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Cytokine Profile at the Beginning of Pregnancy in Mexican Women

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Objectives: To quantify and associate the levels of Th1 pro-inflammatory and Th2 anti-inflammatory cytokines with the anthropometric measurements at the beginning of pregnancy. **Methods:** This prospective clinical and descriptive study included pregnant women 18 years of age and older. Serum levels of IL-4, IL-6, IL-10, IFN-γ, and TNF-α were measured by ELISA. Correlations were determined among the cytokines and anthropometric variables. **Results:** From 83 pregnant women IL-4 was significantly higher in Underweight compared to Overweight patients (97.5 ± 3.5 vs. 66.4 ± 17.6 pg/mL, p = .037), Underweight compared to Obesity Class I patients (97.5 ± 3.5 vs. 60.6 ± 17.2 pg/mL, p = .024) and in Normal Weight compared to Obesity Class I patients (72.2 ± 17.7 vs. 60.6 ± 17.2 pg/mL, p = .026). TNF-α was significantly higher in Normal Weight compared to Obesity Class II patients (41.9 ± 26 vs. 24.9 ± 19.7 pg/mL, p = .031). IL-4 was positively correlated with TNF-α (r2 = .309, p = .005) and negatively correlated with pre-gestational BMI (r^2 = -.243, p = .029). IL-10 was positively correlated with IL-4 (r^2 = .356, p = .001) and TNF-α (r^2 = .308, p = .005). Finally, IFN-γ was negatively correlated with IL-4 (r^2 = -.246, p = .025), IL-6 (r^2 = -.232, p = .035) and TNF-α (r2 = -.289, p = .008). **Conclusion:** In Mexican women, there is a low anti-inflammatory cytokine profile at the beginning of pregnancy.

Keywords: Cytokines, Immune Response, Obesity, Overweight, Pregnancy, Th1-Th2 Balance

Introduction

Obesity is recognized as a global health problem, being of particular interest before and during pregnancy due to its association with increased maternal and neonatal mortality and morbidity. In particular, obesity has been shown to induce metabolic disorders on both, the mother and the baby, even causing a possible transgenerational amplification of the prevalence of obesity, besides the well-known increased incidences of fetal death, macrosomia, shoulder dystocia, and childhood obesity. Even more, obesity in pregnancy has harmful effects on maternal health by increasing the risk of pregnancy-

induced hypertension, gestational diabetes, and thromboembolic disease as well as increasing the risk of miscarriage[1,2].

Among the proposed mechanisms by which obesity increases obstetric complications have been described the induction of oxidative stress, and changes in the balance of T helper (Th) lymphocyte subsets[3,4]. In line with this, the immune system, in general, can be divided into the innate and adaptive immune system. The first is a non-specific system that provides immediate defense against pathogens, while the second is more specific, characterized by T and B lymphocytes. B lymphocytes provide humoral immunity utilizing antibodies, whereas T cells provide mainly immunity mediated by cells[5]. Helper T cells (CD4+) can be grouped into different subsets of T CD4+ lymphocytes, termed T helper 1 (Th1) and T helper 2 (Th2) cells, characterized by their cytokine production profile. But besides this classical division, other subsets of helper T cells have been identified, including Th9, Th17, Th21, Th22, TFr and Treg[6,7].

Th1 cells secrete pro-inflammatory cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), while Th2 cells secrete anti-inflammatory cytokines such as interleukin (IL)-4, IL-10 and IL-13[8]. IL-4 is the dominant factor for the promotion of growth and differentiation from Th0 to Th2, and directly inhibits the development of Th1 cells[9]. IFN-γ, on the other hand, indirectly promotes Th1 differentiation through overexpression of the IL-12 receptor, while inhibiting the growth of Th2 cells at the same time[10]. During healthy pregnancy, an increase in levels of IL-4, along with a decrease in IFN-γ have been shown. By contrast, an increase in Th1 has been demonstrated in cases of recurrent miscarriage and in preeclampsia[11-13].

One of the explanations that would associate obesity with immunological alterations during pregnancy is the fact that oxidized LDL (oxLDL) can exacerbate a chronic inflammatory microenvironment by stimulating the differentiation of naïve T CD4+ cells to Th1 cells, that predominately secrete IFN-γ[14].

Until now, most of the studies describing the cytokines profile of pregnant women have focused on the pro-inflammatory profile, but there is not equivalent information related to the anti-inflammatory cytokines at the beginning of the gestation. We aimed to quantify and associate the levels of Th1 and Th2 cytokines, with the anthropometric measurements at the beginning of pregnancy and to clarify whether at the early stage of gestation in Mexican women a high pro-inflammatory or low anti-inflammatory profile predominates. If either is confirmed, this could add prognostic predictors to a population already known to be at high risk of obstetrical complications.

Materials and Methods

Setting

This prospective clinical and descriptive study was carried out at the "Mónica Pretelini Sáenz" Maternal-Perinatal Hospital (HMPMPS), Health Institute of the State of Mexico (ISEM) during 2017. The hospital is located in Toluca, Mexico, ~60 km west of Mexico City.

Patients

Pregnant women older than 18 years of age were invited to participate and written informed consent was obtained. Despite a large and growing immigration population, in this survey, we selected only Mexican women that have a characteristic miscegenation with elements from indigenous and European ancestry. Women with congenital heart disease, physically disabling conditions, infectious diseases, and autoimmune diseases were excluded and those who were lost to follow-up were not included in the final analysis.

Sample calculation

To ensure we obtained a representative sample of the pregnant women who received care at the clinic during 2017, the required sample size was calculated as follows:[15].

$$\text{Sample size} = \frac{\frac{z^2 \times p\,(1-p)}{e^2}}{1 + \left(\frac{z^2 \times p\,(1-p)}{e^2 N}\right)}$$

Where N = annual population size at the clinic, e = the desired margin of error, z = z-score corresponding to the desired confidence interval, and p = percentage of the population with a given sampling result (p was unknown for our population, the worst scenario of 50% was chosen). Therefore, to obtain a 95% confidence interval for our population of 8500, a z-score of 1.96 was chosen corresponding to a margin of error of 11%. This resulted in a required sample size of 79 patients.

Physical exam

All pregnant women underwent a complete physical examination on each monthly medical appointment, including body weight,

height, body mass index (BMI), and blood pressure. Weight and height were measured using a calibrated adult scale (Seca, Hamburg, Germany). The blood pressure (BP; mmHg) was checked by auscultation using a standard sphygmomanometer (Riester Big Ben® Square; Jangingen, Germany). The patients were classified according to their BMI at each visit as follows: underweight < 18.5, normal weight 18.5 – 24.9, overweight 25.0 – 29.9, class I obesity 30.0 – 34.9, class II obesity 35.0 – 39.9, and class III obesity ≥ 40.0 and their BMI difference (BMI during first prenatal visit– pregestational BMI) was calculated using their recollection of their weight before their last menstrual period as their pregestational weight.

Nutritional & weight management

By protocol of the HMPMPS, all mothers who were overweight or obese were referred to the nutrition department, where they received personalized written nutritional guidance depending on their age, pre-pregnancy weight, current weight and BMI. Based on their daily caloric requirements, changes were made in their diet, appropriate to their caloric and nutritional needs.

Laboratorial analysis

After 8 hours of fasting, blood samples were drawn and routine analyses were performed for albumin (mg/dl), cholesterol (mg/dl), creatinine (mg/dl), glucose (mg/dl), triglycerides (mg/dl), uric acid (mg/dl), liver profile (Dimension R × L Max, Dade Behring, USA), blood cytometry (Advia 120, Bayer Health) and urinalysis.

Quantification of cytokines

The serum levels of IL-10 (Invitrogen Cat. BMS215-2), IL-4 (eBioscience catalog: BMS225/2), IL-6 (Enzo Cat. 80-0625), IFN-γ (Invitrogen Cat. BMS228) and TNF-α (Invitrogen Cat. BMS223-4) were measured by ELISA on an ELx800™ device (BioTek Instruments, Inc.) at the Research Laboratory of Ciprés Grupo Médico S.C. (CGM).

Statistical analysis

Quantitative variables were represented by measures of central tendency. First, the Kolmogorov test was performed to determine the normality of the variables. The one-way ANOVA test was used to contrast the cytokines levels per BMI classification of the patients and independent sample Student's t-tests or the Mann Whitney U tests were used to do multiple comparisons between groups for all BMI classes. The critical p-values for the t-tests and Mann Whitney U tests were adjusted using a Bonferroni correction to control for the increased risk of Type I error associated with multiple comparisons. Based on the Gaussian distribution of the variables, either Pearson or Spearmen correlation were used among the cytokines and anthropometric variables as well as between BMI difference and the cytokines and between the IL-4/TNF-α ratio and the BMI. Also, multiple regression was done for each cytokine, introducing age, BMI and blood pressure levels as independent variables. In all cases $p \leq .05$ was considered statistically significant. The statistical analyses were carried out using SPSS, version 19.

Ethical statements

This project was approved by the Ethics and Research Committee of the HMPMPS (code: 2015-09-415). Negligible risk from this study was attributed to pregnant women or their neonates according to the regulations of the General Law on Health in Research Matters, and our study complied with standards of the Declaration of Helsinki (Fortaleza, Brazil). This project was assigned ClinicalTrials.gov ID: NCT03761966.

Results

During the year 2017, 83 women were enrolled in the study. The general characteristics of included patients are depicted in Table 1. After performing the Kolmogorov test the only two variables that showed a non-parametric distribution were IFN-γ and IL-10.

The BMI distribution of the patients at the first prenatal medical visit was as follows: Underweight 3, Normal weight 27, Overweight 30, Obesity Class I 17, Obesity Class II 5, and Obesity Class III 1. When contrasting the cytokines values according to BMI classification IL-4 was significantly higher in Underweight compared Overweight patients (97.5 ± 3.5 vs. 66.4 ± 17.6 pg/mL, $p = .037$), Underweight comparted to the Obesity Class I patients (97.5 ± 3.5 vs. 60.6 ± 17.2 pg/mL, $p = .024$) and Normal Weight compared to the Obesity Class I patients (72.2 ± 17.7 vs. 60.6 ± 17.2 pg/mL, $p = .026$). TNF-α was significantly higher in Normal Weight than Obesity Class II patients (41.9 ± 26 vs. 24.9 ± 19.7 pg/mL, $p = .031$) (Table 2, Figure 1).

Table 1. General characteristics of the patients

Variable	Minimun	Maximum	Mean	SD
Age (years)	18	43	28.5	7.1
Gestational age (weeks)	6	38	18	6
PG Weight (kg)	46.5	102	66.2	11.5
PG BMI (kg/m^2)	18.6	40.7	27.5	4.3
Pregnancies (number)	1	7	2.7	1.5
Vaginal deliveries (number)	0	6	0.88	1.3
Abortions (number)	0	3	0.47	0.73
Cesareans (number)	0	3	0.47	0.71
Weight (kg)	42.7	102	66	11.9
BMI (kg/m^2)	18.5	40.7	27.2	4.5
SBP (mmHg)	70	125	102.9	11.9
DBP (mmHg)	40	80	63.7	10.2
MAP (mmHg)	50	95	76.8	9.9
IL-4 (pg/mL)	32.5	121.7	67.5	20.2
IL-6 (pg/mL)	13.8	406.1	161.0	84.4
IL-10 (pg/mL)	2.1	244.8	51.9	43.7
IFN-γ (pg/mL)	0.04	94.2	5.9	11.1
TNF-α (pg/mL)	4.3	138.2	35.8	23.0

BMI: Body Mass Index, DBP: Diastolic Blood Pressure, IFN-γ: Interferon gamma, IL: Interleukin, MAP: Mean Arterial Pressure, PG: Pregestational, SBP: Systolic Blood Pressure, SD: Standard Deviation, TNF-α: Tumor Necrosis Factor alpha.

Table 2. Cytokines levels per BMI status

Cytokine	Underweight	Normal	Overweight	Obesity Grade 1	Obesity Grade 2	p-value
IL-4 (pg/mL) Mean ± SD	97.5 ± 3.5	72.2 ± 17.7	66.4 ± 17.6	60.6 ± 17.2	65.1 ± 3.5	.037[a] .024[b] .026[c]
95% CI	65.7-129.2	65.1-79.2	32.5-108	51.8-69.5	21.3-108.8	
IL-6 (pg/mL) Mean ± SD	221.5 ± 110.9	154.4 ± 83.1	151.5 ± 84.1	175.1 ± 82.9	192.4 ± 107.7	
95% CI	-775.3-1218.4	121.5-187.3	20-406.1	40-380.7	50.7-312.3	
IL-10 (pg/mL) Mean ± SD	67.5 ± 35.7	54.5 ± 33.8	50.7 ± 43.5	56.4 ± 62	32.5 ± 24.1	
95% CI	-253.2-388.2	41.1-67.9	34.2-67.3	24.6-88.4	2.6-62.4	
IFN-γ (pg/mL) Mean ± SD	3.8 ± 1.5	4.4 ± 5.8	4.7 ± 4.2	9.7 ± 22.5	8.2 ± 5.3	
95% CI	-10.5-18.1	2-6.7	3.1-6.4	-1.7-21.3	1.6-14.9	
TNF-α (pg/mL) Mean ± SD	49.2 ± 32	41.9 ± 26	32.8 ± 24.2	33.8 ± 14.7	24.9 ± 19.7	.031[d]
95% CI	-238.9-337.4	31.6-52.2	23.5-42	26.2-41.4	0.41-49.5	

IFN-γ: Interferon gamma, IL: Interleukin, TNF-α: Tumor Necrosis Factor alpha, a: statistical difference between Underweight and Overweight, b: statistical difference between Underweight and Obesity Grade I, c: statistical difference between Normal Weight and Obesity Grade I, d: statistical difference between Normal Weight and Obesity Grade II.

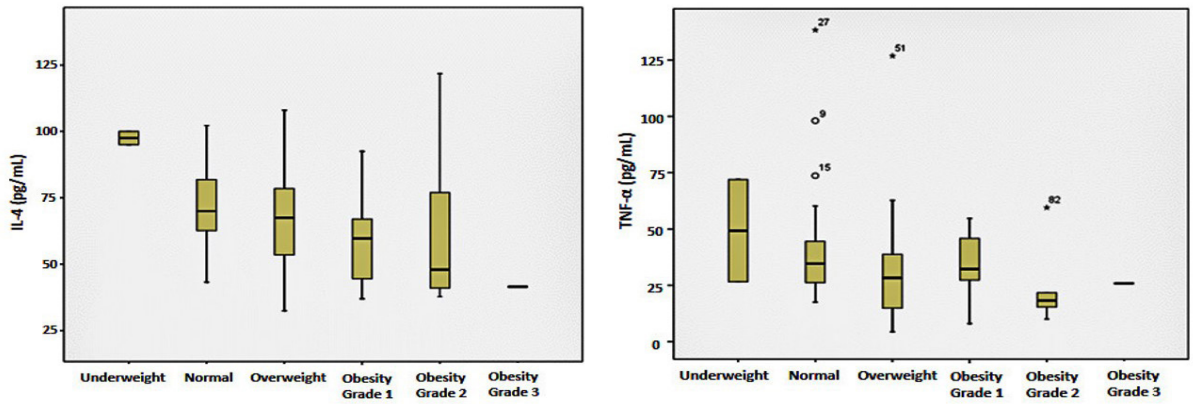

Figure 1. Serum levels of IL-4 and TNF-α per BMI classification.

BMI: Body Mass Index, IL: interleukin, TNF-α: tumor necrosis factor alpha. Data are displayed as minimum, first quartile, median, third quartile, and maximum.

From the physical exam parameters, the BMI was negatively correlated with IL-10 (r^2 = -.247, p = .024), pre-gestational BMI with IL-4 (r^2 = -.225, p = .042) and Diastolic Blood Pressure (DBP) with IFN-γ (r^2 = -.225, p = .047) and the result between Systolic Blood Pressure and TNF-α was almost significant (r^2 = .219, p = .052). Among the cytokines, IL-4 was positively correlated with

Table 3. Correlation matrix

		Age[a]	PG BMI[a]	BMI[a]	SBP[a]	DBP[a]	MAP[a]	IL-4[a]	IL-6[a]	TNF-α[a]	IL-10[b]	IFN-γ[b]
Age	Correlation	1									.005	-.018
	p*										.962	.875
PG BMI	Correlation	.289	1								-.186	.046
	p*	.009									.094	.678
BMI	Correlation	.303	.692	1							-.247	.081
	S p* ig.	.006	.000								.024	.468
SBP	Correlation	.285	.184	.201	1						.142	-.018
	p*	.011	.107	.075							.212	.874
DBP	Correlation	.135	.081	.109	.676	1					.144	-.225
	p*	.238	.478	.340	.000						.204	.047
MAP	Correlation	.147	.084	.577	.863	.955	1				.110	-.138
	p*	.188	.454	.000	.000	.000					.322	.213
IL-4	Correlation	.051	-.225	-.207	.169	.034	-.102	1			.356	-.246
	p*Sig.	.647	.042	.061	.137	.767	.358				.001	.025
IL-6	Correlation	.163	.090	.028	.090	.110	-.087	.084	1		.031	-.232
	p*	.143	.422	.803	.430	.332	.434	.448			.782	.035
TNF-α	Correlation	-.109	-.168	-.187	.219	.094	-.004	.244	.170	1	.005	-.018
	p*	.328	.132	.090	.052	.410	.970	.026	.125		.962	.875

*: Two sided significance, a: Pearson, b: Spearman, BMI: Body Mass Index, DBP: Diastolic Blood Pressure, IFN-γ: Interferon gamma, IL: Interleukin, MAP: Mean Arterial Pressure, PG: Pregestational, SBP: Systolic Blood Pressure, TNF-α: Tumor Necrosis Factor alpha.

TNF-α (r^2 = .244, p = .026) and IL-10 (r^2 = .356, p = .001); by contrast, there was a negative correlation between IFN-γ and IL-4 (r^2 = -.246, p = .025) and IL-6 (r^2 = -.232, p = .035) (Table 3). No linear relationship between the BMI difference with any of the cytokines was identified and it was also absent between the IL-4/TNF-α ratio and the baseline BMI.

Multiple regression analysis with the independent variables age, SBP, DBP, Pregestational BMI and BMI difference was performed for each cytokine and it only reached significance for TNF-α (r^2 = .142, p = .049). For this model the Variance Inflation Factor (VIF) approached 1 with the four cytokines (Table 4) indicating an acceptable degree of multicollinearity between the independent variables.

Discussion

Table 4. Multiple regression

Cytokine	Variables	Standardized coefficients	p-value	Model summary		VIF	ANOVA	
		Beta		R	R^2		F	p-value
IL-4	(Constant)		.003	.365	.133	1.15	2.180	.066
	Age	.012	.918					
	SBP	.333	.037					
	DBP	-.179	.238					
	PG BMI	-.293	.014					
	BMI difference	.090	.431					
IL-6	(Constant)		.452	.254	.065	1.06	.980	.436
	Age	.099	.433					
	SBP	-.028	.864					
	DBP	.088	.577					
	PG BMI	.066	.588					
	BMI difference	.183	.124					
IL-10	(Constant)		.792	.278	.078	1.08	1.193	.321
	Age	.143	.257					
	SBP	.122	.452					
	DBP	.074	.637					
	PG BMI	-.197	.105					
	BMI difference	-.098	.403					
IFN-γ	(Constant)		.702	.127	.066	1.07	2.067	.080
	Age	.085	.487					
	SBP	.327	.041					
	DBP	-.425	.006					
	PG BMI	.075	.525					
	BMI difference	-.032	.781					
TNF-α	(Constant)		.524	.377	.142	1.16	2.355	.049
	Age	-.127	.296					
	SBP	.383	.016					
	DBP	-.118	.433					
	PG BMI	-.198	.092					
	BMI difference	-.150	.187					

BMI: Body Mass Index, BMI difference = BMI in the first medical consultation – Pregestational BMI, DBP: Diastolic Blood Pressure, IFN-γ: Interferon gamma, IL: Interleukin, MAP: Mean Arterial Pressure, PG: Pregestational, SBP: Systolic Blood Pressure, TNF-α: Tumor Necrosis Factor alpha, VIF: Variance Inflation Factor.

In our study, the cytokines IL4-and IL-10 tended to diminish as the patient's weight increased, and the behavior of TNF-α had the same trend. The response of the first two was unexpected, as in normal pregnancy Th2 cells are upregulated at the end of the first trimester[16].

Several authors have published that pregnancies in obese women have a dysregulated maternal cytokine profile with a significant rise in proinflammatory cytokines[17,18]. In contrast, in our study, a predominant Th1 profile not confirmed.

The findings with TNF are disconcerting because their concentrations fall with a higher BMI. TNF-α is probably the most studied cytokine regarding inflammation and obesity, including pregnancy and Gestational Diabetes Mellitus (GDM). However, the reported results are heterogeneous; while some groups have published increased circulating TNF-α in maternal serum correlating with an increasing BMI, several other studies do not report that correlation[19-21]. Increased circulating TNF-α may be related to the development of GDM, but a more extensive an excellent review of these discrepancies can be found with Pantham et al[22]. This leaves the IFN as the primary agent with proinflammatory signals in the initial phase of pregnancy.

Generally speaking, although the correlation analyses produced statistically significant results, the strength of the associations was weak ($r^2<.3$). Further work is required to understand in detail the negative correlations in pregnancy between BMI with IL-10, pre-gestational BMI with IL-4, and DBP with IFN-γ. A handful of factors may contribute to the marked disparities in correlations of cytokines with anthropometric variables, but some may be interpreted as prognostic biomarkers for obstetrical complications[23-25].

Concerning the interaction among the cytokines, the positive correlation between IL-4 and IL-10, and both showing decreasing values with increasing BMI reflects a tendency to a diminished Th2 profile (anti-inflammatory state) at the beginning of pregnancy.

Maintenance of immunological tolerance to fetal antigens is a critical process during pregnancy, which must be accomplished without a detrimental effect in the induction of an immune response to pathogens. The old paradigm that pregnancy is an anti-inflammatory state has been already disregarded as new evidence has shown that pregnancy has specific mechanisms of maintenance of tolerance to fetal antigens, and a particular subset of natural killer (NK) lymphocyte cells, termed uterine NK cells, are central to this phenomenon, along with many other classical tolerance mechanisms[26].

Obese women offspring have a higher risk of developing chronic diseases associated with an altered immune function, characterized by a macrophages M1 (LPS/IFNγ) induction and more than 60% of Mexican women, are unhealthy when becoming pregnant with a BMI ≥ 30, in contrast to other countries e.g. Mongolia in which this percentage is 10%[27,28].

The functioning of the cytokine network, providing the relationships and interactions between the decidual and trophoblast cells, is one of the mechanisms responsible for the maternal immune system tolerance of the fetal antigens of paternal origin. Th1 cytokines are the key mediators of immune reactions associated with graft rejection, while Th2 cytokines mediate the immunological tolerance induction[29]. It has been shown that healthy pregnancy is associated with predominantly Th2 immunological reactions with secretion of the appropriate spectrum of anti-inflammatory cytokines. The development of complications, for example, early embryonic loss, is attributed to the Th1 response and the respective cytokine profile[30].

Low levels of anti-inflammatory cytokines during the early periods of gestation are characteristic of patients with a history of habitual miscarriages; however, an anti-inflammatory shift of the cytokine spectrum develops by the end of the first trimester[31]. A previous work demonstrated that the fetuses of class II–III obese women are exposed in utero to higher cytokine and Matrix metalloproteinases (MMP) levels than fetuses of lean women[32]. The long-term effect of each proinflammatory cytokine on the fetal prognosis is still under scrutiny[33].

Previous authors have postulated that circulating immune markers may be associated with preeclampsia. In this line, a study with Taiwanese women did not support a role of the IL-4 gene in the pathogenesis of preeclampsia[34]. On the contrary, successful pregnancy in humans has been associated with efficient production of IL-4[35]. On the other hand, IL-10 is induced in inflammatory conditions to counteract proinflammatory cytokines, thereby considered as an important homeostatic mechanism to avoid inappropriate T cell activation[36]. In the context of pregnancy, IL-10 is broadly express and shows pleiotropic effects[37].

Research concerning IFN-γ has established that IL-12 production by macrophages is known to induce its production

by CD4+ T cells[38]. Contrasting this information with our results, IFN-γ was confirmed as a critical proinflammatory cytokine, is in fact the most important in the first medical consultation.

In conclusion, in Mexican women, there is a low anti-inflammatory cytokine profile at the beginning of pregnancy, adding to the risk factors of obstetrical complications within this population[39,40].

Together, this information leads to the possibility of designing a Pregestational Healthy Index (PGHI). In an initial attempt, the construction of this PGHI should take into account not only the BMI but also the pre-gestational levels of IL-4, IL-10, IFN-γ, leptin and qualitative variables such as a family history of preeclampsia/eclampsia and gestational diabetes mellitus.

Interestingly, Retnakaran et al. have published a paper concerning the maternal pre-gravid cardiometabolic health and infant birth weight[41]. They concluded that maternal weight before and during pregnancy is the predominant cardiometabolic determinant of infant birth weight, excluding pre-gravid blood pressure, glucose, and lipid profile.

Our paper has some limitations. The pregestational BMI was calculated based on the pre-gestational weight that the patient remembered as closest before she knew she was pregnant, which undoubtedly brings several biases. Other variables that may have an influence on cytokine levels were not studied. Notwithstanding, there is enough information to recognize the challenge that doctors face managing a pregnancy to fruition if patients begin their pregnancy obese and a proper regulation in IL-4 and IL-10 are conducive to that objective.

Finally, it is important to mention that although several studies are underway to evaluate molecules that inhibit the proinflammatory signals of TNF-α and interferon[42]. However, it is unlikely that TNF-α and interferon would approved for studies on pregnant women due to the teratogenic risks and even less so because it is already known that a balanced diet is a key to have a pregnancy with minimal risks.

Based on the above, everything indicates that our objective should be to design strategies help Mexican women have the best health before pregnancy, that because of economic and cultural conditions it is currently a great challenge to maintain a healthy weight after becoming pregnant.

Conflict of Interest

All of the authors declare that there are no competing interests regarding the publication of this paper.

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