ORIGINAL ARTICLE

Salivary levels of Interleukin-6 and Tumor Necrosis Factor-alpha in girls aged 7–17 years practicing volleyball

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Abstract

Introduction: The purpose of the study was to evaluate the salivary concentrations of Interleukin-6 (IL-6) and Tumor Necrosis Factor-alpha (TNFα) and correlate the findings with the caries index, body mass index (BMI), potency of lower limbs (vertical jump), cardiorespiratory fitness, and risk of developing cardiovascular diseases in girls practicing volleyball.

Material and methods: Two studies were performed: 1) a cross-sectional study (*n* = 120) on the association of IL-6 and TNFα with the caries index, anthropometric measures, physical tests, and experience in volleyball practice; 2) longitudinal study (*n* = 63) on the effects of 8 weeks of training on salivary IL-6 and TNFα in girls with intermediate experience in volleyball and competitive girls.

Results: The median levels of IL-6 were 1.98 [1.55–3.11] pg/ml and TNFα, 0.46 [0.28–0.59] pg/ml and these did not correlate with the caries index, BMI, training volume,

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training practice, or cardiovascular risk. A correlation was observed between IL-6 and TNFα ($r = 0.34$; $p < 0.001$), IL-6 and vertical jump height ($r = -0.28$, $p < 0.005$), and TNFα and age ($r = 0.33$; $p < 0.001$). After 8 weeks of training, TNFα levels increased in the intermediate and competitive groups ($p < 0.05$), while IL-6 levels decreased only in the intermediate level group ($p < 0.05$).

Conclusion: the median levels of IL-6 and TNFα did not correlate with the caries index, BMI, training volume, experience practice, or cardiovascular risk. Salivary levels of IL-6 were downmodulated in the group with intermediate experience and TNFα was upmodulated by training.

PALABRAS CLAVE
Saliva;
Citoquinas;
Deporte juvenil;
Adolescentes;
Voleibol

Niveles salivales de interleucina 6 y factor de necrosis tumoral alfa en niñas de 7 a 17 años que practican voleibol

Resumen

Introducción: el objetivo del estudio fue evaluar las concentraciones salivales de interleukina-6 (IL-6) y factor de necrosis tumoral alfa (TNFα) y correlacionarlas con el índice de caries, el índice de masa corporal (IMC), potencia muscular de miembros inferiores (salto vertical), la aptitud cardiorrespiratoria y riesgo de desarrollar enfermedades cardiovasculares en niñas que practican voleibol.

Material y métodos: se realizaron dos estudios: 1) un estudio transversal (n = 120) sobre la asociación de IL-6 y TNFα con índice de caries, medidas antropométricas, pruebas físicas y experiencia en la práctica de voleibol; 2) estudio longitudinal (n = 63) sobre el efecto de 8 semanas de entrenamiento em IL-6 y TNFα salivales en niñas con experiencia intermedia en voleibol y chicas competitivas.

Resultados: los niveles medianos de IL-6 fueron 1.98 [1.55 - 3.11] pg/ml y TNFα fue 0.46 [0.28 - 0.59] pg/ml y no se correlacionaron con el índice de caries, el IMC, el volumen de entrenamiento, la práctica de entrenamiento y el riesgo cardiovascular. Se observó una correlación entre IL-6 y TNFα (r = 0.34; p <0.001), IL-6 y altura de salto vertical (r = -0.28, p <0.005), y TNFα y edad (r = 0.33, p < 0.001). Después de 8 semanas de entrenamiento, los niveles de TNFα aumentaron en los grupos intermedios y competitivos (p <0.05), mientras que los niveles de IL-6 disminuyeron solo en el grupo intermedio (p <0.05).

Conclusión: los niveles medianos de IL-6 y TNFα no se correlacionaron con el índice de caries, el IMC, el volumen de entrenamiento, la experiencia práctica y el riesgo cardiovascular. Los niveles salivales de IL-6 se modificaron a la baja en el grupo con experiencia intermedia y el TNFα se moduló al alza mediante el entrenamiento.

Introduction

Increased serum levels of the inflammatory cytokines Interleukin-6 (IL-6) and Tumor-Necrosis Factor-alpha (TNFα) in children and adolescents are associated with a high body mass index (BMI), low habitual physical activity, low cardiorespiratory fitness, and low muscular strength.[1–5] Consequently, high circulating levels of these cytokines are also associated with the risk of the development of cardiovascular diseases, type II diabetes, and metabolic syndrome.[2,6,7] On the other hand, the practice of physical exercises or sports activities can reduce the serum concentration of these mediators, improving physical fitness and reducing risk factors for metabolic and cardiovascular diseases.[1,5,8–10]

Although high levels of circulating inflammatory cytokines are correlated with obesity, low physical fitness, and time in moderate-to-intense activities in children,[1,11,12] it has not been established whether salivary levels of these mediators might also be associated with these parameters. The use of saliva samples for monitoring risk factors for inflammatory diseases in children and adolescents may present practicality, low cost, and acceptability as the collection is simple and noninvasive.[13,14] Thus, salivary tests could have applicability in the diagnosis and monitoring of individuals as an alternative to blood collection, especially when many consecutive collections are required, and in pediatric patients.[15,16] However, caution should be applied when evaluating the levels of salivary inflammatory cytokines, since these may be altered by the presence of oral lesions.[17–19] Indeed, increased levels of IL-6 and TNFα can be observed in the saliva of children with active dental caries and periodontal inflammation.[17,18,20] Thus,

oral health should be evaluated when using salivary samples for immunological analysis.

Inflammatory cytokines have been investigated in saliva samples as an alternative for monitoring the inflammatory status in pediatric patients.[14,20,21] A low correlation has been reported between salivary inflammatory mediators, including IL-6 and TNFα, and their serum concentrations in healthy subjects.[22] Indeed, production of cytokines seems to be compartmentalized in different body fluids, presenting different values of concentration against immunological challenges.[21,23] However, both serum and salivary IL-6 were associated with BMI in obese/overweight children, class II and III obese adults, patients presenting carotid atherosclerosis, and type-2 diabetic patients, at rest.[7,9,13,16,19,24,25] These studies suggest salivary IL-6 may be a candidate marker for monitoring inflammatory disorders and cardiovascular risk. Although IL-6 is chronically produced by leukocytes and adipocytes in an inflammatory response due to obesity,[7,26] it is also secreted by skeletal muscle contractions acting as an anti-inflammatory cytokine and metabolic sensor.[16,27] An acute bout of exercise can transiently increase serum and salivary IL-6, however, its secretion is not correlated between the two fluids.[27,28] A study demonstrated that increased training load periods can improve strength and increase resting log-transformed salivary levels of IL-6 in healthy adult men,[29] suggesting salivary IL-6 may be modulated by physical training. Considering the potential application of salivary IL-6 to monitor health status, physical fitness, and response to training, it may be a suitable non-invasive method to investigate health benefits of exercise practice in children.

IL-6 induces inflammatory reactions and loss of performance when chronically secreted in association with TNFα.[26,30,31] TNFα is also secreted by inflammatory leukocytes and adipocytes and high circulating levels are associated with cardio-metabolic risk in children and adolescents.[2,7,25] Salivary TNFα concentration was correlated with BMI, obesity, and metabolic syndrome, but not with physical activity patterns in adults and children.[13,25,32,33] An acute session of exercise can increase salivary levels of TNFα over 24 h post-exercise in healthy adults,[34] suggesting saliva cytokine secretion is modulated by exercise. Taken together, these results suggest salivary TNFα may also be a marker of inflammation and cardio-metabolic risk and can be acutely modulated by exercise. Although chronic physical exercise interventions reduce adiposity, improve physical fitness, and decrease serum levels of TNFα in obese children,[9,35] it is not clear if physical
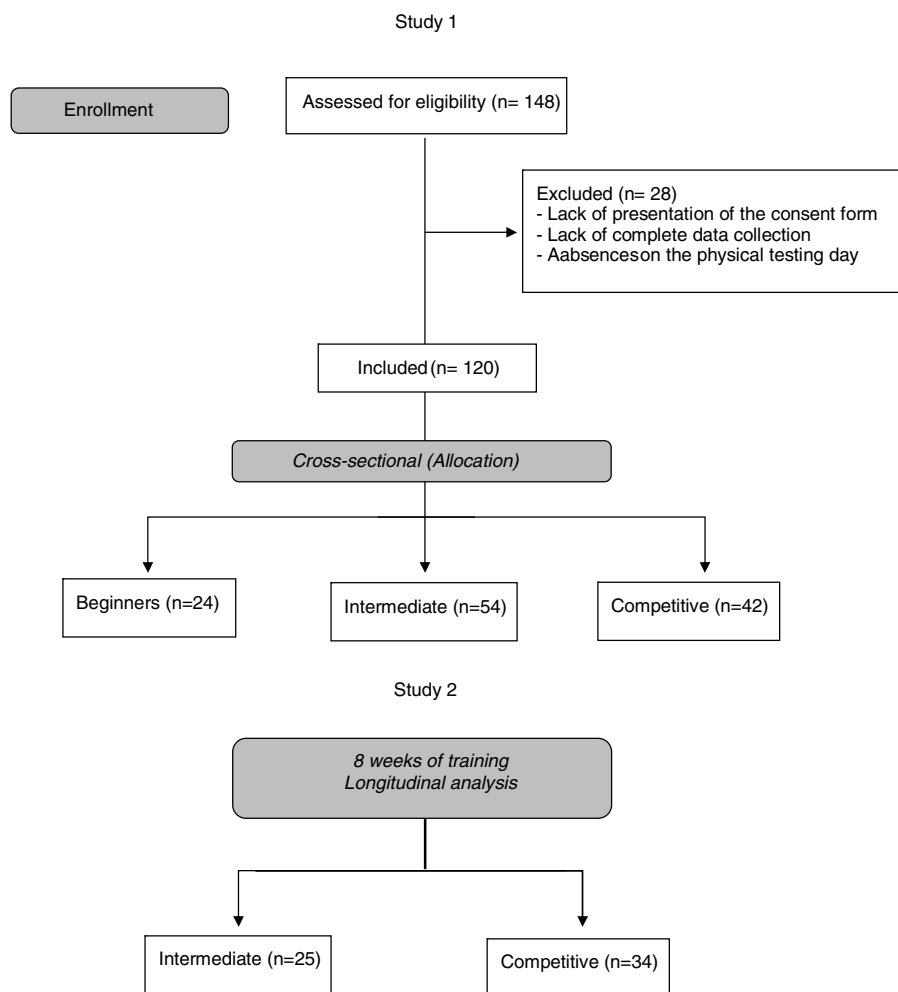

Figure 1 Flow diagram throughout the course of the study.

training could also alter salivary TNFα secretion. The response of salivary TNFα to a training program and its impact on cardiovascular risk factors in children is not known.

Considering the lack of information regarding the relationship between BMI, cardiorespiratory fitness, potency of the lower limbs, and the risk of developing cardiovascular diseases with salivary concentrations of IL-6 and TNFα, the objective of this study was to evaluate the correlation between these variables in children and adolescents enrolled in a volleyball sports activity program.

Materials and methods

Study design

This study was divided into two steps (Fig. 1):

Study 1: a cross-sectional study investigating the association of salivary levels of inflammatory mediators with the caries index (local factor), anthropometric measures, cardiorespiratory fitness, potency of lower limbs, and previous experience in Volleyball practice.

Study 2: a longitudinal study observing the effects of 8 weeks of volleyball training on salivary inflammatory mediators.

The study procedures were approved by the Research Ethics Committee Involving Human Beings of the State University of Londrina (protocol n$^\circ$ 821.804) and ethically conducted according to the Helsinki Declaration (2013).[36] All parents or legal tutors gave their written consent for the children to be included in the study.

To calculate the cross-sectional study sample size, the mean concentration of IL-6 in the saliva of children and adolescents in consecutive samples described by Riis et al. (2015)[20] was taken into account. The minimum number of 35 children per analysis group was initially calculated, considering 20% losses, a maximum α error of 5%, and 80% statistical power, in a context of a 0.5 effect magnitude using the software BioEstat 5.0 (Instituto de Desenvolvimento Sustentável Mamirauá, Tefe, Brasil).

Participants

Study 1

All girls participating in the Project of Sports Schools of the Department of Sports of the Municipality of Cambé - Paraná, Brazil, in August 2016, were invited to participate in the study. The project assists children enrolled in the public school system of the Municipality of Cambé and is carried out during the school semester, excluding the vacation period. The ages of the children ranged from 7 to 17 years, enrolled in a volleyball training program during school counter-hours. The girls were evaluated in the first month of training, after the period of school vacations (July school vacation), with a one month period of detraining. During the vacation period, the girls did not train in the project, or participate in sports classes at school, and reported no physical training or sports training. All girls were eligible after parent's authorization. The exclusion criteria were the presence of chronic inflammatory diseases, mucosal lesions, gingival bleeding, oral abscess, continued use of medication, diabetes, and

participation in other sports training programs. The initial evaluation constituted study 1, and the girls were submitted to anthropometric evaluation, saliva sampling, the counter-movement jump test (CMJ), and 20 m shuttle run test (Léger test). Regarding the experience in volleyball practice, the girls were classified into three groups (Fig. 1):

- Beginners (without previous experience): the girls that had no previous experience in volleyball practice and were not engaged in other sports activities. These girls had just started the training program, with less than 3 weeks of practice.
- Intermediate group ($\leq$12 months): minimum time of three months and maximum of 12 months of practice. The girls had participated in the sports program since the previous semester, but did not participate regularly in competitive events.
- Competitive group (>12 months): minimum of 13 and maximum of 84 months. The girls in the competitive group had participated in volleyball training for more than two school semesters and were engaged in local and state school game competitions.

Study 2

After the initial evaluation (cross-sectional study 1), the girls were followed during 8 weeks of volleyball training (longitudinal study 2) from August to October 2016 (Fig. 1). The girls were submitted to saliva sampling, CMJ, and Léger tests after 8 weeks of training. Only those girls who regularly attended the training sessions were included in the analysis for study 2, with up to two absences allowed during the training program.

Anthropometric evaluation

Height was measured using a stadiometer and body mass was evaluated with a digital scale (Omhron HBF 514C, Omhron Health Care do Brazil, São Paulo, Brazil). The body mass index was calculated using the formula BMI = mass (kg)/height^2 (m). The girls were classified according to BMI into: very underweight, underweight, normal, overweight, and obese according to the criteria adopted by the World Health Organization for girls between zero and 19 years of age.[37]

Oral health

The index of decayed, missing, and filled teeth for permanent or deciduous teeth (DMFT/dmft) was determined according to the criteria described by the World Health Organization (2013).[38] Oral examination was performed using artificial light by three trained examiners (two pediatric dentists and one periodontist) with an inter-examination Kappa coefficient of 0.99. The presence of oral mucous lesions, gingivitis, and spontaneous gingival bleeding were criteria for exclusion from data analysis.

Detection of salivary levels of IL-6 and TNFα

Saliva samples were collected at rest, 1 h after the last meal, between 9:00 AM and 10:00 AM or 2:00 PM and 3:00 PM. The girls were instructed to rinse their mouths for 1 min with drinking water prior to collection. Next, they were instructed to spontaneously salivate into sterile graduated

tubes for 2 min. The saliva samples were immediately placed on ice and frozen at −20 °C prior to use. The saliva samples were analyzed up to 48 h after sampling in order to avoid loss of stability of samples[39] and unstimulated whole saliva was used due to its lower variability and better accuracy in relation to stimulated saliva.[28]

The saliva samples were centrifuged at $4000 \times g$ for 5 min for sedimentation of cell debris. Next, the supernatant was collected and duplicates were submitted to an enzyme-linked immunosorbent assay to determine the salivary concentrations of IL-6 and TNFα, using commercial kits (Human IL-6 ELISA Set, cat. 555220 and Human TNF ELISA Set, cat. 550610, BD OptiEIA™, Becton and Dickinson Biosciences, Franklin Lakes, USA).

Countermovement jump test

Participants were previously familiarized with the CMJ test a week before jump performance analysis. The CMJ height and contact time were recorded on a force plate (Smart jump, Fusion Sports, Summer Park, Australia), according to the validated test.[40] The arms were required to be extended during the flight phase of the jump,[40] simulating the blocking movement in a volleyball game. The girls performed three CMJ, with 1 min intervals between attempts. The best jump was recorded for statistical purposes.

The 20 m shuttle run test (Léger test)

The maximum progressive test developed by Léger & Lambert in 1982[41] and modified by Léger et al. in 1984[42] was applied to estimate the maximum oxygen consumption (VO₂max).[41] The estimation of VO₂max was calculated using the formula described by Léger et al. (1988)[43] for children aged 8–19 years:

$$VO_2\ max = 31.024 + 3.238A - 3.248B + 0.1536AB$$

A is the speed attained in the final stage reached; B is the age.

The familiarization test was performed one week before data sampling. The cardiovascular risk range estimated by performance in the running tests for VO₂max estimation was established as suggested by Ruiz et al. (2016).[44] Children and adolescents with $VO_2max < 35\ mL\ kg\ min^{-1}$, or who did not reach stage 3 of the Léger test were considered to be at risk for cardiovascular diseases.[44]

Volleyball training

The girls performed weekly volleyball training practices, conducted in two to three sessions per week, lasting 120–180 min, depending on the child's previous experience in sports practice or engagement in competitive events.

The training sessions were conducted for 8 consecutive weeks and consisted of 10 min of warm-up, including jogging and running at submaximal speed, and ball throwing. The technical–tactical training consisted of 45 min of exercises, including sprints from the back of the volleyball court to the net, net attack and ball blocking, lateral displacement in the court with serving and passing the ball, and simulated games. The aerobic and anaerobic exercises comprised 20–45 min and included exercises with and without balls, such as 10-m sprints, jumps, agility test, ball throwing, and

resistance exercises. The training session was ended with 5 min of stretching exercises. All sessions were performed by a specialized exercise trainer.

Statistical analysis

The normal distribution of data was evaluated by the Shapiro–Wilks test. Data with Gaussian distribution are expressed as mean and standard deviation. Non parametric data are expressed as median and 25–75% interquartile range. The differences between groups were detected with the ANOVA *one-way test* and post hoc Tukey test (parametric distribution) or Kruskal–Wallis and Dunn tests (non-parametric data). Differences between children with high and low cardiovascular risk were analyzed with the Mann–Whitney U test. Categorical data were expressed in frequency and analyzed with the Chi-squared test with Yates correction or the Fisher exact test. The comparison between the variables before and after 8 weeks of training was performed with the paired-t test or Wilcoxon test. The associations between the salivary IL-6 and TNFα with the other variables were determined by means of multiple linear regression analysis. Predictor variables (age, caries index, BMI, training volume, group, VO₂max, jump height, and cytokine levels) were added to an unadjusted multiple linear regression model to identify the overall value of the determinant coefficient (r^2) and those that presented statistical significance ($p < 0.05$) were added to an adjusted model. Adjustments were performed using a stepwise approach, retaining predictor variables that presented statistical significance. The variance inflation factor did not detect multicollinearity between the predictors inserted in the final model. The correlation between cytokines and significant predictor variables was determined with the Spearman correlation test. Study variables were considered statistically significant if $p < 0.05$. GraphPad Prism 5.0 (GraphPad software, La Jolla, USA) and Epi Info 7.0 (Center for Diseases Control and Prevention, www.cdc.gov/epiinfo) were used for statistical analysis.

Results

Study 1: cross-sectional study

A total of 148 girls were enrolled in the volleyball program, of which 120 (81%) were evaluated; 28 (19%) were excluded due to lack of presentation of the consent form, lack of complete data collection, or absences on the physical testing day. Twenty-four (20%) girls were Beginners, 54 (45%) Intermediate, and 42 (35%) Competitive (Fig. 1).

The girls in the Intermediate and Competitive groups were older in relation to the Beginners (Table 1). The caries index (DMFT/dmft) was not different between the groups (Table 1).

Most of the girls were normal weight ($n = 85$, $19.3 \pm 2.4\ kg/m^2$), followed by overweight ($n = 14$, $23.7 \pm 2.1\ kg/m^2$), obese ($n = 14$, $29.8 \pm 5.7\ kg/m^2$), and underweight ($n = 7$, $14.8 \pm 1.5\ kg/m^2$), with similar frequencies between groups (Table 1).

The Beginners group had started training less than three weeks before the beginning of the study, and the weekly

Table 1 Characteristics of participants involved in volleyball practice ($n = 120$).

	Total	Beginners	Intermediate	Competitive
Age (years)	12.5 ± 2.3	11.8 ± 2.3	12.1 ± 2.1	13.5 ± 2.1[**,#]
DMFT/dmft	1.0 [0.5–2.5]	1 [0.5–1.5]	1 [0.5–2.5]	1 [0.5–3.0]
BMI (kg/m^2)				
Underweight	7 (5.8%)	1 (4.2%)	5 (2.4%)	1 (9.3%)
Normal	85 (70.8%)	17 (70.8%)	35 (78.6%)	33 (64.8%)
Overweight	14 (11.7%)	3 (12.5%)	8 (7.1%)	3 (14.8%)
Obese	14 (11.7%)	3 (12.5%)	6 (11.9%)	5 (11.1%)
Number of weekly training sessions	2 [2.0–2.5]	–	2 [2.0–2.5]	2 [2.0–3.5]
Volume of training sessions (minutes)	240 [180–330]	–	240 [210–240]	270 [240–630][*]
VO$_2$max (mL/kg/min)	39.8 ± 5.1	39.7 ± 5.9	40.3 ± 4.4	39.4 ± 5.8
CMJ height (cm)	28.1 ± 6.0	24.7 ± 3.7	27.2 ± 6.4	30.9 ± 5.0[**,#]
IL-6 (pg/ml)	1.98 [1.55–3.11]	2.08 [1.67–3.05]	1.94 [1.54–3.13]	1.71[1.48–3.33]
TNFα (pg/ml)	0.46 [0.28–0.60]	0.44 [0.25–0.61]	0.43 [0.29–0.56]	0.48 [0.28–0.65]

DMFT/dmft: decayed, missing, and filled teeth in permanent/deciduous dentition; BMI: body mass index; VO$_2$max: estimated maximum rate of oxygen consumption measured during incremental exercise (Léger test); IL-6: Interleukin-6; TNFα: Tumor necrosis factor alpha.

[*] $p < 0.01$.
[**] $p < 0.01$ Competitive vs. Beginners group.
[#] $p < 0.05$ Competitive vs. Intermediate group.

frequency and volume of training sessions were not included in the analysis. Weekly volume of training (minutes) was higher in the Competitive group (Table 1).

The average jump height was higher in the Competitive group, without significant differences in VO$_2$max estimated by the median stage completed in the Léger test (Table 1).

The median salivary concentrations of IL-6 and TNFα were not different between training groups (Table 1). No correlation between BMI and IL-6 ($r = -0.04$, $p = 0.67$, Spearman rank correlation) and BMI and TNFα ($r = -0.04$, $p = 0.67$, Spearman rank correlation) were observed.

The multivariate linear regression analysis, considering the salivary concentration of IL-6 as the outcome variable (Table 2), showed that only the salivary levels of TNFα and vertical jump height were related to salivary IL-6 levels. However, a low value for the coefficient of determination ($r^2 = 0.23$) suggests that the majority of variance expected in salivary IL-6 levels could not be attributed to independent variables. Salivary IL-6 and TNFα presented a moderate positive correlation ($r = 0.34$, $p < 0.001$, Spearman's rank correlation test). There was a weak negative correlation between IL-6 and vertical jump height ($r = -0.28$, $p < 0.005$).

The multivariate linear regression analysis, considering the salivary concentration of TNFα as the endpoint variable (Table 3), showed that only age and IL-6 levels were related to the salivary concentration of this cytokine. The very low value of coefficient of determination ($r^2 = 0.18$) after the model adjustments indicates

that BMI, training experience, and physical fitness had little contribution to variation in salivary TNFα. Spearman's correlation coefficient showed a moderate correlation between salivary TNFα concentration and age ($r = 0.33$, $p < 0.001$).

According to the criteria described by Ruiz et al. (2016), 13 (10.3%) of the girls were at risk of developing cardiovascular disease based on their performance in the Léger test. No differences in median IL-6 and TNFα concentrations were observed in girls with high and low cardiovascular risk (Fig. 2).

Study 2: Longitudinal study in 8 weeks of training

After the training weeks (post), 63 (52.5%) girls completed the evaluations. The number of withdrawals in the Beginners girls was 20 (83.3%), 29 (53.3%) in the Intermediate, and 8 (19%) among the Competitive practitioners. Three girls were excluded due to signs of respiratory tract infections and one had parotiditis. Considering that only four girls in the Beginners group completed the training period, they were not included in the final analysis.

Only the Competitive group presented improved VO$_2$max (Fig. 3a) and jump height (Fig. 3b) after 8 weeks of training (Post).

The concentration of IL-6 significantly reduced in the Intermediate group (Fig. 3c), whereas TNFα increased in both groups at Post (Fig. 3d).

Table 2 Multivariate linear regression analysis of salivary IL-6 concentration and study variables ($n = 120$).

	Multivariate Analysis		Adjusted multivariate analysis	
	Coefficient $\pm$ SD	p value	Coefficient $\pm$ SD	p value
Age (years)	0.10 ± 0.20	0.31		
DMFT/dmft	0.03 ± 0.16	0.84		
BMI (kg/m^2)				
Underweight	-0.42 ± 0.97	0.66		
Overweight	0.83 ± 0.87	0.34		
Obesity	-0.96 ± 0.90	0.29		
Weekly training volume (min)	0.00 ± 0.02	0.65		
VO$_2$max (mL/kg/min)	0.12 ± 0.08	0.15		
CMJ height (cm)	-0.13 ± 0.06	0.006	-0.08 ± 0.03	0.04
TNFα (pg/ml)	2.48 ± 0.68	0.0005	2.52 ± 0.62	0.0001
Training group				
Beginners/intermediate	0.70 ± 0.70	0.31		
Beginners/competitive	0.19 ± 0.75	0.80		
		$r^2 = 0.26$		$r^2 = 0.23$

DMFT/dmft: decayed, missing, and filled teeth in permanent/deciduous dentition; BMI: body mass index; VO$_2$max: estimated maximum rate of oxygen consumption measured during incremental exercise (Léger test); IL-6: Interleukin-6; TNFα: Tumor necrosis factor alpha.

Table 3 Multivariate linear regression analysis of salivary TNFα concentration and study variables.

	Multivariate analysis		Adjusted multivariate analysis	
	Coefficient $\pm$ SD	p value	Coefficient $\pm$ SD	p value
Age	0.03 ± 0.03	0.03	0.03 ± 0.01	0.004
DMFT/dmft	0.00 ± 0.04	0.89		
BMI (kg/m^2)				
Underweight	-0.04 ± 0.20	0.79		
Overweight	0.14 ± 0.14	0.30		
Obesity	-0.05 ± 0.14	0.73		
Weekly training volume (min)	0.00 ± 0.01	0.71		
VO$_2$max (mL/kg/min)	-0.01 ± 0.01	0.54		
CMJ height (cm)	0.01 ± 0.01	0.55		
IL-6 (pg/ml)	0.06 ± 0.01	0.0005	0.05 ± 0.01	0.0005
Training group				
Beginners/intermediate	-0.16 ± 0.11	0.15		
Beginners/competitive	-0.11 ± 0.12	0.33		
		$r^2 = 0.28$		$r^2 = 0.18$

DMFT/dmft: decayed, missing, and filled teeth in permanent/deciduous dentition; BMI: body mass index; VO$_2$max: estimated maximum rate of oxygen consumption measured during incremental exercise (Léger test); IL-6: Interleukin-6; TNFα: Tumor necrosis factor alpha.
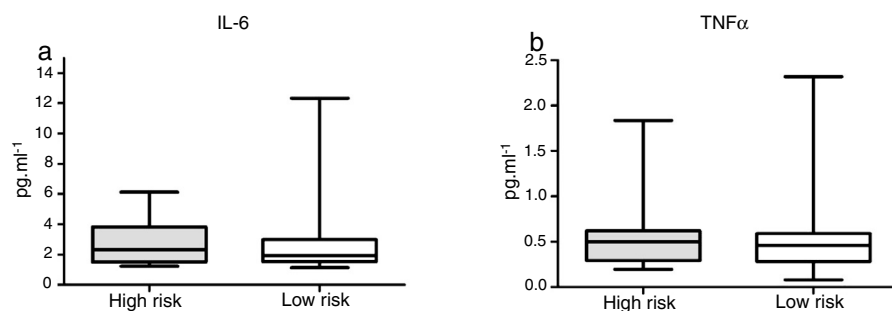
Figure 2 Median IL-6 and TNFα concentrations in girls with high and low cardiovascular risk.
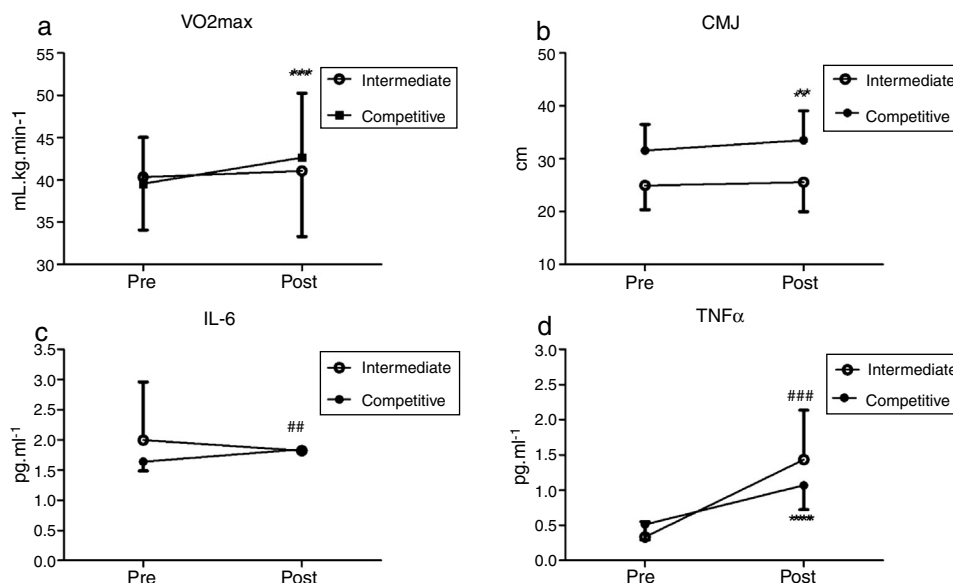
Figure 3 Competitive group for variables VO$_2$max (a), jump height (b); concentration of IL-6 (c), and TNFα (d).

Discussion

The use of salivary cytokines for monitoring inflammatory status in children and adolescents engaged in physical training demonstrated a lack of association with common risk factors for cardiovascular disease, such as BMI and low cardiovascular fitness. Moreover, it seemed to be differently modulated by physical training. The main results of the study demonstrated that local factors (dental caries) and cardiovascular fitness and BMI were not associated with salivary levels of IL-6 and TNFα. On the other hand, vertical jump (IL-6), and age (TNFα), correlated with inflammatory cytokines. Training significantly improved cardiovascular fitness and jump performance in Competitive girls. However, cytokine modulation was observed in both groups, as salivary IL-6 was downmodulated in the Intermediate group, whereas TNFα was significantly increased in both groups. These results suggest salivary inflammatory cytokines may be modulated by regular volleyball training in female children.

It is estimated that 15.3% of Brazilian children and adolescents are overweight and 8.9% are obese.[45] In the initial sample ($n = 120$ girls), approximately 28% of the girls were overweight and obese. In overweight and obese children aged 6 to 19 years, increased serum levels of IL-6 and TNFα were observed, considered inflammatory adipokines.[7,35,45] A study in healthy adults demonstrated that salivary TNFα is associated with increased BMI.[32] Contrary to expected, we did not observe a correlation between salivary cytokine levels and BMI in children. It may be suggested that salivary IL-6 and TNFα do not resemble secretion of adipokines from adipose tissues. However, one of the limitations of the present study was the absence of blood measurements to compare with salivary concentrations.

Plasma IL-6 is a myokine produced during muscle contractions, acting as an energetic sensor and anti-inflammatory stimulus, however, its salivary concentration seemed not to be correlated with circulating levels in healthy subjects and athletes at rest and after exercise.[26,28,46]

Nevertheless, IL-6 may be an important marker for fuel metabolism induced by aerobic exercise, increasing its salivary secretion during and after exercise.[27] Salivary IL-6 has also been suggested as a biomarker of inflammatory status, since it has been moderately correlated with plasma levels in the presence of systemic inflammatory conditions such as diabetes and obesity.[13,14,19,22,24] The measurement of IL-6 by immunoenzymatic assays has been validated using commercial kits designed for blood sampling, showing median values (0.73–4.0 pg/ml) close to those detected in the present study.[27,28,39] Menstrual cycle phase was not controlled since it did not significantly influence secretion of salivary IL-6 at rest and after exercise.[27] However, caution should be taken in evaluating saliva samples, since oral inflammatory diseases, active caries, and periodontal diseases can also increase salivary levels of IL-6,[14,19] suggesting local factors must also be investigated. In the present study, all children were evaluated by pediatric dentists and a periodontist to exclude children with signs of inflammatory oral lesions and to determine if the presence of dental caries altered IL-6 and TNFα levels. Absence of association between inflammatory cytokines and caries indices suggested that caries history did not impact significantly on salivary monitoring of these mediators.

No association was found between the salivary concentration of inflammatory cytokines and cardiorespiratory fitness and the risk range of cardiovascular diseases estimated by low VO$_2$max, suggested by Ruiz et al. (2016).[44] Contradictory results have been reported regarding the association of serum IL-6 with VO$_2$max in obese and normal weight children.[5,11,47] However, we did not observe an association between IL-6 and the cardiovascular disease risk range, cardiorespiratory fitness level, and BMI. Contrary to our initial hypothesis, the results suggest that IL-6 present in saliva is not a good marker of cardiovascular risk and aerobic fitness in children and adolescents. This may be due to the low correlation between IL-6 levels observed between saliva and serum samples from girls (11–17 years old).[21] Indeed,

studies demonstrating associations between BMI and salivary IL-6 reported the presence of diabetes and local periodontal inflammation, which may have contributed to overestimation of local IL-6 production in obese children and those presenting chronic systemic diseases.[14,20,24,25]

Muscle strength presented a negative correlation with serum IL-6 levels in children, adolescents and elderly patients.[5,24,32,48] In the present study, similar effects were detected for muscle strength in the lower limbs, demonstrating a negative correlation with salivary IL-6 levels. Chronic exposure to IL-6 causes activation of molecular signaling pathways of proteolysis (activation of the ubiquitin-proteasome pathway) and inhibition of protein synthesis (inhibition of mTOR-Akt signaling pathway by PGC-1α signaling), resulting in loss of muscle mass in experimental and epidemiological studies.[24,25,33] A study in adolescents (16 ± 1.4 years) of a volleyball team during a pre-competitive period showed that 7 weeks of training promoted a significant increase in vertical jump height and VO$_2$max and promoted a significant reduction in serum levels of IL-6.[10] Another study demonstrated a significant reduction in serum IL-6 after a three-month nutritional and physical education intervention in obese and normal-weight adolescents.[9] The negative association of jump height in the cross-sectional study and the reduction in IL-6 levels in the Intermediate training group suggest that salivary IL-6 levels are also modulated by physical training and may be negatively associated with lower limb potency in children and adolescents. In this group, the Intermediate group reduced salivary IL-6 concentration to values close to the Competitive girls, even when no significant improvement in performance was detected. A study in professional American Football players demonstrated IL-6 and strength performance increased after a high intensity training period, but the mean levels of the salivary cytokine were not statistically significant unless the authors performed log-transformation for statistical analysis.[29] The results of these athletes could not be compared to our study since two levels of training status and cardio metabolic risk were observed in the present study, and the girls were not exposed to high loads to intentionally evoke overreaching, as described by the authors.[29]

TNFα is an inflammatory cytokine, also secreted by adipose cells (adipokine), and its salivary level is increased in obese patients.[32,33] However, it has been demonstrated that increased concentration of salivary TNFα in obese children may be related to the presence of periodontal inflammation, commonly associated with obesity.[14,17,24,25] In the present study, caution was taken not to include children with sign of gingivitis, through systematic control of dental plaque and instructions of oral hygiene during the study. Thus, variation in salivary TNFα could not be attributed to poor oral hygiene or periodontal inflammation.

Salivary TNFα has been associated with local and systemic inflammation and obesity, without association with physical activity levels.[11,13,18,32] Pre-training levels of salivary TNFα found in the present study are close to those reported by other authors,[17] but significantly increased after the training period. Contradictory results have been published regarding the relationship of resting serum levels of TNFα with cardiorespiratory fitness and obesity in children.[8,11,47] In the present study, no association between estimated VO$_2$max and salivary TNFα was found in the pre-training analysis (cross-sectional study 1). Studies in obese and normal-weight adolescents submitted to a training period (5 and 7 weeks) of exercises at moderate to high intensity provoked an increase in serum levels of TNFα.[49,50] On the other hand, a 14-day nutritional and physical exercise combined intervention decreased serum TNFα in normal weight and obese children.[8] Although in the present study we did not control the training load and dietary habits, and no serum cytokine evaluation was performed, it was demonstrated that the training produced positive modulation of salivary TNFα. This modulation was not related to the initial physical status or BMI. Although it is not clear which mechanism increased the secretion of salivary TNFα in trained children, the physical habits of patient should be taken into account for monitoring children's inflammatory status by saliva analysis in healthy children. The results suggest that resting salivary TNFα levels increase in response to a training period.

The present study suggests that salivary IL-6 and TNFα, considered key inflammatory cytokines and markers of cardiovascular risk and metabolic disorders, were modulated by physical training in female children and adolescents, independently of BMI and oral inflammation. This reveals a compartmentalized local response of these inflammatory mediators, suggesting that healthy children engaged in regular physical activities may display elevated levels of salivary TNFα. Using these cytokines to monitor health status in children may not be appropriate, since increases in TNFα might suggest the development of a local inflammation. On the other hand, decreased levels of IL-6 and increased TNFα may be associated with regular engagement in physical activities or training in children, not resembling previous reported effects in serum.[5,32,49,50] Usually, IL-6 and TNFα levels increase during the inflammatory process, acting synergistically and in positive association with other inflammatory cytokines, such as Interleukin-1β, Interleukin-8, and the anti-inflammatory cytokine Interleukin-10.[13,20,23,32,33] As the cross-talk and interplay among these cytokines seemed to be body compartment-dependent,[23] future studies are necessary to investigate other salivary cytokines,[19] their local response to exercise, and their relationship with cardiovascular risk in children.

Conclusion

The salivary levels of IL-6 and TNFα were correlated with each other, but were not correlated with the caries index (local factor). The salivary cytokine levels were not correlated with factors pointed out by other studies as influencing systemic levels of inflammatory cytokines, such as BMI and cardiovascular risk. Salivary IL-6 was negatively related to lower limb muscle strength, and downmodulated by physical training, whereas salivary TNF was not associated with physical performance, but positively modulated by exercise. The results suggested that modulation of salivary IL-6 and TNFα were different, and lower levels of IL-6 could be achieved by training. Thus, caution should be applied when monitoring inflammatory salivary cytokines in children and adolescents engaged in regular physical activities. The upregulation of TNFα could be mistakenly interpreted as the development of

a local inflammation when children are included in a physical training program.

Conflict of interests

Authors declare that they don't have any conflict of interests.

Acknowledgements

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