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Research Article

Expression of Regulatory T Cells (CD4+/FOXp3+/CD25+) and Anti-inflammatory Cytokines IL-4, IL-10, and TGF-β in Pediatric Patients with Obesity

Yanowsky-Reyes, Guillermo[1]*, Garcia-Iglesias Trinidad[2], Sanchez Enriquez Sergio[3], Garcia-Cobian Teresa[4], Gomez-Reyes Manuela[5], Santana-Ortiz Rafael[6], Yanowsky-Gonzalez Carlos Oswaldo[7], Ramírez-Gaytan, Laura Aimee[8]

Universidad de Guadalajara, Centro Universitario de Ciencias de la Salud, Hospital Civil de Guadalajara Fray Antonio Alcalde, Mexico.

Abstract

Objective: To evaluate the expression of Treg cells producing anti-inflammatory cytokines IL-4, IL-10, and TGF-β in pediatric patients with obesity.

Method: An observational, cross-sectional, analytical, and descriptive design was utilized. The sample consisted of a total of 60 children aged 6 to 12 years.

Analysis: Measures of central tendency (mean, median, and mode) and dispersion (standard deviation) were used. For the analysis of immunological variables, analysis of variance was employed, and for distribution, parametric (Student's t-test) and non-parametric tests (Mann-Whitney U test) were used. Pearson correlation was used to analyze the relationships between quantitative variables, and Spearman correlation was used for body mass index and anti-inflammatory cytokines. Data analysis was conducted using WinMDI 2.9 software.

Results: It was determined that the serum cholesterol concentration was higher in the Ob group (148.27 ± 20.75) as well as the triglyceride concentration (121.16 ± 66.55*); however, the HDL quantification was similar in both groups. Additionally, a higher percentage of positive regulatory cells was observed in the Ob group with a percentage of 66.6%. It was reported that the cytokine IL-10 (0.80 ± 0.38 vs 0.26 ± 0.12) showed a significant increase, as well as IL-4 and IL-6, with no significant changes in TNF-α.

Conclusion: It can be inferred that the presence of a regulatory factor for these inflammatory cells could exert a protective effect in pediatric patients against insulin resistance and cellular damage in pancreatic tissue.

Keywords: obesity, inflammation, regulatory T cells, pediatric, cytokines.

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Introduction

In general terms, obesity is defined as an excess of body fat and is considered one of the major public health issues worldwide. [1] This condition is a chronic disease with a heterogeneous clinical presentation making it a complex disorder due to the interaction of various genetic, environmental, and neuroendocrine factors. [2] However, when discussing childhood obesity, it is imperative to be precise and establish that it constitutes a form of malnutrition, positioning itself as the most common nutritional disease among

children and adolescents in both developed and developing countries. [3] Consequently, it is also the most common cause of insulin resistance, thus increasing the comorbidity of metabolic disorders among children, such as dyslipidemias, hyperinsulinemia, and hypertension. [4] This transforms obesity into a significant risk factor for cardiovascular morbidity and mortality in adulthood.

As with any element in clinical practice, understanding the magnitude of a problem requires its classification. To achieve this, it is important to recognize that different diagnostic criteria exist for the pediatric population, primarily based on anthropometric values, as these provide a quick, precise, and non-invasive tool. For the present study, the reference values proposed by the Centers for Disease Control (CDC) were used. This classification (Table 1) is based on the Body Mass Index (BMI) for children aged 2 to 19 years, expressed in growth charts. [5]

Table 1. *Classification of Obesity in Children and Adolescents. Regarding the Body Mass Index for children and adolescents [Source: http://www.cdc.gov], where "Pc" stands for percentile.*

IMC CLASSIFICATION	
> Pc 85	Overweight
> ó = Pc 95	Obesity

As mentioned, obesity is a complex disorder in which body fat content is modulated throughout a person's life by interactions between various factors. Although a polygenic basis exists in 40-80% of obesity cases, this acts through different mechanisms [6]. Therefore, the objective of this study focuses on the immunological factors involved in the development of this disease. Despite knowing that it is an inflammatory condition characterized by chronic activation of the immune system, where pro-inflammatory interleukins such as IL-6 and TNF-α are secreted [7], few studies have examined the relationship between systemic inflammation and levels of different lymphocyte populations in peripheral blood.

Thus, it is crucial to evaluate the response of regulatory immune cells and cytokines in the peripheral blood of obese children, as well as their association with body mass index, to understand the mechanisms leading to obesity and its chronic inflammatory effects, which result in chronic-degenerative diseases.

Objectives

The general objective of this study is to evaluate the expression of Treg cells producing anti-inflammatory cytokines IL-4, IL-10, and TGF-β in pediatric patients with obesity.

Methodology

Design

This research was framed within an observational, cross-sectional, analytical, and descriptive design with the objective of evaluating the expression of Treg cells. A cross-sectional approach was employed to capture information at a specific point in time, providing an instantaneous view of the perspectives of the selected subjects.

The study was conducted between [study dates] in collaboration with the Cardiovascular Research Unit of the Department of Physiology at the Centro Universitario de Ciencias de la Salud, University of Guadalajara, the Immunology Laboratory at the Centro Universitario de Ciencias de la Salud, and the Pediatric Surgery Service at the Antiguo Hospital Civil de Guadalajara Fray Antonio Alcalde.

Sampling Strategy

The sample size consisted of a total of 60 children, of which n=30 were in an obese state and n=30 were of normal weight. The sample size was calculated based on the formula for comparative cross-sectional studies Epi-Info 2000 (Fleis 1981).

The following parameters were used for sample size calculation:

- $\alpha = 0.05$ (confidence level)
- $\beta = 0.20$ (power)
- $\delta =$ (margin of error)
- Standard deviation of 2
- An estimated difference of one standard deviation

Applying the formula for sample size calculation for mean comparisons, used in analytical cross-sectional studies.

$$n = \frac{2(Z\alpha + Z\beta)^2 * S^2}{\delta^2}$$

$$99.96 \div 4 \quad n = 24.74$$
$$24.74 + 5.2 = 29.94$$

The sample size was determined as 30 children per group, with an estimated loss of 20% per group, resulting in 5.2 children [losses per group].

Study Groups

Study Group: n=30 obese children (Ob)

Control Group: n=30 normal weight children (Np)

Population and Selection Criteria

Population

The study population consisted of individuals (children) of both sexes, aged 6 to 12 years, categorized as normal weight and obese.

Inclusion Criteria

- Children of both sexes
- Aged 6 to 12 years
- BMI ≥ 30 (obesity) for the obese group (Ob)
- Normal weight children for the control group (Np)
- Parental consent through informed consent form

Exclusion Criteria

- Children with comorbidities such as hypertension or diabetes
- Those who consumed medications affecting immune response
- Other temporary diseases independent of obesity
- Undergoing pharmacological treatment for weight reduction
- Clinical diagnosis of hypothyroidism, hyperthyroidism, diagnosed psychiatric disorders (bulimia or anorexia), renal damage, endocrinopathies, cardiovascular disorders, or suspicion of syndromic or monogenic obesity.

E***xclusion Criteria***

- Individuals who declined to participate in the study
- Insufficient sample
- Incomplete data
- Those who withdrew from the study during medical history collection, thereby preventing peripheral blood sampling.

Variables

Variables studied in the children included: general characteristics such as age and sex, anthropometric measurements (weight, height, waist circumference, BMI), clinical signs such as acanthosis and other obesity-related stigmata, biochemical parameters including fasting serum glucose, lipid profile (total cholesterol, triglycerides, HDL, LDL, VLDL), and molecular markers such as anti-inflammatory or immunoregulatory cytokines (IL-4, TGF-β, IL-10) and Treg cells (CD4+/FOXp3+/CD25+) in peripheral blood. The same variables were analyzed in the control group of normal weight children.

Measurement Instruments

Anthropometric data collection included waist circumference, body weight, height, and BMI.ELISA method was used to evaluate the serum concentration of anti-inflammatory and immunomodulatory cytokines in both groups. The Kit from Peprotech was used for IL-4 and IL-10 concentration measurements.

Flow cytometry was employed to evaluate cellular populations.CD4+ and CD25+ proteins were labeled with different fluorochromes: CD4 cells were marked with phycoerythrin-cyanine 5 (PCY-5), and CD25 was marked with phycoerythrin (PE). Intracellular proteins were identified using special kits for cell permeabilization, such as Intraprep from Beckman CoulterTM. For FOXp3 identification, a monoclonal antibody labeled with fluorescein isothiocyanate (FITC) was used.

Table 2. *Staining Protocol for FCM: Intracellular Staining*

TUBE	ANTIBODY	OBSERVED CELLS
1	FITC/PE/PC5	Compensation
2	**FOXp3**+/PE/PC5	Compensation
3	FITC/**CD25**+/PC5	Compensation
4	FITC/PE/**CD4**+	Compensation
5	**FOXp3**+/PE/**CD4**+	Compensation
6	**FOXp3**+/**CD25**+/**CD4**+	Regulatory T cells

Lipid Profile was also performed with determination of cholesterol by cholesterol oxidase/peroxidase, determination of triglycerides through glycerol phosphate oxidase/peroxidase, and determination of HDL by cholesterol oxidase.

Ethical Considerations

This study is considered minimal risk according to Article 17 of the Regulations of the General Health Law on Health Research. It was conducted in accordance with the ethical considerations of the Helsinki Declaration (64th General Assembly, Brasilia, 2013). Informed consent was obtained from the parents of the selected children, explaining the study's objectives and procedures, culminating in the signing of the consent form.

Biosecurity aspects were covered, properly discarding and classifying the biological materials used according to NOM-087-SEMARNAT-SSA1-2002.

The project was accepted in the 2009 call for Coecyt-Jal/UdeG under registration number SP-2009-693 and approved by the bioethics, biosecurity, and research committees of the Centro Universitario de Ciencias de la Salud (registration CI/022/2010) and the Secretary of Health of Jalisco (SSA 39/UG-JAL-2010).

Limitations

The main limitations of the study include parental refusal for their children to participate. It is essential for medical personnel to handle patients and their families with care during blood sampling and physical examinations, ensuring their comfort and respecting their culture and decision to participate or not, without compromising their safety.

Analysis and Results

Measures of central tendency such as mean, median, and mode were used, along with measures of dispersion including standard deviation. Analysis of variance (ANOVA) was employed for immunological variables, and for distribution, parametric tests (Student's t-test) were used, supplemented by non-parametric tests (Mann-Whitney U test) when necessary. Pearson correlation was used to analyze quantitative variables such as lipid profile and concentration of anti-inflammatory or immunomodulatory cytokines, as well as regulatory T cells (Tregs). Spearman correlation was used to examine the relationship between body mass index (BMI) and anti-inflammatory cytokines and Tregs. WinMDI 2.9 software (Windows Multiple Document Interface for Flow Cytometry) was utilized for data analysis.

In this study, 59 school-aged pediatric patients aged between 6 and 12 years were included, categorized into 2 groups based on anthropometric evaluation of their anatomical variables: Normal weight Group (Np) and Obese Group (Ob).

For the variable Age, the mean age in the normal weight group was 9.5 ± 2.5 years, and in the obese group it was 9.4 ± 2.2 years, with a non-significant p-value for both groups. Regarding sex distribution, the normal weight group consisted of 16 (53%) females and 14 (47%) males, while the obese group comprised 15 (51%) females and 14 (49%) males, with a non-significant p-value for both groups.

When evaluating body weight, significant differences were found between the groups. The normal weight group had a weight of 21.8 ± 1.16 kg, whereas the obese group had a weight of 53.2 ± 18.7 kg (p = .000*). No significant differences were observed in height between the normal weight group (140.5 ± 15.37 cm) and the obese group (138 ± 10.76 cm). The BMI in the obese group was 34.76 ± 5.93*, significantly higher compared to 20.42 ± 7.0 in the normal weight group (p = 0.05), as shown in Table 3.

Table 3. *Representation of the variables used and their comparison between the normal weight groups (Np) and the obese group (Ob).*

Variables	NP	Ob	p
Age (years)	9.5 ±2.5	9.4 ± 2.2	NS
Sex Fem Mas	16Fem 14Masc	15 Fem 14 Mas	NS
Body weight (Kg)	21.8 ± 1.16	53.2 ± 18.7*	.000*
Height (Mts)	140.5 ±15.37	138 ± 10.76	NS
BMI	34.76 ± 5.93*	20.42 ± 7.0	0.05*
Glucose (gr/mL)	79.43 ± 3.61	83.43 ± 10.12	NS
Cholesterol (gr/mL)	126.68 ± 30.01	148.27 ± 20.75	NS

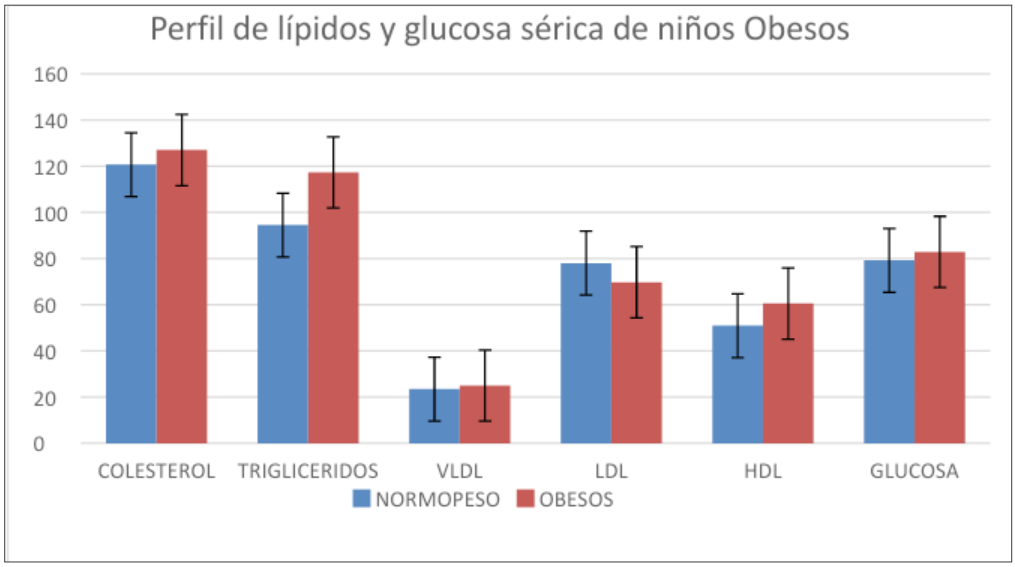
Triglycerides(gr/mL	113.46 ± 64.49	121.16 ± 66.55*	0.013*
HDL (gr/mL)	51.04 ± 1.23	51.54 ± 11.82	NS
LDL (gr/mL)	68.85 ± 19.41	69.70 ± 19.94	NS
VLDL (gr/mL)	26.58 ±18.48	25.24 ± 18.77	NS
$FOXp3^{+}/CD25^{+}/CD4^{+}$	10/30	20/30	0.05*

Regarding the lipid analysis, the following results were observed: The serum concentration of cholesterol was higher in the Obese Group (148.27 ± 20.75) compared to the Normal weight Group (126.68 ± 30.01); however, no significant difference was observed between the two groups.

In terms of triglyceride concentration, the Obese Group showed a higher concentration (121.16 ± 66.55*) compared to the Normal weight Group (113.46 ± 64.49), with a statistically significant difference between the groups (p = 0.013*).

The quantification of HDL was similar in both groups, with values of 51.04 ± 1.23 for the Normal weight Group and 51.54 ± 11.82 for the Obese Group, with a non-significant p-value. Similar findings were observed for LDL cholesterol (68.85 ± 19.41 for Np and 69.70 ± 19.94 for Ob), with no significant difference between groups. The concentration of VLDL was lower in the Obese Group (25.24 ± 18.77) compared to the Normal weight Group (26.58 ± 18.48), and this difference was not statistically significant for both groups. Glucose concentration was higher in the Obese Group (83.43 ± 10.12) compared to the Normal weight Group (79.43 ± 3.61), but no statistical difference was observed between the groups. These data are presented in ***Table 4***.

Table 4. *Percentage results of the concentration of CD4+, CD25+ and FOXp3+ monoclonal antibodies that are part of the regulatory cells in both groups, data represented as average ± Standard deviation significance p>0.05**



The Treg cells were evaluated using flow cytometry, and a higher percentage of positive regulatory cells was observed in the Obese Group, where 20 out of 30 subjects showed positivity for all three markers (FOXp3+CD25+CD4+), accounting for 66.6%. In comparison, 10 out of 29 subjects in the Normal weight Group also showed positivity, accounting for 34.48%. A statistically significant difference was observed with a p-value < 0.05 (***Table 5***).

Table 5. *Percentage of triple-positive T reg cells labeled with fluorochrome-labeled monoclonal antibodies and evaluated by EpicXL flow cytometry.*

GROUPS	CD4+ (%)	CD25+ (%)	FOXP3+ (%)
Np	30.10 ±2.17	0.16 ± 0.73	0.058 ±0.15
Ob	31.46 ±3.93	0.29 ± 1.12	0.106±0.16*

In *Figure 1,* the percentage of each independent marker of Treg cells (CD4+, CD25+, and FOXp3+) is represented for both study groups, showing no differences between the groups.

Percentage expression of Treg proteins with the different fluorochromes

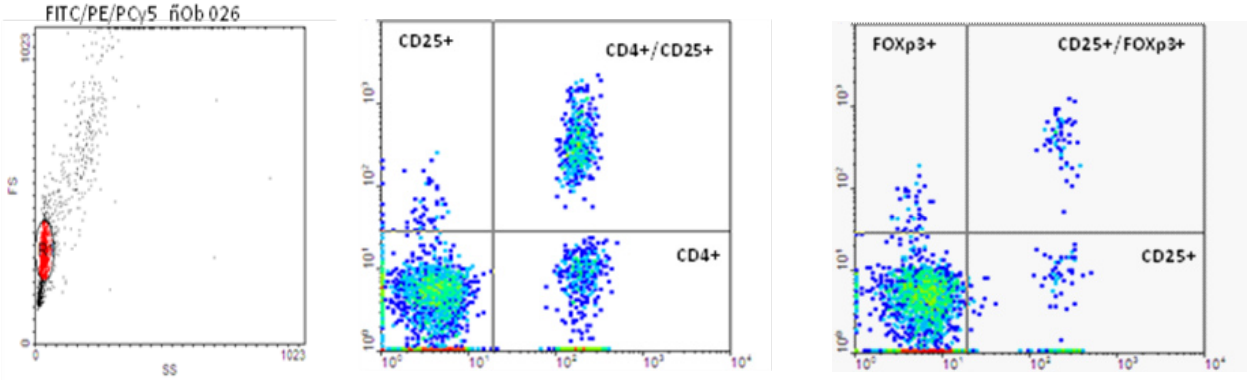


Figure 1. *Percentage expression of T reg cell proteins with the different fluorochromes: CD4+ phycocyanin five (PCY-5) (left), CD25+ phycoerythrin (PE) (center), FOXp3+ monoclonal antibody labeled with fluorescein isothiocyanate (FITC) (right). Results evaluated in the program (WinMDI).*

Regarding the serum concentration of immunoregulatory cytokines evaluated by ELISA, it was reported that IL-10 (0.80 ± 0.38 vs 0.26 ± 0.12) showed a significant increase in the Obese Group compared to the control group, respectively. Similarly, for TGF-β (0.99 ± 0.46 vs 0.49 ± 0.16), results were very similar to IL-10, with children in the Obese Group showing a higher increase. However, individual dispersion within this group was highly variable, leading to statistically significant differences. Representative data can be observed in Figure2**.**

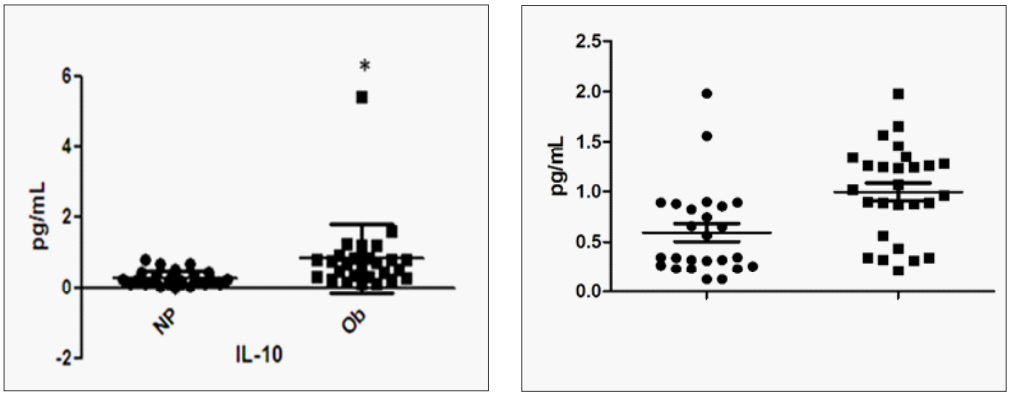


Figure 2. *Serum concentration of anti-inflammatory cytokines** A) (left) TGF-β and B) (right) IL-10 in Normal Weight (Np) vs Obese (Ob) children, data represented as mean and standard error. Protein concentration (pg/mL) between groups was compared using the non-parametric Kruskal-Wallis test. *Values of p < 0.005 are considered significant.**

IL-4 is an anti-inflammatory cytokine derived from T lymphocyte activation, as shown in Figure 3. This cytokine is increased in the group of children with Obesity (0.38 ± 0.23 vs 0.26 ± 0.12) compared to Normal Weight children (0.39 ± 0.23 vs 0.26 ± 0.125).

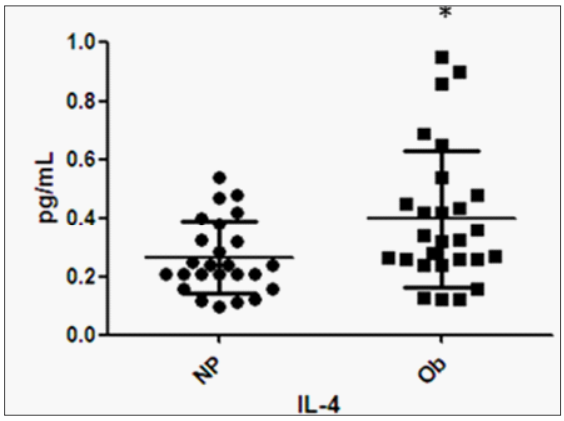


Figure 3. *Serum concentration of anti-inflammatory cytokine IL-4. IL-4 in Normal Weight (Np) vs Obese (Ob) children, data represented as mean and standard error. Protein absorbance (pg/mL) between groups was compared using the non-parametric Kruskal-Wallis test. *Values of p < 0.005 are considered significant.*

As a secondary objective, the concentration of IL-6 and TNF-α was evaluated. For IL-6, higher serum concentration was observed in the Obese group (1.48 ± 1.1 vs 1.07 ± 0.76), which was statistically significant with a p-value of 0.05*. However, no changes were observed in TNF-α (0.71 ± 0.35 vs 0.49 ± 0.28), as described in ***Figure 4.***

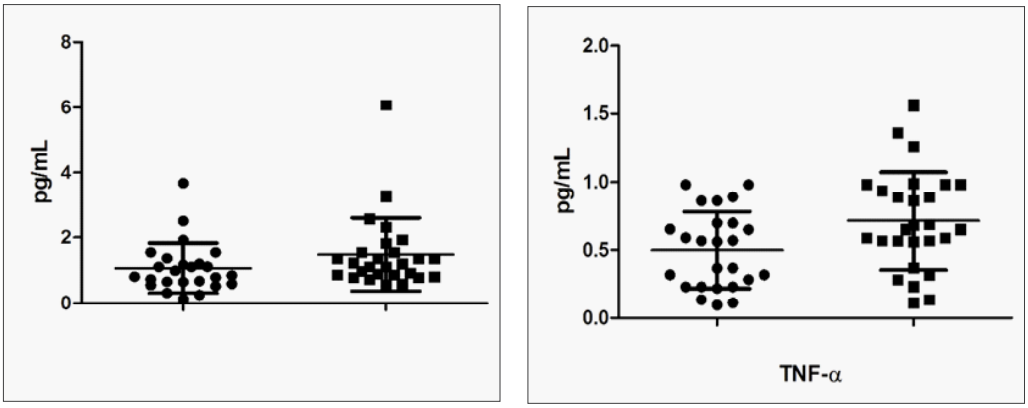


Figure 4. *Serum concentration of pro-inflammatory cytokines. A) (right) IL-6 and B) (left) TNF-α in Normal Weight (Np) vs Obese (Ob) children, data represented as mean and standard error. Protein concentration (pg/mL) between groups was compared using the non-parametric Kruskal-Wallis test. *Values of p < 0.005 are considered significant.*

Using the Lilliefors significance test, the data obtained in the research design were analyzed, as shown in ***Table 6*** and ***Table 7***.

Table 6. *Correlation of variables*

Group	Ob	Np
N=	30 (100%)	29(96.6%)
Expressed cytokines (Il-4,IL-10,TGFβ), TNFα,IL-6	20 (66%)	10 (34.4%)

Table 7. *Correlation of Lilliefors significance*

	Age	Weight	Height	BMI	GlucosE	Trig	VLDL	cHDL	cLDL	TNFα	IL-6	IL-4	IL10	TGFβ
U de Mann_W	322.000	.000	287.500	23.000	240.500	202.000	314.500	316.500	325.000	286.000	217.500	229.000	200.500	124.500
W de Wilcoxon	647.000	325.000	612.5000	348.000	565.000	527.000	639.500	641.500	703.000	611.000	542.500	554.000	525.500	449.500
Z	.287	-6.186	-.917	-5.763	-1.778	-2.482	-.422	-.385	-.229	-.948	-2.200	-1.988	-.2.513	-3.902
Sig.asintol (bilateral)	774	.000	.359	.000	.075	.013	.673	.700	.819	.343	.028	.047	.012	.000

So a correlation with significance was found between the following variables. (***Table 8***)

Table 8. *Correlation between BMI (IL10, TGF-β), triglycerides (VLDL, LDL, IL-6) and weight of the patient with (IL-6)*

Variable	BMI	TriglycerideS	Weight
	IL-10	IL-6	IMC
	TGFβ		IL-6

Discussion

The increase in the prevalence of obesity in the pediatric population has become a global public health issue, particularly in Mexico. This study demonstrates the expression of Treg cells producing anti-inflammatory cytokines in peripheral blood in a group of pediatric patients with obesity, comparing them with a group of age-matched normal-weight children.

It has been reported that adolescents and adults show higher concentrations of lipids, particularly cholesterol and glucose, which serve as indicators of potential insulin resistance and/or cardiovascular

damage [8,9]. Additionally, studies conducted on obese Latino children residing in the United States aged 6-13 years have shown increases in triglycerides, cholesterol, and glucose [10].

In this study, following lipid profile analysis, the only parameter that showed significant differences in children with obesity was the concentration of triglycerides, a qualitative dichotomous variable described by frequencies and determined by Chi-square in inferential statistics, yielding a result of 0.13. It is noteworthy that this positivity is more evident in the patients of the Ob group, although not as high as expected, suggesting the presence of a protective factor in these patients.

The immune system plays a crucial role in health-disease regulation, with nutrition described as a significant factor in protection mechanisms against pathogens [11]. Therefore, children with obesity are more susceptible to triggering opportunistic fungal infections and hyper-response of T cells. Regarding their relationship with inflammation, migration of M1 macrophages, cytokine products, and M2 [12,13] to adipose tissue has been described, along with adipokines such as Leptin, resistin, visfatin, and adiponectin maintaining local inflammatory control. However, excess adipose tissue increases systemic pro-inflammatory cytokine concentrations [14].

Previous studies have highlighted the importance of these two pro-inflammatory cytokines in obesity, with TNF-alpha primarily implicated in insulin resistance [15], while IL-6 is more associated with cardiovascular damage as a primary inducer of heat shock proteins such as C-reactive protein [8,16,17]. Previous studies conducted in our laboratory found that obese youth showed increases in IL-6 and TNF-alpha [17]. Despite being considered a secondary objective, we found it crucial to evaluate the concentration of these two cytokines because their expression in children with obesity remains unknown; we found that IL-6 is increased but not TNF-alpha.

It is well-known that IL-6 is capable of activating the expression of Treg and TH17 cells, and our findings of overexpression of IL-6 also demonstrated evidence of Treg cell expression, CD4+, CD25+, and FOXp3+, in both study groups. It is noteworthy that positivity was observed in 34.4% of the NP group and 66% in the Ob group, which may be due to the presence of a protective factor activating Treg cells. Among them, FOXp3+ is the most important regulator of anti-inflammatory cytokine expression in obese patients. Although acute phase proteins such as CRP and ESR increase, there is also evidence of an immunoregulation factor in pancreatic damage, providing protection to the pancreas and reducing insulin resistance effects [1,14].

In our study, we observed that the concentration of anti-inflammatory cytokines IL-4, TGF-beta, and IL-10 showed increased expression in the Ob group, where they were expressed at double the levels compared to the Np group. The correlation between Treg cell expression and anti-inflammatory cytokines suggests a relationship between them, indicating that the expression of anti-inflammatory cytokines may have a regulatory effect mediated by T cells. These observations in the Ob group are novel in pediatric obesity, and the correlations found in this review are manifested between BMI (IL-10, TGF-beta), triglycerides (VLDL, LDL, IL-6), and patient weight (IL-6), likely due to systemic inflammation in children with obesity.

Conclusion

In this study, no significant differences were found regarding weight and gender between the analyzed groups. However, in the lipid profile, an increase in cholesterol and triglyceride levels was observed, with the latter being significant in the group of obese children. Regarding glucose concentration, a slight increase was noted in the obesity group.

An increase in regulatory T cells (CD4+/CD25+/Foxp3+) was detected in both groups of normal-weight children, but 75% of obese children showed higher activation of these cells. Cytokines induced by regulatory T cells (IL-10, TGF-beta) were also found in higher serum concentrations in the group of obese children. In the analysis of pro-inflammatory cytokines, differences were observed in IL-6 concentration, but no significant changes were found in TNF-alpha concentration.

In conclusion, it can be inferred the presence of a regulatory factor for these inflammatory cells, which could exert a protective effect in pediatric patients against insulin resistance and cellular damage in pancreatic tissue.

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