Sport Sciences for Health (2021) 17:431–439
https://doi.org/10.1007/s11332-020-00719-2

ORIGINAL ARTICLE

Relationship between children physical activity, inflammatory mediators and lymphocyte activation: possible impact of social isolation (COVID-19)

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Received: 30 January 2020 / Accepted: 2 November 2020 / Published online: 23 November 2020

Abstract

Objective Lifestyle and body composition may be simultaneously responsible for immune response modulation. This study aimed to compare plasmatic adipokines concentration and lymphocyte cytokine production in children with different daily steps (DS) range, as well as to discuss the potential negative impact of the social isolation during COVID-19 pandemic in this context. DS can be a useful and low-cost way of monitoring children's health status.

Study design Fifty children were classified into clusters based in DS measured by pedometer: Sedentary Group (DS = 9338 ± 902 steps) and Active Group (DS = 13,614 ± 1003 steps). Plasma and lymphocytes were isolated and cultured to evaluate cytokine production.

Results Sedentary group presented lower adiponectin (7573 ± 232 pg/mL), higher leptin (16,250 ± 1825 pg/mL) plasma concentration, and higher lymphocyte production of IL-17, IFN-gamma, TNF-, IL-2 in relation to active group, suggesting predominance of Th1 response. Otherwise, the active group presented higher lymphocyte supernatant concentration of IL-10 and higher regulatory T cell (Treg) percentage.

Conclusion These results indicate that lymphocytes of children performing higher DS have an anti-inflammatory profile, especially of Treg. Besides, the prolonged social isolation in children during the COVID-19 pandemic, limiting physical mobility and exercise, reduces DS and increases adiposity, which could impair the immune system function and raise the susceptibility to inflammatory diseases.

Keywords Childhood obesity · Physical activity · Inflammation · Adipokines · Social isolation · Mobility reduction

Introduction

Several studies have investigated the association of physical activity, obesity, and immune system. The World Health Organization (WHO) recommends to children and adolescents at least 60 min per day of moderate to vigorous physical activity (PA) aiming health promotion and prevention of diseases, including obesity. However, WHO estimates that more than 80% of adolescents are physically inactive and that 34 million children and adolescents aged between 5 and 19 years old were overweight or obese in 2016. Recent studies have shown that in children and adolescents it was observed a decrease of physical activity levels in the last years in several countries [1, 2].

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Nowadays, the wide world is crossing by an unprecedented pandemic coronavirus disease 2019 (COVID-19), which will undoubtedly have a negative impact on the reduction of physical activity and its protective role of the immune system. The World Health Organization has proposed that the crucial strategy to diminish the COVID-19 pandemic impact is social isolating and distancing (WHO, 2020), keeping people at home, and therefore limiting their physical mobility and exercise. Previous studies have indicated that the immune system function is modulated by physical exercise, depending on the intensity and duration of the exercise effort, promoting different adaptations, acutely, and chronically [1]. Although current statistics show an alarming and increasing number of people under physical inactivity and obesity under normal conditions, the great concern that this number will worsen during the quarantine period [2], this proposition is supported by studies that observed weight gain during school vacation periods with a consequent increase in the prevalence of overweight and obesity [3, 4].

Obesity deregulates energetic metabolism and hormonal function resulting in a low-grade inflammation [3, 4]. Adipocytes release several adipokines, such as leptin, adiponectin, and resistin, which lead to inflammation and insulin resistance [5]. In response to inflammation, the innate immune system activates naïve T cells, inducing them to differentiation [6, 7]. Regulatory T cells (Tregs) are important in the maintenance of tolerance, prevention of autoimmune disease, and control of inflammation. The correct responsiveness of these cells is import to immune homeostasis. Interleukin (IL)-6 and leptin inhibit regulatory T cells (Treg), and adiponectin induces IL-10 production. Nevertheless, in obese people, the opposite occurs, resulting in a lower production of IL-10 [8]. It is important to consider that obesity is the result of a complex interaction of genetic, environmental, social, hormonal factors in addition to physical activity [4]. Therefore, physical activity can bring a direct effect to overweight and obese children and adolescents, promoting a reduction of inflammation [9, 10].

In the last years, pedometer has been widely used in the scientific and practical contexts as a simple and accessible device able to indicate PA levels in different population groups, including children. However, there is no consensus on the most appropriate range of daily steps to indicate physically active children. Researchers have to aim to establish a relationship between the number of daily steps and physical activity level [11–15] and with anthropometric parameters [16, 17].

Herein, we hypothesized that the clusters composed of children who perform different mean of daily steps have different plasmatic concentrations of leptin and adiponectin and altered inflammatory responses through cytokines produced by lymphocytes. This study aimed was to compare leptin and adiponectin plasmatic concentration and cytokine production by lymphocytes in children with different daily steps range, as well as to discuss the potential negative impact of the social isolation during COVID-19 pandemic in this context.

Methods

Sample

The experimental procedures were conducted according to the Declaration of Helsinki after approval from the Ethics Committee of Cruzeiro do Sul University (Protocol CU/UCS-169/2012). All legal representatives were informed about the research and provided written consent before children being enrolled in the study. Fifty children participated in this study, composed of twenty-nine males and twenty-one females, all classified as pre-pubertal according to Tanner board proposed by Matsudo & Matsudo [18]. Children who presented a previous diagnosis of diseases (such as infections in general, leukemia, asthma, and autoimmune diseases) or characteristics that could interfere in the results (use of medicines chronically) were not included.

The determining mean of daily steps

The number of daily steps (DS) was determined using *Digiwalker-Yamax* pedometers *SW 200* [19]. Children were evaluated for seven consecutive days and recorded the number of daily steps. It calculated the mean over this period. The children and responsible respective legal guardians were instructed about the correct use of the pedometer, and only the children who fulfilled all the guidelines were considered.

Determination of anthropometric parameters

Body mass index (BMI) was calculated using the formula: BMI = Body Weight (Kg)/height^2 (m). For the nutritional profile, it was used benchmarks in *z score* based on BMI curves for boys and girls, proposed by the WHO in 2007 [20]. Body composition (percentage of fat, fat body mass, and lean body mass) was estimated using the bioimpedance analysis (BIA). The BIA measurement was performed on the right side of the body, with the child lying supine on a non-conductive surface in a room with a normal temperature (~ 22 °C), and the protocol followed the instruction manual.

Blood collection

After overnight fasting (8 h), blood was collected from the antecubital vein into tubes containing ethylenediaminetetraacetic acid (EDTA, 1 mg/mL). After, the blood was

centrifuged at 4 °C, 400 g, for 10 min. Plasma samples were separated and frozen (− 80 °C) for further assays.

Lymphocyte isolation

Lymphocytes were isolated from peripheral blood. After plasma separation, blood was diluted in phosphate-buffered saline (PBS, pH 7.4) (1:1), suspended in Histopaque-1077 (Sigma Chemical Co., St. Louis, MO, EUA), and centrifuged for 30 min, 400 g, at room temperature. Peripheral blood mononuclear cells (PBMC; a mixture of monocytes and lymphocytes) were collected from the interphase. Remnant erythrocytes were lysed with 150 mM NH_4CI, 10 mM $NaHCO_3$, and 0.1 mM EDTA, at a pH of 7.4. PBMCs were washed once with PBS and maintained in RPMI-1640 medium to allow adherence of the monocytes to the plates for further isolation of pure lymphocyte population from the supernatant (approximately 98% lymphocytes). The number of lymphocytes was determined using a Neubauer chamber under an optical microscope (Nikon, Melville, NY).

Primary culture of human lymphocytes

The isolated lymphocytes were cultured in RPMI-1640 supplemented with 10% fetal bovine serum-containing antibiotics (10,000 U penicillin and 10 mg/L streptomycin medium) at the concentration of 1×10^6 cells per mL. At the beginning of the culture, human lymphocytes were stimulated with concanavalin A (5 µg/mL) (ConA) at 37 °C and 95% air/5% CO_2 atmosphere for 24 h. ConA is a mitogenic agent of T lymphocytes that binds to glycoproteins complexed to T cell receptor (TCR), promoting the activation of these cells. After this time, the cell suspension was centrifuged (400 g for 10 min), and the supernatant was collected and stored at − 70 °C.

Determination of cytokines in the supernatant

TNF-alpha, IL-17, IL-4, IL-6, IL-2, IL-10, and IFN-gamma concentrations in the supernatant were determined by Cytometric Bead Array (CBA), using the BD™ CBA Th1Th2Th17 Human Cytokine Kit (BD Biosciences) and a BD Accuri flow cytometer. Briefly, 25 µL of particles containing different fluorescent beads and covered with specific antibodies for the cytokines were added to 25 µL of diluted culture supernatant and incubated for 1 h, at room temperature in the dark. Afterward, 25 µL of the secondary antibody conjugated to a fluorochrome was added to the suspension, followed by the incubated for 2-h, at room temperature. At the same time, the standards for each cytokine were similarly used in the absence of the samples. The particles were washed to remove the unbound antibodies, suspended in washing buffer, and analyzed using the *BD Accuri* (BD Biosciences). The acquisition was made in BD-Accuri C6 Software, and the cytokine concentrations determined using the FCAP Software v.3.0 (BD, Biosciences). The limit of detection was 0.1 pg/mL for IL-2, 0.03 pg/mL for IL-4, 1.4 pg/mL for IL-6, 0.5 pg/mL for IFN-gamma, 0.9 pg/mL for TNF-alpha, 0.8 pg/mL for IL-17A, and 16.8 pg/mL for IL-10.

Determination of T regulatory cells (Treg)

Lymphocytes were suspended in PBS and labeled with FITC-conjugated anti-CD4 and APC-anti-CD25 (1:50) (Becton Dickinson, San Juan, CA). Cells were incubated for 30 min at room temperature in the dark. Negative control cells were incubated with isotype-matched nonreactive IgG1 antibody. Thereafter, lymphocytes were washed with PBS and analyzed using a BDAccuri flow cytometer (Becton Dickinson, San Juan, CA). Twenty thousand events were analyzed per experiment. Fluorescence from cells presenting FITC was evaluated using Diva software (Becton Dickinson). The percentage of Treg cells was determined by the evaluation of $CD4^+$, $CD25^+$, and $Foxp3^+$-positive cells. Intranuclear staining of Foxp3 was performed after fixation and permeabilization, according to the manufacturer's protocol, and subsequently incubated with the specific antibody (Becton Dickinson).

Adiponectin and leptin plasma concentration

Plasma levels of adiponectin and leptin were determined by the ELISA method using the Kit Human Total Adiponectin/Arcp30 Kit (R and D Systems) and Human Leptin Kit (R and D Systems) according to manufacturer's instructions.

Statistical analysis

A two-step cluster based in Euclidean distance and Schwartz's Bayesian criterion was used to classify the sample into the different groups according to the mean of daily steps. It was used Kolmogorov–Smirnov test with the correction of the Lilliefors test to verify the normality of the sample. The null hypothesis for normality was rejected for all analyzed variables. Data are presented as the mean ± standard deviation for differences between groups and were analyzed by the nonparametric test, unpaired *T* test (Kolmogorov–Smirnov). Spearman was used for correlation analysis. Statistical significance was set at 0.05, and the statistical analysis was carried in IBM SPSS Statistics for Windows (Armonk, NY: IBM Corp).

Results

The children were classified into two clusters based in the mean of daily steps (DS), where the cluster 1 (Sedentary Group) was characterized by lower mean of DS (9338 ± 902) and cluster 2 (Active Group) by highest mean of DS (13,614 ± 1003). The silhouette measurement of cohesion and separation pointed to good cluster quality (between 0.5 and 1.0) to a used method (two-steps cluster). Table 1 shows the means ± standard deviation for the daily steps, age, and anthropometric parameters (body mass, height, body mass index, fat percentage) for the whole sample and the two groups.

Based on BMI, 100% of overweight ($n=8$) and obese ($n=9$) children were allocated in the sedentary group, and 100% of the eutrophic children ($n=33$) were allocated in the active group. Additionally, all anthropometric parameters evaluated were significantly greater to the sedentary group compared to the active group. This tendency was confirmed by correlation analysis, a moderate inverse correlation ($r=-0.60$, $p<0.0001$) was observed between mean of DS and BMI (Fig. 1a) and between mean of DS and body fat ($r=-0.48$, $p=0.0004$) (Fig. 1b).

Table 1 Anthropometric data, body composition, and age from children separated according to daily step count

	Sedentary group ($n=17$)	Active group ($n=33$)
Daily steps	9,338 ± 902	13,614 ± 1,003*
Age (years)	9.00 ± 1.96	7.54 ± 1.70*
Body mass (Kg)	44.81 ± 13.15	25.41 ± 5.78*
Height (m)	1.37 ± 0.12	1.26 ± 0.11*
BMI (Kg/m^2)	22.98 ± 3.08	15.83 ± 1.54*
Fat percentage (BIA)	30.22 ± 0.99	18.17 ± 1.07*

Mean ± standard deviation

BMI body mass index, *BIA* bioimpedance

*$P<0.05$ *vs.* group 1

Analysis of adiponectin and leptin plasmatic concentration

We found differences for the concentrations of adiponectin (Fig. 2a) and leptin (Fig. 2b) between sedentary and active children. Sedentary group presented lower concentrations of adiponectin (7573 ± 232 pg/mL) when compared to active group (9995 ± 161 pg/mL). Leptin was higher for sedentary group (16,250 ± 1825 pg/mL) compared to active group (7816 ± 721 pg/mL).

These differences were reinforced when the total sample adiponectin plasma concentration and DS showed moderate correlation ($r=0.61$, $p<0.0001$) between adiponectin and DS (Fig. 3a) and moderate inverse correlation ($r=-0.52$, $p<0.0001$) between leptin plasma concentration and mean of DS (Fig. 3b).

Analysis of regulatory T cells (Treg)

Comparison of Treg cell percentage of children separated in the cluster according to the mean of daily steps demonstrated that cluster 1 (2.0 ± 0.3 pg/ml) shows lower Treg cells percentage than cluster 2 (8.0 ± 0.2 pg/ml) (Fig. 4). For the total sample, a strong positive correlation ($r=0.77$, $p<0.0001$) was found between plasma concentration of Treg cell and mean of DS, demonstrating that as the daily amount of steps increases, Treg cell population also increases (Fig. 5).

Analysis of cytokine concentration in cell supernatant

Concerning plasma cytokines analysis, no differences were found between the clusters (data not shown). However,

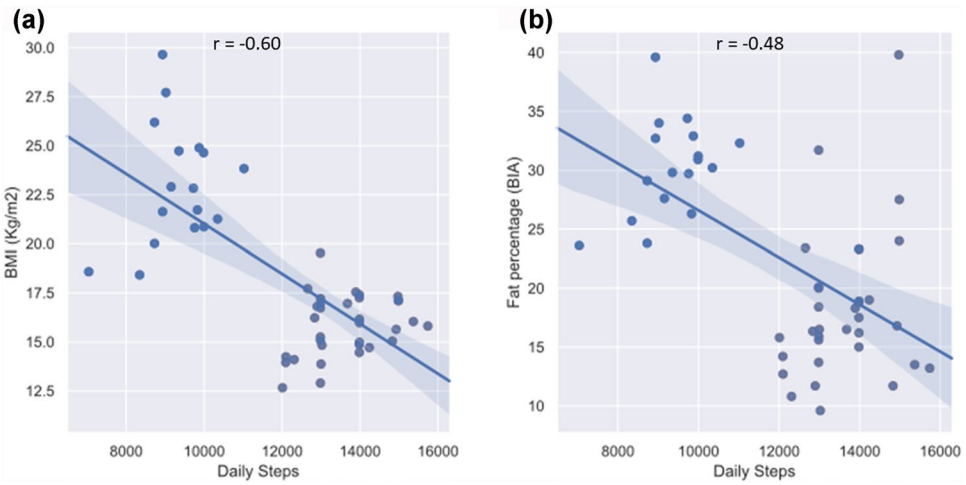

Fig. 1 **a** Correlation between BMI (Kg/m^2) and mean of daily steps for the total sample ($n=50$). The correlation analysis ($r=-0.59$; $p<0.0001$) indicates a moderate inverse correlation. **b** Correlation between percentage of fat (%) and mean of daily steps for the total sample ($n=50$). Correlation analysis ($r=-0.48$, $p=0.0004$) indicates moderate inverse correlation

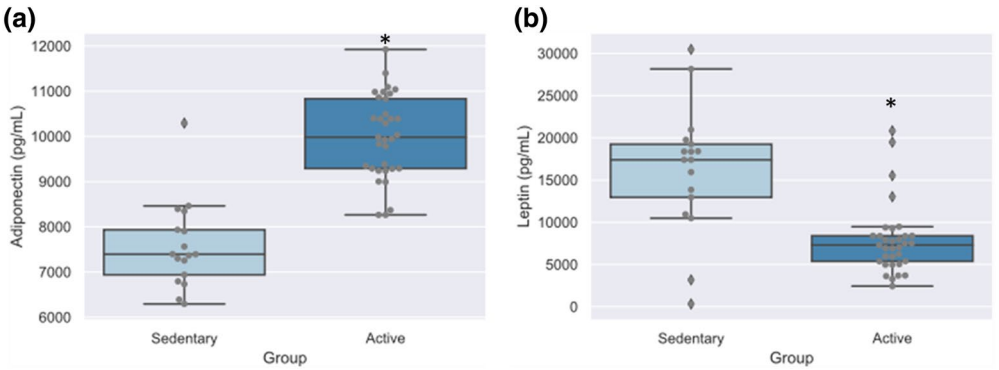

Fig. 2 Comparison (pg/mL) of adiponectin (**a**) and leptin (**b**) plasmatic concentration between sedentary and active groups. The plasma concentrations of leptin and adiponectin were determined using an Enzyme-linked immunosorbent assay (ELISA) method. The values presented are the mean ± standard error of mean (*$p < 0.0001$ versus sedentary group)

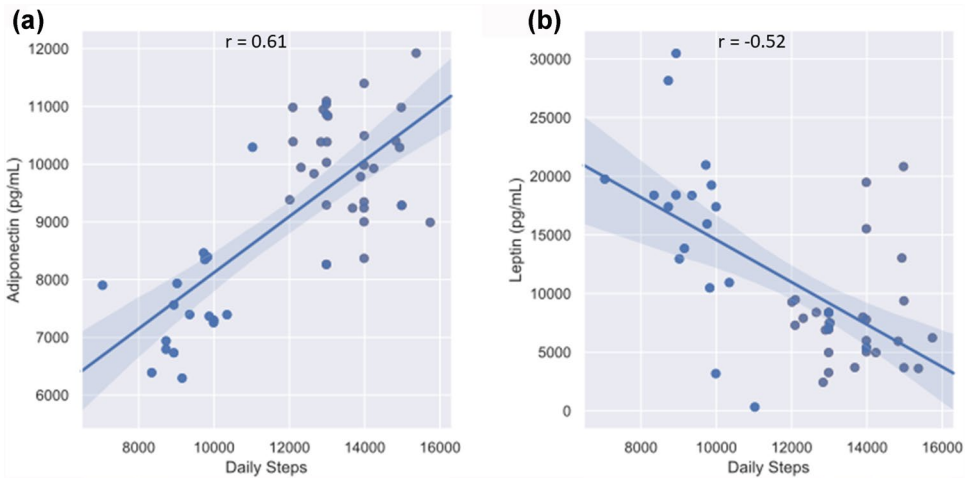

Fig. 3 **a** Correlation between plasma levels of adiponectin (pg/ml) and number of daily steps for the 50 children evaluated. Values of $r = 0.61$ and $p < 0.0001$ were found, indicating a moderate correlation between mean of daily steps and adiponectin plasma levels. **b** Spearman correlation between plasma levels of leptin (pg/ml) and the mean of daily steps for the 50 children evaluated. Values of $r = -0.52$ and $p < 0.0001$ were found, indicating a moderate inverse correlation between number of daily steps and plasma leptin values

it was found differences between sedentary and active group by lymphocytes in the presence of ConA. Sedentary group presented higher lymphocyte production of IL-17 (89,820 ± 29,500 pg/mL), IFN-gamma (18,010 ± 2013 pg/mL), TNF-alpha (1169 ± 220 pg/mL), IL-2 (292 ± 72 pg/mL) in relation to active group of IL-17 (49,840 ± 16,270 pg/mL), IFN-gamma (1643 ± 178 pg/mL), TNF-alpha (265 ± 36 pg/mL), IL-2 (27 ± 3 pg/mL), suggesting predominance of Th1 response. Otherwise, the active group presented higher lymphocyte supernatant concentration of IL-10 (3369 ± 775 pg/mL) that sedentary group (426 ± 165 pg/mL) For IL-4 and IL-6 concentrations, no significant differences were observed.

For the total sample analysis, correlations were found between cytokine concentrations in the lymphocyte supernatant and DS, with a moderate inverse correlation for IL-17 ($r = -0.55$, $p < 0.0001$), IFN-gamma ($r = -0.68$, $p < 0.0001$), TNF-alpha ($r = -0.46$, $p = 0.0009$), and IL-2 ($r = -0.62$, $p = 0.0186$), and moderate correlation for IL-10 ($r = 0.48$, $p < 0.005$).

Discussion

This is the first study to associate serum adipokynes/cytokines, lymphocyte activation parameters from children with daily steps. According to our data, daily steps can be a useful noninvasive and low-cost way of monitoring children's health status. Daily steps performance and physical activity levels are correlated [12, 21, 22]. A compliance of 12,000 steps as a threshold for achieving the WHO recommendations for physical activity [23]. In this study, the cluster analysis results allowed the definition of these two different groups as function of their lower (sedentary) or higher (active) level of physical activity grounded on the WHO recommendations.

Children's physical activity levels were distributed in two groups, based on the daily steps performed per day in a typical 7-day week. One of the groups showed limit values between 7039 and 11,023 daily steps, while the other showed daily steps limits between 12,009 and 15,739.

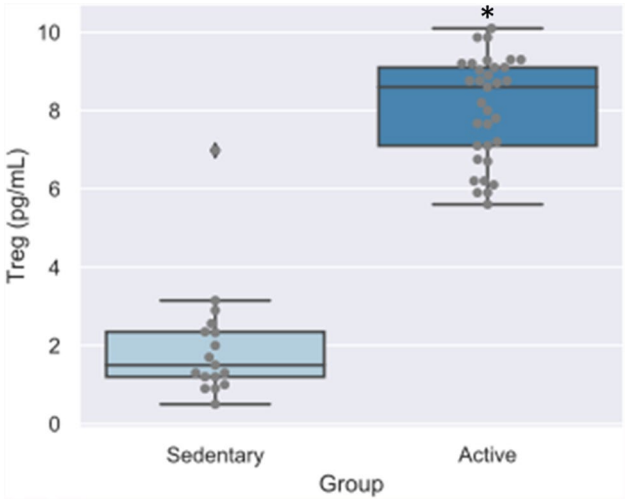

Fig. 4 Comparison of T regulatory cells percentage of children separated in cluster according to the mean of daily steps. Cells were pelleted and labeled with FITC-conjugated anti-CD4, APC-conjugated anti-CD25, and PE-conjugated anti-Foxp3 and analyzed by flow cytometry. The values are presented as the mean ± S.E.M. *$p < 0.0001$ vs. cluster 1

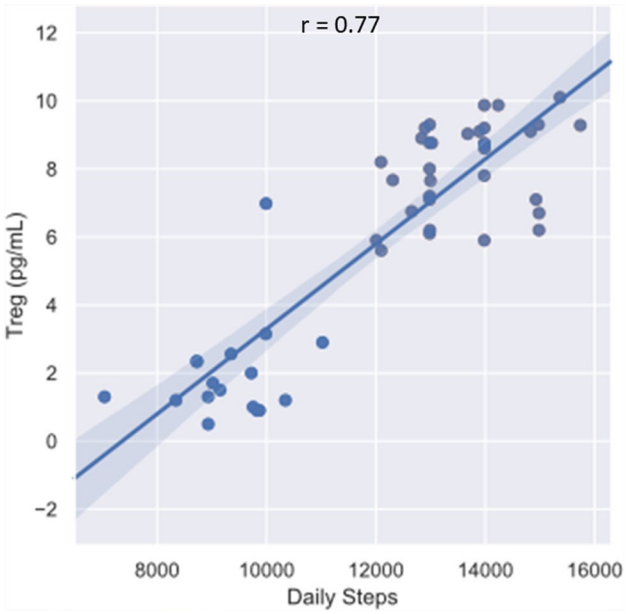

Fig. 5 Correlation analysis between Treg cells and the mean of daily steps for the 50 children evaluated. Cells were pelleted and labeled with FITC-conjugated anti-CD4, APC-conjugated anti-CD25, and PE-conjugated anti-Foxp3 and analyzed by flow cytometry. Values of $r = 0.77$, $p < 0.0001$ indicating a strong correlation between mean of daily steps and Treg cells

According to cluster division, 100% of overweight and obese children were allocated in the cluster 1–100% of the eutrophic children were allocated in cluster 2. Also, we found higher body fat in cluster 1 compared to cluster 2 and an inverse correlation of daily steps in the total sample with body fat and BMI corroborating with previous data [16, 17].

Another important fact observed in the population studied is that children from cluster 1 presented a lower concentration of the adiponectin and higher of leptin in relation to children from cluster 2. We also found a positive correlation between mean of DS and adiponectin and negative correlation with leptin concentration. The increase of leptin concomitant with adipose tissue has been widely reported in the literature [5, 24, 25]. However, there is still a gap to be explored between the influence of physical activity on adipokines production, especially about adiponectin and leptin. A previous meta-analysis indicated an association of exercise with increased adiponectin levels in more extended duration exercise programs, through body fat reduction [26]. The present study findings corroborate to this meta-analysis, even though we focused on how the levels of physical activity (PA), defined by DS, would affect these parameters.

We also investigated how different levels of PA could interfere in the cytokine production by lymphocytes from children analyzed in this study. Regarding plasma levels, we did not find any significant difference between the groups for any of the cytokines determined. When lymphocytes were stimulated with ConA, it was found a significant positive difference between the sedentary and active groups for IL-17, INF-gamma, TNF-alpha, IL-10, and IL-2. This relationship is even more evident when we observe a negative correlation between the concentration of IL-17, IFN-gamma, TNF-alpha, and IL-2 with daily steps. INF-gamma and TNF-alpha are cytokines with inflammatory characteristics secreted by Th1 cells [27]. Besides that, it has been reported that IL-2, as well as other cytokines, such as INF-gamma and TNF-alpha, are elevated in obese individuals [28], which presents low levels of PA. On the other hand, only IL-10 was higher in lymphocytes supernatant for the active group. IL-10 is related to anti-inflammatory response and promotes the differentiation of Treg cells [29]. Its expression is also stimulated for adiponectin [30].

Treg cell percentage was also higher for the active group and presented a higher positive correlation with DS. Treg cells are an anti-inflammatory T cell subtype with a unique phenotype controlling proliferation of effector T cells [3]. The present findings of Tregs show the important relationship between higher PA level and Treg cell concentration. Similar results previously found an association between lifestyle factors, such as PA, with immune system homeostasis and shift the balance of the immune system toward a more anti-inflammatory state by increasing Treg cells [31]. Although there are reports of a lower percentage of Treg cells in overweight children when compared with eutrophic, there was no evidence on the effect of physical exercise or PA levels on the Treg cells [32]. Thus, the present study

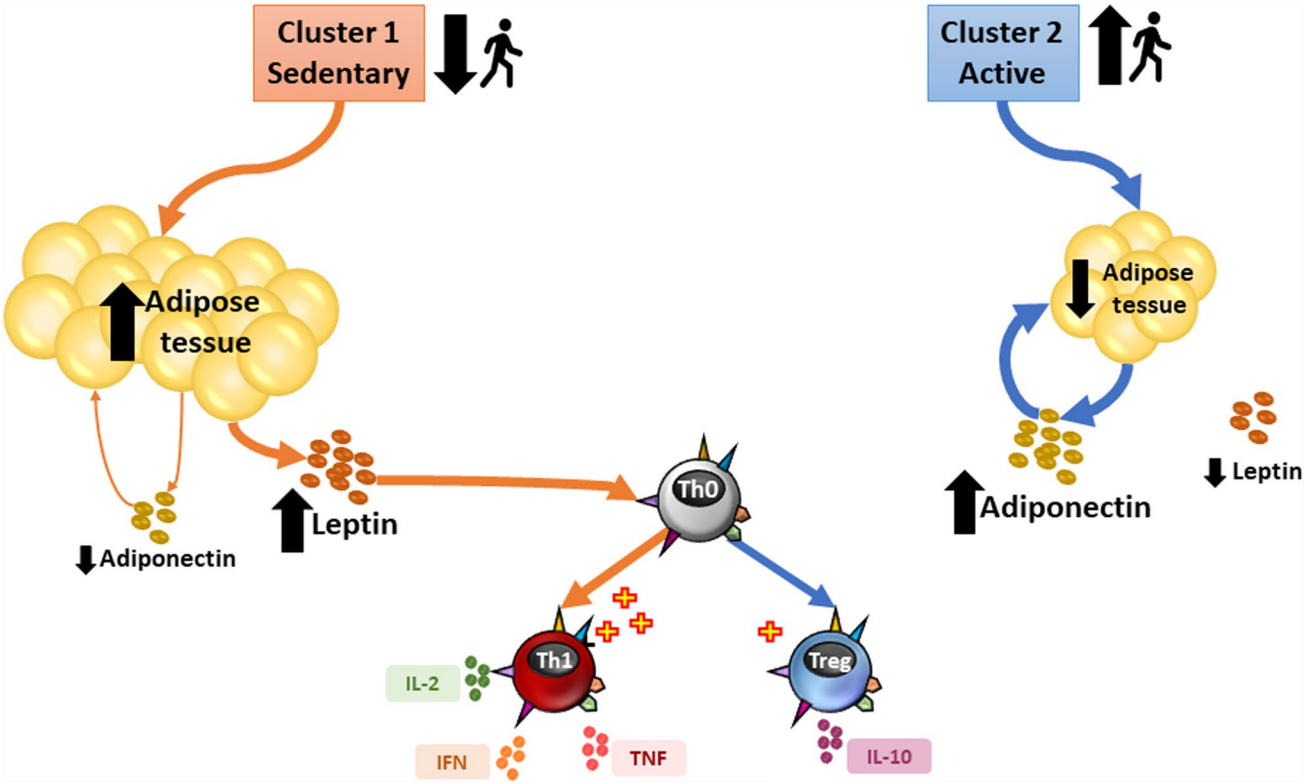

Fig. 6 Summary of the results involving analysis of relation between children daily steps, adipokines and lymphocyte response

brings novel information about the interaction between PA and the immune system.

The results about the production of inflammatory mediators secreted by lymphocytes or adipokines according to different levels of PA are also new information related to this issue. The sedentary group presented lower adiponectin and higher leptin plasmatic concentration, as well as higher lymphocyte supernatant concentration of IL-17, IFN-gamma, TNF-alpha, and IL-2. It suggests that children less physically active tend to present predominance of Th1 response for lymphocyte differentiation. The leptin produced by adipose tissue may contribute to the higher production of IFN-gamma and TNF-alpha by lymphocytes. Lymphocytes have receptors for leptin that favor their proliferation [33]. These findings partially confirm previous results about the significant correlation between leptin levels and IFN-gamma levels in obese asthmatic children, suggesting that leptin may mediate the increase of this cytokine, contributing to the Th1 response [34]. On the other hand, the active group presented higher adiponectin and lowered leptin plasmatic concentration, higher lymphocyte supernatant concentration of IL-10, and higher Treg cells concentration. In this case, the results suggest that children physically active tend to present anti-inflammatory response, especially of regulatory T cells. Nevertheless, it is important to highlight that level of PA and anthropometrics may simultaneously promote important immune responses, once the sedentary group presented mean of BMI value referent to overweight and obese children. In contrast, the active group showed BMI mean rated for eutrophic children. A summary of the results is represented in Fig. 6.

Considering our results found with the current scenario of the COVID-19 pandemic, while children are under social isolation [35] and consequent reduced physical mobility and exercise, we have an alarming situation. Children are further precluded from having physical activities, including those practiced in school and extracurricular environments, significantly reducing the daily physical activity level. Also, prolonged isolation of children at home could promote increased sedentary behavior and decreased regular physical activity and energy expenditure. Children could spend a long time sitting or lying down for sedentary activities, including playing games, watching television, and using mobile devices [36]. All these factors will be accentuated during a prolonged period of quarantine, exacerbating the problems caused by physical inactivity, such as those observed in the sedentary group, including overweight, obesity, and related diseases. Therefore, it is important to establish alternative ways to increase daily step counts during this period. As mentioned by [37] there are numerous exercise programs planned for practice with limited gym equipment and these

programs could be adapted by schools in the home lesson strategies.

In conclusion, children present mainly two different levels of PA based on daily steps mean, and these differences reflect on their body composition and inflammatory responses. The sedentary group children characterized by lower PA level presented higher BMI, body fat, and increased Th1 cells that and can be modulated by the higher production of leptin. The active group characterized by higher levels of PA have lower BMI, body fat, as well as higher Treg cell differentiation. These results suggest that DS can be a useful and low-cost way of monitoring children's health status. Besides, we discuss the adverse effects of the prolonged social isolating in children during the COVID-19 pandemic in reducing physical mobility and exercise, increasing sedentary behavior, and elevating the adiposity, which could impair the immune system function and raise the susceptibility to infectious diseases.

Acknowledgements We grateful Paula Bresciani Martins Andrade, and Rebeca dos Passos Costa for technical assistance. Cruzeiro do Sul University, Curtin School of Pharmacy and Biomedical Sciences and CHIRI (Curtin University), São Paulo Research Foundation (FAPESP, Process No.: 2018/07283-1), Coordination for the Improvement of Higher Level Personnel (CAPES), and National Council for Scientific and Technological Development (CNPq) supported this study.

Compliance with ethical standards

Conflict of interest The authors declare no conflict interest.

Ethical approval The experimental procedures were conducted according to the Declaration of Helsinki after approval from the Ethics Committee of Cruzeiro do Sul University (Protocol CU/UCS-169/2012).

Informed consent All legal representatives were informed about the research and provided written consent before children being enrolled in the study.

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