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Original Article

Chlorella improves inflammatory profiles and quality of life of prediabetes and diabetes patients

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ABSTRACT — The long-term effects of Chlorella doses on the inflammatory status and quality of life (QoL) of individuals with type-2 diabetes (T2D), and prediabetes (pre-T2D), and of nondiabetic controls were investigated. Chlorella was administered for 12 months; 1.6 g/day for the first six months and 3 g/day for the following six months. The inflammatory profile was studied by quantification of cytokines, adipokines and incretins. QoL was evaluated using the Short Form-36 health survey questionnaire (SF-36). Evaluations were performed at baseline, 6 (T6) and 12 (T12) months after initiating Chlorella intake. At baseline, QoL was more deeply impacted in T2D, a similar proinflammatory profile was observed in T2D and pre-T2D. In both, at T6 and T12, Chlorella modulated the altered levels of adipocytokines and incretins towards healthy values, and significantly improved QoL. Moderate correlations between the modulation by the alga and enhancement in QoL were observed only in the T2D group. In the nondiabetic control group, Chlorella improved QoL vitality and mental health scores. No differences were found between the two doses. Our results illustrate Chlorella adaptogen activity on inflammatory pathways and suggest its promising use as a complementary alternative in treating diseases related to insulin resistance in a wide range of chronic low-grade systemic inflammation-related diseases. Moreover, Chlorella increased QoL in all groups, the ultimate goal of all healthy interventions. Altogether, our findings suggest that one core mechanism involved in the homeostatic response produced by Chlorella is related to its rich content of carotenoids, operating mainly through inhibition of the NF-κB signalling pathway.

Key words: Chlorella, Type-2 diabetes, Prediabetes, Adipocytokines, Incretins, Quality of life

INTRODUCTION

Type-2 diabetes (T2D) is a state of chronic low-grade systemic inflammation that is deeply involved in the activation of inflammatory pathways and the development of insulin resistance, characterised by increased circulating concentrations of proinflammatory cytokines (Makki *et al.*, 2013; Gonzalez *et al.*, 2018). It is associated with high mortality, morbidity, and loss of quality of life (QoL) (Al Hayek *et al.*, 2014). Therefore, improved QoL is considered one of the primary goals in treating this disease (Trikkalinou *et al.*, 2017). In this context, the search for alternative therapies capable of improving QoL by modulating the disturbances observed in T2D is receiving increasing attention (Yang *et al.*, 2013; Chang *et al.*, 2013; Ríos *et al.*, 2015). Chlorella, a microscopic single-

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celled freshwater alga, containing all the ingredients necessary to promote human health (Bito *et al.*, 2020), has emerged as an alternative prophylactic agent for diseases related to insulin resistance (Torello *et al.*, 2016b; Vecina *et al.*, 2014).

Studies from our laboratory clearly illustrate the adaptogen modulation produced by the alga Chlorella, as shown by its ability to restore homeostasis (Vecina *et al.*, 2014; Torello *et al.*, 2016b; Queiroz *et al.*, 2008b; Torello *et al.*, 2016a). Using different experimental models of immunosuppression (Queiroz *et al.*, 2008a; Queiroz *et al.*, 2003; Ramos *et al.*, 2010; Queiroz *et al.*, 2011) and chronic low-grade systemic inflammation, represented by obesity in T2D mice (Torello *et al.*, 2016a), we demonstrated that the core mechanism by which the alga acts as an adaptogen is direct modulation of cytokine production. In the immunosuppression models, the alga restored the reduced production of proinflammatory cytokines as well as the increased production of anti-inflammatory cytokines to healthy control levels, thus inducing a shift towards the T helper-1 pattern of response, which is the main mechanism that governs the resolution of pathological processes responsible for the recovery of an immunosuppressed host. In contrast, in the obesity/T2D model, the alga restored the increased production of proinflammatory cytokines, as well as the reduced production of anti-inflammatory cytokines to healthy control levels, thus recovering the homeostatic response pattern that mitigates the obesity/T2D-associated state of chronic low-grade systemic inflammation.

Another relevant finding in our study in the obesity/T2D model is related to the fact that the production of proinflammatory cytokines is directly involved in the development of insulin resistance by disrupting the insulin signalling pathway through the phosphorylation of insulin receptor substrate (IRS)-1 in the serine residue, thus activating the nuclear factor-κB (NF-κB) signalling pathway (Makki *et al.*, 2013; Gual *et al.*, 2005). As discussed in more detail later, our findings demonstrate that the ability of Chlorella to prevent insulin resistance is related to its ability to increase phosphorylation of IRS-1 on tyrosine residues in the liver, skeletal muscle, and adipose tissue of obese mice, thereby lowering the phosphorylation levels of IRS-1^{ser307}, which is used as a marker of insulin resistance in obesity (Vecina *et al.*, 2014). Evidence suggests that a central mechanism by which Chlorella produces its homeostatic anti-inflammatory modulation in the organism is its rich content of carotenoids since these phytochemicals have anti-inflammatory properties through inhibition of the translocation of NF-κB to the nucleus and consequent inhibition of the downstream production of inflammatory cytokines (Murillo and Fernandez, 2016).

Based on these findings, our study evaluated the long-term modulating effects of low doses of Chlorella on T2D, pre-T2D, and nondiabetic controls. The results presented here highlight the promising use of Chlorella as an adjuvant in treating diseases related to insulin resistance, thus providing a venue for promoting the translation of this understanding into effective prevention strategies and new treatment regimens to improve human health.

MATERIALS AND METHODS

Ethics

This study was approved by the Ethics Committee of the University of Campinas (UNICAMP) and was conducted in accordance with the ethical standards of the Declaration of Helsinki (CAAE 30981114.4.0000.5404) and the Ethical Guidelines for Medical and Health Research Involving Human Subjects. The study was registered in the Brazilian Registry of Clinical Trials. All participants provided written informed consent before starting the study.

Study design and participants

T2D and pre-T2D patients were recruited from the Diabetes Group of the Community Health Center (CECOM) of UNICAMP, Brazil. They were diagnosed with T2D and pre-T2D by the clinical staff of the CECOM, according to the guidelines of the Brazilian Society of Diabetes Endocrinology (Sociedade Brasileira de Diabetes, 2014). To participate in the study, clinicians were required to be licensed in Campinas, Brazil, and practiced medicine for an average of 12 years.

Inclusion criteria, as required by the Ethics Committee, were as follows: only T2D and pre-T2D patients participating in the Diabetes Group of the CECOM were enrolled in the study; their biochemical parameters were within the normal reference range; participants would continue taking their prescribed treatment, which should be used for over 12 months at the onset of the investigations. The exclusion criteria were severe psychiatric illness, cancer, nephropathy, advanced cardiopathy, lung disease, type-1 diabetes, and a history of drug or alcohol abuse. Nondiabetic individuals were recruited from the clinical and administrative staff of CECOM, and the requirements were good health and unaffected by T2D and pre-T2D.

Chlorella tablets were administered for 12 months, during breakfast, eight tablets/day (1.6 g) for the first 6 months, and 15 tablets/day (3 g) for the next 6 months. Evaluations were performed at baseline, 6 (T6), and 12 (T12) months after initiating Chlorella intake. This dos-

age regimen was determined based on previous clinical trials (Mizoguchi *et al.*, 2008; Panahi *et al.*, 2012; Noguchi *et al.*, 2014; Azocar and Diaz, 2013; Merchant and Andre, 2001; Nakano *et al.*, 2007, 2010; Nagayama *et al.*, 2014; Miyazawa *et al.*, 2013b).

The Chlorella tablets were composed of 100% Chlorella powder (*Chlorella vulgaris* CK-5) that was cultured, manufactured, and supplied by Chlorella Industry Co. Ltd., Fukuoka, Japan. According to analysis, each tablet is composed of the following (/100 g): protein, 57.4 g; lipid, 8.6 g; total carbohydrates, 21.8 g; calories, 394 kcal; iron, 95 mg; saturated fatty acid, 1.67 g; monounsaturated fatty acid, 1.60 g; polyunsaturated fatty acid, 4.09 g; omega-3, 1.49 g; omega-6, 2.0 g dietary fiber, 11 g; vitamin B_1, 1.8 mg; vitamin B_2, 5 mg; vitamin B_{12}, 500 µg; vitamin C, 60 mg; folic acid, 2,500 µg; biotin, 300 µg; α-tocopherol, 30 mg; vitamin K_1, 3,000 µg; potassium, 1,000 mg; magnesium, 350 mg; chlorophylls, 3.2 g; lutein, 265 mg; β-carotene, 99.7 mg; zeaxanthin, 28 mg; α-carotene, 13.9 mg; linoleic acid, 2.0 g; and α-linoleic acid, 1.9 g (Vecina *et al.*, 2014; Maruyama *et al.*, 2018).

Sample collection and analytical methods

Blood samples were collected in sodium citrate, EDTA, or heparin between 8:00 and 10:00 a.m. after overnight fasting and processed immediately. Serum levels of tumour necrosis factor (TNF)-α, interleukin (IL)-6, and IL-10 (BD Biosciences, San Diego, CA, USA), monocyte chemoattractant protein (MCP)-1, adiponectin, leptin, resistin, and glucagon (R&D Systems, Minneapolis, MN, USA), glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), and insulin (Ray Biotech, Norcross, GA, USA) were quantified by ELISA in microtiter plates at baseline, T6, and T12. All parameters were determined according to the manufacturer's protocol; titers were expressed as pg/mL or ng/mL and calculated by reference to the respective standard curves.

Biochemical parameters (glycated haemoglobin [HbA1c], cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL), and triglycerides) were determined using a colorimetric method. Fasting blood glucose was analysed using the Clinical Chemistry System ERBA XL200-Mannheim. The insulin resistance index (HOMA-IR) was calculated using the formula: insulin (µU/mL) × glucose (mmol/L)/22.5, as previously described (Geloneze and Tambascia, 2006).

Clinical measurements

A questionnaire was administered to evaluate demographic and clinical characteristics, pharmacotherapy, habits, and use of drugs or alcohol. Side effects of Chlorella consumption were also evaluated. Body mass index was calculated according to the guidelines of the Brazilian Association for the Study of Obesity and Metabolic Syndrome (ABESO, 2016).

QoL evaluation

The Brazilian standardised version of the Short Form-36 Health Survey Questionnaire (SF-36) (Ciconelli *et al.*, 1999) was used to evaluate QoL. It is a physically and emotionally based instrument that consists of 36 items corresponding to eight health concepts: physical functioning (health limitations in all physical activities), physical limitation (problems with work or other activities related to physical health), pain (presence or absence of pain), general health perception (health from the patient's perspective), vitality (presence or absence of fatigue), social functioning (interference of physical or emotional difficulty in social activities), emotional limitation (problems with work or other activities related to emotional factors), and mental health (frequency of subjective feelings such as nervousness, depression, happiness) (Ware and Sherbourne, 1992; Ciconelli *et al.*, 1999). Eight subscales were constructed using the Likert method of summated ratings. Sub-scale scores were calculated according to standard procedures, with scores ranging from 0 to 100, with higher scores indicating better QoL. SF-36 was applied at baseline, T6, and T12.

Statistical analysis

Demographic and clinical characteristics are expressed as frequencies and percentages for categorical variables. For continuous variables, we used the mean and SD. As the study population did not follow a Gaussian distribution, the Wilcoxon signed-rank test for non-parametric paired data was used to compare the differences between the three time points evaluated (baseline, T6, and T12) in terms of QoL, biochemical parameters, and the levels of cytokines, adipokines, and incretins. The Mann-Whitney test for non-parametric unpaired data was used to compare differences between groups. Spearman's test was used for the correlations. Differences were considered statistically significant at $p < 0.05$. Analyses were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Population

As shown in Table 1, in the T2D group (n = 25), 80% were female, with a mean age of 58 ± 8 years. The prevalence of overweight/obesity was 84%, and 68% had

Table 1. Population characteristics.

Characteristics	Type-2 diabetes (T2D) n = 25	Prediabetes (pre-T2D) n = 20	Non-diabetic n = 20
Gender			
Female	20	8	16
Male	5	12	4
Age (years)			
< 60	12	16	20
≥ 60	13	4	0
mean ± SD	58 ± 8	53 ± 9	41 ± 11
min–max	36–71	33–73	21–58
Body mass index (kg/m^2)			
Normal ≤ 25	4	2	7
Overweight/obese > 25	21	18	13
Occupation			
Employed	14	17	20
Retired	11	3	0
Drug use			
None	0	5	20
Metformin only	10	13	0
Hypoglycemic therapy	8	2	0
Insulin	7	0	0
Hypertension			
Yes	17	9	0
No	8	12	0

hypertension. Twenty-eight percent used insulin, 32% used hypoglycaemic therapy (metformin plus glibenclamide, gliclazide, glimepiride, pioglitazone, or sitagliptin), and 40% used metformin alone. Fifty-six percent were employed, and 44% retired.

In the pre-T2D group (n = 20), 60% were men, with a mean age of 53 ± 9 years. The prevalence of overweight/obesity was 90%, and 45% had hypertension. Sixty-five percent used metformin alone, 10% used hypoglycaemic therapy (metformin plus glibenclamide, gliclazide, glimepiride, pioglitazone, or sitagliptin), and 25% received a treatment based on lifestyle changes. Eighty-five percent were employed, and 15% were retired. In the nondiabetic group (n = 20), 80% were female, with a mean age of 41 ± 11 years. The prevalence of overweight/obesity was 65%, with no cases of hypertension. All of them are listed in Table 1.

No significant (p > 0.05) changes in biochemical parameters were observed in any group throughout the study (Table 2), as well as in body weight and blood pressure (data not shown). In addition, the participants did not report any side effects related to gastrointestinal or other system during the study.

Cytokine, adipokine, and incretin levels

Although significant statistical differences ($p < 0.05$), compared to baseline, were observed at T6 and T12 between the changes produced by Chlorella intake on the serum levels of proinflammatory cytokines, adipokines, and incretins in the T2D and pre-T2D group, the same response pattern was produced by the alga in both groups, and no statistical differences were observed between them ($p > 0.05$). The results were as follows: TNF-α, IL-6, and MCP-1 were reduced at T6 and T12, whereas IL-10 levels were increased only at T6 (Fig. 1A–B); leptin and resistin levels were reduced at T12, whereas adiponectin levels were increased only at T6 (Fig. 2A–B). GLP-1 and GIP were increased at T6 and T12, whereas glucagon levels were reduced in both periods (Fig. 3A–B). In the nondiabetic group, no changes in the relatively lower values of the above-mentioned mediators were observed throughout the study ($p > 0.05$) (Fig. 1–3C).

QoL scores

At T6 and T12, out of the eight concepts evaluated by the SF-36 questionnaire, significant ($p < 0.05$) improvements were found in six in the T2D group (physical limitation, pain, general health perception, vitality, social functioning, and mental health) (Fig. 4A), two in the pre-T2D group (pain and mental health) (Fig. 4B), and two in the nondiabetic group (vitality and mental health) (Fig. 4C).

Table 2. Biochemical parameters (mean ± SD) in the study population following Chlorella intake.

PARAMETERS	Type-2 diabetes (T2D)			Prediabetes (pre-T2D)			Non-diabetic		
Reference values	Baseline	6 months	12 months	Baseline	6 months	12 months	Baseline	6 months	12 months
Glucose (mg/dL) *< 99*	119 ± 33	123 ± 38	139 ± 78	108 ± 24	105 ± 12	102 ± 12	86 ± 7	84 ± 7	83 ± 8
Insulin (uUI/mL) *3.2–16.4*	14 ± 9	17 ± 13	19 ± 9	13 ± 6	13 ± 6	15 ± 5	8 ± 4	8 ± 4	9 ± 8
HOMA-IR *2.71*	5.4 ± 7.6	5.3 ± 5.0	5.8 ± 6.5	3.7 ± 1.8	3.7 ± 2.0	3.9 ± 4.0	2.0 ± 0.9	2.0 ± 1.0	2.0 ± 0.5
HbA1c (%) *< 6.4*	7.8 ± 2.5	7.6 ± 2.0	7.7 ± 2.3	6.1 ± 1.0	6.0 ± 0.7	6.0 ± 0.5	5.4 ± 0.5	5.5 ± 0.4	5.5 ± 0.4
Cholesterol (mg/dL) *< 200*	172 ± 38	166 ± 39	169 ± 35	182 ± 38	179 ± 31	170 ± 26	188 ± 35	182 ± 36	184 ± 40
LDL (mg/dL) *100–129*	94 ± 29	91 ± 34	91 ± 31	104 ± 24	108 ± 29	97 ± 25	110 ± 31	109 ± 28	112 ± 31
HDL (mg/dL) *men ≥ 40; women ≥ 50*	50 ± 9	47 ± 9	48 ± 8	42 ± 11	40 ± 9	40 ± 9	55 ± 13	52 ± 12	50 ± 9
Triglycerides (mg/dL) *< 150*	148 ± 78	141 ± 66	160 ± 129	173 ± 78	156 ± 68	174 ± 81	106 ± 43	106 ± 53	108 ± 49

Correlations

The following moderate correlations between adipokines/cytokine modulation by Chlorella and improvements in QoL were present only in the T2D group: at T6, reduced levels of leptin correlated with better SF-36 scores on the concepts of vitality ($p = 0.02$; r = 0.502), reduced pain ($p = 0.022$; r = −0.495), and mental health ($p < 0.001$; r = −0.835), and lower levels of IL-6 correlated with better SF-36 scores in the concept of role limitation due to physical problems ($p = 0.046$; r = −0.441) (Fig. 5A). At T12, reduced levels of MCP-1 correlated with better SF-36 score in the concept of role limitation due to physical problems ($p = 0.022$; r = −0.551), reduced levels of TNF-α (p = 0.025; r = −0.527), and increased levels of IL-10 correlated with better scores in the concept of general health ($p = 0.032$; r = 0.506) (Fig. 5B). No correlations were found between the responses of the pre-T2D and the nondiabetic groups.

DISCUSSION

T2D is a state of chronic low-grade systemic inflammation deeply involved in the activation of inflammatory pathways and the development of insulin resistance (Gonzalez *et al.*, 2018). Chlorella significantly ameliorated the altered serum levels of proinflammatory and anti-inflammatory proteins released by adipocytes in both T2D and pre-T2D individuals. Considering that the production of proinflammatory cytokines is directly involved in the development of insulin resistance by disrupting the insulin signalling pathway through the phosphorylation of IRS-1 in the serine residue (Makki *et al.*, 2013; Gual *et al.*, 2005), our experimental findings showing the ability of Chlorella to upregulate the phosphorylation of IRS-1 on tyrosine residues and downregulate its phosphorylation on serine residues (IRS-1^{ser307}) in the liver, skeletal muscle, and adipose tissue, thus preventing the development of insulin resistance (Vecina *et al.*, 2014), are relevant to clarify the mechanisms involved in the protective effects of the alga in conditions related to insulin resistance.

We observed that, despite the pre-T2D condition being an intermediate metabolic state between healthy and diabetic glucose homeostasis, the levels of these proteins at the onset of the study were similar in this group to those observed in individuals with T2D. Thus, the activation of proinflammatory pathways and a state of chronic low-grade systemic inflammation, which is well characterised in the established condition of T2D, is already well developed in this intermediate state of pre-T2D. Moreover, the same pattern of modulatory response to Chlorella supplementation was observed in both groups, and no significant differences were observed between them. However, as described below, symptom scores indicative of QoL were higher and milder in the pre-T2D group, and correlations between these scores and the modulation by the alga on the levels of pro- and anti-inflammatory proteins were present only in the T2D group. These results corroborate findings in the literature showing that impairment of glucose metabolism is associated with increased diabetes-related symptom distress (Adriaanse *et al.*, 2008). Furthermore, self-reported data, in addition to being easily accessible and reliable, might correlate with disability

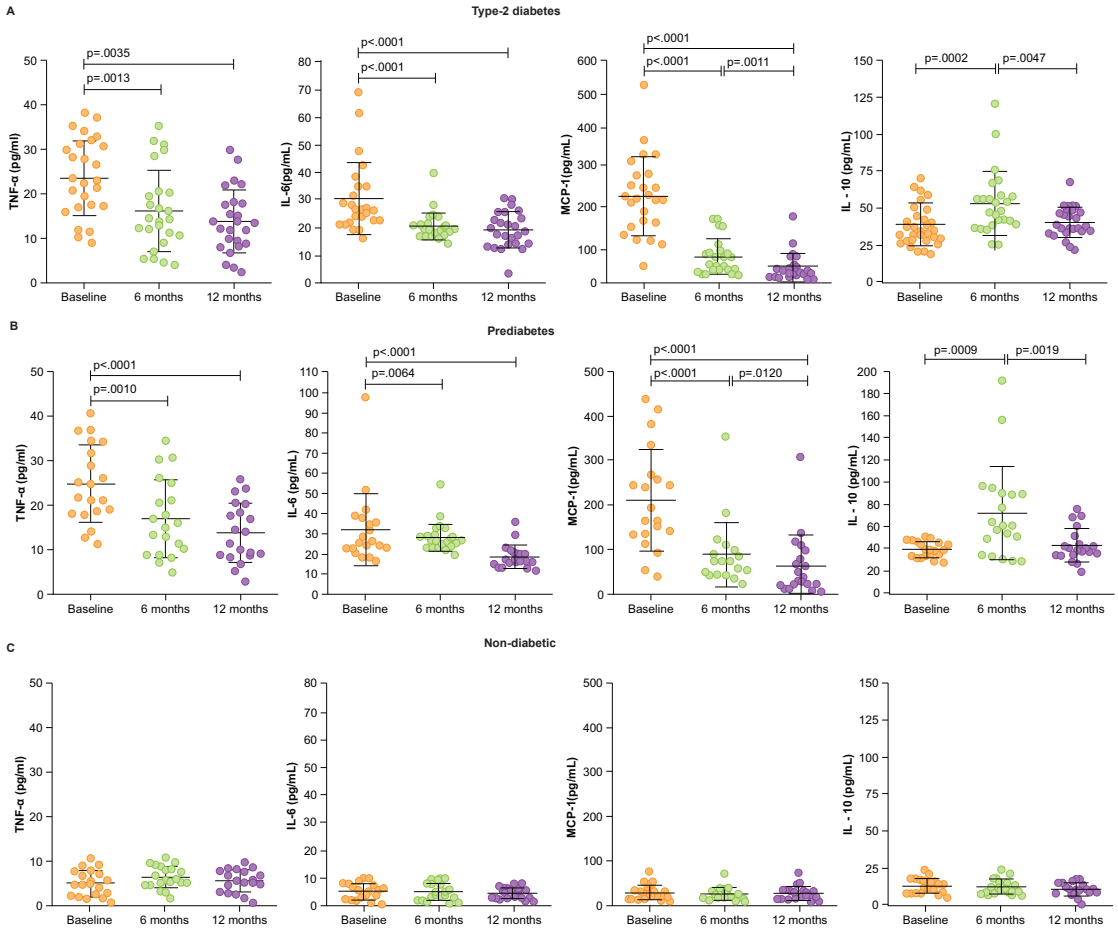

Fig. 1. Chlorella intake modulates inflammatory status in patients with type-2 diabetes (T2D) and prediabetes (pre-T2D) without affecting the nondiabetic group. Serum levels (mean ± SD) of cytokines (TNF-α, IL-6, MCP-1, and IL-10) in T2D (n = 25), pre-T2D (n = 20), and non-diabetic (n = 20) patients at baseline, and 6 and 12 months after initiating Chlorella supplementation. The values of "p" are represented in the figure; Wilcoxon signed-rank test.

burden at least as strongly as disease scores, thus supporting this kind of approach in comorbidity research and the clinical care of complex patients (Whitson *et al.*, 2009).

A similar pattern of response in both groups, observed after Chlorella intake at both doses, was represented by reduced TNF-α, IL-6, and MCP-1 at T6 and T12. These findings corroborate several studies that emphasise the pathological role of these cytokines in the development of insulin resistance, obesity, T2D, and diabetic complications (Panee, 2012; Liu *et al.*, 2016). Elevated levels of TNF-α are strongly related to chronic low-grade systemic inflammation and insulin resistance, which is aggravated by the induction of IL-6 (Makki *et al.*, 2013). It has been reported that IL-6 plasma levels may be up to three times higher in patients with obesity and T2D than in lean individuals (Padilha *et al.*, 2011). Recent findings showing the ability of Chlorella to downmodulate adipose tissue hypertrophy (Noguchi *et al.*, 2013) corroborate our findings of downregulation by the alga on the levels of IL-6, which is considered a marker for visceral adiposity (Fried *et al.*, 1998). Studies that have highlighted the significance of MCP-1 in the development of T2D and its complications suggest that its downregulation is a viable therapeutic target (Panee, 2012).

The same kinetic profile was also observed for both anti-inflammatory proteins, adiponectin, and IL-10, in both T2D and pre-T2D groups, which were upregulated by the alga only at T6. This finding may be partly explained by the fact that adiponectin enhances the expression of IL-10 (Menzaghi and Trischitta, 2018).

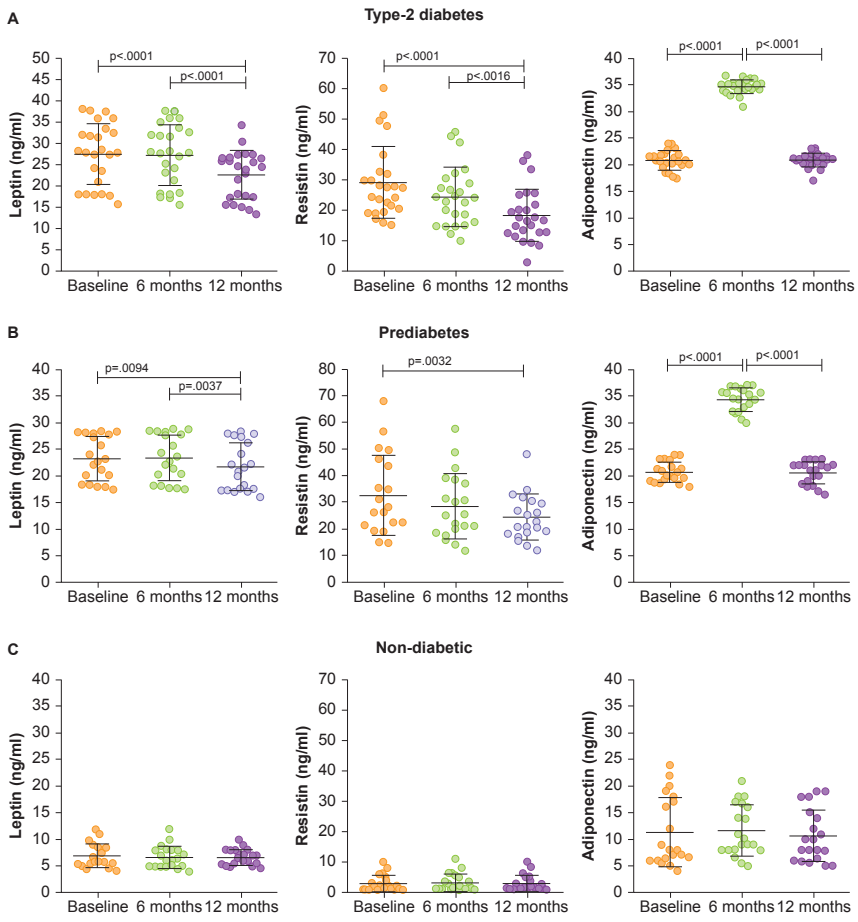

Fig. 2. Chlorella intake modulates adipokines in patients with type-2 diabetes (T2D) and prediabetes (pre-T2D) without affecting the nondiabetic group. Serum levels (mean ± SD) of adipokines (leptin, resistin, and adiponectin) in T2D patients (n = 25), pre-T2D (n = 20), and nondiabetic (n = 20) patients at baseline, and 6 and 12 months after initiating Chlorella supplementation. The values of "p" are represented in the figure; Wilcoxon signed-rank test.

These results are relevant because low concentrations of both proteins are related to increased insulin resistance (Calle and Fernandez, 2012). Adiponectin is associated with the regulation of insulin sensitivity, and its levels in serum are affected by altered metabolic homeostasis, mediating a wide spectrum of reactions, including gluconeogenesis and fatty acid oxidation (Akingbemi, 2013). Reduced serum adiponectin levels during insulin resistance allow for the recruitment, retention, and activation of lymphocytes in the liver, which mediates the induction of a chronic inflammatory environment (Arita *et al.*, 1999). It has also been reported that adiponectin promotes β-cell function and survival (Turer and Scherer, 2012). Adiponectin also regulates insulin signalling by increasing the translocation of glucose transporter type-4 (GLUT-4) to the membrane, thus enhancing the peroxisome proliferator-activated receptor (PPAR) α/γ-mediated pathways in the liver and skeletal muscle by the selective activation of anti-inflammatory cytokines such as IL-10 (Dasari and Raghunath, 2018; Itakura *et al.*, 2015).

Chlorella reduced the levels of leptin and resistin in the T2D and pre-T2D groups at T12. These findings with leptin might seem contradictory in the face of this protein's antidiabetic action and the relative success of long-term leptin-replacement therapy (Meek and Morton, 2016). However, although increased leptin reduces food intake and body weight in healthy individuals, it leads to leptin resistance in obese individuals (Myers *et al.*, 2010). Resistin is a proinflammatory cytokine that is considered a link between obesity and insulin resistance. Important-

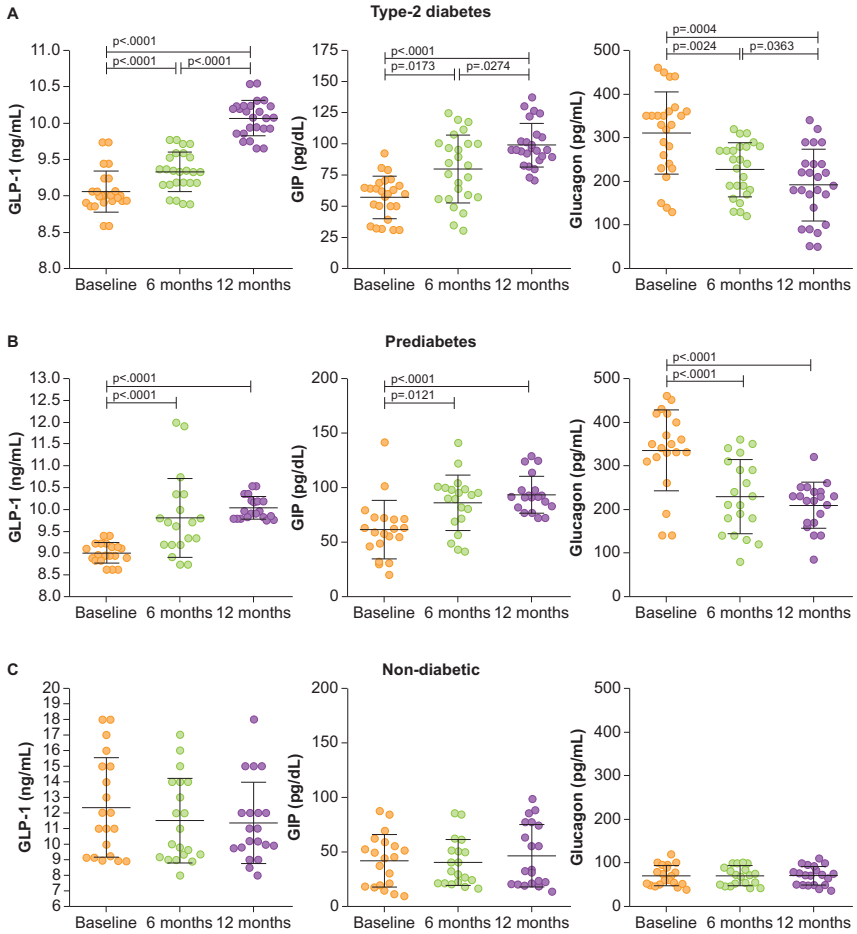

Fig. 3. Chlorella intake modulates incretins in patients with type-2 diabetes (T2D) and prediabetes (pre-T2D) without affecting the nondiabetic group. Serum levels (mean ± SD) of incretins (glucagon, GLP-1 [glucagon-like peptide-1] and GIP [glucose-dependent insulinotropic polypeptide]) in T2D (n = 25), pre-T2D (n = 20) and nondiabetic (n = 20) patients at baseline, and 6 and 12 months after starting Chlorella supplementation. The values of "p" are represented in the figure; Wilcoxon signed-rank test.

ly, it also upregulates the expression of proinflammatory cytokines such as TNF-α, IL-6, IL-12, and MCP-1 via activation of the NF-κB pathway (Dasari and Raghunath, 2018). Chlorella intake (8.0 g/day) for 12 weeks suppresses resistin gene expression in peripheral blood cells of patients with borderline diabetes (Itakura *et al.*, 2015). Evidence from epidemiological, genetic, and clinical studies indicates that human resistin plays pleiotropic inflammatory roles throughout the body and is involved in the pathogenesis of inflammation, insulin resistance, diabetes, and many other chronic low-grade systemic inflammatory diseases (Park *et al.*, 2017).

As for the incretins, Chlorella increased the release of GLP-1 and GIP and consequently decreased glucagon levels at T6 and T12 in both groups. Incretins are hormones with an insulinotropic activity that regulates glucose metabolism after nutrient intake and stimulates insulin secretion. GLP-1 decreases glucagon secretion by pancreatic α-cells and increases insulin sensitivity and biosynthesis, glucose uptake and storage, and circulating insulin availability, thus reducing glucose production by the liver. GIP acts mainly on β-cells, stimulating insulin secretion (Campbell and Drucker, 2013). Glucagon is a counter-regulatory hormone that neutralises insulin actions, promoting the mobilisation of glucose from the liver through glycogenolysis and gluconeogenesis (Gaisano *et al.*, 2012; Habegger *et al.*, 2010). Therefore, the modulating effect of Chlorella on the production of incretins

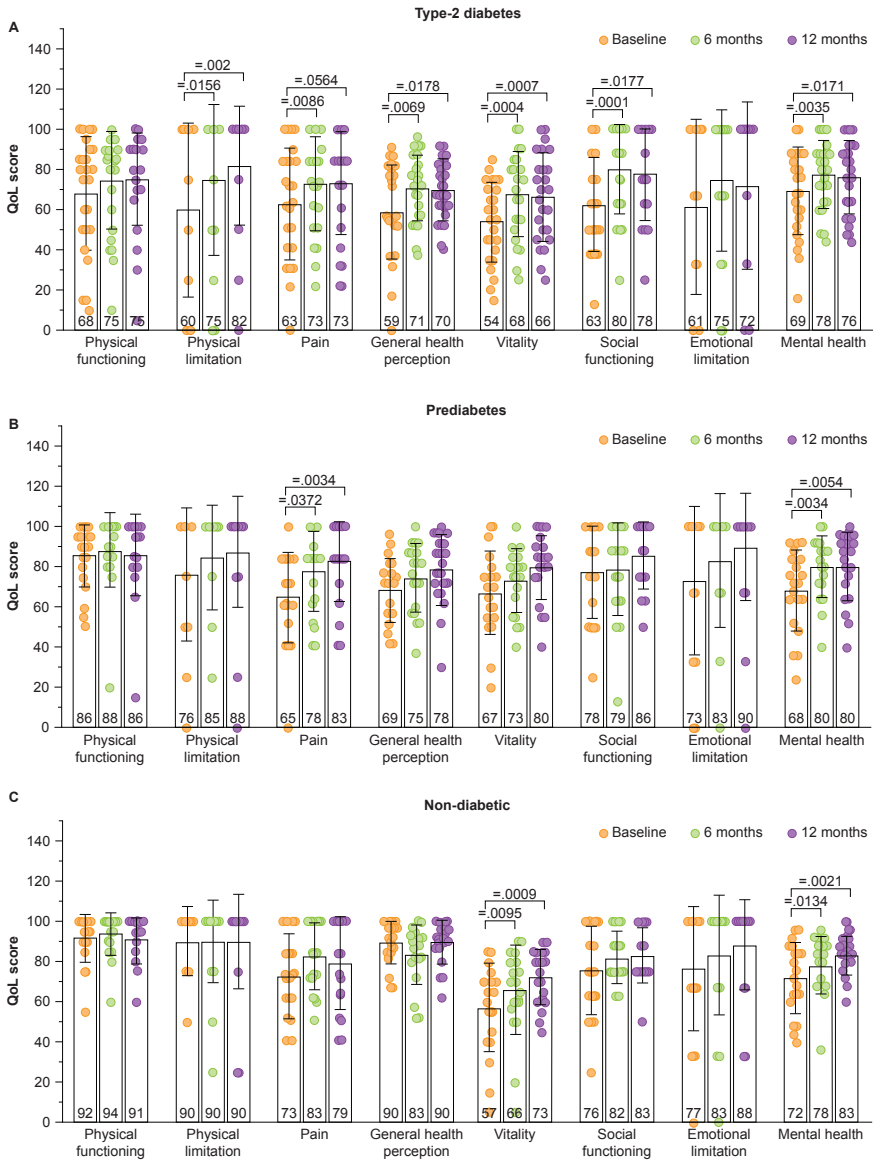

Fig. 4. Chlorella intake improves the quality of life (QoL) in type-2 diabetes (T2D), prediabetes (pre-T2D), and in nondiabetic patients. QoL scores (mean ± SD) in eight concepts of SF-36 in T2D (n = 25), pre-T2D (n = 20) and nondiabetic (n = 20) patients at baseline, and 6 and 12 months after initiating Chlorella supplementation. The mean values of each concept are demonstrated at the bottom of bars; "p" values are represented in the figures; Wilcoxon signed-rank test.

is relevant since these hormones are regulators of hormone secretion, glucose concentrations, lipid metabolism, intestinal motility, appetite, and body weight (Campbell and Drucker, 2013). In this context, our results corroborate experimental studies showing that diabetic animals fed a Chlorella-supplemented diet showed reduced fasting glucagon levels similar to those of exendin-4, a GLP-1 receptor agonist resistant to dipeptidyl peptidase-IV (Jeong *et al.*, 2009).

Improvement in QoL is another important attribute of Chlorella intake and was manifested throughout our study in both T2D and pre-T2D group, in accordance with the degree of diabetes-related symptom distress, was considerably higher in the T2D group. Notably, the baseline score for vitality and mental health in the nondiabetic group was similar to that in the T2D group, thus reflect-

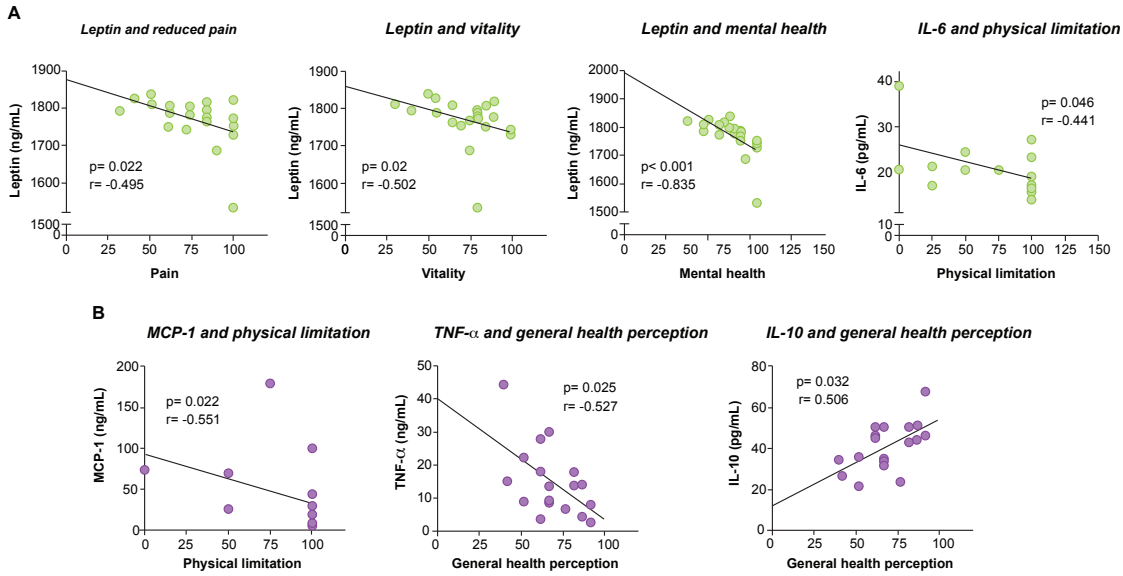

Fig. 5. Correlations of SF-36 concepts and serum levels of adipokines/cytokines in type-2 diabetes (T2D) patients (n = 25) after 6 (A) and 12 (B) months after starting Chlorella supplementation. The values of "p" and "r" are represented in the figures; Spearman's test.

ing the profile of our control group, which is composed of active health professionals with a very intense work routine, standing and moving most of the time, with irregular breaks and dealing with difficult challenges. In this context, Chlorella intake significantly improved the vitality and mental health scores of nondiabetic subjects, thus corroborating the adaptogen ability to restore homeostasis attributed to the alga previously (Vecina *et al.*, 2014; Torello *et al.*, 2016b; Queiroz *et al.*, 2008b; Torello *et al.*, 2016a). Moreover, despite the similar vitality scores observed at baseline in both T2D and nondiabetic groups, the improvement produced by the alga in nondiabetic individuals was significantly higher than that in the T2D group. This finding seems to be related to the connection between the impairment of glucose metabolism and the increased diabetes-related symptom distress observed in the T2D group (Adriaanse *et al.*, 2008).

The impact of Chlorella on QoL has been demonstrated in other clinical conditions, such as breast cancer, hepatitis C, and major depression (Azocar and Diaz, 2013; Noguchi *et al.*, 2014; Panahi *et al.*, 2015). These results are relevant since the improvement in QoL positively reflects treatment adherence, reduced morbimortality rates, and disease progression (Gusmai *et al.*, 2015). Moreover, significant moderate correlations between improved QoL and the levels of pro- and anti-inflammatory proteins were observed after Chlorella intake only in T2D patients. Attenuation of the systemic inflammatory response by the reduced levels of IL-6 and MCP-1 produced by the alga correlated with improvements in role limitations due to physical problems. A reduction in leptin levels correlated with reduced pain, increased vitality, and improvement in mental health, which corroborates reports showing that leptin-mediated inflammation is associated with increased pain sensitivity (Perruccio *et al.*, 2014; Fietta and Fietta, 2006) and with physical and psychological diseases that are observed more often in obese individuals (Vuolteenaho *et al.*, 2014; Milaneschi *et al.*, 2012). Improvement in general health perception was correlated with increased levels of IL-10 and reduced levels of TNF-α. Studies have demonstrated that, in conditions of autoinflammatory syndromes, one of the mechanisms involved in the development of fatigue and is associated with severely compromised QoL includes increased pro-inflammatory cytokines as IL-1, IL-6, and TNF-α (Yadlapati and Efthimiou, 2016).

Altogether, our findings indicate that a key mechanism by which Chlorella prevents the development of inflammatory responses, insulin resistance, and low QoL in humans is related to its rich content of carotenoids, which are lipid-soluble phytochemicals with anti-inflammatory properties linked to their effect on intracellular signalling cascades by blocking the translocation of NF-κB to the nucleus and inhibiting the downstream production of

inflammatory cytokines. By interacting with the nuclear factor erythroid 2-related factor 2 (NRF2) pathway, carotenoids can also block oxidative stress and activate phase II enzymes and antioxidants (Kaulmann and Bohn, 2014; Murillo and Fernandez, 2016). HPLC analysis of the Chlorella used in this study corroborates findings in the literature showing that the alga is a rich source of bioactive carotenoids, especially lutein. It has been demonstrated that after a single 6 g ingestion of the alga containing 15 mg of lutein by healthy volunteers, blood serum lutein concentrations increased 1.5-fold under fasting conditions, and these levels were maintained for over 3 days (Shibata and Hayakawa, 2009). In another study, a dietary intake of 9 g of Chlorella administered daily for 2 months to healthy volunteers led to a 4-fold increase in the concentration of lutein in erythrocytes. Moreover, one month after cessation of Chlorella intake, lutein returned to basal levels, suggesting that daily Chlorella intake effectively improves and maintains erythrocyte lutein concentration in humans (Miyazawa *et al.*, 2013a).

The fact that NF-κB is a pleiotropic proinflammatory transcription factor ubiquitously expressed in the organism and a key event in the pathobiology of systemic low-grade chronic inflammatory diseases adds to the relevance of these findings. In this context, the NF-κB signalling pathway has become a pivotal target for pharmacological interventions. However, although a large number of molecules have been tested as inhibitors of this transcription factor, the highly pleiotropic nature of their effects, associated with the toxicity of systemic and indiscriminate blockade of NF-κB signalling, which interferes with cellular homeostasis, prevents their approval for clinical use (Herrington *et al.*, 2016; Gupta *et al.*, 2010). Therefore, the complementary use of Chlorella as a possible NF-κB inhibitor that can address specific pathways without interfering with cell homeostasis and improving QoL seems to be a promising target.

Studies regarding the interactions of Chlorella with antidiabetic drugs are scarce in the literature. In a 3-month randomized, open-label clinical trial, individuals with nonalcoholic fatty liver disease were randomly assigned to receive either Chlorella (1200 mg/day) + metformin (750 mg/day) + vitamin E (200 mg/day) or metformin (1250 mg/day) + vitamin E (200 mg/day) only. The results demonstrated that weight and body mass index were decreased in both groups, whereas serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides, uric acid, glycated haemoglobin (HbA1c), and HOMA-IR were reduced only in the group receiving Chlorella. In addition, significant changes in total cholesterol, LDL, and HDL were observed only in the metformin + vitamin E group. Although the authors did not describe the interactions between Chlorella and metformin, they highlighted Chlorella as a promising haepatoprotective supplement because the findings of this trial showed that the addition of Chlorella to the therapeutic regimen was associated with favourable effects on serum levels of transaminases, triglycerides, and insulin sensitivity (Panahi *et al.*, 2012).

The choice of the doses to be used in the present study was based on clinical studies from the literature. Although improvement with Chlorella ingestion has been observed in different pathologies and the lack of reported side effects is a well-recognized attribute of the alga (Miyazawa, *et al.*, 2013b; Nakano *et al.*, 2007, 2010; Shimada *et al.*, 2009; Lee *et al.*, 2012; Otsuki *et al.*, 2012; Kwak *et al.*, 2012; Otsuki *et al.*, 2011; Nagayama *et al.*, 2014; Merchant *et al.*, 2000; Lee *et al.*, 2010; Mizoguchi *et al.*, 2008; Noguchi *et al.*, 2014; Azocar and Diaz, 2013), no consensus has been reached on the choice of the low effective clinical doses to be used. In this context, studies showed that in patients with nonalcoholic hepatic steatosis, a dose of 1.2 g/day for 3 months, complementary to conventional treatment, produces significant improvements in serum transaminase levels and triglycerides as well as increased sensitivity to insulin (Panahi *et al.*, 2012). In patients with major depression, a dose of 1.8 g/day for 6 weeks led to better control of cognitive symptoms of depression (Panahi *et al.*, 2015). In subjects with chronic hepatitis C, 5 g/day for 12 weeks resulted in improvement of QoL in 77% of patients (Azocar and Diaz, 2013). Beneficial effects, such as reduction in the incidence of anaemia, proteinuria, and oedema as well as increased levels of lutein, zeaxanthin, and carotenoids in breast milk, were also reported in healthy pregnant women who received 6 g/day for six weeks (Nakano *et al.*, 2007, 2010). In another study, the administration of 7 g/day for 30 days to women with breast cancer who received radiotherapy and/or chemotherapy for at least 6 months increased QoL in 50% of these women (Noguchi *et al.*, 2014). In a study conducted in healthy volunteers and in people with a high-risk factor for the development of lifestyle-related diseases, the intake of 8 g/day for 12 weeks produced an improvement in fat and glucose metabolism in addition to increased gene expression of factors essential for ideal glycaemic maintenance, such as Akt and IRS (Mizoguchi *et al.*, 2008). In healthy volunteers, 8 g/day for a period of 2 months increased the antioxidant activity in erythrocytes by increasing the plasma and erythrocyte concentrations of lutein and decreasing oxidative injury by reducing the phospholipid hydroperoxide concentration in the erythrocyte membrane (Lee *et al.*,

2010). The modulating effects of 10 g/day over a period of 3 months were verified in three different clinical conditions as follows: in hypertensive patients, there was a decrease in high blood pressure and cholesterol levels; in patients with fibromyalgia, there was decreased pain; and in patients with ulcerative colitis, the rectal mucosal region was less inflamed, and patients also noted a reduction in the frequency of evacuations (Merchant and Andre, 2001). Merchant *et al*. (1990) reported the use of 20 g/day algae for a period of 2 years in patients with primary brain tumours. Although improvement in the immunological response was observed during immunosuppressive therapy with this high dosage, some cases of gastrointestinal discomfort, such as abdominal cramps, decreased intestinal peristalsis, and nausea, were reported.

Because no consensus has yet been reached regarding the choice of low effective doses of Chlorella, the comprehensive modulatory effects of the alga observed in this study with a dose as low as 1.6 g/day in the context of chronic low-level systemic inflammation, which seems to be at the root of a wide range of diseases (Hunter, 2012), are relevant and point to the therapeutic efficacy of relatively low doses of Chlorella. In this context, experimental studies from our laboratory (Justo *et al.*, 2001; Queiroz *et al.*, 2003; de Souza Queiroz *et al.*, 2004) show that once homeostasis is reached, higher Chlorella doses do not produce more or less intense physiological or therapeutic effects, consistent with the response profile of an adaptogen (Panossian, 2017). Moreover, our choice of a 12-month Chlorella intake allowed for the reproduction of the long-term condition's characteristic of dietary supplementation (Block *et al.*, 2007) and brought to light modulating effects of the alga occurring over time.

In conclusion, the findings presented here point to the promising use of Chlorella as an adjuvant in treating diseases related to insulin resistance. Moreover, the well-known fact that the role of chronic low-grade systemic inflammation is crucial in the context of infectious diseases, as well as in a wide range of chronic non-infectious diseases (Hunter, 2012), further supports the use of Chlorella as an adjuvant in the treatment of a wide range of pathological conditions.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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