [39].

All participants received the same physical conditioning and pro-
fessional skills program during 3 months prior to experimentation,
and presented a remarkable similarity in physical fitness in-between
groups at the beginning of the study (►Table 1). Dietary intake was
assessed on 2 weekdays and one weekend day record (one week
before the intervention) using a photo album with 134 images con-
taining average raw/cooked food portions. Mean daily food intake
was converted to nutrients using Food Processor Plus [1] and no
differences between groups regarding pre-supplementation val-
ues were observed (►Table 2). As groups had similar nutritional
intake before intervention and firefighters had the same meals dur-
ing the intervention, they were not tested again for these variables.

Venous blood was drawn from the antecubital vein in a fasting
state after 2 resting days in pre- (3 months after the start of train-
ing activities) and post-5 weeks of supplementation. Complement
components concentration in serum was determined using specif-
ic antisera to human C3 and C4 (Codes OSAP and OSAO), with the
immune complexes formed measured in a nephelometer (Dade
Behring Marburg GmbH, Newark, USA). C3 and C4 were calculated
by comparison with known concentration standards. Total haemo-
lytic complement activity (CH100) was determined in human
serum by enzyme immunoassay in conjunction with the DiaSorin
CAE Kit (Stillwater, Minnesota 55082-0285, USA). Activation level
was expressed in complement activation by enzyme immunoassay
units. In an attempt to avoid data analysis bias due to analytical vari-
ability, all blood samples were analysed in a single laboratory fol-
lowing the same analytical procedures. Main reference values in
the literature are 86–184 and 90–180 mg/dl for C3, 20–58 and
10–40 mg/dl for C4, and 63–145 U/ml for CH100 [8, 19].

► Table 1 Means plus SD values of age, anthropometrical and physical
conditioning characteristics of the participants.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Supplemented group (n = 12)</th>
<th>Placebo group (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>22.1 ± 1.9</td>
<td>23.9 ± 0.3</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>174.5 ± 3.8</td>
<td>174.3 ± 3.5</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>68.9 ± 7.4</td>
<td>69.3 ± 12.3</td>
</tr>
<tr>
<td>Fat mass (% body mass)</td>
<td>9.6 ± 2.0</td>
<td>10.2 ± 12.0</td>
</tr>
<tr>
<td>Bench press with 50 kg (reps)</td>
<td>12.0 ± 7.1</td>
<td>13.7 ± 5.7</td>
</tr>
<tr>
<td>Chin-ups (reps)</td>
<td>15.8 ± 0.7</td>
<td>15.7 ± 2.1</td>
</tr>
<tr>
<td>Sprint 50 m (s)</td>
<td>7.00 ± 0.0</td>
<td>6.97 ± 0.05</td>
</tr>
<tr>
<td>Cooper test (m)</td>
<td>3007 ± 127</td>
<td>3120 ± 106</td>
</tr>
<tr>
<td>Relative peak power output (watt/kg) *</td>
<td>10.5 ± 0.5</td>
<td>10.8 ± 0.1</td>
</tr>
<tr>
<td>Relative mean power output (watt/kg) *</td>
<td>7.8 ± 0.5</td>
<td>8.1 ± 0.3</td>
</tr>
<tr>
<td>Relative minimum power output (watt/kg) *</td>
<td>5.7 ± 0.8</td>
<td>5.8 ± 0.6</td>
</tr>
<tr>
<td>Fatigue index (%) *</td>
<td>45.9 ± 6.4</td>
<td>45.2 ± 6.8</td>
</tr>
<tr>
<td>Squat jump (cm)</td>
<td>37.3 ± 5.7</td>
<td>37.8 ± 3.5</td>
</tr>
<tr>
<td>Countermovement jump (cm)</td>
<td>38.3 ± 6.2</td>
<td>39.4 ± 5.8</td>
</tr>
</tbody>
</table>

* Determined through the Wingate test
Statistical analysis
A sample size of 24 subjects was deemed adequate (software G*Power 3.1.9.2© Heinrich-Heine-Universität Düsseldorf, Germany), assuming 85 % of statistical power and 0.05 α error probability. Data were first tested for distribution normality and variance homogeneity. A paired measures t-test was used to compare C3, C4 and CH100 values on pre- and post-supplementation conditions for each group. Then, the effects of treatment (supplemented vs placebo) and time (pre- vs post-supplementation) were assessed for each variable using a 2-way ANOVA. When a significant effect was found, the Bonferroni post hoc procedure was performed to localize the difference. Statistical Package for Social Sciences 19.0 was used, with results presented as mean plus standard deviation and statistical significance set at p < 0.05.

Results
Regarding micronutrient supplementation (concurrent with heavy physical training), differences for pre- and post-test were observed only for CH100 in the placebo group (p = 0.004; mean diff = -26.92; 95 %CI = -43.58 to -10.25; cf. ▶ Fig. 2).

ANOVA showed no interaction, treatment or time effect for C3 and C4 (▶ Table 3). Although interaction accounted for 8.8 % of the total variance in CH100 (F(1,44) = 4.249, p = 0.045, partial η² = 0.088; observed power = 0.522), with time effect accounting for 19.5 % of the total variance in CH100 (F(1,44) = 10.662, p = 0.0021, partial η² = 0.195; observed power 0.891), the treatment effect was not significant (F(1,44) = 0.144, p = 0.670, partial η² = 0.04; observed power = 0.522).

Discussion
Literature relating to physical training, nutrition and immune humoral system is very scarce, with the current study giving new insights into the influence of several nutrients in humoral immune response in very exhaustive training. The hard physical loads that typical elite athletes (and firefighters) experience induce necrotic material proliferation that can be cleared by complement system activation [23]. This allows opsonisation of damaged tissue prior to its ingestion by phagocytic leukocytes [38, 43]. Complement pathway activation seems to be independent of exercise type, since aerobic and anaerobic exercises induce similar changes (C3 and C4 serum levels decrease after both a 30 s anaerobic test and 30 min treadmill running [14, 18]). However, some data are contradictory, because C3 and C4 rose after maximal cycling [10] but no alterations were detected immediately after long-lasting exercise [37]. In addition, C3 and C4 values rose during and immediately after 2.5 h of running (with C4 continuing to rise some hours after the...
quickly to basal levels when exercising at moderate intensity [33]. It was observed that runners had lower basal C3 and haemolytic activity values than non-exercising controls [14, 40], with literature differences justified by diverse subjects’ physical condition or methodological discrepancies.

In general, exhaustive physical loads induce an acute increase of several complement system components [4, 7, 43], with systematic heavy physical training depressing their basal values [14, 44]. This was observed in the current study’s C3, C4 and CH100 data, evidencing a chronic adaptation to high-intensity training loads. Our subjects’ average basal serum C3 levels were close to the lowest reference values [8, 19] and similar to those found in professional cyclists [36]. The current study’s serum C4 levels were lower than some clinical references [19] but within the normal range [8, 36]. Although not observed in our experiment (cf. Fig. 2), basal C3 and C4 levels tend to be lower in well-trained subjects [27, 44]. However, differences between sports should be considered, because no modification of C3 and C4 serum levels was observed during a volleyball season [6] despite decreased basal C3 values through aerobic cardiovascular training [27, 30].

In the current study, total complement activity (CH100) at pre-supplementation was lower than laboratory references. This provides insight into the integrity level of the entire classical complement pathway, which is usually elevated in inflammation and infection situations, and decreases with fasting and malnutrition [24]. Although haemolytic activity decreases after short-term aerobic exercise [40], analysis of CH100 alterations induced by heavy physical training is required. From the current data, we speculate that low basal CH100 values, in the absence of any known disease or nutritional deficiency, evidence haemolytic activity attenuation subsequent to systematic training. Moreover, CH100 increased in the placebo group, but no differences between groups were observed both at pre- and post-intervention. However, an increasing tendency after the intervention period was seen for this variable in both groups, suggesting that physical training continuity accentuated haemolytic activity independently of the supplementation.

Complement system enhancement through nutritional interventions is not new, with reduced nutritional intake promoting a decrease in serum C3 and C4 levels [15, 20], whereas diets inducing elevated serum low-density lipoprotein cholesterol increased C3 [21]. It is also known that vitamin D deficits are inversely related to C3 serum concentration [31], whereas exercise-induced dehydration increases C3 and C4 serum levels [5]. Dehydration was not taken into consideration in the current study because blood samples were drawn after a 48 h post-exercise recovery period.

It is accepted that complement system response can be improved by supplementation (at least in situations of nutritional deficits), with cyanocobalamin treatment improving the immune status of vitamin-B12-deficient patients by increasing C3 and C4 levels [11]. However, in well-fed subjects, nutritional supplementation effects are equivocal, because ingestion of mangosteen, multitamins and essential minerals increased C3 and C4 serum concentration [42] but arginine supplementation did not modify their values [25]. It is also known that histidine-rich glycoprotein binds strongly to several complement proteins, contributing to the maintenance of normal immune function, inhibiting the formation of

**Fig. 2** Micronutrient supplementation in both groups

![Graph showing changes in C3, C4, and CH100 levels before and after supplementation.](image)
insoluble immune complexes (enhancing complement activation) and promoting the faster clearance of necrotic materials [23]. Furthermore, supplementation with a substance isolated from Greenland shark liver increased C1q, C3 and C4 serum levels, improving innate immunity [17].

In the current study, vitamins C and E, β-carotene, selenium, zinc and magnesium did not enhance C3, C4 and CH100, meaning that humoral immunity responds differently than cellular immunity to supplementation even in situations of great immune stress. Similarly, cellular immunity in severely burned patients was enhanced by glutamine supplementation, whereas humoral immunity did not change [1]. Some reasons might justify the current study data: (i) in well-fed subjects, nutritional supplements do not improve humoral innate immunity; (ii) the selected supplements unlikely improve innate immune system in situations of adequate nutritional status; and/or (iii) the selected micronutrient doses were not sufficient to elicit immune changes.

No differences between groups were observed both at pre- and post-intervention, except for CH100 that increased in the placebo group. Although time effect accounted for 19.5 % of its total variance, treatment effect was not significant, suggesting an accentuated haemolytic activity with physical training, independent of supplementation. Although it was not possible to verify the same behaviour for all studied variables, the current study’s low basal CH100 values can be the outcome of systematic heavy physical training loads that professional firefighters are accustomed to (worldwide they are typically engaged in this type of heavy physical conditioning). Therefore, these low values are not related to any nutritional inadequacy, because they did not change after micronutrient supplementation (neither did body or fat mass values). Therefore, albeit some studies point out complement system participation haemolytic activity with physical training, independent of supplementation due to the low values obtained. In fact, the higher value obtained in the placebo group was close to the inferior limit, evidencing that this indicator was depressed in both groups in the beginning of the intervention and that supplementation did not alter the situation. We might conclude that 5 weeks of multivitamin and multi-mineral supplementation during heavy training in well-trained and well-fed individuals did not elicit any relevant changes in serum concentration of humoral immunity biomarkers. Future research should further investigate the underlying mechanisms and test the immunity responses to this kind of supplementation in subjects who are not so heavily trained.

### Acknowledgements

To the Immunology Service of St John Hospital (Porto, Portugal) where the laboratory procedures took place.

### Conflict of Interest

The authors declare that they have no conflict of interest.

### References


### Table 3

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Post</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>95 % CI</td>
<td>95 % CI</td>
</tr>
<tr>
<td></td>
<td>Supplemented</td>
<td>Placebo</td>
</tr>
<tr>
<td>C3 (mg/dL)</td>
<td>88.2 ± 7.3</td>
<td>82.1 to 94.2</td>
</tr>
<tr>
<td></td>
<td>85.4 ± 15.0</td>
<td>79.3 to 91.5</td>
</tr>
<tr>
<td>C4 (mg/dL)</td>
<td>18.8 ± 3.1</td>
<td>16.1 to 21.4</td>
</tr>
<tr>
<td></td>
<td>17.3 ± 4.1</td>
<td>14.5 to 19.9</td>
</tr>
<tr>
<td>CH100 (U/mL)</td>
<td>49.6 ± 8.1</td>
<td>39.4 to 59.8</td>
</tr>
<tr>
<td></td>
<td>55.7 ± 14.3</td>
<td>45.5 to 65.8</td>
</tr>
</tbody>
</table>

Values (mean ± SD and 95 %CI) of C3, C4 and CH100 before and after the training period for supplemented and placebo group.

![Table 3](image-url)


