Journal of Diabetes Research

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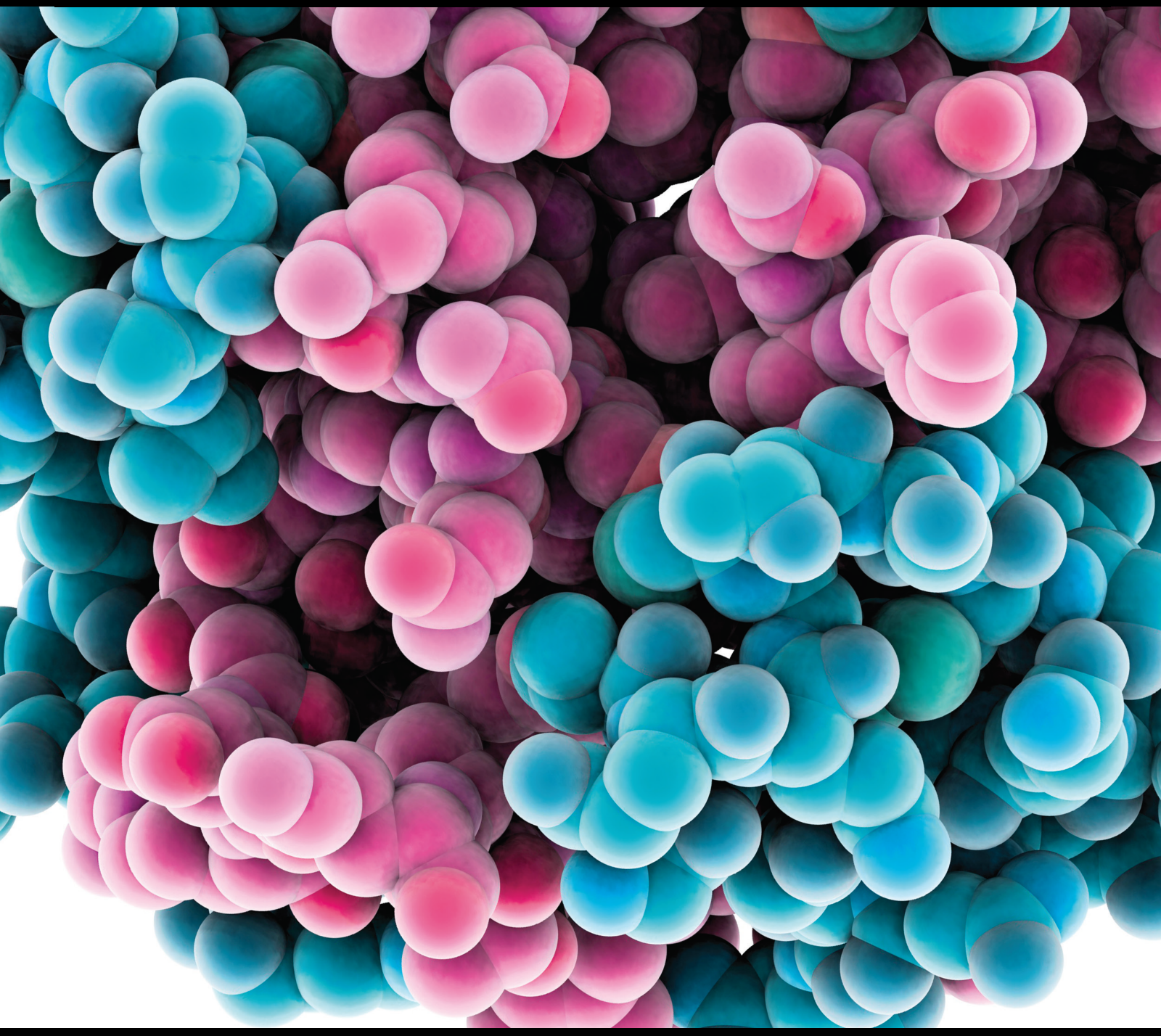

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Research Article

Myeloid-Derived Suppressor Cells Show Different Frequencies in Diabetics and Subjects with Arterial Hypertension

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Type 2 diabetes mellitus (DM2) is strongly associated with other comorbidities such as obesity, atherosclerosis, and hypertension. Obesity is associated with sustained low-grade inflammatory response due to the production of proinflammatory cytokines. This inflammatory process promotes the differentiation of some myeloid cells, including myeloid-derived suppressor cells (MDSCs). In this study, two groups of individuals were included: DM2 patients and non-DM2 individuals with similar characteristics. Immunolabeling of CD15+ CD14- and CD33+ HLA-DR-/low was performed from whole peripheral blood, and samples were analyzed by flow cytometry, and frequencies of MDSCs and the relationship of these with clinical variables, cytokine profile (measured by cytometric bead array), and anthropometric variables were analyzed. The frequency of CD33+ HLA-DR-/low MDSCs (that produce IL-10 and TGF-β, according to an intracellular detection) is higher in patients with DM2 ($P < 0.05$), and there is a positive correlation between the frequency of CD15+ CD14- and CD33+ HLA-DR-/low MDSC phenotypes. DM2 patients have an increased concentration of serum IL-5 ($P < 0.05$). Also, a negative correlation between the frequency of CD15+ CD14- MDSCs and LDL cholesterol was found. Our group of DM2 patients have an increased frequency of mononuclear MDSC CD33+ HLA-DR-/low that produce TGF-β and IL-10. These cytokines have been associated with immune modulation and reduced T cell responses. DM2 and non-DM2 subjects show a similar cytokine profile, but the DM2 patients have an increased concentration of IL-5.

1. Introduction

According to the American Diabetes Association (ADA), diabetes mellitus is a metabolic disease characterized by severe hyperglycemia due to defects in insulin secretion or the lack of proper action of this hormone in the target tissues. Type 2 diabetes mellitus (DM2) is the one that has the greatest impact worldwide, accounting for 90-95% of all reported cases of diabetes worldwide as reported by the ADA [1, 2]. The World Health Organization (WHO) estimated that 347 million cases of DM2 exist worldwide as of 2014 [3], and also, recent estimations suggest that DM2 will be the 7th cause of mortality by 2030 [4]. Data from the International Diabetes Federation suggest 4.9 million deaths associated with

diabetes and its related complications [5]. In Mexico, the National Institute for Statistics and Geography (INEGI, acronym in Spanish) has reported that 70 out 1000 deaths were caused by diabetes and its complications causing a great economic burden to the national health institutions [6].

It has been described that the main factors associated with an increased risk of developing DM2 are obesity, unhealthy eating habits, sedentarism, advanced age, family history of diabetes, ethnicity, etc. [7–9]. The relationship between diabetes and obesity has been widely documented, and around 90% of diabetics are overweight or obese [10]. Obesity has also been associated with low-grade chronic inflammatory processes, and several cytokines such as tumor necrosis factor alpha (TNF-α) have been shown to be elevated in obese individuals due to an increased activity of adipose tissue-derived cytokine production and insulin resistance [11, 12]. Other bioactive molecules such as leptin, IL- (interleukin-) 6, resistin, and monocyte chemoattractant protein 1 (MCP-1) have been associated with insulin resistance [13–17].

Chronic inflammatory processes have been associated with alterations in leucocyte composition. In the seventies, a subset of myeloid cells with suppressor properties was described in patients with several types of cancer. These cells were at the moment described as "natural suppressor cells," but the functional properties and markers of such cells have been recently described and named as myeloid-derived suppressor cells (MDSCs) [18–20]. These cells are a heterogeneous group of myeloid cells such as myeloid progenitors, immature myeloid cells, immature granulocytes, and immature dendritic cells. In humans, the main MDSCs have been described as immature monocytic and granulocytic cells, and several reports suggest an elevation in the frequency of MDSCs in some pathologic conditions, including cancer, sepsis, and acute and chronic inflammatory processes [19, 20]. Several phenotypes for these cells have been described such as CD15+ CD14- and also CD33+ HLA-DR-/low [19] to mention some [21]. It has been described that these cells have immune suppressive functions [22], and they are producers of interferon gamma (IFN-γ), IL-10, and transforming growth factor beta (TGF-β) [23].

As described previously, obesity is the biggest risk factor for developing DM2. This has also been associated with low-grade chronic inflammation with overproduction of TNF-α, IL-6, and IL-1β and other bioactive molecules in the adipose tissue such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), vascular endothelial growth factor (VEGF), or IFN-γ [24–27]. Taking all the previously described data and the fact that diabetic individuals with DM2 have a higher predisposition to infection due to diminished immune responses [28–30], it becomes really important to understand if several cell populations that suppress the immune response such as the MDSCs are major conditioning factors that promote deficiencies in the development of several immune-mediated mechanisms in DM2 individuals [19]. The aim of the present paper was to compare the frequency of myeloid cells with the phenotypes CD15+ CD14- and CD33+ HLA-DR-/low producers of IL-10 and TGF-β in peripheral blood of patients with DM2 and non-DM2 subjects. The correlation between the MDSC immunophenotypes with common comorbidities to diabetics, laboratory, cytokine levels, cell suppressive function, and anthropometric data was also analyzed.

2. Material and Methods

2.1. Participants' Inclusion Criteria. DM2 patients of the study were recruited at the medical family care unit # 4 (Zacatecas, Mexico) according to approved protocols (R-2011-785-063); the visits were made between March 19 and May 19 in 2015. DM2 subjects ($n = 23$, age range of 35-62 years old) were invited to participate. DM2 subjects complied with the ADA criteria for DM2 diagnosis as follows: random glucose measurement of >120 mg/dl and/or glucose tolerance test > 200 mg/dl (for newly diagnosed individuals) and/or glycated hemoglobin (HbA1c) > 6.5%. Nondiabetic subjects (non-DM2) ($n = 21$) were negative for diabetes according to ADA criteria and were recruited at the same clinic with that from a similar age group. Clinical and laboratory data from all participants was collected from the routine follow-up at their clinic. Common comorbidities (reported by the treating physician) were also recovered from the medical records.

2.2. Ethics Statement. The present study was approved by the national commission on scientific investigation (CNIC) at the Mexican Institute for Social Security (IMSS) as well as the national ethics commission with registration number R-2011-785-063. All protocols were based on the International Declaration of Helsinki and in the principles of not malevolence, justice, and equality. All participants that agreed to participate signed an informed consent, and blood samples were drawn from only these individuals.

2.3. MDSC Phenotype and Flow Cytometry Analysis. Immunophenotypes were identified according to Ko et al. [31]. Briefly, blood samples were obtained from the participants by means of venipuncture. 4 ml of blood was collected. 100 μl of whole blood was used with a lyse/wash protocol for staining. For the identification of the MDSC phenotypes, a combination of the following antibodies was used: CD14 PECy7 and CD15 PE-Cy5 (BD Biosciences, USA); CD33 APC and HLA-DR PerCP-Cy5.5 (BD Biosciences, USA). All antibodies were titrated before use. Immunostained samples were analyzed by flow cytometry in a FACSCanto II (Becton Dickinson, USA) with 488 and 633 laser lines and a standard configuration of 4-2 detectors. Data acquisition was performed by FACSDiva software v. 6.1 (BD Biosciences, USA), and 50000 events were acquired for each sample. The time parameter was used as quality control, observing stability between the performed analyses. Analysis of flow cytometry data was carried out in the FCS 3.0 files after export in FlowJo Software v. 8.7 (FlowJo LLC, USA).

2.4. Intracellular Staining of IL-10 and TGF-β. Blood samples were obtained from 4 DM2 patients and 4 nondiabetic

Table 1: Clinical features of patients.

Variable	Group		P value
	Non-DM2 ($n = 21$)	DM2 ($n = 23$)	
Gender (female/male)	11/10	12/11	1.000[a]
Age (years)	43 (q1 = 42, q3 = 50)	50 (q1 = 40, q3 = 62)	0.264[b]
Years with diabetes		6.0 (q1 = 2, q3 = 14)	
BMI (kg/m^2)	27.55 (q1 = 26.07, q3 = 30.22)	27.4 (q1 = 24.2, q3 = 28.7)	0.275[b]
Waist to hip ratio	0.94 (q1 = 0.91, q3 = 0.96)	0.93 (q1 = 0.89, q3 = 0.98)	0.913[b]
Glucose (mg/dl)	112 (q1 = 93, q3 = 117)	185.4 (q1 = 111, q3 = 233)	0.001*[b]
Hb1Ac (%)	5.9 (q1 = 5.8, q3 = 6.2)	8.5 (q1 = 6.3, q3 = 10)	0.000*[b]
Total cholesterol (mg/dl)	207 (q1 = 260, q3 = 231)	209.4 (q1 = 170, q3 = 230)	1.000[b]
cHDL (mg/dl)	48.3 (q1 = 40.9, q3 = 52.6)	49 (q1 = 38.2, q3 = 55.8)	0.869[b]
cLDL (mg/dl)	119.8 (q1 = 99.7, q3 = 156.3)	130.6 (q1 = 81.1, q3 = 160.9)	0.860[b]

[a]Fisher's exact test. [b]Mann-Whitney U test. $^*P < 0.05$.

participants by venipuncture. 100 μl of whole blood was used with a lyse/wash protocol, and for cellular permeabilization, we used the BD Cytofix/Cytoperm™ Kit (BD Biosciences, USA) according to the manufacturer's instructions. Intracellular staining was performed immediately afterwards with the antibodies for TGF-β PE and IL-10 PE in separate tubes (BD, Biosciences, USA). Fluorescence minus one (FMO) was used as the control. Immunostained samples were analyzed by flow cytometry in a FACSCanto II (Becton Dickinson, USA), and analysis of flow cytometry data was carried out in the FCS 3.0 files after export in FlowJo Software v. 8.7 (FlowJo LLC, USA).

2.5. Cytokine Quantification by Cytometric Bead Array. The concentration of TNF-α, IFN-γ, IL-1α, IL-5, IL-10, IL-12p70, IL-17, and eotaxin was analyzed by cytometric bead array using the manufacturer's specifications (BD Biosciences, USA). Briefly, starting with cytokine standard preparation and cytokine capture bead mixture, 50 μl serum samples from each individual and dilutions for the standard curve were placed in a multiscreen 1.2 μm, 96-well filter plate (Merck Millipore, Germany), and the mixed capture beads and phycoerythrin detection reagent were added. After 3-hour incubation and repeated wash steps, the data was acquired in a FACSCanto II flow cytometer (Becton Dickinson, USA) and analyzed using FCAP Array v3.0 (BD Biosciences, USA) to convert fluorescent intensity values to concentrations using a standard curve.

2.6. Statistical Analysis. Normality of data was verified by means of a Kolmogorov-Smirnov test or a D'Agostino-Pearson normality test. Group comparisons for continuous quantitative variables were made with a Mann-Whitney U test for the nonparametric data or a Welch corrected t-test for normally distributed data. Categorical variables were analyzed with a Fisher exact test. A correlation analysis was made to explore the correlation of MDSC, laboratory, and anthropometric data by using a Spearman correlation (non-normal distribution was verified). The two-tailed level of significance used for all analysis was $\alpha = 0.05$. All statistical analysis was carried out in the GraphPad Prism software v6.0 (GraphPad Software, USA).

3. Results

3.1. Clinical Features of Patients with DM2 and Nondiabetic Controls. Clinical features, laboratory data, and anthropometric data were analyzed in order to determine whether differences were present due to heterogeneity of the population or whether differences in MDSC cells were associated with other parameters such as metabolic markers of disease or cardiovascular risk factors. As shown in Table 1, no major differences were identified in the groups in the variables such as age, sex, body mass index, waist to hip ratio, low-density lipoprotein cholesterol (cLDL), high-density lipoprotein cholesterol (cHDL), and total cholesterol. As expected, there were significant differences between DM2 subjects for both Hb1Ac% and glucose levels due to the inclusion criteria. No differences were identified either for markers associated with alterations in lipid metabolism or cardiovascular risk.

3.2. MDSCs with CD15+ CD14- and CD33+ HLA-DR-/Low Phenotypes Are Present in Both DM2 and Non-DM2 Subjects. As mentioned above, several phenotypes of MDSCs have been identified and thoroughly characterized. The CD33+ HLA-DR-/low MDSCs are a subtype of myeloid cells reported by Ko et al. to have suppressive functions in renal cell carcinoma [31]. As shown in Figure 1(a), we identified such population with a similar gating strategy, and differences in both diabetics and nondiabetic subjects were also identified. For the CD15+ CD14- MDSC population (which has been described as another important subpopulation regulating in a negative manner the immune responses in some cancer patients) [31], samples of diabetics and nondiabetics show the presence of this population in low frequency (Figure 1(b)).

3.3. Patients with DM2 Have Different Frequencies of MDSCs. Given the suppressive role of MDSCs over other immune cells, one of the main goals of this study was to evaluate the frequency of MDSCs in patients with DM2 compared to non-DM2 subjects. For this purpose, the frequency of total cells was evaluated for both the CD33+ HLA-DR-/low and the CD15+ CD14- phenotypes. Significant differences were identified for the CD33+ HLA-DR-/low mononuclear

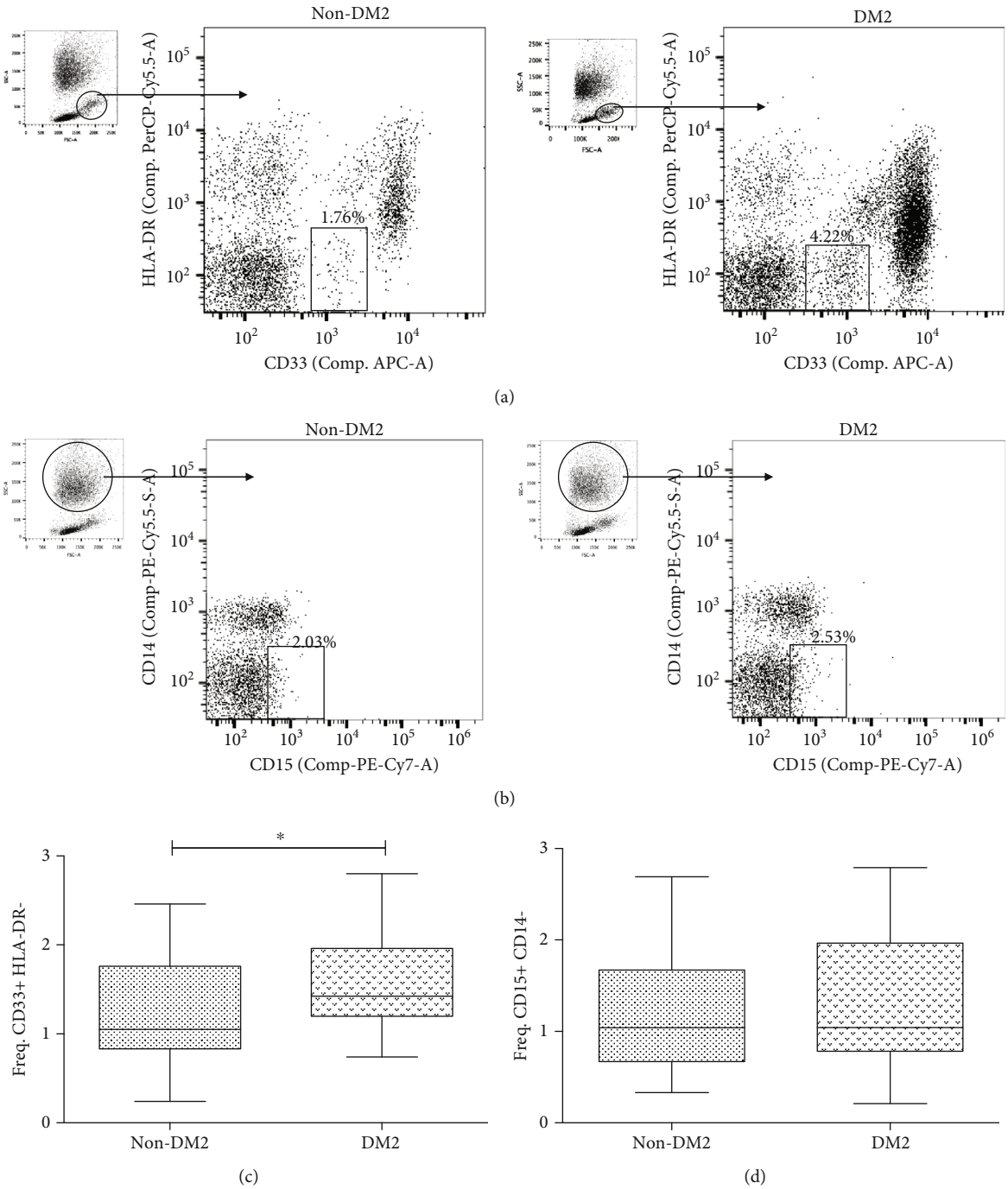

Figure 1: Gating strategy and frequencies of MDSCs in DM2. (a) Dotplot APC vs. PerCP-Cy5.5 shows PBMCs and the gate mark of the CD33+ HLA-DR-/low MDSCs. (b) Dotplot PE-Cy7 vs. PE-Cy5 shows PBMCs and PMNs and the gate mark of the CD15+ CD14- MDSCs. (c) Frequency of CD33+ HLA-DR-/low MDSCs, comparing the group of DM2 ($n = 22$) with the non-DM2 group ($n = 21$). The graph shows the median with interquartile ranges. (d) Frequency of CD15+ CD14- MDSCs, comparing the group of DM2 ($n = 20$) with the non-DM2 group ($n = 21$). The graph shows the mean and standard deviation. A P value < 0.05 was considered as statistically significant which was calculated using the statistical test of Mann-Whitney. Statistical analysis was performed with the GraphPad Prism® v5.0 software. Data were obtained in a BD FACSCanto II® Flow Cytometer and were analyzed with the software FlowJo® v10.0.

MDSCs in patients with DM2 as compared to non-DM2 ($P < 0.05$, Figure 1(c)). For the CD15+ CD14- MDSC cell population, no differences between groups were found ($P > 0.05$, Figure 1(d)).

3.4. CD33+ HLA-DR-/Low MDSCs Produce TGF-β and IL-10. One of the major mechanisms by which MDSCs regulate the T cell-mediated immune response is by the production of IL-10 and TGF-β. Therefore, we evaluated the production of

these cytokines in DM2 patients and controls. To demonstrate that the CD33+ HLA-DR-/low MDSCs are an immunoregulatory subset of cells, we did an intracellular staining of IL-10 and TGF-β and we found that this subpopulation actually produces these cytokines (Figure 2), suggesting that the immunosuppressive function of the MDSCs is also increased in the DM2 patients.

3.5. DM2 and Non-DM2 Subjects Show a Similar Cytokine Profile. Several reports suggest an association of increased frequencies of MDSCs and overproduction of inflammatory cytokines. Due to the elevation of the frequencies in the CD33+ HLA-DR-/low MDSCs in the diabetic group, we wondered whether this increased frequency of CD33+ HLA-DR-/low MDSCs could be related to cytokine overproduction. Therefore, several proinflammatory cytokines such as IL-1α, TNF-α, IFN-γ, IL-5, IL-12p70, and IL-17 and one anti-inflammatory IL-10 were quantified by cytometric bead array and analyzed through FCAP Array. The concentrations were classified for groups and compared between groups. Only significant differences were found in the comparison of IL-5 ($P = 0.02$); the other cytokines did not show any significant differences (Table 2). To corroborate, a correlation analysis was made between the concentrations of the cytokines and the frequencies of CD33+ HLA-DR-/low MDSCs. No correlation between these parameters was found ($P > 0.05$) (Supplementary Table 1). Finally, the same correlation analysis was made; considering only the DM2 group, no significant correlations were identified either (Supplementary Table 2).

3.6. CD33+ HLA-DR-/Low MDSC Increased Frequency Is Associated with Individuals with Hypertension. It is known that some cell subtypes in the blood are associated with an increase in cardiovascular alterations and comorbidities, such as the case of T helper 17 (Th17) cells that respond to sodium increase in essential hypertension and have been recently linked to the pathogenesis of arterial hypertension [32, 33]. In order to identify if other variables were also associated with the increase in MDSCs, we evaluated if hypertension had any effect on the frequency of MDSCs. For this purpose, all subjects were polled for analysis and categorized according to having or not hypertension (reported in their clinical history) in two groups: hypertensive (which includes diabetic and nondiabetic) and nonhypertensive (which includes diabetic and nondiabetic). When the analysis was performed on these assumptions, we found a difference in the frequencies of CD33+ HLA-DR-/low MDSC phenotype ($P < 0.05$, Figure 3(a)) between hypertensive (mean = 1.620, SD = 0.602) and nonhypertensive (mean = 1.209, SD = 0.483) groups. These differences are not observed for the frequency of CD15+ CD14- MDSCs, although a slight increase is observed in the group that has hypertension ($P > 0.05$, Figure 3(b)).

3.7. MDSC Phenotypes Correlate with Cardiovascular Markers of Disease. Given the previously shown results, MDSCs are elevated in the DM2 group and also in hypertensive individuals. To further clarify whether differences are associated with cardiovascular markers or diabetes markers, a correlation analysis was carried out. It was decided to perform the analysis for nonparametric data; of all the variables analyzed (age, BMI, WHR, glucose, Hb1ac%, cholesterol, cHDL, and cLDL), only the LDL cholesterol levels have a negatively statistically significant ($P < 0.05$) correlation with the frequency of CD15+ CD14- MDSCs (Figure 4(b)). Additionally, the frequency of CD33+ HLA-DR-/low and CD15+ CD14- MDSCs has a statistically significant correlation ($P < 0.001$, Figure 4(a)), and this correlation is maintained when only the DM2 group is analyzed ($P = 0.029$, Supplementary Table 3), so this correlation is directly associated with DM2 condition. Data correlations with Spearman's rho value are summarized in Table 3.

4. Discussion

It is known that subjects with diabetes have an increased susceptibility to infectious diseases. For active tuberculosis infection, a risk of more than 5-fold has been reported in diabetics compared to nondiabetic subjects [34, 35]. Recent reports suggest that there are alterations in the immune cells such as neutrophil chemotactic and phagocytic functions that contribute to this increased susceptibility to infection in diabetics [36]; however, other cell populations might be associated with this phenomenon. MDSCs were linked in the last few years with increased susceptibility to infectious diseases; particularly, it was recently described that frequencies of these cells are elevated in patients with tuberculosis [37]. Recently, it has been shown that low-grade inflammation induced by obesity and infiltrating cytokine-producing adipose tissue immune cells is associated with several complications of diabetics such as insulin resistance [38]. Given that chronic inflammation is present in these individuals and due to the previously described reports that suggest that this might be associated with MDSC expansion, the present report evaluated whether or not diabetes-associated low-grade inflammatory processes might be associated with increased frequencies of MDSCs.

It has been described that there is a basal frequency of MDSCs in healthy individuals, although very small (around 1%) for both monocytic and polymorphonuclear MDSCs [31]; consistent with these findings, similar frequencies in our groups are reported. The physiological role of these cells is thought to be associated with immune regulation by several mechanisms, such as arginine depletion which causes a reduced activity of T cells and a decrease in CD62L in lymphocytes caused by ADAM metallopeptidase domain 17 (ADAM17), and through the expansion of regulatory T (Treg) cells by TGF-β secretion and IL-10 [20], we confirm that our population of CD33+ HLA-DR- mononuclear MDSCs produces IL-10 and TGF-β in both DM2 and nondiabetic donors.

A statistically significant rise in the frequency of CD33+ HLA-DR-/low MDSCs was found in the DM2 group; these findings are similar to those reported by Wang et al. [39] where they find an increased frequency of MDSC CD11b+ CD33+ and probe how these MDSCs inhibit T cell proliferation; although the phenotype is a little different (CD11b+

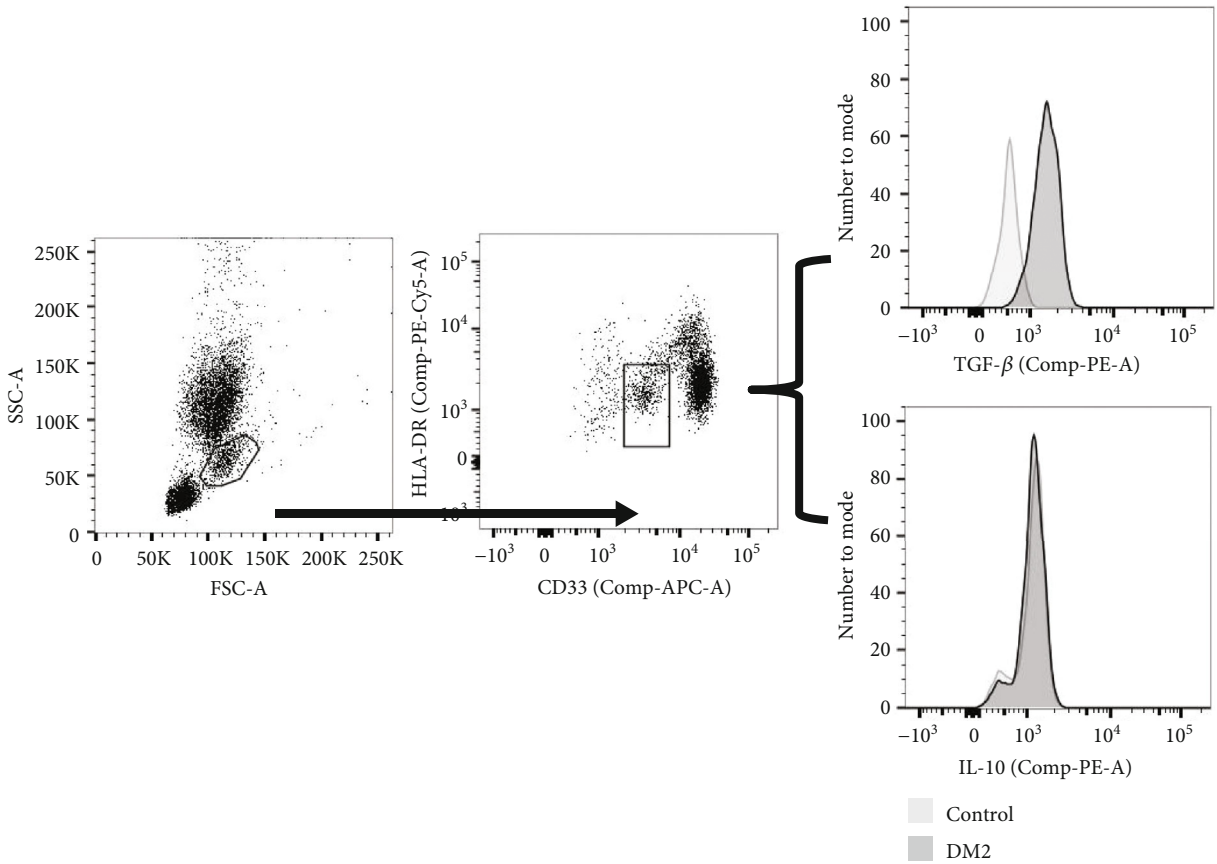

Figure 2: CD33+ HLA-DR-/low MDSCs produce IL10 and TGF-β. Representative data of the intracellular staining of IL-10 and TGF-β on CD33+ HLA-DR-/low MDSCs. Data were obtained in a BD FACSCanto II® Flow Cytometer and were analyzed with the software FlowJo® v10.0.

Table 2: Comparison of serum cytokine concentrations among study groups[#].

Variable	Non-DM2	DM2	P value
Eotaxin ($\bar{x} \pm$ SD)	4475 pg/dl ± 1987	5338 pg/dl ± 2409	0.2127[a]
IL-1α ($m \pm$ IQR)	482.1 pg/dl q1 = 365.8, q3 = 665	415.1 pg/dl q1 = 323.1, q3 = 487.8	0.1741[b]
TNF-α ($m \pm$ IQR)	69.64 pg/dl q1 = 14.16, q3 = 149.4	42.75 pg/dl q1 = 34.94, q3 = 133.5	0.8591[b]
IFN-γ ($m \pm$ IQR)	642.8 pg/dl q1 = 397, q3 = 1534	400.1 pg/dl q1 = 83.98, q3 = 1252	0.4202[b]
IL-5 ($m \pm$ IQR)	2.850 pg/dl q1 = 0.4175, q3 = 15.89	38.4 pg/dl q1 = 4.42, q3 = 74.72	0.0200*[b]
IL-10 ($m \pm$ IQR)	16.65 pg/dl q1 = 13.03, q3 = 32.66	26.68 pg/dl q1 = 11.49, q3 = 46.71	0.5652[b]
IL-12p70 ($m \pm$ IQR)	22.18 pg/dl q1 = 2.40, q3 = 60.23	22.65 pg/dl q1 = 16.11, q3 = 34.25	0.9004[b]
IL-17 ($m \pm$ IQR)	117.9 pg/dl q1 = 10.76, q3 = 298.4	93.97 pg/dl q1 = 65.10, q3 = 434.9	0.9999[b]

[#]Only the concentrations obtained above detection limits for each cytokine were considered for analysis. [a]Student's t-test. [b]Mann-Whitney U test. *$P < 0.05$.

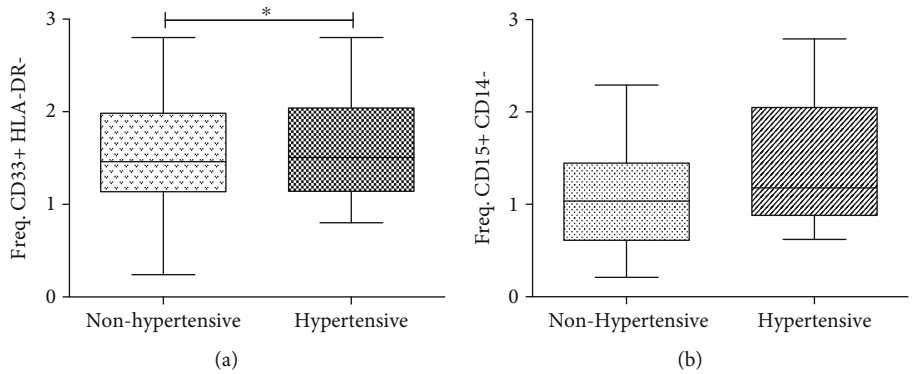

Figure 3: Frequency of CD33+ HLA-DR-/low and CD15+ CD14- MDSCs comparing if the subjects have hypertension in both groups (DM2 and non-DM2). (a) Frequency of CD33+ HLA-DR-/low MDSCs (hypertensive $n = 16$, nonhypertensive $n = 27$). (b) Frequency of CD15+ CD14- MDSCs (hypertensive $n = 15$, nonhypertensive $n = 26$). The graphs show the median and interquartile ranges. It was considered as statistically significant if P value < 0.05* which was calculated using the statistical t-test for unpaired samples. Statistical analysis was performed with the GraphPad Prism® v5.0 software.

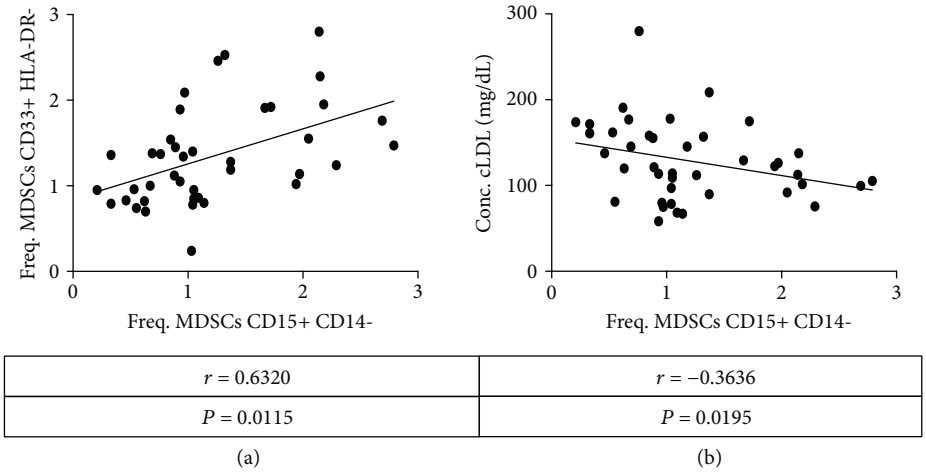

Figure 4: Correlation of MDSC frequency with cardiovascular markers. (a) Correlation between Freq. CD15+ CD14- and CD33+ HLA-DR-/low MDSCs. Data from 44 subjects, including groups in DM2 and nondiabetic controls. (b) Correlation between the concentration of LDL cholesterol and Freq. CD15+ CD14- MDSCs. Shown data are from 44 subjects (DM2 and nondiabetic controls). It was considered as statistically significant if value of $P < 0.05$. Statistical analysis was performed with the GraphPad Prism ® v5.0 software.

CD33+ vs. CDD33+ HLA-DR-/low), both correspond to a mononuclear immunophenotype; our findings reaffirm the importance that MDSCs could play in the pathophysiology of DM2 suppressing T cell response making patients susceptible to some infections and also provide a link to cardiovascular alterations in DM2 patients. The causes behind such increase could be associated with the production of TGF-β and IL-5. Several reports have described that the production of such cytokines in diabetics and obese people is increased and linked to insulin resistance [26]. Also, in diabetics, an increase in signaling pathways such as JNK and IκB kinase beta (IKKβ) has been described to be associated with insulin resistance, and these same signaling pathways are responsible of MDSC expansion in bone marrow [40–43]. Therefore, the low-grade inflammation process characteristic of diabetics could be associated with such observed expansion. Surprisingly, our results did not show differences in the cytokine profile, perhaps because all the patients were already under medical oversight and treatment. In our sample, there were no differences in the BMI (which is one of the variables associated with low-grade inflammation). Interestingly, the only cytokine that is elevated in our sample of DM2 was IL-5. This cytokine that has been reported to be increased in those patients with DM2 and tuberculosis [44], common comorbidity associated with immunodeficiency in DM2 patients; nevertheless, further investigation is needed to confirm the role of IL-5 in susceptibility to infections in DM2.

Systemic arterial hypertension (SAH) is a common comorbidity of DM2 patients, according to the National Health and Nutrition Survey (ENSANUT, acronym in Spanish) in 2016; in Mexico, patients with a previous diagnosis of diabetes have a prevalence of 57.6% with hypertension [45]. Therefore, when we analyzed according to SAH, a significant increase in MDSC frequency was found. Recent reports have

Table 3: Correlation analysis of variables with the frequency of MDSCs[#].

		Freq. MDSCs CD15+ CD14-	Freq. MDSCs CD33+ HLA-DR-
Age	Correlation coefficient	-0.083	-0.117
	Significance (two-tailed)	0.607	0.456
	N	41	43
Time of diagnosis (years)	Correlation coefficient	-0.080	0.039
	Significance (two-tailed)	0.744	0.869
	N	19	20
BMI (kg/m^2)	Correlation coefficient	0.180	0.054
	Significance (two-tailed)	0.259	0.731
	N	41	43
Waist-hip ratio	Correlation coefficient	-0.259	-0.139
	Significance (two-tailed)	0.107	0.378
	N	40	42
Fasting glucose	Correlation coefficient	-0.097	0.252
	Significance (two-tailed)	0.547	0.103
	N	41	43
HbA1c (%)	Correlation coefficient	-0.040	0.255
	Significance (two-tailed)	0.802	0.100
	N	41	43
Total cholesterol (mg/dl)	Correlation coefficient	-0.284	-0.083
	Significance (two-tailed)	0.071	0.597
	N	41	43
cHDL (mg/dl)	Correlation coefficient	0.104	-0.044
	Significance (two-tailed)	0.518	0.779
	N	41	43
cLDL (mg/dl)	Correlation coefficient	-0.364*	-0.110
	Significance (two-tailed)	0.019	0.482
	N	41	43
Triglycerides (mg/dl)	Correlation coefficient	-0.068	-0.068
	Significance (two-tailed)	0.675	0.665
	N	41	43
Freq. MDSCs CD15+ CD14-	Correlation coefficient	1.000	0.511**
	Significance (two-tailed)		0.001
	N	41	40
Freq. MDSCs CD33+ HLA-DR-	Correlation coefficient	0.511**	1.000
	Significance (two-tailed)	0.001	
	N	40	43

[#]Correlations were calculated with Spearman's rho. Differences in the sample size for each correlation may differ depending on the availability of the data for such patients or controls. $^{*}P < 0.05$; $^{**}P < 0.01$.

documented that hypertension has an important inflammatory component particularly associated with Th17 cells that have a high response to salt concentrations [46]. Given that these cells are able to produce IL-6 and other cytokines [47] that are able to induce proliferation of MDSCs, our hypothesis is that such phenomena are intertwined, meaning that MDSC increased frequencies are probably a consequence of inflammatory processes from diabetes, obesity, and hypertension. This is further supported by our own results given that increased frequency of MDSC cells correlates between the two phenotypes; high levels of LDL cholesterol correlated negatively with CD15+ CD14- MDSCs, and this could be related to the generation of atheromatous plaques in blood vessels given that an increase of such inflammatory cells with

phagocytic functions such as these is able to adhere to such plaques. These hypotheses need to be confirmed by experimental observations.

5. Conclusion

Our group of DM2 patients have an increased frequency of mononuclear MDSCs CD33+ HLA-DR-/low that produce TGF-β and IL-10. DM2 and non-DM2 subjects show a similar cytokine profile, but the DM2 patients have an increased concentration of IL-5. CD33+HLA-DR-/low MDSCs are associated with other complications of diabetics such as hypertension and cardiovascular markers of the disease (cLDL).

Data Availability

The data used in this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

JECD conceived and designed the experiments; JCFR, JCGA, and MAVA performed the experiments; JCFR and JCGA analyzed the data; JECD, MLMF, IGV, ARCV, CJSE, MHGH, and JAEM contributed reagents/materials/analysis tools; JCFR and JECD wrote the paper; JCFR, JCGA, MLMF, IGV, ARCV, MAVA, CJSE, MHGH, JAEM, and JECD critically reviewed the manuscript.

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Supplementary Materials

Supplementary Table 1: correlation analysis of cytokine concentrations and MDSC frequency (CD33+ HLA-DR- phenotype). Supplementary Table 2: correlation analysis of cytokine concentrations and frequency of MDSCs CD15+ CD14- in the DM2 group. Supplementary Table 3: correlation analysis of variables with the frequency of MDSCs (only DM2 group). *(Supplementary Materials)*

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Research Article

Pathological Features of Diabetic Retinopathy in Spontaneously Diabetic Torii Fatty Rats

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Objective. The Spontaneously Diabetic Torii (SDT) fatty rat, established by introducing the *fa* allele (obesity gene) of the Zucker fatty rat into the SDT rat genome, is a new model of obese type 2 diabetes. We studied the pathologic features of diabetic retinopathy (DR) in this animal. *Methods*. The eyes of SDT fatty, SDT (controls), and Sprague Dawley (SD) rats (normal controls) were enucleated at 8, 16, 24, 32, and 40 weeks of age (n = 5-6 for each rat type at each age). The retinal thicknesses, numbers of retinal folds, and choroidal thicknesses were evaluated. Immunostaining for glial fibrillary acidic protein (GFAP) and vascular endothelial growth factor (VEGF) was performed. Quantitative analyses of the immunopositive regions were performed using a cell-counting algorithm. *Results*. The retinas tended to be thicker in the SDT fatty rats and SDT rats than in the SD rats; the choroids tended to be thicker in the SDT fatty rats than in the SD rats. The retinal folds in the SDT fatty rats developed earlier and were more severe than in the SDT rats. Quantitative analyses showed that the GFAP- and VEGF-positive regions in the retinas of the SDT fatty rats were significantly larger than those of the SDT rats. *Conclusions*. SDT fatty rats developed more severe DR earlier than the SDT rats. The SDT fatty rats might be useful as a type 2 diabetes animal model to study DR.

1. Introduction

Diabetes has reached nearly epidemic levels worldwide. Many patients with diabetes with long histories of morbidity have one or more diabetic complications, such as diabetic nephropathy, diabetic peripheral neuropathy, or diabetic retinopathy (DR), all of which impact the patients' quality of life.

Among the ocular complications associated with diabetes, DR is a leading cause of visual loss and blindness in adults in developed countries [1]. Researchers need to determine how DR develops and preventative measures using animal models of diabetes. To that end, an animal model of diabetes with ocular complications that mimic human DR should be established.

Many diabetic animal models have been reported [2]. The Goto-Kakizaki (GK) rat is a nonobese animal with mild type 2 diabetes [3, 4]. Although electroretinography (ERG) showed functional abnormalities of photoreceptors in GK rats [5], no significant differences were observed in the retinal arterial and venous diameters [6]. Énzsöly et al. reported that degenerative changes in the photoreceptors and pigment epithelium developed in streptozotocin-induced diabetic rats [7]. Using male Wistar and Sprague Dawley (SD) rats, those investigators found no significant differences in the retinal

thicknesses between the normal and diabetic rats. Long-Evans Tokushima Lean rats have been used as a model of type 1 diabetes [8, 9]. Although pancreatic changes and genetic analysis were discussed in those studies, no ocular complications were mentioned. Yang et al. [10] reported that the retinas of Otsuka Long-Evans Tokushima Fatty rats, a well-known model of type 2 diabetes, were significantly thinner than normal Long-Evans Tokushima Otsuka rats, and that tendency was apparent in the retinal nerve fiber layer using spectral-domain optical coherence tomography (OCT). While the ocular findings in the diabetic animal models in those studies are important to the understanding of the diabetic ocular complications, the ocular changes in those models differ markedly from those in humans. In particular, no retinal thickening occurs in most diabetic animals, unlike in patients with diabetic macular edema (DME).

A spontaneously type 2 diabetic strain of the SD rat, the Spontaneously Diabetic Torii (SDT) rat, was established in 1997 [11]. Hyperglycemia, nephropathy, and peripheral neuropathy have been reported in this rat [12]. We reported the severe diabetic ocular complications in this model [13–16]. The retinas tended to be thicker in SDT rats than in SD rats [16]. Mature diabetic cataracts and proliferative DR, especially, resemble human diseases in SDT rats [11, 15] and appear only in this diabetic rat model. We also studied the effect of ranirestat, an aldose reductase inhibitor, on DR in the SDT rats [17].

However, the systemic features and DR in the SDT rat differ somewhat from those in humans. It takes a long time to develop DR in the SDT rat. It has been reported that severe DR is found in 80% of the SDT rat at 51 to 60 weeks of age [13]. For that reason, a more suitable experimental animal model of DR is needed.

The SDT fatty rat, established in 2004 by introducing the *fa* allele (obesity gene) of the Zucker fatty rat into the SDT rat genome, is a new model of obese type 2 diabetes. The prominent findings of hyperglycemia, overt obesity, hyperlipidemia, and diabetes-related complications (nephropathy, peripheral neuropathy, etc.) develop at a younger age in SDT fatty rats compared to SDT rats [18, 19]. It is noteworthy that nephropathy develops in SDT fatty rats at 8 weeks of age, which is much earlier than in SDT rats at 24 weeks of age [18]. The SDT fatty rat is presumed to be a suitable animal model to reproduce clinical diabetes cases that have multiple metabolic disorders. In the retina, the peak latencies of the oscillatory potentials in ERGs in SDT fatty rats are prolonged compared with the age-matched normal SD rats, demonstrating retinal dysfunction [18]. In our preliminary study, the SDT fatty rats exhibited increases in the vascular endothelial growth factor (VEGF) concentrations in the vitreous humor and both retinal vascular hyperpermeability and retinal thickening, and those findings were normalized by phlorizin [20]. In the current study, we evaluated quantitatively and chronologically the pathological features of DR that developed in SDT fatty rats.

2. Methods

2.1. Animals. The care and handling of animals were in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research, the Guidelines for Animal Experimentation of Japan Tobacco Inc., and the Guidelines for Animal Experimentation of the Animal Care and Committee of Jichi Medical University, the last of which approved all experiments (study number, 17095-01). We used colonies of male SDT fatty rats ($n = 30$), SDT rats ($n = 30$), and normal SD rats ($n = 25$) purchased from CLEA Japan Inc. (Tokyo, Japan). All SDT fatty and SDT rats were confirmed to be diabetic based on a nonfasting blood glucose concentration exceeding 350 mg/dL. The SDT rats were diagnosed with diabetes by 8 to 16 weeks after birth. The SDT fatty rats were diagnosed with diabetes by 8 weeks after birth. All rats were fed standard rat chow (CRF-1, Oriental Yeast Inc., Tokyo, Japan). The eyes were enucleated in SDT fatty and SDT (control) rats at 8, 16, 24, 32, and 40 weeks of age ($n = 6$ for each rat type at each age). The eyes of age-matched male SD rats (normal controls) also were enucleated ($n = 5$ at each age).

2.2. Measurement of Body Weight, Blood Glucose, Blood Insulin, Blood Triglycerides, and Blood Total Cholesterol. Body weight and blood chemistry parameters, including glucose, insulin, triglycerides (TG), and total cholesterol (TC), were measured when each rat was sacrificed. Blood samples were collected from the tail vein of nonfasting rats. The serum glucose, TG, and TC levels were measured using commercial kits (Roche Diagnostics, Basel, Switzerland) and an automatic analyzer (Hitachi 7180, Hitachi High-Technologies Corp., Tokyo, Japan). The serum insulin was measured using a rat-insulin enzyme-linked immunosorbent assay kit (Morinaga Institute of Biological Science, Yokohama, Japan).

2.3. Ocular Histopathology. The procedures of the histopathological study were the same as we reported previously [17]. Under deep isoflurane anesthesia (isoflurane inhalation solution, Pfizer Inc., New York, NY, USA), the eyes were enucleated for conventional histopathologic studies and placed in a fixative (Super Fix KY-500, Kurabo Industries Ltd., Osaka, Japan). The fixed eyes were washed in 0.1% mol/L cacodylate buffer and embedded in paraffin. The paraffin block was cut into 4 μm sections that were stained with hematoxylin and eosin (HE) for conventional histopathologic examination. The immunohistochemical procedures were based on the standard avidin-biotin horseradish peroxidase method using each antibody and performed with 3,3′diaminobenzidine substrate-chromogen. Glial fibrillary acidic protein (GFAP) mouse monoclonal antibody (Cell Signaling Technology Inc., Danvers, MA, USA) and rat VEGF antibody (R&D Systems Inc., Minneapolis, MN, USA) were used at a dilution of 1 : 100 as the primary antibody.

2.4. Measurement of Retinal Thickness, Retinal Folds, and Choroidal Thickness. To quantify the pathological features of the specimens, we used the BZ-X700 digital microscope system (Keyence Corp., Osaka, Japan). One high-resolution image of an entire specimen was created using the BZ-H3XD image stitching system (Keyence). After staining with HE, the retinal thicknesses, numbers of retinal folds, and

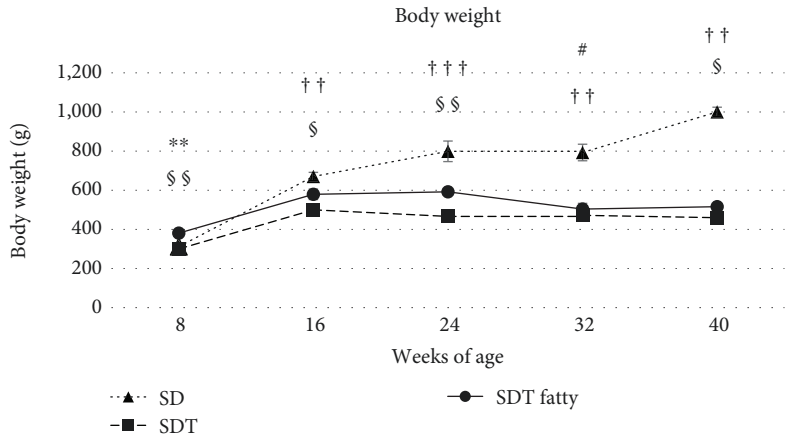

Figure 1: Body weight of the study animals. Compared with the Sprague Dawley (SD) rats, the Spontaneously Diabetic Torii (SDT) rats are significantly lighter. The SDT fatty rats are significantly heavier than the SDT rats. The data are expressed as the mean ± standard error. $^{**}p < 0.01$, SDT fatty rats vs. SDT rats; $^{\#}p < 0.05$, SDT fatty rats vs. SD rats; and $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$, SDT rats vs. SD rats by Scheffe's test. $^{\S}p < 0.05$ and $^{\S\S}p < 0.01$, SDT fatty rats vs. SDT rats by Mann–Whitney U-test.

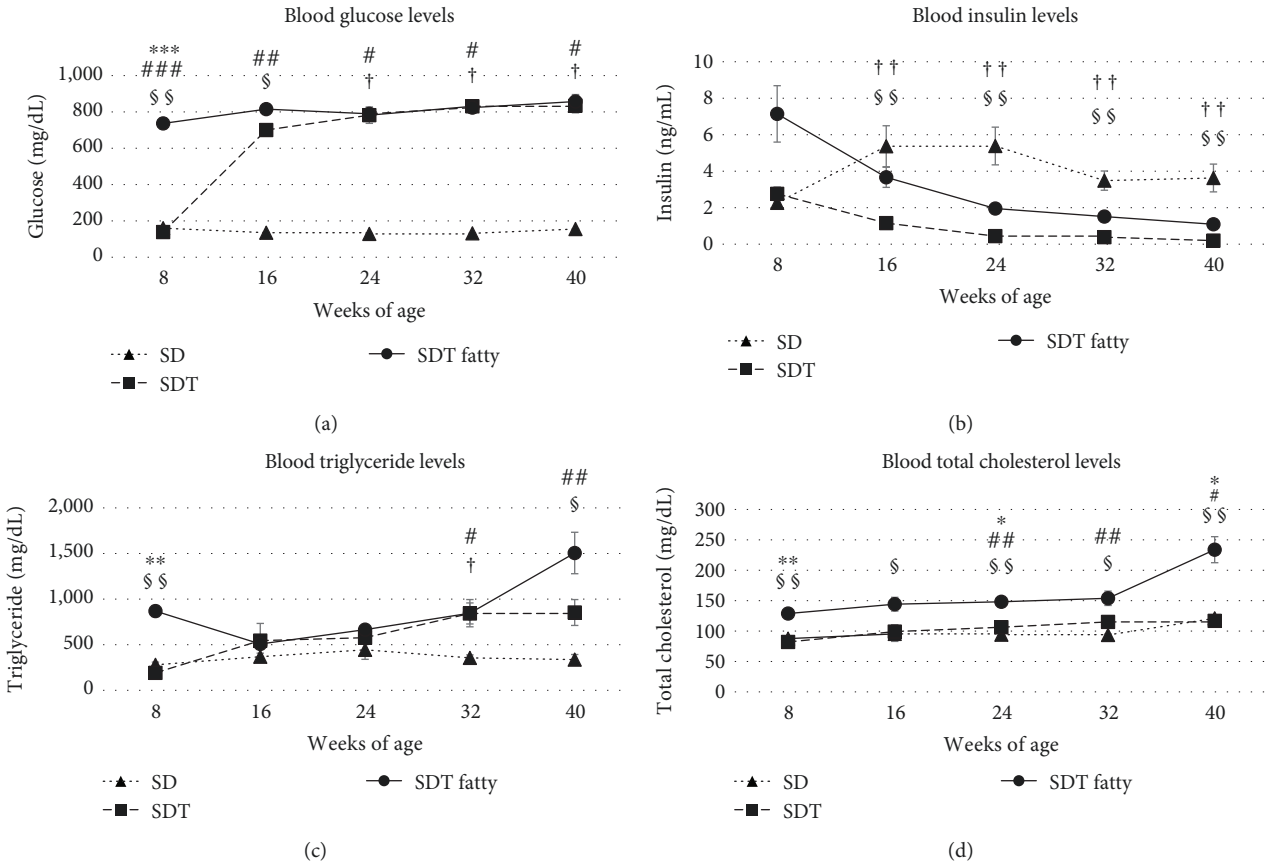

Figure 2: Changes in biologic parameters ((a) glucose, (b) insulin, (c) triglycerides, and (d) total cholesterol) in the three rat types. Glycolipid disorders in the Spontaneously Diabetic Torii (SDT) fatty rats are obviously prominent compared with those in the SDT rats. The data are expressed as the mean ± standard error. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, SDT fatty rats vs. SDT rats; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$, SDT fatty rats vs. Sprague Dawley (SD) rats; $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$, SDT rats vs. SD rats by Scheffe's test. $^{\S}p < 0.05$ and $^{\S\S}p < 0.01$, SDT fatty rats vs. SDT rats by Mann–Whitney U-test.

choroidal thicknesses were evaluated in the images. The total retinal thickness was defined as the distance between the retinal internal limiting membrane and the photoreceptor layer (PL). The mean retinal and choroidal thicknesses were measured 500, 1,000, and 1,500 microns from the optic nerve disc. The numbers of retinal folds, defined as deformations from the outer nuclear layer (ONL) to the PL, were measured within 1,500 microns of the optic nerve disc.

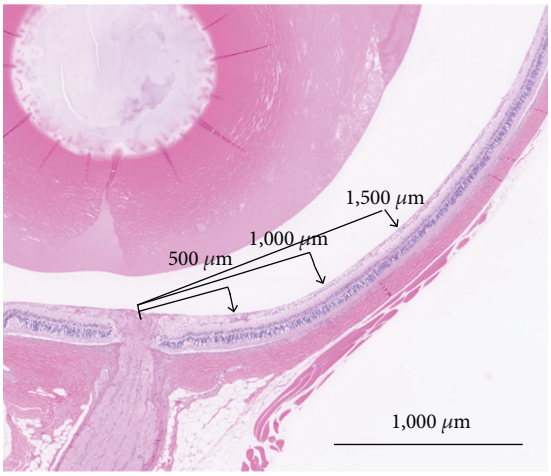

Figure 3: The retinal and choroidal thicknesses were measured 500, 1,000, and 1,500 microns from the optic nerve disc. The scale bar indicates 1,000 microns.

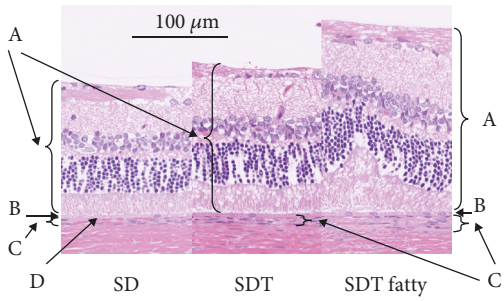

Figure 4: Comparison of the retinal and choroidal thicknesses 500 microns from the optic nerve disc at 40 weeks of age in each rat type (hematoxylin and eosin stain). A: retina; B: retinal pigment epithelium; C: choroid; D: choriocapillaris. The scale bar indicates 100 microns.

2.5. Measurement of the Area of Immunostained GFAP and VEGF. Quantitative analyses of the GFAP- and VEGF-positive regions, which we referred to as the immunopositive regions, were performed using the Hybrid Cell Count Module/BZ-H3C software (Keyence). The entire specimen was marked with a magenta stain, and the immunopositive regions were marked with dark blue over them. The boundary was marked with light blue. The color coding of the immunopositive and immunonegative regions can be selected freely in this software. The ratio of the immunopositive areas to the entire specimen was calculated automatically in each specimen.

2.6. Statistical Analysis. The measurements of the parameters are expressed as the mean ± standard error. For statistical analysis, we used the Excel Tokei 2006 software (Social Survey Research Information Co. Ltd., Tokyo, Japan). The Mann–Whitney U-test and Scheffe's test were used for the numerical parameter test of nonnormal distribution. $p < 0.05$ was considered significant.

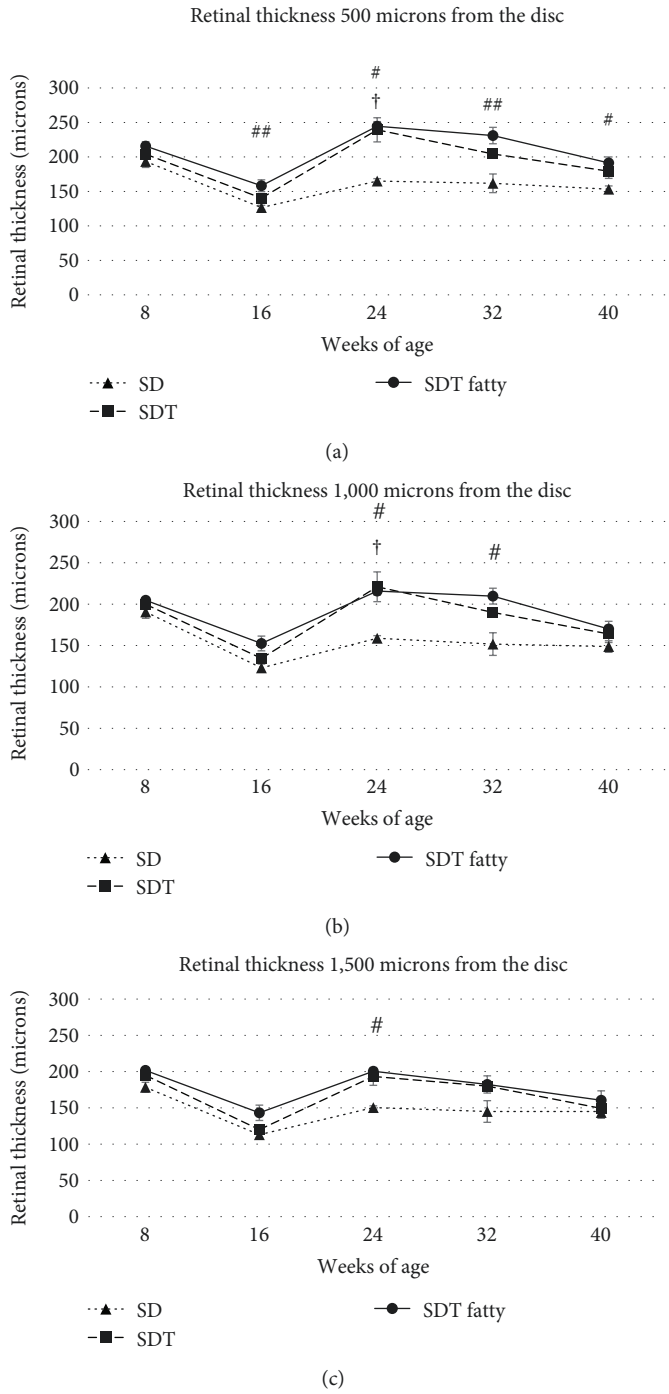

Figure 5: The retinal thicknesses ((a) 500 microns from the disc; (b) 1,000 microns from the disc; (c) 1,500 microns from the disc) in each rat type. The retinas tend to be thicker in the Spontaneously Diabetic Torii (SDT) fatty rats and SDT rats than in the Sprague Dawley (SD) rats. The data are expressed as the mean ± standard error. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, SDT fatty rats vs. SD rats; $^{\dagger}p < 0.05$, SDT rats vs. SD rats by Scheffe's test.

3. Results

3.1. Body Weight, Blood Glucose, Blood Insulin, TG, and TC. Figure 1 shows the changes in body weight. Compared with the SD rats, the SDT rats were significantly lighter

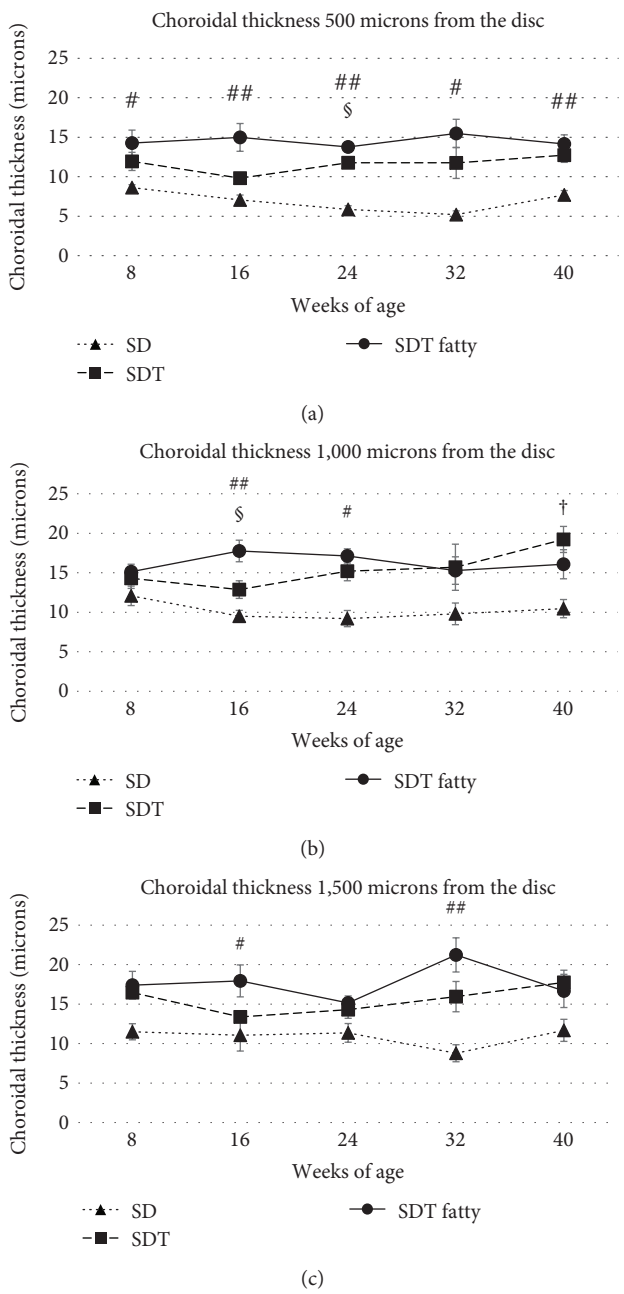

Figure 6: The choroidal thicknesses ((a) 500 microns from the disc; (b) 1,000 microns from the disc; (c) 1,500 microns from the disc) in each rat type. The choroids tended to be thicker in the Spontaneously Diabetic Torii (SDT) fatty rats than in the Sprague Dawley (SD) rats. The data are expressed as the mean ± standard error. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, SDT fatty rats vs. SD rats; $^{\dagger}p < 0.05$, SDT rats vs. SD rats by Scheffe's test. $^{\S}p < 0.05$, SDT fatty rats vs. SDT rats by Mann–Whitney U-test.

($p < 0.01$ at 16, 32, and 40 weeks of age; $p < 0.001$ at 24 weeks of age by Scheffe's test). The SDT fatty rats were significantly heavier than the SDT rats ($p < 0.05$ at 16 and 40 weeks of age; $p < 0.01$ at 8 and 24 weeks of age by Mann–Whitney U-test).

Figure 2 shows the changes in blood glucose, blood insulin, TG, and TC. The SDT rats were hyperglycemic from 16 weeks of age and the SDT fatty rats from 8 weeks of age. The mean insulin levels in the SDT fatty rats were higher than those in the SDT rats from 16 weeks of age. The mean serum TC levels in the SDT fatty rats were higher than those in the SDT rats at each age.

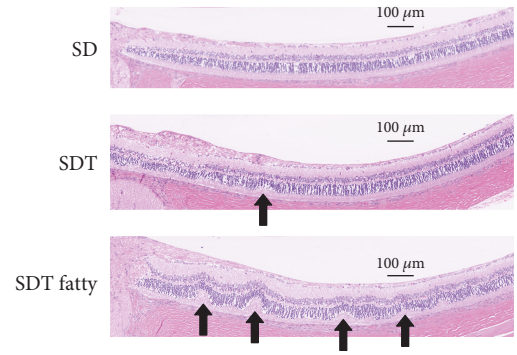

Figure 7: Comparison of the retinal folds in the study animals at 40 weeks of age. The numbers of retinal folds, defined as deformations from the outer nuclear layer to the photoreceptor layer, were measured within 1,500 microns of the optic nerve disc. The retinal folds (arrows) in the Spontaneously Diabetic Torii (SDT) fatty rats are more severe than in the SDT rats. The scale bar indicates 100 microns.

3.2. Retinal Thickness, Retinal Folds, and Choroidal Thickness. Figures 3–6 show the retinal and choroidal thicknesses. The retinas tended to be thicker in the SDT fatty rats and SDT rats than in the SD rats. No significant differences in the retinal thicknesses were seen between the SDT fatty rats and SDT rats. At 24 weeks, the mean retinal thicknesses 500 microns from the optic nerve disc in the SDT fatty rats, SDT rats, and SD rats, respectively, were 244.5 ± 6.7, 239.3 ± 17.5, and 165.0 ± 3.5 microns (SDT fatty rats vs. SDT rats, $p = 0.90$; SDT fatty rats vs. SD rats, $p < 0.05$; and SDT rats vs. SD rats, $p < 0.05$ by Scheffe's test; SDT fatty rats vs. SDT rats, $p = 0.52$ by Mann–Whitney U-test). The choroids tended to be thicker in the SDT fatty rats than in the SD rats. The choroidal thicknesses did not differ significantly between the SDT fatty rats and SDT rats, except for the choroidal thicknesses 500 microns from the optic nerve disc at 24 weeks and choroidal thicknesses 1,000 microns from the optic nerve disc at 16 weeks. At 24 weeks, choroidal thicknesses 500 microns from the optic nerve disc in the SDT fatty rats, SDT rats, and SD rats, respectively, were 13.8 ± 0.2, 11.8 ± 0.6, and 5.9 ± 0.5 microns (SDT fatty rats vs. SDT rats, $p = 0.19$; SDT fatty rats vs. SD rats, $p < 0.01$; and SDT rats vs. SD rats, $p = 0.16$ by Scheffe's test; SDT fatty rats vs. SDT rats, $p < 0.05$ by Mann–Whitney U-test).

Figures 7 and 8 show the retinal folds. The retinal folds in the SDT fatty rats developed earlier and were more severe than those in the SDT rats; no retinal folds developed in the SD rats. At 24 weeks, the mean numbers of retinal folds in the SDT fatty rats, SDT rats, and SD rats, respectively, were 2.8 ± 0.5, 0.5 ± 0.2, and 0 ± 0 (SDT fatty rats vs. SDT rats, $p < 0.05$; SDT fatty rats vs. SD rats, $p < 0.01$; and SDT rats vs. SD rats, $p = 0.58$ by Scheffe's test; SDT fatty rats vs. SDT rats, $p < 0.01$ by Mann–Whitney U-test). The peaks of

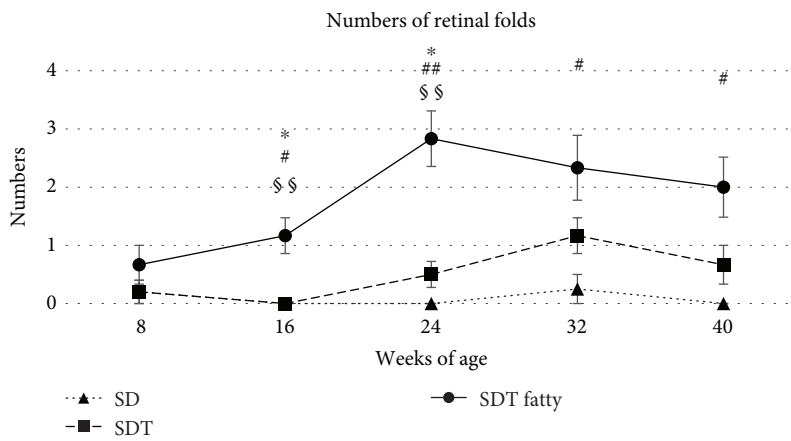

Figure 8: The numbers of retinal folds in the study animals. No retinal folds are seen in the Sprague Dawley (SD) rats. The retinal folds in the Spontaneously Diabetic Torii (SDT) fatty rats developed earlier and are more severe than those in the SDT rats. The data are expressed as the mean ± standard error. $^{*}p < 0.05$, SDT fatty rats vs. SDT rats; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, SDT fatty rats vs. SD rats by Scheffe's test. $^{\S\S}p < 0.01$, SDT fatty rats vs. SDT rats by Mann–Whitney U-test.

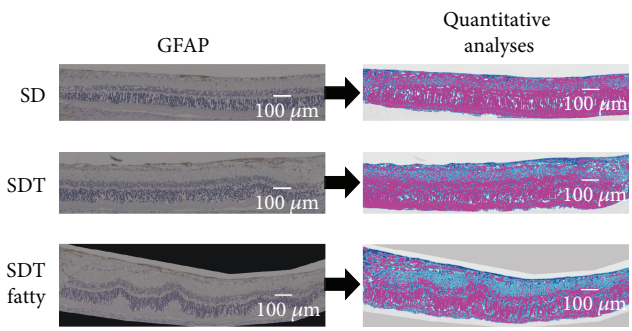

Figure 9: Quantitative analyses of the glial fibrillary acidic protein (GFAP) immunopositive regions were performed within 1,500 microns of the optic nerve disc. The entire specimen is marked with a magenta stain, and the immunopositive regions are marked with dark blue over them. The boundary is marked light blue. The scale bars indicate 100 microns.

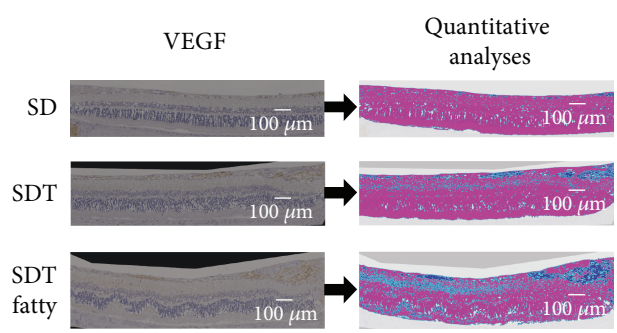

Figure 10: Quantitative analyses of the vascular endothelial growth factor (VEGF) immunopositive regions were performed within 1,500 microns of the optic nerve disc. The entire specimen is marked with a magenta stain, and the immunopositive regions are marked with dark blue over them. The boundary is marked light blue. The scale bars indicate 100 microns.

the retinal folds occurred at 32 weeks of age in the SDT rats (1.2 ± 0.3) and at 24 weeks of age in the SDT fatty rats (2.8 ± 0.5) ($p < 0.05$, by Mann–Whitney U-test).

3.3. Areas of Immunostained GFAP and VEGF. Figures 9 and 10 show the areas of immunostained GFAP and VEGF in the rat models. Figure 11 shows the mean area ratios of immunostained GFAP and VEGF. Quantitative analysis showed that the GFAP and VEGF immunopositive regions in the retinas of the SDT fatty rats were significantly larger than those of the SDT rats. At 40 weeks, the mean area ratios of GFAP positivity in the specimens from the SDT fatty rats, SDT rats, and SD rats, respectively, were 8.0 ± 0.5%, 5.7 ± 0.5%, and 4.3 ± 0.5% (SDT fatty rats vs. SDT rats, $p < 0.05$; SDT fatty rats vs. SD rats, $p < 0.001$; and SDT rats vs. SD rats, $p = 0.26$ by Scheffe's test; SDT fatty rats vs. SDT rats, $p < 0.01$ by Mann–Whitney U-test). At 40 weeks, the mean area ratios of VEGF positivity in the SDT fatty rats, SDT rats, and SD rats, respectively, were 8.2 ± 1.4%, 4.0 ± 0.4%, and 1.5 ± 0.2 % (SDT fatty rats vs. SDT rats, $p = 0.29$; SDT fatty rats vs. SD rats, $p < 0.0001$; and SDT rats vs. SD rats, $p < 0.05$ by Scheffe's test; SDT fatty rats vs. SDT rats, $p < 0.05$ by Mann–Whitney U-test).

4. Discussion

In patients with diabetes, longstanding hyperglycemia causes retinal and choroidal thickening because of leakage of the blood components from the retinal and choroidal vessels. Thickened retinas and choroids are seen frequently in clinical observations using OCT. DME, a component of DR, causes visual loss, and this condition is the target of anti-VEGF therapy. We reported previously that the retina and choroid were thicker in the SDT rats compared to the normal nondiabetic SD rats [16, 17]. The choroidal thickening in diabetic eyes remains controversial. Patients with DR have many complications such as hypertension and have been treated with many kinds of treatment such as laser photocoagulation and hypertensive medications. Multiple factors should affect the choroidal vasculature in clinical cases of DR [21–24]. In this particular animal model, the animals had not received any treatment such as laser photocoagulation or medication. Therefore, the animal model has its value. In the current

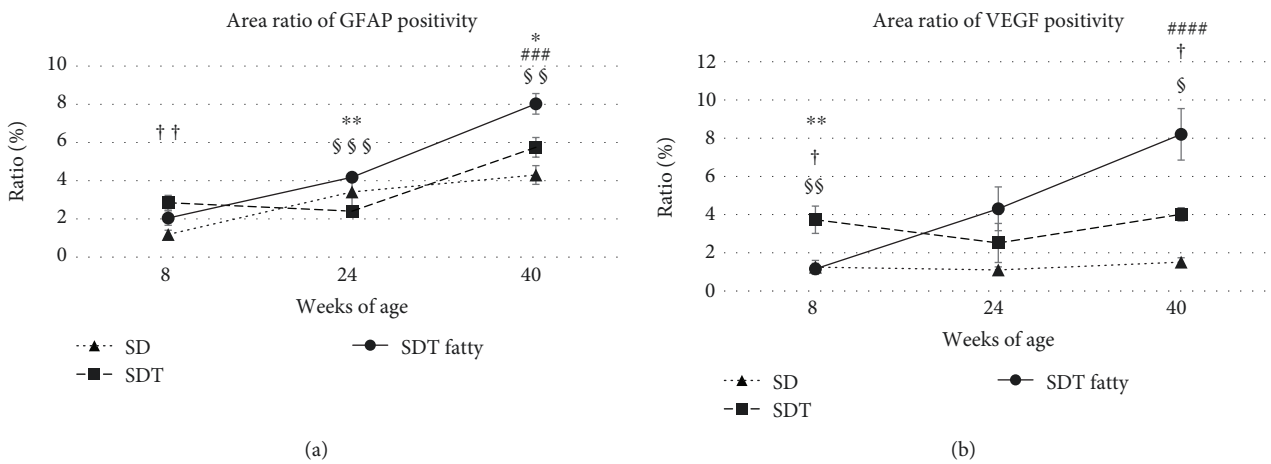

Figure 11: The mean area ratios of glial fibrillary acidic protein (GFAP) and vascular endothelial growth factor (VEGF) positivity ((a) GFAP; (b) VEGF) in the three rat types. Quantitative analysis shows that the GFAP and VEGF immunopositive regions in the retinas of Spontaneously Diabetic Torii (SDT) fatty rats are significantly larger than those of the SDT rats. The data are expressed as the mean ± standard error. $^{*}p < 0.05$ and $^{**}p < 0.01$, SDT fatty rats vs. SDT rats; $^{\#\#\#}p < 0.001$ and $^{\#\#\#\#}p < 0.0001$, SDT fatty rats vs. Sprague Dawley (SD) rats; $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$, SDT rats vs. SD rats by Scheffe's test. $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, and $^{\S\S\S}p < 0.001$, SDT fatty rats vs. SDT rats by Mann–Whitney U-test.

study, the retina and choroid were thicker in the SDT fatty rats than in the SD rats (normal controls). The retinal and choroidal thicknesses did not differ significantly between the SDT fatty rats and SDT rats (controls). However, the numbers of retinal folds and quantitative analysis of the immunohistochemistry showed the progression of DR in SDT fatty rats compared with SDT rats.

The retinal folds in the SDT fatty rats developed earlier and were more severe than those in the SDT rats. In the current study, the retinal folds were defined as deformations observed from the ONL to the PL, changes that did not include the entire retinal layer. Fibrous proliferations and tractional changes were reported at 70 weeks of age in SDT rats, and these changes included the entire retinal layer [11]. These changes usually are seen in older SDT rats; the rats in the current study were too young to have tractional changes. No preretinal membranes, which frequently cause retinal folds in clinical cases, were found in the rats in the current study; the retinal folds did not develop as the result of tractional force but might have resulted from volume changes with edema and/or increasing proliferation in the retina. It has been reported that retinal folds in SDT fatty rats were prevented with phlorizin and ipragliflozin, sodium glucose cotransporter inhibitors [25, 26]. Therefore, the retinal folds would be a phenomenon associated with DR. As mentioned, VEGF and glial cell proliferation should play an important role in retinal edema and retinal folds in both the SDT rats and SDT fatty rats. These mechanisms should have affected the SDT fatty rats more than the SDT rats. We think that retinal folds might be a new indicator for the quantitative assessment of DR in both SDT rats and SDT fatty rats.

Using ImageJ software (National Institutes of Health, Bethesda, MD, USA), we reported that the areas of GFAP and VEGF immunopositivity in the retina were larger in the SDT rats than in the SD rats [17]. In the current study, quantitative analysis of the immunohistochemistry using the Hybrid Cell Count Module/BZ-H3C software showed that the immunopositive regions of the GFAP and VEGF in the retinas were significantly larger in the SDT fatty rats than in the SDT rats, indicating that the SDT fatty rats develop more severe DR earlier than the SDT rats.

To promote drug development, repositioning, and treatments for DR, it is important to objectively evaluate the progress of DR using an appropriate animal model. However, there are few quantitative evaluation methods and few animal models that mimic human DR, which may be responsible for delayed DR research compared to diabetic nephropathy and neuropathy. We quantitatively analyzed the pathological features of DR in SDT fatty rats by measuring the retinal thicknesses, retinal folds, and area ratios of the immunopositive regions. These seem to be useful to objectively evaluate DR. SDT fatty rats are expected to be most useful as a type 2 diabetes animal model to study DR.

For now, the DR found in this SDT fatty rat is the best indicator to represent DR, including DME.

Data Availability

The data used to support the findings of this study are included within the article.

Disclosure

We presented our results at the Association for Research in Vision and Ophthalmology (ARVO) Annual Meeting in 2018.

Conflicts of Interest

All authors declare that there are no conflicts of interest regarding the publication of this paper. Drs. Ohta and Sasase are employees of Japan Tobacco Inc.

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Clinical Study

Propolis as an Adjuvant in the Healing of Human Diabetic Foot Wounds Receiving Care in the Diagnostic and Treatment Centre from the Regional Hospital of Talca

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Objective. Diabetic foot wounds are a relevant diabetes complication and a major health problem. It has been described that propolis has health benefits due to its anti-inflammatory, antioxidant, and support in the healing process. The current study assessed the effect of propolis as an adjuvant in the healing of human diabetic foot ulcers. This was evaluated in a randomized placebo-controlled study of subjects receiving care in the Diagnostic and Treatment Centre from the Regional Hospital of Talca, Chile. *Research Design and Methods*. Randomized subjects received ambulatory healing treatment for diabetes foot wounds with propolis spray (3%), which was applied to cover the entire wound surface each time it was dressed from week 0 until cicatrization or 8 weeks as a maximum. Two serum samples were taken (day 0 and end of the study) for cytokine and oxidative stress analyses. Also, macro- and microscopy were analyzed in the process of wound healing. *Results*. The study comprised 31 subjects with type 2 diabetes in treatment for diabetic foot wounds in the Diagnostic and Treatment Centre from the Regional Hospital of Talca. Propolis promotes a reduction of the wound's area by an average of $4\,cm^2$, related to an increase in the connective tissue deposit compared to the control. Also, propolis increased the glutathione (GSH) and GSH/glutathione disulfide (GSSG) ratio ($p < 0.02$), depleted tumor necrosis factor- (TNF-) α, and increased interleukin- (IL-) 10 levels. Topical propolis did not modify the biochemical parameters in the serum of the studied subjects. *Conclusions*. The topical use of propolis turned out to be an interesting therapeutic strategy as an adjuvant in the care of diabetes foot wounds due to its ability to improve and promote healing based on its anti-inflammatory and antioxidant profile. This trial is registered with NCT03649243.

1. Introduction

Diabetes Mellitus is a chronic disease with high prevalence in the world; it is estimated that on an average, 7% of the world population are diabetics [1]. Diabetes presents global mortality rates of 9%, which equals to 4 million deaths per year [2]. Patients with diabetes have higher rates of premature death, functional disability, and coexistence with other diseases compared to healthy subjects. The progressive increase of this pathology has been associated with a rise of diabetic's chronic complications such as foot amputations [3, 4]. Overall, diabetic foot is the first cause of nontraumatic amputation [5] and affects about 15% of all patients with Diabetes Mellitus, even though most cases are preventable [6].

Diabetic foot ulcers are not only a patient problem but also a major healthcare concern throughout the world and are one of the common and serious complications in diabetic patients. The treatment of complications in diabetic ulcers is

difficult and expensive. Patients usually need to take long-term medications or become hospitalized for an extended period of time [7]. These diabetes complications are associated with cardiovascular risk factors such as high blood pressure, dyslipidemia, and obesity, which can contribute to arterial obstruction. This together with orthopedic deformation is one of the most important pathological conditions that lead to development of diabetic foot [8, 9]. Nevertheless, the hyperglycemia status is the most relevant factor in the development and worsening of diabetic foot pathology, producing multiple metabolic and molecular changes such as sorbitol gain with increased glycation-end products and oxidative damage and increased in kinase C protein activity. These factors are directly related to diabetic microangiopathy [10]. All of these processes could be present in the eyes, kidneys, nervous system, and others. Specifically, the skin presents most of these alterations with changes in temperature, hydration, and dermis perfusion. These symptoms are caused by the neuropathy (autonomic and sensitive) that seriously affects the extremities of these patients. As a whole, both macro- and microangiopathy are responsible for the difficulty in healing wounds and favor infections in the diabetic foot. The presence of aggressions and/or trauma in diabetic foot, even slightly, can lead to development of ulcerations that in a high percentage proceed to amputations [11].

Stimulating ulcer cicatrization represents a permanent challenge for health services around the world. This healing is determined by multiple factors that involve molecular reactions influenced by the microenvironment of the wound, the persistent inflammation, ischemia, oxidative stress, and infections [12]. There are multiple factors that impair the recovery of a diabetic wound, including vascular insufficiency, deregulation of inflammatory processes, and angiogenic responses, among others. Persistence of inflammation and neutrophil infiltration is characterized by the chronic upregulation of proinflammatory cytokines and superoxide anions in the diabetic wounds [13]; all of these may interfere with the normal process of wound recovery.

There are multiple natural products with potential benefits in the healing process such as propolis (a resin produced by bees), which has been attributed to beneficial effects on human health, specifically for its antioxidant, antimicrobial, and immunomodulatory capacity [14, 15]. In diabetes clinical studies, there is a lack of evidence that shows the specific effects and mechanisms of these natural products. Previous published studies of this research group demonstrated the beneficial effect of propolis on oxidative stress in subjects treated for three months with oral propolis [16]. Considering this, the objective of the present study was to evaluate the effect of propolis as an adjuvant on the healing of human diabetic foot wounds receiving care in the Diagnostic and Treatment Centre from the Regional Hospital of Talca, Chile.

2. Research Design and Methods

2.1. Participants. All of the diabetic patients with foot wounds receiving ambulatory treatment from the Diagnostic and Treatment Centre at the Regional Hospital of Talca in October 2015 to March 2016 who met the inclusion criteria were invited to participate in this study. The ethics committee of the Maule Health Service approved this project on September 11th of 2015 (Folio number 2015-c03), and it was also approved by the Bioethical Committee of Universidad de Talca (Folio number 2015-095-EL). All included patients signed an informed consent. The inclusion criteria were type 1 or 2 diabetes with complicated foot diabetic wounds under complete treatment in the diabetes program and between 18 and 80 years of age (only type 2 diabetes subjects accepted to be part of the study). The exclusion criteria were (i) propolis allergy, (ii) critical ischemia, (iii) uncontrolled severe infection, and (iv) psychosocial conditions that impede regular attendance for health assistance. A total of 31 subjects were eligible for this study and follow-up for a maximum of 8 weeks. Twenty voluntary subjects were allocated in the propolis group, and 11 voluntary subjects were allocated in the control group. At the end, three patients discontinued the study (flow chart of enrolment in supplementary Figure S1).

2.2. Propolis. The propolis (Beepolis®) used was 3% in propylene glycol preparation manufactured by a bee product company in the Maule Region of Chile (Health Authorization no. 639-18/08/2009, Laboratories Rotterdam, Maule, Chile). Propolis spray was applied to cover the wound surface in each dressing from week 0 until cicatrization or 8 weeks as a maximum, whichever occurred first.

2.3. Wound Evaluation. Macroscopic aspect (*wound area measurement*): the nurse who dressed the wound and applied the propolis was the same for all subjects and did not participate in the result analysis. Control subjects received the same nursing care without the addition of propolis spray (diabetic foot wound care medications are summarized in Table S1). The nurse evaluated the wound by taking a photograph and measured the area (large × width = cm^2) with acetate tracing at the beginning of the treatment (week 0) and at the end of the study. Microscopic aspect (*histopathology evaluation*): representative fragments of wound sections (biopsy) were fixed in 10% buffered formalin and embedded in paraffin. Formalin-fixed paraffin-embedded tissues were stained with Masson's trichrome according to the fabricant's instructions (Merck, Germany). The collagen fibers were marked with light blue, while the cellular component was marked with red stain. Fibrous tissue areas were quantified using an arbitrary scale (ACT) based on the Ishak fibrosis score [17] (see Supplemental Table S2).

2.4. Serum and Tissue Evaluation

2.4.1. Biochemical Parameters in Serum. Glycaemia was measured using a colorimetric enzymatic hexokinase test (Glucose-Custom Biotech), insulin by electrochemiluminescence immunoassay (insulin ECLIA), HbA1c (glycosylated hemoglobin A1c) by a turbidimetric inhibition immunoassay (Tina-quant hemoglobin A1c Gen.2®), and C-reactive protein (CRP) by highly sensitive turbidimetric immunoassay (cardiac C-reactive protein (Latex) High Sensitive®). The analyses were measured in a Cobas c311 autoanalyzer (Roche, Switzerland).

2.4.2. Oxidative Status

(1) TBARS Measurement. Thiobarbituric acid reactive substances (TBARS) were measured according to Knodell et al. [18]. Briefly, 250 μL of each serum was incubated with 0.67% thiobarbituric acid and 50% trichloroacetic acid for 30 min at 90°C and centrifuged at 2500 rpm for 15 min. The supernatant was used to measure TBARS at 530 nm in a Multiskan Go microplate reader (Thermo Fisher Scientific, USA). The results were expressed in nmol/mL using malondialdehyde (MDA) as a standard curve (Sigma-Aldrich, USA).

(2) Glutathione (GSH) Measurement. The GSH level was measured using metaphosphoric acid for protein precipitation and 5,5-dithiobis 2-nitrobenzoic acid (DTNB) (Sigma-Aldrich) for color development at 412 nm. 40 μL of whole blood from EDTA tube was mixed with 760 μL of distilled water. 1200 μL of precipitating solution (1.67 g of glacial metaphosphoric acid, 0.2 g of EDTA, and 30 g of NaCl in 100 mL of distilled water) was added to this mixture and centrifuged at 3500 rpm for 10 minutes. To 250 μL of the supernatant, 1000 μL of phosphate buffer and 125 μL of DTNB were added. This solution was used to measure GSH spectrophotometrically (Multiskan Go, Thermo Fisher Scientific). The values were expressed as mg/dL for serum analysis and μmol/gr for tissue analysis.

2.4.3. Inflammatory Status

(1) Serum Cytokine. Serum tumor necrosis factor- (TNF-) α and interleukin- (IL-) 10 were measured by an ELISA technique (Thermo Scientific) according to the manufacturer's instructions and were determined spectrophotometrically (Multiskan Go, Thermo Scientific) at 450 nm, and the concentration was calculated against a standard curve; the levels were expressed as pg/mL.

(2) Tissue Cytokine. TNF-α and IL-10 were analyzed by quantitative real-time PCR (RT-qPCR). Before PCR, total RNA of each sample was processed with a RNase-free DNAse kit (Ambion, Life Technologies, USA), NanoDrop (Thermo Scientific) RNA was reversed by Revert Aid Reverse Transcriptase (Thermo Scientific), and RT-qPCR was performed for the following genes: TNF-α 5′-GGTTCCGTCCCTCT CATACA-3′ forward and 5′-AGACACCGCCTGGAGT TCT-3′ reverse primer and IL-10 5′-TGGAGTGAAGA CCAGCAAAG-3′ forward and 5′-GGCAACCCAAGTAA CCCTTA-3′ reverse primer, with GAPDH as a housekeeping gene 5′-TTGTGAAGCTCATTTCCTGGTA-3′ forward and 5′-GGCCTCTCTCTTGCTCTCAGTA-3′ reverse primer. The assay was performed in a thermo cycler (Stratagene Mx3000P, Agilent Technologies, USA). The thermal cycle conditions were 95°C for 5 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 45 seconds, and finally, a dissociation cycle. Efficiency of every primer set was calculated through a serial dilution of a cDNA sample from 10^{-1} to 10^{-8}. The gene expression level was measured on a standard curve; additionally, relative change was calculated using $2^{-\Delta\Delta Ct}$ methods and normalized to GAPDH.

Table 1: Demographic and wound characteristics.

	Control	Propolis	*p value*
Subjects number	8	20	
Age (mean ± SD)	58.8 ± 6.34	60 ± 11.2	0.739
Gender			
Female	3 (37.5%)	4 (20%)	
Male	5 (62.5%)	16 (80%)	
Diabetes duration (year) (mean ± SD)	7.6 ± 3.5	11.8 ± 6.4	0.154
Reduction wound area (cm^2)			
Mean	3.03	4.0	*0.0317
Median	2.25	2.68	
IQR	0.7-5.3	1.1-5.5	

*Significant differences. SD: standard deviations; IQR: interquartile range.

2.5. Statistical Analysis. All values correspond to mean ± SEM or standard deviation (SD). The data were evaluated with GraphPad Prism 6® software (La Jolla, USA). The statistical analysis included intragroup *t*-student analysis and one-way ANOVA followed by the Mann-Whitney test for unpaired data. A *p* value of <0.05 was considered statistically significant.

3. Results

3.1. Description of General Characteristic Demographic. A total of 31 patients were eligible for this study and provided informed consent. Table 1 summarizes the demographic characteristics of the study population and the treated ulcer details. The control group consisted of five men and three women with an average age of 58.8 ± 6.34 years and a diabetes diagnosis of 7.6 ± 3.5 years ago. The propolis group consisted of 16 men and 4 women with an average age of 60 ± 11.2 years and a diabetes diagnosis of 11.8 ± 6.4 years ago. These parameters did not show significant differences between groups. All the subjects were screened for the presence of other pathologies and concomitant therapies (see Table S3); pharmacological treatment remained constant throughout the entire study and the control of all their pathologies with the appropriated specialist physician. Hematological and serum parameters were measured for the groups' pre- and posttreatment (Table 2); postprandial glycaemia was measured at the beginning and at the end of the study; the average for the control was 320 mg/dL (range of 157 to 716 mg/dL) at time 0 and 196 mg/dL (range of 87 to 349 mg/dL) at the time of wound healing with nonstatistical significance ($p = 0.0794$). For propolis-treated subjects, the average at time 0 was 213 mg/dL (range of 65 to 384 mg/dL) and 215 mg/dL (range of 71 to 598 mg/dL) at the end of the study with nonstatistical significance among the data. The mean value of HbA1c (glycosylated hemoglobin A1c) was 10.3 and 9.1% for the control group but 9.8 and 9.3% for the propolis-treated patients at the beginning and at the end of the study, respectively, with nonstatistical significance among the

Table 2: Evaluation of hematological and serum biochemical parameters.

Cases	Control			Propolis		
Subjects' number	8		*p value*	20		*p value*
	Initial	Final		Initial	Final	
Creatinine (mg/dL)	1.53 ± 0.76	1.5 ± 1.14	0.9545	1.57 ± 2.1	1.73 ± 1.83	0.9828
Total cholesterol (mg/dL)	174 ± 70	179 ± 55	0.9560	161 ± 53	137 ± 24	0.6862
Triglycerides (mg/dL)	170 ± 40	170 ± 85	0.9999	165 ± 123	143 ± 53	0.8719
Glycemia (mg/dL)	321 ± 162	197 ± 98	0.5231	210 ± 87	215 ± 135	0.9756
HbA1C (%)	10.3 ± 3.2	9.2 ± 2.9	0.8026	9.6 ± 2.7	9.3 ± 2.4	0.9350
Hematocrit (%)	37.5 ± 7.1	36 ± 5.9	0.8732	37.5 ± 5.3	34.5 ± 7.2	0.7422
Hemoglobin (g/dL)	12.3 ± 2.33	11.8 ± 2.1	0.8756	12.5 ± 1.86	11.3 ± 2.5	0.7059
White blood cells (mm^3)	9543 ± 3703	8726 ± 4917	0.8963	8811 ± 3215	9286 ± 1774	0.8916

groups. Other parameters measured were creatinine, total cholesterol, triglycerides, hematocrit, hemoglobin, and white blood cells, with no significant differences in time. Also, the levels of high sensitive C-reactive protein (usPCR) were analyzed; no differences were found among the groups (Figure S2).

3.2. Wound Analysis. Macroscopic aspects: the data shows a significant difference ($p = 0.0317$) in the wound healing of the propolis group in relation to the control group; specifically, there was a decrease in the wound area by an average of 4 cm^2 in the propolis group compared with the control group, which reduced 3 cm^2 (Figure 1(a)). *Microscopic analysis* (*histopathological*): to evaluate whether the propolis treatment had an effect on collagen deposition and formation of fibrotic tissue (potential scar), histological staining with Masson's trichrome was performed, as observed in Figures 1(b) and 1(c). At the beginning, wound tissues showed the presence of 70 to 80% of connective tissue in the biopsies from both groups. At the end of the study, the presence of fibrosis and connective deposit was increased to 95% in propolis groups compared to an average of 80-85% in controls. When the ACT scale was applied (Figure 1(d)), it was possible to observe that the control group changed score III to IV and propolis from II-III to V. Together with the biopsy, the presence of microorganisms in the wound was analyzed (see Supplemental Table S4); the most prevalent bacteria found was *S. aureus* with almost 30% in both groups, and none of the subjects had infections derived from fungi.

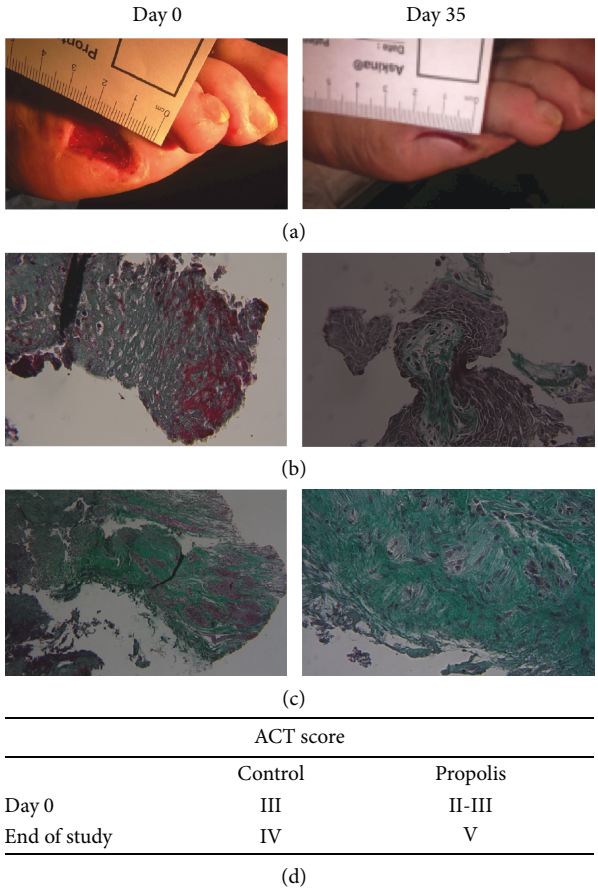

Figure 1: Representative images of a representative photograph of wound healing from a subject treated with propolis (a), control (b), and propolis (c) of foot wound biopsies stained with Masson's trichrome. (c) Day 35 means the last tissue biopsy sample for that patient, and (d) is the average of the ACT score determinates for all the samples.

3.2.1. Oxidative Status. The serum oxidative stress analysis shows that GSH increased in the time in both groups ($p < 0.02$ and $p < 0.04$, respectively) (Figure 2(a)). The control group displayed a higher increase of GSH than the propolis group at the end of the study ($p < 0.01$). Serum TBARS showed nonsignificant differences between the control and propolis groups ($p < 0.66$) (see Figure 2(b)), and the differences showed no significant statistical changes. GSH and GSH/glutathione disulfide GSSG were determined in tissue (see Figures 2(c)–2(e)); GSH increased in time in the control and propolis groups ($p < 0.03$ and $p < 0.0001$, respectively), and GSH increased more in the propolis group than the control at the end of the study in all subjects ($p < 0.01$). Also, GSH tissue content was determined for analyzing the net change of GSH (Figure 2(d)), evidencing and increasing the total content of GSH in the subjects

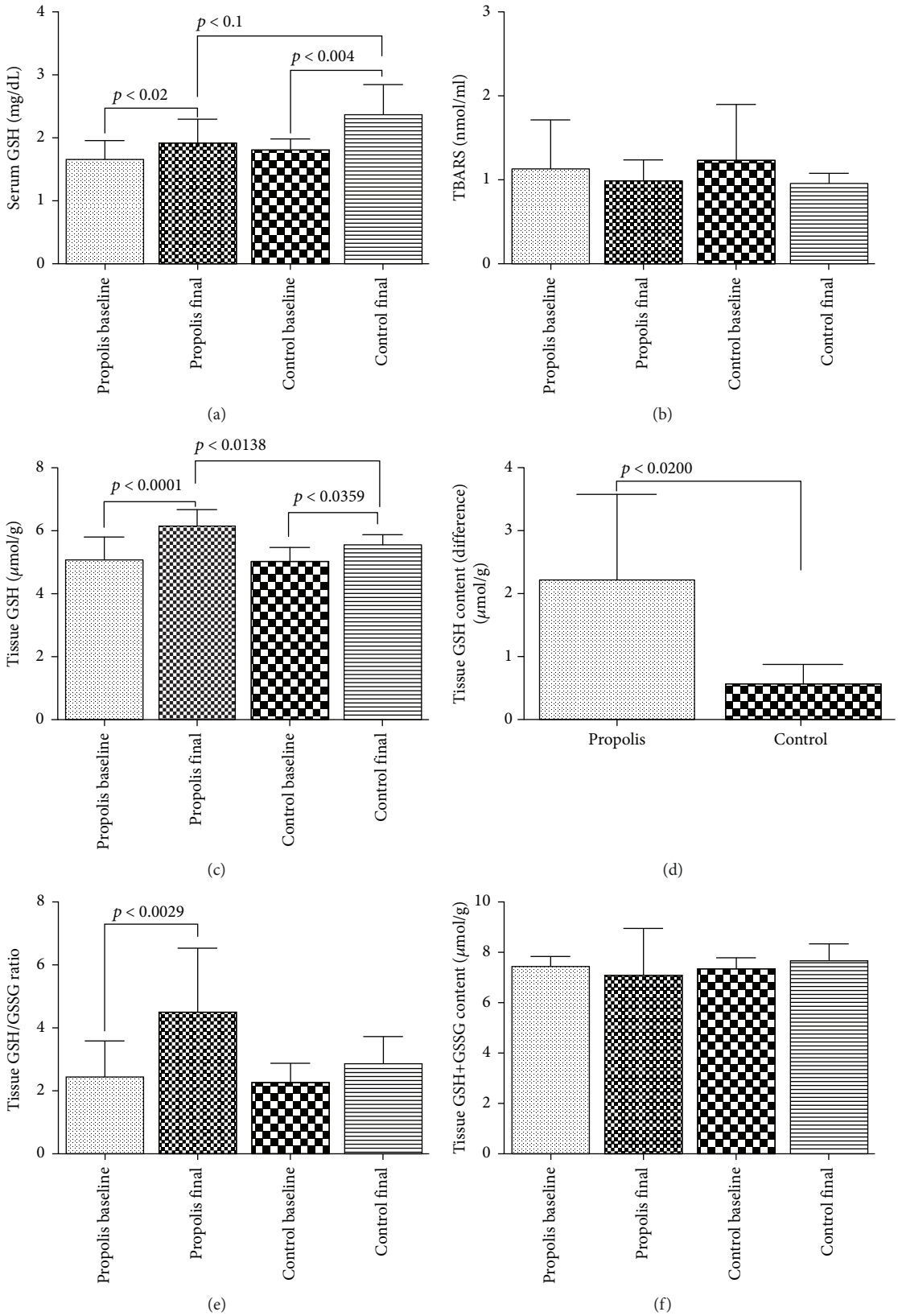

Figure 2: Oxidative status. Serum analysis of GSH (a) and TBARS (b). Tissue analysis of GSH (c), net change of GSH (d), GSH/GSSG (e), and total tissue content of GSH+GSSG (f). Results are expressed as mean ± SD for 8 control subjects, and 20 propolis subjects (t-student and Tukey's posttest). $p < 0.05$ was considered significant.

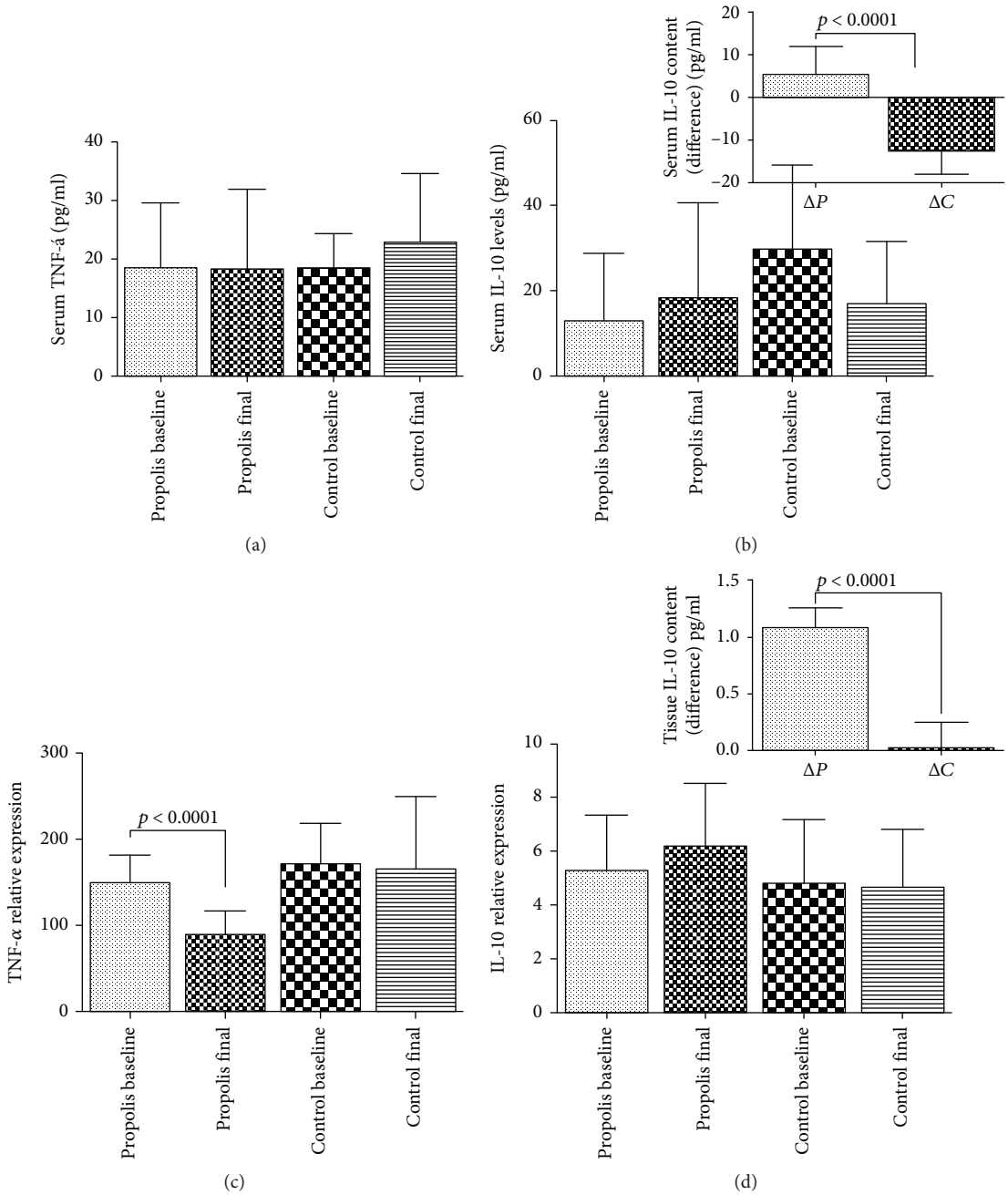

Figure 3: Cytokine pattern. Serum analysis of TNF-α (a) and IL-10 (b). Tissue analysis of TNF-α (c) and IL-10 (d). In the insets, it is possible to observe the net change of IL-10: ΔP equal propolis final minus propolis at time zero; ΔC equal control final minus control at time zero. Results are expressed as mean ± SD for 8 control subjects and 20 propolis subjects (t-student and Tukey's posttest). $p < 0.05$ was considered significant.

treated with propolis ($p < 0.05$). The GSH/GSSG ratio was enhanced in time in the propolis group ($p < 0.002$) (Figure 2(e)). The control patients showed nonsignificant differences in the GSH/GSSG ratio. Additionally, it was verified that GSH or GSSG were not lost during the entire study (Figure 2(f)).

3.2.2. Inflammatory Status (Cytokines Analyses): TNF-α and IL-10. The inflammatory status was determined by the levels of TNF-α and IL-10 in serum and in the wound tissue (Figures 3(a) and 3(b), respectively). The levels of TNF-α and IL-10 showed no serological changes both in the control and in the propolis-treated group, but when these cytokines were extracted from the site of the injury, it was possible to observe that (i) TNF-α decreased in time in the propolis group ($p < 0.0001$), a situation not observed in the control group, where the TNF-α levels remain constant over time ($p < 0.5197$) (Figure 3(c)). (ii) IL-10 did no show significant changes over time in the control and/or propolis group ($p < 0.9744$ and $p < 0.2281$, respectively), but when the net

change over time was analyzed, it was possible to observe in the treatment group a 100% increase of IL-10 (see inset in Figure 3(d)), an increase not seen in the control group.

4. Discussion

Chronic wounds are a rising problem in healthcare systems worldwide as the population ages and experiences increases in the incidence of obesity and diabetes. These wounds are difficult to heal, and treatment is often lengthy and expensive [19]. Multiple efforts have been made to improve the treatment. Wound research, therapies, and treatment options are a critical challenge. Nevertheless, some studies have been carried out for understanding the molecular and cellular *milieu* of chronic wounds in order to remove the main barriers that prevent their healing [20].

There is an increasing interest in the use of natural products in modern medicine as part of disease and patient management. Bee products are natural and have diverse applications in medical fields for the treatment of various diseases. The identification of bee products that may enhance skin repair can contribute to a better understanding of the wound healing process and generate a new strategy to combat chronic wounds [21]. The present study shows favorable changes in the patients that received topical treatment of propolis at the site of the wound, healing better than those not treated. Previously, it has been reported that propolis is well tolerated and reduces the area of the ulcer by an average of 41% vs. 16% in controls (applied Wagner's classification weekly) [15] and reduces the size of ulcers (four weeks) with grades 1 and 2 [22], proving for the first time that topical propolis may enhance wound closure. The evolution of patients treated with topical propolis in our research for an average of 8 weeks showed a 25% reduction in the wound area. We also analyzed the histopathological deposit of the extracellular matrix in the foot wound biopsies by Masson's trichrome stain. Masson's trichrome has been extensively used to analyze collagen remodeling and histological examination in the promotion of wound healing in models of diabetes [23]. Thus, it takes into consideration that the regeneration phase of the wound involves extensive tissue remodeling by replacing proteoglycan and collagen molecules. This results in stronger tissues where the collagen is one of the most important events in wound healing. In diabetes, collagen fibber synthesis was impaired and it was accompanied by an increased apoptosis of fibroblasts [24]. Topical propolis administration allowed a better connective tissue deposit with a favorable trend to regeneration in comparison with the nontreated group.

The persistence of hyperglycemia in diabetes is an important cause of increased production of reactive oxygen species (ROS), which enhance oxidative stress and become the main factor of cardiovascular complications in diabetes. Moreover, diabetes is characterized by the presence of inflammatory mediators such as cytokines, growth factors, and free radicals that may accelerate the development of diabetes. Thus, inflammatory and oxidative events seem to act together in the development of chronic pancreatic inflammation leading to the deterioration of its function. Several lines of evidence indicate that ROS production activates signaling pathways that promote angiogenesis [25]. Hyperglycemia is an important factor for the intense oxidative stress in diabetes, and the toxicity induced by glucose autoxidation is likely to be one of the important sources of ROS. Additionally, lipid peroxidation plays an important role in the production of free radicals and oxidative stress in diabetes [26]. Preceding authors have shown that bee honey and their variants can ameliorate the oxidative parameters in diabetic animal models. Particularly, bee honey reduced superoxide dismutase (SOD) and decreased catalase (CAT) and MDA levels [26–29]. Also, GSH levels and GSH/GSSG ratio were significantly elevated, and honey did not increase the levels of glutathione peroxidase [28]. On the other hand, it has been demonstrated that propolis administration has beneficial effects. El-Sayed et al. [30] showed that the ethanolic extract of propolis in (streptozocin) STZ-treated rats generated a marked reduction of GSH and CAT (66% and 31%, respectively) in serum and SOD (54%) in the pancreas. The same parameters were measured in kidney tissues of animals induced by diabetic nephropathy where the oral administration of propolis extract in doses of 100, 200, and 300 mg/kg improved the serum glucose, lipid profile, MDA, and renal function tests. Kidney GSH, SOD, and CAT were significantly increased while MDA was markedly reduced [31]. This would suggest a strong antioxidant effect of propolis, which can ameliorate oxidative stress and delay the occurrence of diabetic complications.

Propolis is rich in antioxidants such as polyphones and flavonoids. Antioxidant activity of propolis occurs with high amounts of phenolic compounds, and weak activity occurs in low amounts. It has also been reported that flavonoids reduce blood glucose levels [26]. The topical administration in our protocol generated a change in the tissue oxidative parameters with a significant increase in tissue GSH levels and GSH/GSSG ratio, which is related to an action of propolis over the site of the wound and not a systemic action, taking in consideration that the hyperglycemia was not modified in the serum of the subjects, but in the serum, the oxidative parameters have a trend to normalization, must probably this is due to an improvement in the wound healing observed in all the patients. According to the previously discussed, these parameters are high predictive criteria for focal oxidative stress although there are no previous studies that evaluated the diabetic foot with these markers. We can propose that the improvements in foot wounds are directly related to the local antioxidant potential previously described for propolis.

Together with the changes described for oxidative stress, we found changes in the local inflammatory parameters with significant modification in TNF-α levels and an increase in IL-10. It should be considered that diabetes-induced ulcers, at least in experimental models, display impaired profiles of proinflammatory/anti-inflammatory factors. This phenomenon is associated with a delay in the resolution phase of the healing process because aberrant messages are sent to T- and B-lymphocytes and macrophages, thereby impairing reepithelialization and remodeling. This is normally carried out by platelets, macrophages, epitheliocytes, and fibroblasts, representing the final phase of healing associated with

physiological inflammation [32]. Natural antioxidants play various biological roles in the treatment of diabetic complications, including impaired wound healing and T-cell immune responses in offspring born to diabetic mothers, as well as the treatment of other diseases [32–34]. Previously, the group of Al Ghamdi et al. [33] showed that the ethanol-soluble derivative of propolis administered to mice with diabetes induced by STZ significantly increased the circulating lymphocyte count. This was associated with the restoration of the aberrant elevated levels of proinflammatory cytokines IL-1β, IL-6, and TNF-α and the normalization of the reduced levels of IL-2, IL-4, and IL-7, concluding that propolis impaired lymphocyte proliferation and migration towards chemokines to maintain an efficient lymphocyte immune response.

There are several lines of evidence posing the Nuclear Factor kappa B (NFκB) as a key regulator in the crosstalk among the pathways leading to type 2 diabetes. It was documented that NFκB is activated via phosphorylation of inhibitor NFκB (IκB) leading to its ubiquitination and proteasomal degradation. Such a reaction will unmask the nuclear localization signal of NFκB, and once in the nucleus, it will activate several genes that regulate proliferation, apoptosis, angiogenesis, and inflammation [35]. Furthermore, obesity activates the transcription factor NFκB, which increases the risk for diabetes. It has been shown that NFκB pathway inhibition exerts a beneficial effect on type 2 diabetes [36]. Considering the above, it was discovered that TNF-α is overexpressed in the adipose tissues of obese mice, thereby establishing a clear link between obesity, type 2 diabetes, and chronic inflammation [35]. Propolis and its constituent caffeic acid showed a higher antioxidant activity and inhibited nitric oxide production in macrophages without cytotoxicity by blocking NFκB and Mitogen-Activated Protein Kinase (MAPK) activation in macrophages. This did not induce hepatotoxicity at concentrations with strong anti-inflammatory potential [32]. It would be interesting to analyze the role of propolis over NFκB in diabetic wound foot.

It is important to highlight that we have not found any changes in the values of hemoglobin A1c (HbA1c) nor glycaemia among the groups. Thus, this would respond to the topical administration of propolis and that all the patients in both groups maintained the normal adjustment of their pharmacological treatment. These findings support that the observed tissue levels are due to the effect of the topical propolis and are not derived from systemic interventions.

5. Conclusion

Propolis promotes the closure of diabetes foot wound and the reduction of the injury area related to an increase in the extracellular matrix deposit, which helps the cicatrization. Topical propolis contributes to oxidative stress equilibrium by enhancing GSH and GSH/GSSG ratio and decreasing inflammation mediated by the depletion of TNF-α and the enhancement of IL-10 in the injury area. Propolis seems to be an attractive adjuvant tool for the management of diabetic foot wounds that could offer a wide cost-benefit ratio.

Data Availability

Due to ethical concerns, supporting data cannot be made openly available and are restricted by the committee of the Maule Health Service according to Chilean Law No. 25584 in order to protect patient privacy. The data are available from Dra. Verónica Mujica as the treating doctor and Jessica Zuñiga-Hernandez as the corresponding author, for researchers who meet the criteria for access to this confidential data.

Conflicts of Interest

The authors declare no conflicts of interest relevant to the study.

Authors' Contributions

Verónica Mujica, Roxana Orrego, Elba Leiva, and Jessica Zuñiga-Hernandez designed this study for which Elba Leiva secured funding. Verónica Mujica, Roxana Orrego, Roberto Fuentealba, and Jessica Zuñiga-Hernandez performed experiments, and Roxana Orrego and Jessica Zuñiga-Hernandez performed analysis of data. Jessica Zuñiga-Hernandez wrote the manuscript, which was critically revised by Verónica Mujica, Roxana Orrego, Elba Leiva, and Jessica Zuñiga-Hernandez. All authors read and approved the final paper.

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Supplementary Materials

Figure S1: consolidate standard of reporting trial (CONSORT) flow chart with the participant recruitment and progress through a study. Table S1: foot care and dressing. List of dressings used for wound care by a specialist nurse in advanced healing. Both groups received the same type of protocol. The protocols were adjusted to the technical standards of the Ministry of Health, Minsal, Chile, and were ranked according to the degree of exudate and/or presence of infection. Table S2: arbitrary connective tissue (ACT) score. Based on Ishak17. Table S3: concomitant pathologies and chronicled pharmacology therapy. Figure S2: serum laboratory analysis of glycaemia (A), HbA1c (B), and usPCR (C). Table S4: determination of microorganisms present in the diabetic foot wound. Negative culture means biopsy culture does not show growth of bacteria or fungi. *(Supplementary Materials)*

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Review Article

Oxidative Stress as the Main Target in Diabetic Retinopathy Pathophysiology

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Diabetic retinopathy (DR) is one of the most common complications of diabetes mellitus (DM) causing vision impairment even at young ages. There are numerous mechanisms involved in its development such as inflammation and cellular degeneration leading to endothelial and neural damage. These mechanisms are interlinked thus worsening the diabetic retinopathy outcome. In this review, we propose oxidative stress as the focus point of this complication onset.

1. Introduction

Diabetes mellitus (DM) is expected to affect around 550 million people all over the world according to global estimates of the prevalence of diabetes [1]. DM is characterized by constant hyperglycemia that damages various organs and manifests in macrovascular complications like premature atherosclerosis resulting in strokes, peripheral vascular disease, and myocardial infarctions and microvascular complications such as nephropathy, neuropathy, and retinopathy [2].

Diabetic retinopathy (DR) is the number one cause of blindness in people between 27 and 75 years of age. Prevalence of DR is around 25% and 90% at 5 and 20 years, respectively, from diagnosis; it is calculated that 191 million people will be diagnosed with this microvascular complication by the year 2030 [3]. It consists of progressive retinal structure and function loss due to vessel damage such producing blood-retina barrier rupture and promoting new vessel formation in the presence of chronic hyperglycemia [4].

The first clinical signs of DR are microaneurysms in the retina found in the mild version of the disease. In moderate diabetic retinopathy, exudates, hemorrhages, and minimum intraretinal microvascular abnormalities are present up to being prominent in severe stages among with more than 20 hemorrhages and venous rosaries in at least 2 quadrants. Neovascularization is the main clinical change in proliferative diabetic retinopathy (PDR) [5].

Through the last three decades, extensive scientific reports have shown ROS to play an important role in DM complications such as diabetic neuropathy, nephropathy, and retinopathy due to alterations on the biomechanisms involved in the instauration and progression of microvascular complications [6]. These three microvascular complications share high glucose levels as a starting point; nonetheless, according to Barret et al., such condition is

necessary, but may not be enough to initiate the damage present in the peripheral nervous system (neuropathy), kidneys (nephropathy), and retinas (retinopathy) of diabetic patients [7, 8]. In addition, the activation of various pathways involving proinflammatory factors and reactive oxygen species overproduction has been linked to vascular injury in the structures previously mentioned [9–11]. With this in mind, multiple molecules and nutraceuticals have been studied in recent years by their antioxidant effects due to their apparent benefits over diabetes and its complications [12–15].

As will be seen in this document, hyperglycemic states favor the activation of alternative pathways leading to reactive oxygen species (ROS) formation and augmented concentrations locally and in the rest of the body even at the point of surpassing the antioxidant capacity, a state known as oxidative stress affecting retinal integrity.

2. Pathophysiology of Diabetic Retinopathy

The retina is a high energy-demanding organ, which makes it susceptible to high levels of free radicals or ROS. Multiple factors are implicated in DR pathophysiology. Along with hyperglycemia that promotes changes in vascular and neuronal structures through ischemic or hyperosmotic damage, it also leads to oxidative stress (OS). Oxidative stress produces inflammation, mitochondrial dysfunction, and cell death, via pyroptosis, apoptosis or autophagia, and neurodegeneration that in conjunction leads to neural, vascular, and retinal tissue damage. In recent years, it has been found that such damages are present in a sequential order, in which neurodegeneration takes place before microvascular dysfunction, then clinical characteristics may be found, and finally symptoms appear. However, one could believe that these steps occur in a timely manner and that each biomechanism happens only in one direction; study findings show that different biomechanisms are active at the same time and have an influence between them. As seen in Figure 1, the retina consists different types of cells that form identifiable layers, from the endothelial layer in the inner side of the eye through the retinal pigmented cell layer in the outer side close to the choroidal surface. At each layer, various biomechanisms such as inflammation, pyroptosis, and neurodegeneration could appear simultaneously and have an intricate relationship with high levels of reactive oxygen species and oxidative stress.

2.1. Hyperglycemia in Diabetic Retinopathy. Through the glycolytic pathway, glucose suffers various biotransformations up to pyruvate that enters the Krebs cycle in the mitochondria to follow the respiratory chain in order to synthesize adenosine triphosphate (ATP). It is known that high concentrations of serum glucose can cause damage to cell structure and function. In the retina, pericytes are key cells in normal retinal function. As shown in Figure 2, these cells suffer from edema due to intracellular accumulation of sorbitol, which is formed by aldose reductase in the presence of high blood sugar through the polyol pathway, leading to a blood-retinal barrier (BRB) dysfunction [16, 17]. Edema causes vessels to swallow impeding adequate perfusion especially in the inner retina where blood supply is sparse compared to the outer retina [18]. Ischemia upregulates the expression of vascular endothelial growth factor (VEGF), known to play a role in angiogenesis, increased permeability, and activation of proinflammatory proteins [19]. All of them are important mechanisms involved in the development of diabetic retinopathy [18, 19]. On the other hand, the presence of glucose forms glyceraldehyde-3 phosphate (DHAP) through the glycolysis pathway; these two phosphates are very reactive to the nonenzymatic formation of methylglyoxal (MG) [20]. Such dicarbonyl (methylglyoxal) has been implicated in the activation of the hexosamine pathway, loss of pericytes, and decreased function of bipolar cells in the retina even in the absence of hyperglycemia [21]. The hexosamine pathway transforms fructose 6-phosphate into UDP-N-acetyl glucosamine (UDP-GlcNAc). When this very last molecule exceeds its normal concentrations, it promotes protein modifications by O-glycosyl-N-acetylation (O-GlcNAc) inducing an exacerbated activity; one of those proteins is nuclear factor-κB (NF-κB), a factor known to be implicated in DR worsening [22–24].

Methylglyoxal activates the advanced glycation pathway, AGE formation, and receptor activation (RAGE). AGEs can promote VEGF activation which alters tight junctions between retinal pigmented endothelial (RPE) cells. Such alterations lead to increased vascular permeability and leakage of blood components into the retina [25]. VEGF also mediates angiogenesis, so when chronic hyperglycemia persists, this factor deviates from physiological functions onto the formation of pathologic new vessels as happens in proliferative diabetic retinopathy among other cytokines, proinflammatory, proangiogenic, and prooxidative factors [26].

Hyperglycemia augments thioredoxin-interactin protein (TXNIP) levels, an inflammation mediator in Müller glia. TXNIP upregulation activates cellular defense mechanisms including autophagy, hypoxic-like HIF-1α induction and inflammasome formation [27].

According to many studies, the principal cause of DR is the lack of or poor glycemic control, but hypertension and dyslipidemia management has been proven to be beneficial in reducing progression and incidence of this complication [28, 29].

2.2. Reactive Oxygen Species in Diabetic Retinopathy. ROS are free radicals, oxidant molecules that contain one extra electron conferring them great instability and reactivity. By trying to regain stability, they obtain electrons from other molecules in the vicinity, therefore creating an oxidative chain [30].

As presented in Figure 2, ROS are formed in a physiological manner through the electron transport chain in the mitochondria derived from oxygen; some of the most common ROS are superoxide anion ($O^{\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) [31]. By antioxidant enzymatic defenses, such as catalase, glutathione peroxidase, superoxide dismutase, hemoxygenase 1, peroxiredoxins, and glutaredoxins, and nonenzymatic antioxidants, the body is capable of maintaining a redox balance. When the production of ROS is higher than the antioxidant defenses, OS occurs

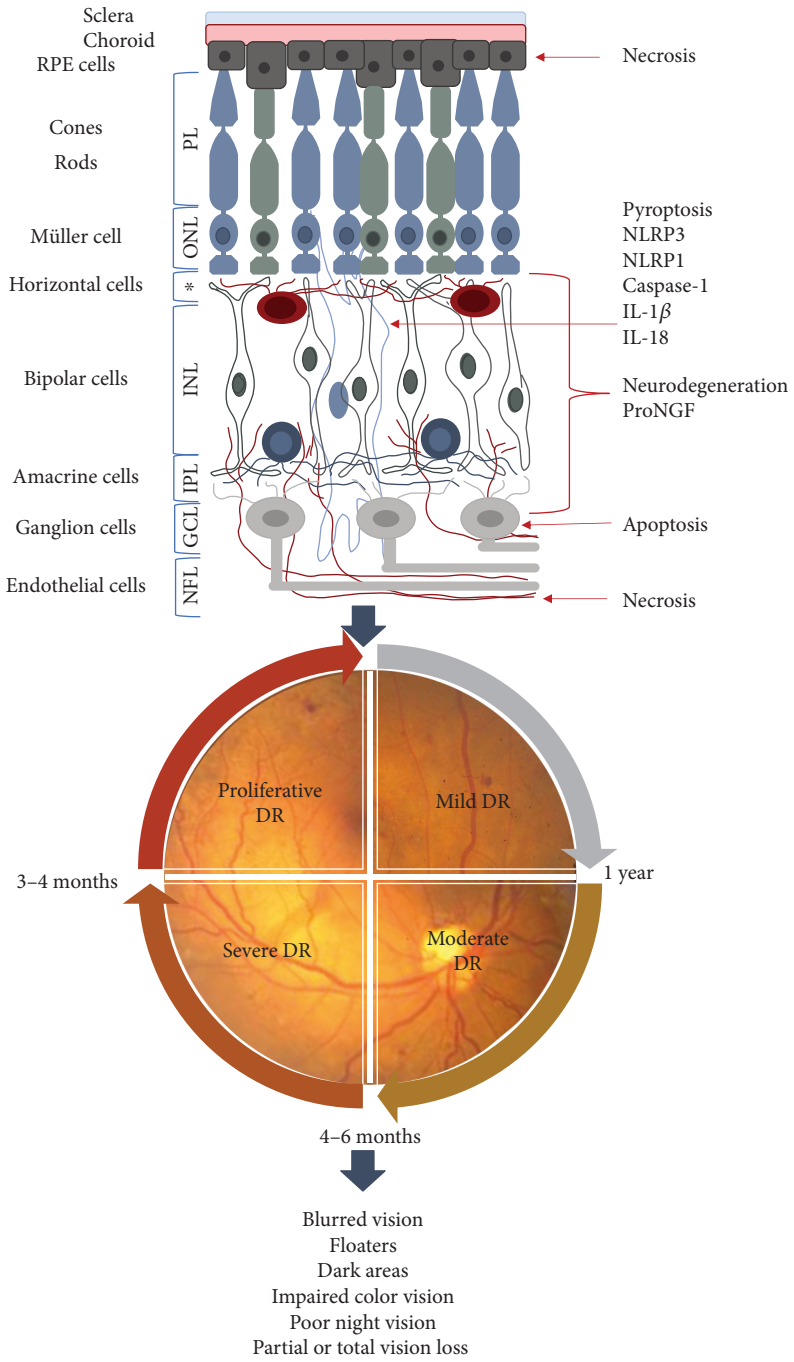

Figure 1: Damage at each retinal layer. A series of events occur in early DR development. Neurodegeneration of horizontal, bipolar, amacrine, and ganglion cells. These damages may be determined by proNGF concentrations as NLRP3 and NLRP1 are related to eye degenerative diseases. NFL: nerve fiber layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; *OPL: outer plexiform layer; ONL: outer nuclear layer; PL: photoreceptor layer.

and, at that point, cellular and mitochondrial function get affected [17, 32].

OS has been considered one of the most important factors in the development of DR and chronic hyperglycemia and also plays a role in the formation of ROS due to the activation of the secondary pathways like the polyol and the protein kinase C (PKC) and overactivity of the hexosamine pathways [32, 33].

Glucose metabolism is known to involve redox reactions as the main purpose in energy production by extraction, storage, and transport of electrons. When glycemic conditions are normal, glucose undergoes transformation through the glycolysis pathway to produce ATP by the Krebs cycle in the mitochondria, where electrons are stored in NADH and FADH2. Then, in the respiratory chain, they donate the electrons to the complex I or complex II. In complex IV, oxygen

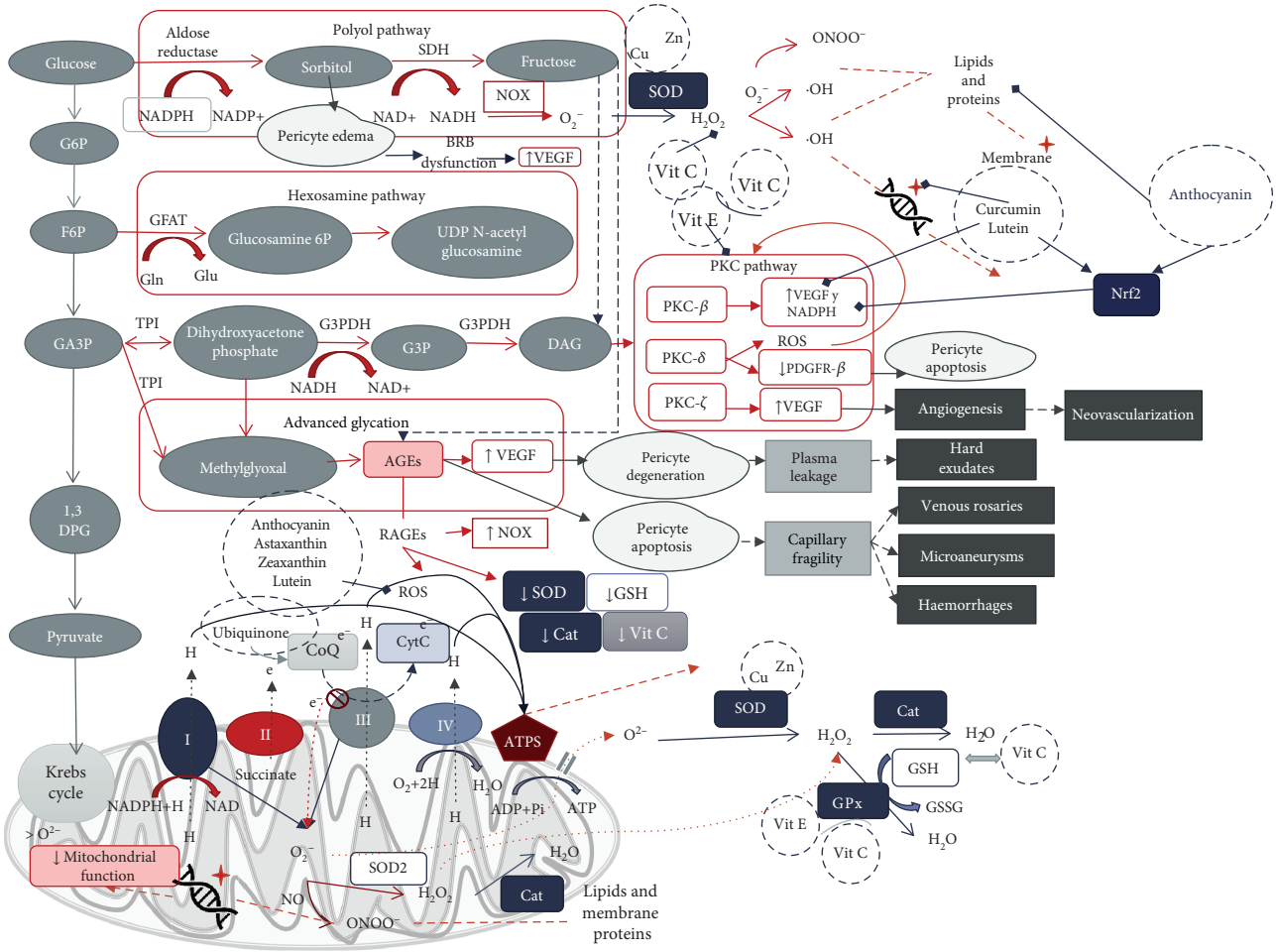

Figure 2: Glucose metabolic pathways in the hyperglycemic milieu, oxidative stress in diabetic retinopathy, and antioxidant targets. In hyperglycemic states, different pathways were activated producing ROS which enhance inflammatory, apoptotic, and degeneration pathways, ultimately leading to the appearance of diabetic retinopathy clinical characteristics. Some antioxidant substances are able to interact with ROS (xanthophylls, vitamins C and E, and anthocyanin); others function as cofactors to enhance antioxidant enzymes (Cu, Zn, and vitamins E and C), and others are capable of inhibiting the expression of proinflammatory and prodegeneration factors (curcumin and lutein). Finally, all of them interfere in diabetic retinopathy development.

is used again to receive electrons from cytochrome c [34]. Nonetheless, the polyol pathway is increased during diabetes; it consumes 30% of the systemic glucose. It consists of the production of sorbitol by two main reactions dependent of aldose reductase (AR) and sorbitol dehydrogenase (SDH) by the consumption of NADPH [34]. As mentioned before, sorbitol leads to osmotic stress and damage in the capillaries; also, in the reaction of converting sorbitol to fructose by SDH, reduced Nicotinamide Adenine Dinucleotide (NADH) is formed. As we can see in Figure 2, NADH now serves as substrate of Nox family enzymes to produce superoxide [35], contributing to redox imbalance and oxidative stress. Another way that NADH may contribute to the redox imbalance is by reductive stress creating pseudohypoxia and overwhelming mitochondria complex I function [36]. Complex I is not able to oxidize all NADH available, though by trying, it pumps more electrons to partially reduce oxygen leading to superoxide formation instead of adequate usage of oxygen and electrons [37]. In this case, NADH concentrations would still be higher than NAD+ which is needed to transport electrons to oxygen; this alteration in the appropriate consumption of oxygen is known as pseudohypoxia [38].

Fructose upregulates the formation of AGE [34, 39]. Endogenous fructose from the polyol pathway (Figure 2) suffers a rearrangement in carbon 2 by a reaction called Heyns reaction. Afterwards, the products undergo processes of rearrangement, dehydration, and condensation to form AGEs. By the Maillard reaction and Amadori rearrangement, glucose ends up forming AGEs yet the fructose-specific AGEs have not been yet described [40].

When the polyol pathway is activated during diabetes, OS is increased, then the increase in the activity of the polyol pathway is postulated to deplete NADPH by competing with glutathione reductase, and the availability of NADPH may be reduced and less available to regenerate intracellular antioxidants [41]. Accordingly, NADPH and ATP are decreased in lenses of diabetic rats with higher concentrations of sorbitol and fructose than healthy rats, supporting the findings on the activation of the polyol pathway in sustained hyperglycemic states [42, 43].

Through the hexosamine pathway, glutamine:fructose-6-phosphate amidotransferase (GFAT) oxidizes glutathione as a cofactor in order to transform F6P into glucosamine-6-phosphate; GFAT activity is significantly higher in diabetic subjects inducing to a lower pool of such endogenous antioxidant (glutathione) [44].

Diacylglycerol (DAG) is formed from 6-phosphate dihydroxyacetone phosphate, the second metabolite from fructose 6-phosphate (from the polyol pathway or glycolysis). DAG, in turn, activates the PKC pathway. PKCs are calcium and DAG-dependent kinases; the activation of these molecules has been associated to increased vascular permeability and abnormal angiogenesis in hyperglycemic and hypoxic conditions [45, 46].

PKCβ and PKCς are involved in the VEGF-dependent retinal barrier changes [47]. PKCβ also increases the activity of NADPH oxidase that produces superoxide [48, 49]. On the other hand, activation and translocation of PKCδ have proven to promote proliferation in the retinal tissue even in the absence of hypoxia [46]. In cell cultures, PKCδ activation by phosphorylation is able to inactivate complex IV of the mitochondria, thus augmenting ROS production [50].

At high glucose levels, glyceraldehyde-3-phosphate transforms to methylglyoxal, a precursor of AGE formation which is implicated in pericyte apoptosis and VEGF elevation. The activation of receptors for AGEs (RAGEs) leads to Nox augmentation, increase of ROS production, and decrease in SOD, catalase, glutathione, and vitamin C antioxidant activities [51] (see Figure 2).

Next, we discuss the following biomechanisms implicated in DR that have been described to be upregulated or closely related to oxidative stress, from inflammation to neurodegeneration.

2.3. ROS, Inflammation, and Pyroptosis in Diabetic Retinopathy. It has been proven that diabetes is an inflammatory state since hyperglycemia leads to cell malfunction and elevation of several cytokines and inflammatory mediators. Reactive oxygen species such as H_2O_2 and superoxide anion promote NF-κB production which in turn mediates VEGF expression; at the same time, it is activated by VEGF and translocated to the nucleus to promote the expression of proinflammatory mediators such as ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), monocyte chemotactic protein-1 (MCP-1), and cyclooxygenase-2 (COX-2) [19, 52]. It is known that COX-2 increases prostaglandin synthesis; prostaglandins stabilize hypoxia-induced factor-1 (HIF-1) which favors VEGF expression and NF-κB activation for COX-2 expression. This way an inflammatory mediator loop is formed [53–56]. ICAM-1, VCAM-1, and VEGF are implicated in BRB disruption that causes microaneurysms and leakage in the retina [57].

As inflammatory factors are activated, an inflammasome is formed recruiting the adaptor apoptosis speck-like protein containing a CARD (ASC); this cleaves caspase-1 activating IL-1β and IL-18 and leading to cell death, and this particular death process that includes damage and rupture of the cell membrane is known as pyroptosis [58].

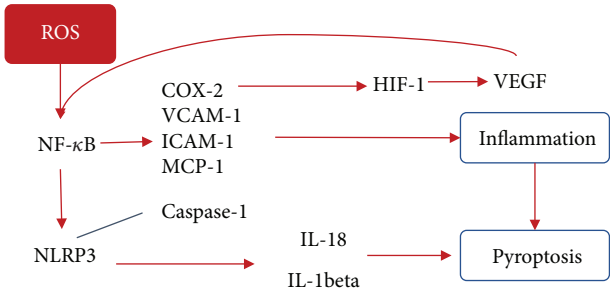

Figure 3: The ROS role in inflammation and pyroptosis. ROS augments NF-κB production which promotes proinflammatory mediators favoring the expression of VEGF. VEGF translocates NF-κB into the nucleus, and NF-κB activate NLRP3 with caspase cleavage leading to cytokine release. NLRP3 inflammasome has been associated to diabetic retinopathy by Müller pyroptosis by the caspase-1/IL-1beta pathway. NF-κB: nuclear factor kappa B; COX-2: cyclooxygenase-2; VEGF: vascular endothelial growth factor.

Pyroptosis is a type of caspase-1-dependent death cleaved by inflammatory molecular platforms called inflammasomes, also called pyroptosomes [59]. Such platforms contain oligomers of ASC adaptor proteins with a sensor of danger-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) [59] and are assembled by a variety of toll-like receptors (TLRs), either one of the six nod-like receptors (NLRs) or IFNγ-inducible protein absent in melanoma 2 (AIM2) or retinoic acid-inducible gene-I- (RIG-I-) like helicase. According to recent studies, inflammasomes NLRP3 (NOD-like receptor pyrin domain-containing 3) and NLRP1 (NOD-like receptor pyrin domain-containing 1) are associated to retinal diseases [58, 60].

It has been explained how ROS have a very important role in the DR development and progression, as synthesized in Figure 3; they also promote the assembly of inflammasomes or pyroptosomes leading to pyroptosis [58] (see Figure 3).

2.4. ROS and Autophagy in Diabetic Retinopathy. Diabetes is related to different forms of cell death (apoptosis, pyroptosis, and autophagic death) affecting retinal cells like pericytes, ganglion, and Müller cells [61]. In retinal cells, apoptosis may be triggered by the excess of ROS that upregulate matrix metalloproteinases 9 and 2 (MMP-9 and MMP-2) which impair mitochondrial membrane potential leading to apoptosis via mitochondrial pathway [62].

Autophagy is the process of degradation and recycling of proteins and organelles. Autophagy's main function is to regulate processes such as maintenance of organelle integrity, control of protein quality, and regulation of stress and immune responses [63]. Two forms of autophagy may be cited: (a) nonselective autophagy, triggered by nutrient deficiency in order to acquire metabolic components, and (b) cargo-specific autophagy, employed to remove impaired or nonfunctional organelles like ribophagy (ribosome elimination), pexophagy (peroxisome elimination), and mitophagy (mitochondrial removal). There are three types of autophagy in mammalian cells: (1) macroautophagy, (2) chaperone-mediated autophagy, and (3) microautophagy [61].

Macroautophagy is basically done in four steps: (1) ubiquitination labeling of the molecules or structures to be recycled, (2) autophagosome formation, (3) fusion to lysosomes (autophagolysosomes) that provide hydrolytic enzymes, and (4) release of products. It consists of the sequestration of the cargo (organelles and macromolecules) into the lysosome [64, 65].

Chaperone-mediated autophagy transports the cargo (protein complexes or unfolded proteins) across the lysosomal membrane while microautophagy uptakes the cargo (protein remains or small molecules) into the lysosome via an invagination, without a phagosome formation [65].

Macroautophagy is activated under normal conditions to maintain cellular homeostasis though it is also induced by stress conditions whether it is starvation or OS to protect the cell [66]. In diabetes, an overload to the mitochondria leads to mitochondria dysfunction (MD) which is the loss of efficiency in the electron transport chain; it promotes ROS production creating a vicious cycle in which ROS damage mitochondrial structures and machinery; when the cell detects this malfunctioning, it induces mitophagy to survive [11]. At high oxidative stress levels, caspases inactivate autophagy and activate apoptosis [67] (see Figure 4). Moreover, it has been shown that autophagy deficiency in beta cells creates a reduced insulin production but chronic activation of autophagy leads to autophagic cell death [63, 68].

As mentioned above, ROS production, hyperglycemic states, and ischemia are implicated in the upregulation of VEGF. This growth factor activates mammalian target of rapamycin (mTOR) which in physiological conditions prevents autophagy promoting RPE cell dedifferentiation and photoreceptor preservation, though in energy deficiency intracellular conditions, whether by lack of ATP (mitochondrial dysfunction) or lack of glucose (vascular disruption), other growth factors such as insulin-like factor induce autophagy via modulation of mTOR/AMPK (AMP-activated protein kinase) by the activation of caspase-3, reduction of glutathione, and photoreceptor cell death [61].

VEGF, ICAM, and nitric oxide have been associated with retinal photoreceptor disruption and severity of diabetic retinopathy. Photoreceptor cells release factors that control neuronal survival and angiogenesis, such as the pigment epithelium-derived factor (PEDF), which promotes the survival of photoreceptors and has an antiangiogenic action [69].

The unbalanced expression of VEGF seems to be implicated in important human pathologies, such as choroidal neovascularization (VNC) in diabetic retinopathy [69]. Vascular endothelial growth factor (VEGF) induces the expression of retinal intercellular adhesion molecule 1 (ICAM-1) and initiates the adhesion of retinal leukocytes, which leads to an early rupture of the retinal barrier and generates no capillary perfusion, injury, and death of endothelial cells [70, 71]. DR causes the interruption of the external limiting membrane (ELM) and the junction of the internal segment and the external segment of the photoreceptor, which is related to DR severity and affects visual acuity [70, 72].

ICAM-1 has been implicated in leukostasis development, a prominent DR feature. Its specific inhibition prevents leukocyte adhesion on the diabetic retina and the rupture of the hematoretinal barrier. ICAM-1 is eliminated by the cell and is the key mediator of the effect of VEGF on retinal leukostasis [70, 73].

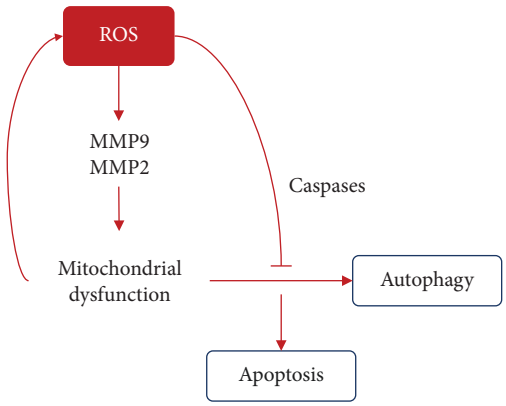

Figure 4: The ROS role in autophagy. ROS upregulate MMP9 and MMP2 that leads to mitochondrial membrane potential impairment. When a mithochondrion malfunctions, autophagy (mitophagy) is activated, though in high stress conditions, caspases inactivate mitophagy and activate apoptosis pathways.

The neuronal nitric oxide synthase (NOS) may be responsible for the production of NO in photoreceptors and bipolar cells which has significant effects on the blood flow, neutrophil aggregation, and platelet aggregation [74].

Inducible NOS, found in Müller cells and in the retinal pigment epithelium, can participate in normal phagocytosis of the outer segment of the retina, in infectious and ischemic processes, and in the pathogenesis of the diabetic retinopathy. Nitric oxide is involved in maintaining rest in the uveal and retinal circulations, which contributes to the basal tone in the latter [74, 75]. Retinal ischemia occurs because of a primary ocular disease, such as vascular occlusion of the retina or as a consequence of a systemic disease, such as diabetes mellitus. NO significantly affects the blood flow, neutrophil activation, and platelet aggregation [74].

2.5. ROS and Neurodegeneration in Diabetic Retinopathy. Let us recall that the retina is formed by various layers; one of which is the neural retina, composed of ganglion, amacrine, horizontal, and bipolar cells as well as light-sensitive photoreceptors. These cells interact with each other to transmit visual signals to the brain [76]. Neural retina cells are altered in their function in patients with diabetes as many studies in the past have shown. According to a longitudinal study performed by Kim et al., patients with diabetic retinopathy who had at least 2-step progression in a 4-year follow-up presented a greater thinning rate of macular ganglion cell-inner plexiform layer [77].

In the last decade, it has been demonstrated that constant high glucose concentrations lead to death of neurons in the retina even before apoptosis of pericytes begins [78–80]. These alterations may result from hypoxia and inflammation [81]. The retina is a highly energy- and oxygen-demanding tissue; hypoxia is a mechanism known to induce neuronal degeneration [82].

A number of cytokines and neurotrophic factors related to hypoxia have been described to be implicated in the diabetic retinopathy onset; some of them are also responsible for neurodegeneration [83, 84]. Secretion of IL-1β, IL-6, IL-8, MCP-1, TNF-α, and VEGF factors known to play an important role in inflammation pathways and pyroptosis may have a role in neurodegeneration as well [85]. As shown in Figure 5, TNF-α is also induced by H_2O_2 by activating caspases in numerous nerve cells [86]. Oxidative stress has been implicated in axonal degeneration and neuronal apoptosis in traumatic and nontraumatic nerve degeneration, via ZNRF1 activation by oxidative stress [87].

There appear to be some factors that protect amacrine, ganglion, and Müller cells from degradation like brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and mesencephalic astrocyte-derived neurotrophic factor (MANF). Müller cells produce NGF increasing the expression of VEGF contributing to angiogenesis in physiological conditions in order to protect neuronal cells from the oxygen-glucose-deprived milieu [88, 89]. Oxidative stress in the retina is capable of preventing NGF activation from its precursor form proNGF which is known to promote apoptosis of neural retina cells [90] (see Figure 5). An imbalance of NGF/proNGF in vitreous correlates to retinal damage [91]. Other factors that may contribute to retinal neuron protection are ciliary neurotrophic factor (CNTF) and fibroblast growth factor (FGF) [92]. Reactive oxygen species lowering has shown to be helpful in protecting neuronal degeneration and favoring the expression of protective factors like compact myelin proteins [93]. Retinal ganglion cell survival is also promoted by PEDF (pigment epithelium-derived factor) via STAT3 (signal transduction and activator of transcription 3) activation secreted by Müller cells [94].

2.6. Oxidative Stress-Related Genetics in Diabetic Retinopathy. Studies have shown that DR has a genetic component by observing higher prevalence in certain ethnic groups: Hispanics, Asians, and African Americans.

It is worth noting the complexity of DR as a complication of diabetes and that this is influenced by hereditary factors and the environment [95]. Some DR phenotypes show that changes in the neural retina and the associated microvascular network resulting in abnormal and leaking vessels are a distinctive feature of this pathology [95].

Genetic predisposition of some ethnic groups who suffer from retinopathy is suggested in some studies. It has been found a higher prevalence among Hispanic and African American individuals than in non-Hispanic whites [96, 97]. Knowledge about genetics of this disease will be useful to identify the genome variants that are associated with the higher possibility of complications among individuals with DM; this would allow generating strategies or guidelines for the early identification of diabetic individuals with a high risk of developing DR. In this sense, three main research strategies have been discussed: linkage studies in families, candidate genes, and complete genome association studies (GWAS) [97]. It has been estimated that inheritance is as high as 27% for DR and 52% for PDR [98]. Relatives of patients with DR have a 2-4 times higher risk of developing DR compared to family members of patients without retinopathy [99].

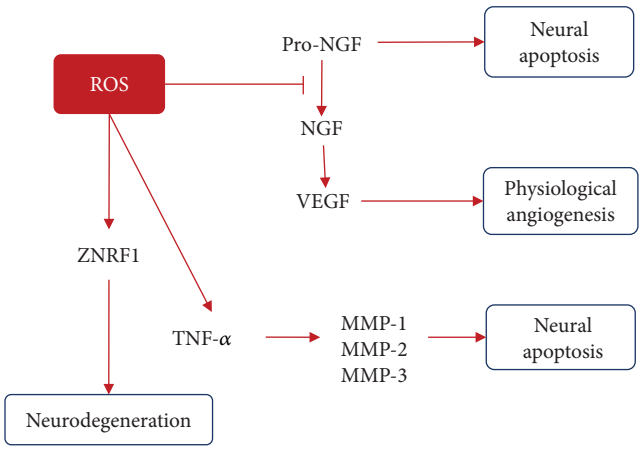

Figure 5: The ROS role in neurodegeneration. In physiological conditions, NGF activates VEGF to promote angiogenesis and protect nerves from hypoxia and ROS inhibits NGF formation from its precursor which leads to neural apoptosis. ROS activate ZNRF1 that provokes neurodegeneration; at the same time, TNF-α activates apoptosis via metalloproteinase/caspase pathway. ZNRF1: zinc and ring finger-1; NGF: nerve growth factor; VEGF: vascular endothelial growth factor; MMP: matrix metalloproteinases; TNF-α: tumor necrosis factor-α.

2.6.1. Polymorphisms Linked to DR. It is said that the DR is a complex genetic disease which means it is commonly associated with multiple genetic and environmental factors. These factors are commonly called polymorphisms instead of mutations [100].

Thus, a polymorphism can increase or decrease the risk of suffering from the disease. Some of the advances about DR genetics involve the following genes as part of the DR pathogenesis [101].

(1) Aldose Reductase (ALR). Aldose reductase (ALR) is the first limiting enzyme in the polyol pathway responsible of inducing vascular and hemodynamic pathogenic changes that contribute to DR, as well as the result of sorbitol accumulation, oxidative damage, and protein kinase C activation [102]. ALR is found in high concentrations in Schwann cells and in retinal pericytes where glucose is converted to sorbitol; polymorphisms of ALR have been significantly associated in some populations [100]. Vascular retinal changes, such as the degeneration of retinal pericytes and the development of microaneurysms, can be induced in rats and dogs that have become hyperglycemic with a galactose-rich diet, but galactose is reduced by aldose reductase (AKR1B1) to form galactitol [103]. Consequently, search of pharmacological inhibitors of this enzyme for the treatment of DR is taking an important course [104].

(2) Receptor for Advanced Glycation End Products (RAGE). A state of sustained hyperglycemia can promote protein and lipid glycation in consequence producing AGE, which promote the alteration of the structure and function of other

proteins; AGE has affinity for receptors known as receptor for advanced glycation end product (RAGE). The RAGEs are immunoglobulins that when activated promote the secretion of cytokines, which further stimulate the complications of diabetes by increasing vascular permeability and inflammatory processes [105, 106]. These effects will promote a hypoxic state in the microcapillaries of the retina leading to the beginning of the angiogenic process in the PDR [106]. It has also been found that AGEs and RAGE are overexpressed in DR, which leads to think that genetic polymorphisms of RAGE are probably involved in the DR pathophysiology [107]. In a meta-analysis conducted by Yu et al. in 2016, they found that Gly82Ser in RAGE showed a significant association with DR; however, it was important to perform more studies with better control over the risk factors and duration of diabetes in patients [107]. There are other polymorphisms such as -429T/C, -374T/A, and 1704G/T, of which other studies have had contradictory results; therefore, without significant evidence, it has not been possible to associate them with DR [100, 105, 107].

(3) Vascular Endothelial Growth Factor (VEGF). High levels of VEGF have been detected in the eyes of patients undergoing vitrectomy operations in patients with PDR; it is an important growth factor responsible for vascular permeability [103]. That is, high levels of VEGF promote a greater vascular permeability and neovascularization; therefore, it is said that the inhibition of this factor has shown an improvement of these events at the level of the retina. High levels of VEGF promote a greater vascular permeability and neovascularization, which is consistent with what several studies have shown, where patients with DR have a high expression of VEGF [103, 108]. Therefore, it is said that the inhibition of this factor has shown the improvement of these events at the level of the retina [103]. In a study conducted by Gonzalez-Salinas et al. [109] in the Mexican population, they aimed to associate the polymorphisms rs3025035, rs3025021, and rs2010963 that just increase the expression of VEGF and that were previously associated with PDR in other populations; however, their results did not allow them to create a significant association. It requires new studies with a larger sample size, knowledge about pharmacological treatment, and fewer restrictions on the patient's clinical information which is highlighted [109].

(4) Nitric Oxide Synthase (NOS) Genes. Nitric oxide has been detected in internal segments of photoreceptors, in some amacrine cells, in ganglion cells, and in the inner plexiform layer of the retina of adult rats [110]. The formation of NO is catalyzed by the enzyme endothelial nitric oxide synthase (eNOS) from L-arginine, which also takes a role in angiogenesis [97, 111, 112]. Therefore, eNOS is an important enzyme that contributes to vascular homeostasis in which overproduction can cause damage to the retina, by increased cell death, vascular permeability, and neurodegeneration mainly; eNOS polymorphisms have been related to increased risk to DR progress [110]. The decrement in the production of endothelial NOS can lead to the decrease of NO and vascular dilation [113].

Several analyzes have been made about the a/b polymorphism of the eNOS gene, and it has been argued that there is an association between this polymorphism and the risk of DR development [97, 112, 114]. A significant association was found between the intron 4a allele of the 4b/a polymorphism and a reduced risk of DR [114]. However, a meta-analysis indicates that the eNOS 4b/a polymorphism is not associated with an increased risk of DR among subjects with type 2 diabetes [97, 112].

As discussed here, many pathways and biomechanisms are implicated in DR; therefore, it is important to explore gene polymorphisms in enzymes and factors that play a role whether in redox balance, vascular function, or inflammation. Previously, we have discussed some of the most important polymorphisms linked to diabetic retinopathy; over the years, various studies have been done in this regard though results have been inconsistent. We present some examples of polymorphisms that have been associated with the diabetic retinopathy onset but have yet to be confirmed (see Table 1).

3. Influence of Antioxidants in Diabetic Retinopathy

Optimizing glycemic and lipid controls are the first-line therapies in diabetes control, which also reduce the DR progression [130]. Specific recommendations on diet as well as some of the dietary components or food intakes have already been reviewed on its effect on type 2 DM. Mediterranean diet is a recognized healthy dietary pattern [131] and has shown to have a protective effect against DR [131, 132] which contains a high amount of fish and extra virgin olive oil containing omega-3 fatty acids [133] and mixed nuts, which are rich on polyphenols that may reduce the risk of developing diabetes [134] and lowers insulin resistance [135]; also, it is rich in protective factors such as the Nrf2 [136] diet. Finally, the intake of vitamin-rich food such as fruits and vegetables as well as supplements has also been related to a risk reduction of chronic diseases [137] or DR itself [138], and they also have some hypoglycemic effects carried out by their bioactive compounds such as, flavonoids, alkaloids, and anthocyanins [139], the latter being present in wild blueberry, bilberry, cranberry, elderberry, raspberry seeds, and strawberry which have shown to have powerful antioxidant activity [140] while other micronutrients, such as vitamin C and E, have not shown any association between risk and intake [141] in contrast to Tanaka's prospective study on fruit consumption [142] but have yet to be explored on full potential in a possible combined-antioxidant therapy.

Nutrients in diet can play a massive role in diabetic patients who are resistant to conventional treatment; these nutritional strategies can reduce the risk of prognosis and attenuate progression preserving the normal function as well as structure of the retina [143].

As a complementary therapy to the existing conventional one, we propose the use of some supplements with antioxidant properties since they have protective effects at different points in the pathways involved in DR prognosis (see Figure 2).

Table 1: Polymorphisms implicated in diabetic retinopathy. Many genes have been associated with diabetic retinopathy; some polymorphisms in them have, apparently, protective effects while others worsen its progression.

	Author (year)	Population	Polymorphism	Conclusions
Aldose reductase (Alr)	Abhary et.al. (2010) [102]	Australian	rs9640883	Association with duration of diabetes rather than a direct association to DR
	Wang et al. (2003) [115]	Chinese	Rs759853 T allele	Protective effect against DR in DM type 1
	Santos et al. (2003) [116]	Euro-Brazilian	ALR C(-106)T	No association to DR
Nitric oxide synthase (NOS)	Zhao et al. (2012) [114]	Chinese	NOS3 4b/a	Negative association with DR (protective effect)
	Cheema et al. (2012) [117]	Asian Indian	rs3138808	No association with DR
	Santos et al. (2012) [118]	Caucasian-Brazilian	NOS3b/a	No association to DR
Receptor for advanced glycation end products (RAGEs)	Ng et al. (2012) [119]	Malaysian	-429T/C and -374T/A	No association with DR
	Vanita (2014) [120]	Indian	Gly82Ser	Positive association with DR
	Yang et al. (2013) [121]	Chinese	Gly82Ser	Associated to DR risk
Vascular endothelial growth factor (VEGF)	Kangas-Kontio et al. (2009) [122]	Multiethnic	rs3095039	No association
	Abhary et al. (2009) [123]	Multiethnic	rs3025021	Positive association
	Qiu et al. (2013) [124]	Chinese	rs2010963	Positive association
Gluthatione S-transferase (GST)	Dadbinpour et al. (2013) [125]	Iranian	GSTM1	Positive association with DR
Manganese superoxide dismutase (MnSOD)	Haghighi et al. (2015) [126]	Iranian	A16V	Positive association with DR
	Vanita (2014) [120]	Indian	Val16Ala	No association with DR
Intercellular adhesion molecule1 (ICAM-1)	Fan et al. (2015) [127]	Asian	rs5498	Negative association with DR
			Rs13306430	Positive association with DR
Transforming growth factor beta 1 (TGF-β1)	Rodrigues et al. (2015) [128]	Brazilian	Rs1800471	Positive association with DR
	Bazzaz et al. (2014) [129]	Caucasian	+869 C/T +915 G/C	No association with DR

3.1. Antioxidant Supplements

3.1.1. Xanthophylls. Xanthophylls are natural pigments derived from carotenoids that contain oxygen. This family includes lutein and zeaxanthin. Both substances are found in the fovea; lutein concentration is superior to zeaxanthin which differs from lutein in its double link in one of the hydroxyl groups. They have antioxidant effect by alternating their single and double links reducing blue light wavelength and protecting the eye from light-induced oxidative stress. Around 90% of the blue light is absorbed by these pigments [144].

Astaxanthin is another xanthophyll which is extracted from H. pluvialis to be used as an alimentary supplement. According to a study, astaxanthin presents a larger biological activity compared to other antioxidants since it is able to bind both sides of the cell membrane [145].

According to various studies, lutein, xanthophylls, and other carotenoids have demonstrated to be useful in protecting the retina from OS in chronic hyperglycemic conditions and ameliorating oxidative stress states [146, 147]. Lutein quenches free radicals leading to the blockade of NF-κB pathway activation and has effects on inflammation, by the inhibition of arachidonic acid release keeping prostaglandins, thromboxanes, and leukotrienes from being formed [144]. Lutein inhibits PI3K activity when it is increased secondary to oxidative stress via PI3K/Akt pathway which is capable of inhibiting the PDGF-induced RPE cell migration [148]. Lutein also is able to inhibit IL-8 secretion [149]; besides, zeaxanthin and lutein supplementation augments retinal pigment epithelial cell viability [150], and the former has been related to restoring VEGF concentrations [151]. Astaxanthin plays a role in the inhibition of proinflammatory molecule expression such as VEGF, ICAM-1, and MCP-1 [152]. In preclinical studies, astaxanthin has been shown to promote the expression of heme oxygenase-1 (HO-1) in the retina and greater glutamine synthase concentrations in Müller cells along with the reduction of H_2O_2-induced retinal ganglion cell apoptosis as well as improvement of MnSOD activity and decrement of oxidative damage markers [153, 154].

3.1.2. Vitamin C. Vitamin C exists in two main forms, ascorbic and dehydroascorbic acid; it is a ubiquitous metabolite in plants and animals. Ascorbic acid acts as a cofactor alongside many human enzymes and as a water-soluble antioxidant [155]. Vitamin C is present in higher concentration in

healthy patients, contrary to those with DR who have lower concentrations than those diabetic patients who have not developed this complication [156]. Vitamin C prevents the propagation of free radical-induced chain reactions [157], and thus, directly scavenging ROS preventing breakdown of NO and decreasing low-density lipid oxidation [143, 158, 159] protects the endothelial barrier permeability by the inhibition of VEFG [160]; however, caution is indeed needed since ascorbate can act as a prooxidant in the presence of transition metal such as ionic iron or ferritin, both associated with diabetes [161]. On the other hand, a supplementation with 1000 mg/day of ascorbic acid relates directly by reducing the activity of the enzyme aldose reductase and this way, it acts by inhibiting the polyol pathway [162]. Advanced glycation end products tend to decrease intracellular ascorbate; however, vitamin C also prevents the apoptosis of vascular pericytes [163]. It may have a role in autophagy, by the induction of autophagosome formation [164], increasing the rate of protein degradation lysosomes [165], and expressing Bcl-2 family proteins between hypoxia and reoxygenation statuses [166]. However, vitamin C has yet to be explored in DR since nothing similar has been reported in this diabetes complication.

3.1.3. Vitamin E. Vitamin E is, contrary to vitamin C, a fat-soluble vitamin, and the predominant isomer found in human's body is alpha-tocopherol; because of this, it parts to lipid storage organelles and membranes [167]. Vitamin E has roles in many different explored mechanisms, one of them being on lipid peroxidation by inhibiting the formation of malondialdehyde [168, 169]; at concentrations as high as 2000 mg/day, it has been shown to reduce fasting plasma glucose in diabetes [159]. Also, the oxidative formation of N-epsilon-carboxymethyl-lysine in damaged proteins by long-term exposure to high-glucose concentration can be reduced by it [170]. Tocopherols can also modulate transduction and gene expression by modulating nuclear receptors for peroxisome proliferator-activated receptors [171]. Alpha-tocopherol at a concentration of 10 and 50 μM was shown to inhibit smooth muscle proliferation as well as inhibit protein kinase C activity [172]. In a similar way, vitamin E has some effects on hemodynamic diabetes by decreasing the total diacylglycerol level, thus preventing the abnormal retinal flow [173]; furthermore, using the unsaturated vitamin E, tocotrienol, has an effect as an anti-angiogenic agent by increasing apoptosis of signal-regulating kinase and p38 in the fibroblast growth factor [174]. As mentioned above, although vitamin E by itself has not proven its efficacy as a treatment for DR [175], more clinical studies are needed specially as a combined therapy, since it may have some more beneficial properties administered alongside other antioxidant compounds [176].

3.1.4. Copper and Zinc. Zinc (Zn) is a nutritional element essential for the structure and function of numerous macromolecules, such as lipids, nucleic acids, and the enzymes, that regulate cellular processes and cellular signaling pathways [177]. Zn is widely distributed in foods and beverages, but as with other elements, the contents are variable and generally low [178].

Zinc exhibits antioxidant and anti-inflammatory activities, delaying oxidative processes in the long term by inducing the expression of metallothioneins (MT), and acts as a cofactor of the cytosolic and extracellular Zn/Cu SOD enzyme, which scavenges ROS by catalyzing the dissociation of the O_2^- radical in the less harmful forms O_2 and H_2O_2 [177]. Copper (Cu) participates in the production of energy in the mitochondria and functions as a cofactor to superoxide dismutase (SOD) found in the cytosol and intracellular space. Over the years, copper imbalances have been linked to chronic inflammatory diseases [179].

Oxidative stress (OS) influences the molecular mechanisms responsible for the development of many inflammatory diseases, such as DM [177]. It has been shown that zinc supplementation is beneficial for the balance between the content of free radicals and antioxidant enzyme systems in rats with systematic inflammatory response [180]. It is possible that these supplements improve the absorption in food of vitamin E and therefore prevent deficiency [181].

Zn supplementation increases insulin sensitivity and antioxidant capacity [182]. In these models in which diabetes was induced, the antioxidant enzymes catalase, GPx (glutathione peroxidase), and superoxide dismutase (SOD) are diminished in comparison with normal animals. Zn supplementation in these animals restored the activity of the enzyme and the synthesis of glutathione [182] and also attenuates the OS induced by diabetes in the circulation, as well as in cardiac and hepatic tissues in diabetic rats [183]. Renal oxidative damage induced by diabetes and inflammation has been significantly attenuated by Zn supplementation, mediated through MT expression [182]. Regarding the metabolism of glucose and lipids, the blood glucose level is also reduced in type 2 diabetic rats given with ZnO nanoparticles, with better glucose tolerance and a 70% increase in insulin levels. In addition to the significant reduction of circulating triglycerides and free fatty acids [184], Zn deficiency can have serious implications on the elderly; therefore, it is important to maintain adequate nutrition of Zn in this population [185].

It is known that more than 100 specific enzymes require Zn for their catalytic function, which indicates the critical role of Zn in cellular processes [186], including events of genomic stability, cognitive functions, depression, and oxidative stress [185]. Zinc alone is not actively redox, and therefore, Zn^{2+} does not interact directly with ROS or with free radicals centered on carbon [187, 188]. Zinc then contributes to the antioxidant status through its ability to compete with transition metals and copper for binding sites in the cell membrane [183]. Iron and copper ions catalyze the production of lipoperoxides; therefore, their replacement by zinc under conditions of insulin resistance in the plasma membrane could inhibit lipoperoxides [189]. Several studies in animals and humans have found that high levels of supplemental Zn over long periods of time can result in a decrease in the absorption of Cu leading to Cu deficiency [190].

3.1.5. Alpha Lipoic Acid. Alpha lipoic acid also called thioctic acid is a natural compound found primarily in vegetables (broccoli, spinach, and tomatoes) and meats and nowadays

in many additives. Alpha lipoic is both hydrophilic and hydrophobic and widely distributed both in cellular membranes and the cytosol and is essential for mitochondrial function [191]. It has been named as the "universal antioxidant" [192] since once consumed it is reduced to dihydrolipoic acid and both lipoic and dihydrolipoic acid can inhibit lipid and protein oxidation, as well as ROS scavengers [193]; not only that, lipoic acid also induced Nrf2 binding to antioxidant response elements and thus higher gamma glutamylcysteine ligase and its catalytic subunit, and this way, it ameliorates this antioxidant loss related with age [194]. Finally and importantly, as of why combined antioxidant therapies are not only viable but also synergized, dihydrolipoic acid can regenerate endogenous antioxidants, particularly vitamins C and E, two of the revised antioxidants in this article, and glutathione [195]. Alpha lipoic acid has antiangiogenic activity; it has proven to be effective in reducing the VEGF, angiopoietin 2, and erythropoietin by blocking superoxide formation in diabetic rat's retina [196] and by protecting the retinal ganglion cells by preserving its thickness [197], but it also has a direct antiangiogenic role, by inhibiting endothelial cell apoptosis and proliferation (not related to pericytes) through a probable inhibition of NF-κB, activating protein kinase B and upregulating p27 activity (inhibiting cell cycle progression) [198]. Alpha lipoic acid has even been formulated as an aqueous solution and administered intravenously, intraperitoneally, and intravitreally to evaluate the activity on microvascular complications in the eye by fluorescein leakage and by direct observation that concluded to reduce these complications and slow the progression of diabetic retinopathy [199]. Alpha lipoic acid's beneficial properties were also assessed on mitochondrial metabolism; in one study, mitochondrial function and regulation, measured by its transcriptional factor, peroxisome proliferator-activated receptor-γ coactivator-1α, and nuclear respiratory factor 1 was benefited by lipoic acid by preventing the loss of the mitochondrial copy number and increasing gene transcripts of PPARγ and NRF1 [200]. Some preclinical studies have shown efficacy of lipoic acid therapy in DR [201, 202], and clinically, it may have a protective role [203] but has yet to show efficacy on patients who have already developed DR, as it has shown no effect on macular edema at a daily dose of 600 mg [204].

3.1.6. Manganese. Manganese (Mn) is a heavy metal present in nature and is the fifth most abundant metal in the environment. Mn is essential for humans and animals; daily requirements are usually met with a proper diet. High levels of manganese can be found in legumes, rice, nuts, and whole grains [205]. Mn is transported by simple diffusion in the large intestine and is absorbed by active transport in the small intestine. Only about 5% of the Mn in the diet seems to be absorbed [206]. Mn is involved in cellular antioxidant defense mechanisms, but it is known that it participates in the generation of ROS and has prooxidative properties [207]. Mn is an essential nutrient that is required as part of a healthy diet; however, exposure to excessive levels results in toxicity in human development leading to hyperactivity, inferior intellectual function, impaired motor skills, and reduced olfactory function in children [205].

Mn is a cofactor in the key mitochondrial antioxidant enzyme [207] and a component of metalloenzymes such as Mn superoxide dismutase (MnSOD), glutamate synthetase, and pyruvate carboxylase and is associated with oxidative phosphorylation and mucopolysaccharide metabolism [206]. MnSOD main function is the detoxification of superoxide free radicals [208]. Mn can provide resistance to oxidative stress through the formation of manganese-based nonprotein antioxidants and also function safely as a cofactor for the enzyme superoxide dismutase (SOD) [209]. Given the similar physical properties between Fe and Mn, most transporters are capable of transporting both metals, which are competent to bind to the plasma membrane [205]. Several proteins involved in the transport of Mn have been identified, including the putative uptake proteins divalent metal transporter-1 (DMT1), transferrin receptor (TfR), and ATP13A2, as well as the efflux protein Fpn [206].

It was found that Mn is required for synthesis and secretion of normal insulin from an initial study in rats. Rats that are on a high-fat diet can improve glucose tolerance and insulin secretion. The fact that Mn results in insulin secretion induced by glucose is consistent with the improvement of mitochondrial function with glucose metabolism [210].

3.1.7. Curcumin. Curcumin is one of the main substances of *Curcuma* spp.; it is a crystalline orange-yellow color compound [211]. World Health Organization (WHO) recommended a minimal diary intake of 0-3 mg/kg as a food additive [212]. In recent years, it has been shown that curcumin has beneficial properties in DR treatment. (1) Curcumin acts as an antioxidant agent by reducing free radicals [213]. (2) Curcumin increases mRNA expression of antioxidant enzymes like SOD and catalase by reducing oxidative and regulating nitrosative DNA damage [214]. (3) Curcumin activates a mitochondrial pathway by regulating the respiratory function on mitochondrial complexes I, II, III, and V and simultaneously activates Nrf2 [215]. (4) Curcumin can increase antioxidant capacity in the retina of diabetic rats and hypoglycemic and preventive anti-inflammatory activity by reducing the levels of proinflammatory cytokines like IL-1β, tumor necrosis factor alpha, VEGF [216, 217], and 5-hydroxyeicosatetraenoic acid being a dual inhibitor of arachidonic acid [218]. (5) Curcumin acts as an antiangiogenic agent by decreasing stromal cell-derived factor 1 alpha that inhibits the migration of retinal human endothelial cells [219].

As seen in Figure 2, curcumin induces Nrf2 pathway activation, helping into a better defense against oxidative stress in retinal cells [220, 221]. All these effects related to diabetes and more specifically DR on curcumin are a promising alternative for the treatment of DR [222, 223]. Attention is needed in the presence of high concentrations since curcumin can act as a prooxidant agent and induce apoptosis [224].

3.1.8. Anthocyanins. Anthocyanins belong to the flavonoid group; they are six polyphenolic pigments in which 90% of the composition are found in nature: pelargonidin, cyaniding, peonidin, delphinidin, petunidin, and malvidin [225] while in the body, they are mostly metabolized to phenolic

acid and degradation products and are a stable water-soluble compounds [226], and they can be found deposited in the eye [227]. These compounds have been studied recently and extensively, and their effects are primarily on cardiovascular diseases; here, we try to summarize those related to DR pathogenesis. Anthocyanins alongside other bioactive compounds have a role as antioxidants by scavenging ROS [228], by inhibiting lipid peroxidation [229] and induction of Nrf2 expression (see Figure 2) [230], and by their antioxidant properties; anthocyanins induce the downregulation of the NF-κB signaling pathway exerting an anti-inflammatory response, and it may be as well partially involved in the mitogen-activated protein kinase pathways [231, 232]. Given all of these beneficial effects, more clinical interventions are needed to prove or assess these effects on diabetic retinopathy, rather than diabetes itself [233].

3.1.9. Ubiquinone. Ubiquinone or coenzyme Q10 (CoQ10) is ubiquitous in nature and widely distributed in plants, animals, and microorganisms. Ubiquinone can be obtained through exogenous sources, such as food. The richest dietary sources are meat, migratory fish, some oils, and nuts, but in the diet of the populations of western countries, these sources contribute in total to only 3-5 mg of CoQ10 per day [234]. A dose that varies from 50 to 150 mg is recommended in food supplements; however, there are also products with higher levels available [234]. In diabetes, the resulted hyperglycemia state induces the overproduction of superoxide by the electron transport chain in the mitochondria; this leads to vascular damage mediated by glucose [235].

Coenzyme Q10 (CoQ10) or ubiquinone is an essential compound found naturally in all cells of the human body. It is particularly known for its role in the chain of electron transport in mitochondrial membranes during aerobic cellular respiration. It is the only lipid-soluble antioxidant that animal cells synthesize de novo in the body [236] and is able to recycle and regenerate other antioxidants such as tocopherol and ascorbate [235]. Coenzyme Q10 is part of the process of oxidative phosphorylation in mitochondria, where it converts energy into carbohydrates and fatty acids into ATP to boost cellular machinery and synthesis. In addition to facilitating the transfer of electrons during oxidative phosphorylation, CoQ10 acts by inhibiting certain enzymes involved in the formation of free radicals, thereby reducing the consequences of oxidative stress [237].

One of the most important mechanisms offered by coenzyme Q10 to protect against diabetes is through the "recoupling" of the endothelial NOS. Increased oxidative stress in diabetes can cause diabetics to reduce the biological availability of nitric oxide [238]. Coenzyme Q10 acts by blocking endothelial dysfunction by activating endothelial nitric oxide synthase and mitochondrial oxidative phosphorylation. Thus, supplementation with coenzyme Q10 shown to alleviate the symptoms in animals and humans, by decreasing blood pressure in hypertensive individuals [238]. The treatment with CoQ10 presented several benefits, among them are the significant decrease in the high levels of glucose, triglycerides, very low-density lipoproteins, low-density lipoproteins, and atherogenic index and increase in the levels of high-density lipoproteins in diabetic rats. It also reduced lipid peroxidation and increased antioxidant parameters such as superoxide dismutase, catalase, and glutathione in the homogenates of diabetic rats [237].

3.1.10. Resveratrol. Resveratrol (3,5,4′-trihydroxystilbene (RSV)) is a natural phenol produced by several plants in response to damage when the plant is under attack by microorganisms. RSV is found in red wine and the skin of grapes, but also in blueberries, raspberries, and mulberries. RSV has antiproliferative, antiangiogenic, antioxidant, endothelial, anti-inflammatory, antiplatelet, and neurogenic activity [239, 240]. Resveratrol exists in both *cis* and *trans* form, and it is believed that the *trans* form is more stable [240]. RSV is absorbed by 75%, mainly by transepithelial diffusion, but when taken orally, bioavailability is very low, less than 1%; this is because in the intestine and liver, the metabolism is rapid and glucuronidated compounds are involved and sulfated to generate key metabolites that are easily eliminated. However, bioavailability is very variable between one individual and another due to factors such as age and gender [240–242]. As RSVl is a hydrophobic compound, it has been shown to be absorbed by intestinal epithelial cells, hepatocytes, and breast tumor cell lines [240]. It was found that the treatment with resveratrol causes an increase in the levels of reduced glutathione (GSH) in erythrocytes and the ocular level in rats, where GSH has a protective function against oxidants; also, significantly lower concentrations of malondialdehyde were found, which is a marker of peroxidation lipid [243, 244]. RSV also suppresses the action of endothelial nitric oxide synthase in the eyes of rats, an enzyme associated with neovascularization and with inflammatory processes in diabetes. Resveratrol use as a treatment creates a beneficial effect on the increase in vascular leaks, in the loss of pericytes, and the levels of VEGF [245, 246]. A study conducted by Luna et al. [247] showed that resveratrol also inhibited the production of reactive oxygen species (ROS), which in turn prevented the induction of proinflammatory markers such as interleukin-1a (IL-1a), interleyukin-6 (IL-6), and interleukin-8 (IL-8).

3.1.11. Omega-3. Lipids are important for cellular signals and metabolism, since they are part of the structure of the membranes and storage energy, so lipids and their metabolites are of great importance in ocular diseases because they are regulators in neovascularization [248]. The omega-3 are a family of healthy fats and are within the monounsaturated and polyunsaturated fatty acids. They are obtained from marine sources and also have anti-inflammatory and antiangiogenic properties which have been investigated in various parts of the human body, including the retina. There are three types of omega-3 fatty acids: alpha-linoleic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) [249]. Because the retina is a tissue with high lipid content, it receives high amounts of oxygen, so it is highly vulnerable to oxidative stress; reactive oxygen species carry out lipid peroxidation causing damage to membranes, proteins, and the nuclear DNA. It is also known that the deficient consumption of omega-3 contributes to the degeneration of the retina [250]. In the trial, PREDIMED (Prevention

with Mediterranean Diet), which followed a 6-year follow-up of middle-aged and older individuals with diabetes mellitus type 2 with adherence to a "healthy" Mediterranean diet and demonstrated a subset of patients whose diet includes omega-3 polyunsaturated fatty acids, show a 48% decreased incidence in DR [248, 251]. Both hyperglycemia and dyslipidemias are associated with DR, and although a strict diet control could delay the onset of retinopathy in patients with T2DM, this is not always achieved; however, in a trial, it was shown that a diet rich in foods with omega-3, similar to a Japanese diet, effectively reduced pathological neovascularization in the retina when compared to a diet rich in omega-6, apparently similar to an American diet [252]. Given that current treatments to counteract DR are costly and generally invasive, nutritional interventions have the potential to significantly improve microvascular complications resulting from diabetes. For this, diets rich in omega-3 can diminish the visual deterioration that appears in the first stages of the DR in a safe and accessible long before clinical manifestations [251].

4. Conclusion

At the beginning, there was a debate whether diabetic retinopathy was mainly a neuropathy or a vasculopathy. Through years of investigation, neural damage have shown to occur before vascular changes in the retinal tissue. Nevertheless, both have similitudes in the mechanisms involved and are present at different stages of the disease, and they continue; at the same time, hyperglycemia leads to inflammatory response causing cellular degeneration, endothelial insult, and hypoxia which in turn leads to more inflammatory response. At the same time, hyperglycemia induces ROS generation. Nevertheless, it is known that lowering glucose levels in diabetic patients remains the best way to avoid complications from diabetes as many studies have shown; however, this goal is hard to achieve for many patients; for that reason, we propose a multitarget therapy including oxidative stress-lowering strategies. Studies have demonstrated that oxidative stress plays an important role in all the described mechanisms by enhancing inflammatory responses, mediating the expression of prodegenerative and proinflammatory proteins, causing damage in cellular structures and functions. Genetic alterations involving antioxidant defenses are found to be linked to DR worsening or speeding up the onset, supporting the importance of oxidative stress as a pillar of diabetic retinopathy pathophysiology thus endorsing antioxidant supplementation as an adjuvant therapy along with diabetes management.

Conflicts of Interest

Authors declare that they have no conflicts of interest to report.

Authors' Contributions

All authors have significantly contributed in the present review. All authors are in agreement with the content of the manuscript.

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Research Article

Distribution of the Highest Plantar Pressure Regions in Patients with Diabetes and Its Association with Peripheral Neuropathy, Gender, Age, and BMI: One Centre Study

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The abnormal plantar pressure distribution and value play a key role in the formation of plantar calluses and diabetic foot ulcer. The prevalence of the highest pressure different distribution and its association with various factors among patients with diabetes is not well known. The study purpose was to evaluate the prevalence of different regions for the highest pressure on the sole and its association with selected factors among patients with diabetes. Medical records of nonulcer patients were retrospectively analysed. The relationship between pressure patterns on the sole obtained during a pedobarographic test as a semiquantitative assessment with colourful print analysis and neuropathy, gender, age, and BMI was searched. The most common location of the highest pressure was the central part of the forefoot. No association was found between the different highest pressure regions and age, sensory neuropathy, calluses, and foot deformities. The highest pressure on the lateral part of the foot and midfoot was observed more often in females and in patients with a BMI ≥ 35. The prevalence of the highest pressure on the forefoot was more common in patients with a BMI < 35. *Conclusions*. The most frequent regions of the highest pressure on the sole in patients with diabetes were the central part of the forefoot (2-3 metatarsal heads) with no simple relationship to the assessed variables other than BMI < 35. Female gender and higher BMI seem to be responsible for shifting the place of the highest pressure to other places of the foot.

1. Introduction

The foot is the most inferior-located part of the body that bears weight. The arrangement of bones, muscles, and joints allows mobility by absorbing and supporting vigorous pressure during standing and walking. Despite the initial overthrow of the "tripod" theory of load distribution (three points where the foot contact the ground) in 1987 by Cavanagh et al. [1], based on the description of human anatomy as well as on work presented by Taha et al. in 2016 [2], it seems that physiologically, there are three main points of the highest load on the sole: central part of the heel and the 1st and 4th-5th metatarsal heads. The physiological pressure distribution pattern on the sole is nearly symmetric and gives the foot, and thus our body, optimal stabilization.

Pressure distribution and value beneath the plantar surface depends on, e.g., body weight [3], age [4], and foot abnormalities secondary to disease [5, 6]. In population with diabetes, also poor control of the disease seems to be responsible for unnormal foot pressure [7, 8]. It is a known fact that the increased pressure in patients with diabetes peripheral neuropathy may be responsible for foot ulceration [9–19] and that foot ulcers occur mainly under the metatarsal heads [9, 20, 21]. In one of the recent big meta-analysis [22] which

was dedicated to the relationship between ulceration and neuropathy, the authors stressed that people with diabetes peripheral neuropathy and previous ulceration demonstrate higher plantar pressure compared to those with diabetes peripheral neuropathy but no ulceration history, which seems to be understandable. However, in this meta-analysis, the authors also demonstrated results which showed that patients with diabetes and active foot ulceration do not demonstrate an elevation in plantar pressures compared to those with neuropathy and no ulceration history. These surprising results of meta-analysis confirm how complicated are the relationships between the various studied dependencies on the foot. The most likely explanation of such an observation which the authors' proposed is the offloading theory which suggest that people with active ulceration protect the part of the foot where the ulceration exist which has an impact on the pressure measurement results. It is assumed currently that, appeared to foot ulcers, high plantar pressure must coexist with neuropathy [23–25]. The other author [26], however, observed 28% incidence of ulceration in patients with peripheral neuropathy and high plantar pressure but did not confirm the presence of ulcers in patients with neuropathy but without abnormal plantar pressure. This suggests that abnormal value of foot pressure as well as neuropathy could play an important role in the formation of plantar ulcers independently.

Pathological foot structure and abnormalities during the gait are responsible for incorrect pressure on the sole, and it should be emphasized that both are responsible for the risk of diabetic foot [27]. The result of the first is a disorder of standing and walking mechanics, which can have an important impact on inappropriate foot peak plantar pressure value and location [28]. The plantar pressure pattern and value can be determined by a pedobarographic examination [29]. The pedobarograph is a device which converts the applied pressure into a visible light pattern with a pressure measurement. The important role of pedobarography, as a diagnostic procedure, in plantar pressure pattern and value creating during stance and/or gait is confirmed in many studies. Unfortunately, as was mentioned by Fernando et al. [30], there is no standardized protocol for this assessment up to now. In summary, because of the complexity of the problem and due to the lack of good quality studies, there is no clear information on how to assess the plantar pressure in the guidelines which is dedicated to the diabetic foot prevention and treatment [31]. Studies most often emphasize the role of the dynamic test (during walking) which requires special insoles with pressure sensors or floor-based foot pressure measurement devices and the computer software to interpret the results. The subjects need special training for the optimal measurements before the dynamic pressure assessment, which is time consuming. The evaluation also requires additional skills [30]. For this reason, most dynamic pressure measurement devices are not routinely used as screening tools in daily clinical practice in patients with diabetes and are rather used for clinical research. Additionally, despite of this clinical research, the research problems with study interpretation could also be found, e.g., as in one study [32], 12 steps per foot were required; the other authors in previous studies analysed less number of steps or gave no detailed information about the procedure. Moreover, the patients walking speed (sometimes defined by researchers and sometimes by patient's choice) can interfere the meta-analysis if one study results are compared to another and probably does not reflect everyday walking characteristics of individuals [33–35]. Similarly, the application of footwear insoles with different sensor locations does not reflect the "daily work" of the foot because people walk at different speeds during everyday activities and use several types of footwear. The changes observed during standing are mainly the result of foot pathology, while during the walk, the abnormal image of pressure on the foot may be affected by other disorders: pain in the knees, hips, or spine [30]. Some authors [36] found that dynamic measurement is identical or even inferior to the static one because during walking, antalgic gait is promoted, so the real forces on the patients' soles could be misinterpreted. The static pedobarography could be helpful to detect structural changes within the foot without the influence of the other factors that may be revealed only during gait. The advantages and disadvantages of using different pressure measurement devices based on the current literature were clearly discussed and commented by Fernando and coauthors [30].

Although the value of both peak plantar pressure and pressure-time integral is reported in most of the studies, the systematic review by Bus and Waaijman showed that the added value of reporting pressure-time integral data is limited [37]. One of the most advanced peak plantar pressure classifications based on the results from pressure platform is the assessment done by Bennetts et al. [38]. The authors proposed regional pressure distribution for a total number of clusters set to seven, but as mentioned by the authors of this work, such mapping of the soles is possible only for specialists and difficult to use in everyday practice. Moreover, in this study, the obtained results were not compared to the physiological model, which may make difficult to interpret the result obtained in daily practice for the physician. In 2016, Deschamps et al. proposed the plantar pressure-based classification system in diabetic foot medicine [39]. This classification takes into consideration the possible 4 patterns for the highest pressure location, and the differences between the four clusters were coded with colour. The analysis was based on gait assessment, and within the examined patients were also subjects with previous (but not active) foot ulcer.

Static pedobarography, which is easier to administer for patients and medical staff than dynamic one, gives us important and sufficient data about foot structure and function [30, 40, 41]. In such cases, the pictures from static pedobarography could be helpful to detect abnormal distribution of plantar pressure which results from invisible foot structure deformity. This way, it can become a simple tool in the daily practice of diagnosing patients with diabetes [42].

The intensity of print colours, according to the device's software, without pressure value from static pedobarography analysis shows us abnormal load distribution on the soles which may predispose to calluses and those to ulcers, especially due to the loss of plantar pad thickness in these places [43]. The regions of high pressure are marked using "warm"

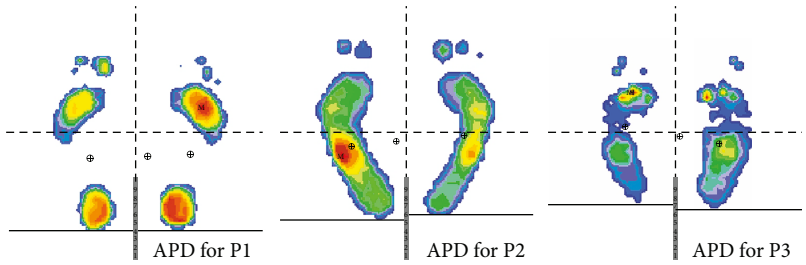

Figure 1: Examples of abnormal plantar pressure distribution (APD) for P1, P2, and P3.

colours (red or yellow), and the regions of low pressure are marked using "cold" colours (blue or green). This simply facilitates the identification of places of abnormal plantar loading distribution that can be corrected by insoles, injection of liquid silicone, or surgery interventions [44, 45]. The other pathologies within the foot are also more related to the distribution of the plantar pressure than to the absolute values of the pressure, e.g., forefoot pain [46]. Thus, it appears that the knowledge of the absolute pressure is not necessary to confirm the existing dysfunction within the foot structure.

In the last years, innovation like local temperature monitoring in the prevention of foot ulcer is proposed [47]. An unquestionable advantage of this technique is its objectivity and ease of performance. Nevertheless, the temperature rise seems to occur just before the development of the ulcer according to the five classical signs of inflammation (heat, pain—this can be masked by neuropathy, redness, swelling, and loss of function). Analysis of plantar pressures seems to indicate earlier disorders, when inflammation is not yet occurred.

The aim of our study was to evaluate the prevalence of different regions for the highest plantar pressure (HP) on the sole among patients with diabetes, based on images from static pedobarography interpreted every day in clinical practice and to explore its possible association with selected, available factors among patients with diabetes without previous diabetic foot diagnosis.

2. Material and Methods

We retrospectively investigated the distribution of the regions of the highest plantar pressure defined as a static, peak-plantar pressure. Then, we assessed the prevalence of the APD (abnormal pressure distribution) which was defined as the warmest colour obtained in the pedobarographic image, in a place on the sole other than physiological (defined according to tripod theory mentioned in Introduction). The results were obtained by colourful print analysis (Figure 1) among patients with diabetes mellitus (DM), and the association between this pressure and selected factors: neuropathy, gender, age, and BMI (body mass index) was also explored. Patients were qualified as APD positive (+) only if symmetrical APD was observed, as neuropathy, age, BMI, or gender, which were analysed in the context of their impact on APD, potentially have an impact on both feet.

2.1. Patients. Nine hundred seventy-four medical records of nonulcer patients with DM were retrospectively analysed. All of the documents which were obtained from the Diabetic Foot Centre (DFC), where the patients had consultations, covered a period of fifteen months of work.

Inclusion criteria: cases were defined as subjects who had a diagnosis of DM, live in the city where the DFC exists (consultations are sponsored from the city budget), and came spontaneously to be examined despite the absence of neuropathy signs or symptoms. None of the patients had previous foot ulcerations or operative procedures involving the foot. The evaluated group was representative of a large urban area in the country.

2.2. Examination and Data Subdivision. According to the standards of studies and papers on the prevention and management of foot ulcers in diabetes [31], we analysed necessary data dedicated to the lower extremity, coming from patients' medical records. A typical foot examination in the DFC consists of the ankle-brachial pressure index (ABPI), visible foot deformities, calluses, peripheral neuropathy, and plantar pressure assessment. The examination is always carried out at room temperature ranging from 24°C to 26°C. In addition to the foot examination, BMI, age, and gender data are collected.

Related to the physiological possible changes in foot structure [48] in the study, we subdivided patients into six groups to analyse the abnormal plantar pressure distribution (APD) with respect to age.

To study the prevalence of the APD among patients with different BMI, we subdivided subjects for two groups: patients with $\mathrm{BMI} \geq 35\,\mathrm{kg/m^2}$ and with $\mathrm{BMI} < 35\,\mathrm{kg/m^2}$. We expected that severe obesity ($\mathrm{BMI} \geq 35$) can influence the plantar pressure pattern.

ABPI analysis was not taken into account in this part of the study as it is not connected with APD and, therefore, has been omitted in the following text.

Peripheral neuropathy: motor component of the peripheral neuropathy causes muscle atrophy within the feet with subsequent abnormal distribution of the plantar pressure, feet deformity, and calluses [49]. Information about visible deformities and calluses were derived from physical examination. Deformities included hammer or claw toes, hallux valgus, visible flat feet, or "other visible deformities".

Calluses were defined as thick, hardened layers of the skin.

Peripheral neuropathy was assessed with questions (see below) and clinical evaluation in accordance with the local

recommendations, and the tools used in the centre are similar to those recommended in the document prepared by Jeffcoate and coauthors [31]. A skilled, board-certified, nurse asked patients about stinging, numbness, tingling, or burning of the foot for the questionnaire items. Ten-gram monofilament and tuning fork (128 MHz) tests were administered. Monofilament was applied in 10 locations on the sole (calluses were avoided) and one on the dorsal part of the foot for checking the loss of protective sensation. A positive monofilament test was considered to be the lack of sensation of tightness in at least 6 of 11 tested sites. The tuning fork was applied for vibration detection to both ankles, the first metatarsophalangeal joint, and the anterior aspect of the shin bone sites. A positive vibration test was considered to be no detection of vibration in three of four test sites [50].

Two positive test results and typical symptoms of neuropathy were the basis for confirmation of symmetric, peripheral, sensory polyneuropathy based on the local, internal guidelines in the centre. The condition required for the occurrence of these disorders was symmetry. Analyzing the current knowledge of the principles of diagnosis of sensory diabetic neuropathy as a risk factor for foot ulcers, these tests are sufficient for its identification. International Working Group on the Diabetic Foot [51] recommend monofilament, tuning fork, and cotton wool tests for sensory neuropathy detection, while data from PODUS [52] and Inlow's 60-second Diabetic Foot Screen [53, 54] suggest only monofilament test, as representative for damage to the sensory components (in combination with interview) to assess risk for foot ulceration.

All of the above information that was analysed came from history cards held by skilled diabetes nurse with several years of experience in the study of feet in patients with diabetes and subsequently confirmed by a physician, if in doubt.

Pedobarograms: the PEL-38 Medicapteurs SAS device, Balma, France (https://www.medicapteurs.com/diabetic-tool/), is used in DFC. The patient stands on a special platform with pressure sensors connected to the computer to produce a static pressure profile. If necessary, the patient repeats the test until a correct impression of the foot (symmetric with the corresponding location of the centre of the mass) to avoid, consciously or subconsciously, off-loading one foot, e.g., due to hip pain. This is a standard procedure in the DFC.

For the purpose of this analysis, the authors assessed plantar pressures using a semiquantitative method, like static barefoot pedobarographic records with colourful print analysis. The intensity of colour was proportional to the pressure received. Warm colours indicated the greatest pressure, while cold colours indicated the least plantar pressure (starting with red, then yellow, green, and blue). Pedobarograms from the centre were assessed, for the internal purpose of this study, by an independent physician (diabetologist), who was blind to the subjects' status. This physician has been previously trained in the evaluation of pedobarograms to the extent necessary for the study. Moreover, 20 randomly selected prints were similarly tested for verification by an orthopaedist with experience in this field (internal validation). The results were consistent at 100%. APDs were analysed for the forefoot (P1—about 25% of the foot length) and midfoot (P3—about 28% of the foot length), with a separate evaluation for the lateral (P2) part of the midfoot (the edge of the foot) (Figure 1). The length of the foot come from pedobarograms' documents and was measured as a line length with a ruler. This line connected two points: one end of the foot (the most forward point of the foot) to the other end of the foot—located on the heel. The line was run parallel to the central, vertical line visible on the mat (similar as is visible on the pedobarograms' pictures, Figure 1), and then its length was the basis to calculate the % of the foot length [55].

The load of the hallux (about 20% of foot length), which is a part of forefoot and rearfoot (about 27% of the foot length), was not analysed. In the assessment, we used colour intensity, not values of the pressure evaluation (semiquantitative method), to demonstrate the presence of the maximum pressure (peak pressure) represented by the hottest colour—e.g., the red one. For the heel, the hottest colour does not constitute pathology, because this colour always indicates a site of greatest pressure (typically presented within the heel according to the tripod theory). The heel load evaluation is therefore only useful if it includes absolute pressure value assessment.

2.3. Statistical Analysis. The Statistica 9 PL (StatSoft) software package was used for statistical analysis. The Kołmogorowa-Smirnowa test was used in the distribution analysis, according to the result of the analysis, the parametric, T-test, or nonparametric; U Mann-Whitney test was used in further calculations. The chi-square test was used to determine the association between two categorical variables. Data are presented as means (±S.D.). A P value < 0.05 was considered statistically significant.

The study was approved by the Commission of Bioethics at local Medical University.

3. Results

3.1. Population of the Study. The authors retrospectively analysed 974 medical records (974 history cards and 1948 feet pedobarograms from 451 males and 523 females). The mean patient age was 64.6 years (±11.1): 63.8 (±10.9) for men and 65.3 (±11.3) for women, $P > 0.05$. The mean BMI was 29.9 kg/m^2 (±5.2): 29.4 (±4.7) for men and 30.4 (±5.6) for women, $P > 0.05$.

3.2. The Prevalence and the most Frequent Location of the APD according to the HP Analysis. In the cohort, 80 patients (8.21%) had a typical region of HP according to the tripod theory, with no APD (37 females and 43 males (7.07% and 9.53%, respectively)). In 894 cases (91.79%), at least one, symmetrical location of the APD was noted (P1 ($N = 806$) and/or P2 ($N = 216$) and/or P3 ($N = 26$))—most of the APDs were found within the forefoot (metatarsophalangeal joints); the least frequent location was within the midfoot.

3.3. Peripheral Neuropathy and APD. Peripheral, symmetric, sensory foot neuropathies (PSSN) were shown in 6.88% ($N = 67$) of the subjects. No association existed between the APD and the presence of the PSSN (Table 1).

Table 1: Association between APD and PSSN on physical examination; the presence of calluses and visible deformities.

NT (%)	Patients with abnormal planter pressure distribution (%)	Patients without abnormal planter pressure distribution (%)	NT	P
Patients without peripheral symmetric sensory neuropathy	833 (91.8)	74 (8.2)	907	0.82
Patients with peripheral symmetric sensory neuropathy	61 (91.0)	6 (9.0)	67	
Patients without calluses	588 (91.2)	57 (8.8)	645	0.32
Patients with calluses	306 (93.0)	23 (7.0)	329	
Patients without foot deformity	728 (91.2)	70 (8.8)	798	0.17
Patients with foot deformity	166 (94.3)	10 (5.7)	176	

A P value of < 0.05 is considered statistically significant. Data are presented as number (percentage). NT: total number of patients, APD: abnormal plantar pressure distribution, PSSN: peripheral, symmetric, sensory foot neuropathy.

The calluses and feet deformities were demonstrated in 33.78% ($N = 329$) and 18.07% ($N = 176$), respectively. Deformities and secondary calluses are considered an expression of motor neuropathy, which results in muscle weakness. The next stage of our study was to assess the relationship between these abnormalities and the presence of APD revealed during the pedobarographic examination. No association was found between the APD and the presence of calluses ($P = 0.32$), as well as between the APD and the presence of visible deformities ($P = 0.17$) (Table 1). In exceptional cases, the presence of callus or deformation was found without APD ($N = 23$ and $N = 10$, respectively), but many patients with APD had no calluses ($N = 588$) or visible deformities ($N = 728$).

3.4. APD and BMI. When authors assessed this relationship for BMI and APD without dividing for P1, P2, and P3, the P value was 0.27. Only after dividing the patients into groups, we noted that APD for P1 was more common in patients with a BMI $< 35\,\mathrm{kg/m^2}$, while P2 and P3 were more common in the BMI $\geq 35\,\mathrm{kg/m^2}$ group ($P = 0.0015$, $P < 0.0001$, and $P < 0.0001$, respectively (Table 2)).

3.5. APD and Gender. Tripod load distribution within both feet existed with near-equal prevalence in both genders (43 males and 37 females (9.53% and 7.07%, respectively); $P = 0.16$). APD within the forefoot (P1) had a similar prevalence for males and females (84.26% and 81.45%, respectively; $P = 0.25$). APD on the lateral part of the foot (P2) and midfoot (P3) occurred significantly more often in females ($P = 0.00066$ and $P = 0.005$, respectively) (Table 3).

3.6. APD and Age. There was no association between age and the presence of the APD for total points ($P = 0.44$) (Table 4), as well as for P1, P2, and P3 separately ($P = 0.4$, $P = 0.06$, and $P = 0.34$, respectively).

4. Discussion

The consequence of pathological changes in the feet is a disturbance of standing and walking mechanics. The first one is evaluated during a static pedobarographic examination. For quick analysis of the foot structure, pedobarographic images are used in everyday medical practice [56]. Based on them, a decision is made on whether or not there is a need for specialist consultations (podologist, orthopedist) and/or insoles.

In our observational, descriptive, retrospective analysis, the pattern of loading across the sole showed that the most common region for the high plantar pressure was the central metatarsal heads (II-IV metatarsophalangeal joints). This area appears to be strongly associated with the formation of ulcers, e.g., Eurodiale study showed that about 55% of diabetic ulcers are located on patients' toes but 22% of all ulcers concerns the forefoot/midfoot area [57]. In other studies, APD and higher pressure (detected for feet calluses and deformities) led to ulcerations also, particularly at the height of the metatarsophalangeal joints [58–60] and even half of the plantar foot ulcers were described as located under metatarsal heads and hallux [57, 61, 62]. From a clinical point of view regardless of the foot inspection results, APD can be detected as the first pathology which precedes visible abnormality.

The most common type of neuropathy in the population of patients with diabetes is peripheral, sensorimotor, symmetric polyneuropathy [63]. The sensory and the motor neuropathy both play an important role in the foot ulcer formation [22, 26, 64–67]. According to our very strict criteria, the sensory component of this disorder occurred in nearly 7% of the subjects, but visible deformation (18.07%) and calluses (33.78%) (resulting from motor component of the neuropathy) were more frequent. The above findings may result from the fact that the motor disturbances can be more common than sensory as was shown by Ishpekova et al. [68] or that foot deformation and/or calluses can occur independently of the peripheral neuropathy. According to Farndon [69], there was no statistically significant difference in diabetic versus nondiabetic patients concerning the incidence of toe deformity (claw/hammer toes), although the prevalence of sensory neuropathy was significantly greater in the diabetic population. Data from the other study also showed that neuropathy is not simply related to calluses, foot deformities, or joint mobility [70]; however, this neurologic, motor-related pathologies are dangerous for patients with diabetes as can provoke injury.

To summarise the connections between neuropathy and abnormal pressure distribution, the major finding in our study of patients with DM was that sensory and equivalents of motor components of the peripheral neuropathy were not connected with APD (APD was more common than PSSN, calluses, and foot deformity) which was also

Table 2: Association between APD for the forefoot, lateral part of the foot, midfoot, and BMI.

NT (%)	Patients without abnormal plantar pressure distribution within the forefoot (%)	Patients with abnormal plantar pressure distribution within the forefoot (%)	Patients without abnormal plantar pressure distribution within the lateral part of the foot (%)	Patients with abnormal plantar pressure distribution within the lateral part of the foot (%)	Patients without abnormal plantar pressure distribution within the midfoot (%)	Patients with abnormal plantar pressure distribution within the midfoot (%)
BMI < 35	128 (15.6)	693 (84.4)	665 (81.0)	156 (19.0)	808 (98.4)	13 (1.6)
BMI ≥ 35	40 (26.1)	113 (73.9)	93 (60.8)	60 (39.2)	140 (91.5)	13 (8.5)
P	*0.0015*		*<0.0001*		*<0.0001*	

A *P* value of <0.05 is considered statistically significant. Data are presented as number (percentage). BMI: body mass index; NT: total number of patients; APD: abnormal plantar pressure distribution.

Table 3: Prevalence of APD in the forefoot, lateral part of the foot, and midfoot for gender.

NT	Abnormal plantar pressure distribution within the forefoot (%)	Abnormal plantar pressure distribution within the lateral part of the foot (%)	Abnormal plantar pressure distribution within the midfoot (%)
Male $n = 451$	380 (84.3)	78 (17.3)	5 (1.1)
Female $n = 523$	426 (81.4)	138 (26.4)	21 (4.0)
NT = 974	806	216	26
P	0.25	*0.00066*	*0.005*

A P value of <0.005 is considered statistically significant. Data are presented as number (percentage). NT: total number of patients. APD: abnormal plantar pressure distribution.

Table 4: Prevalence of APD for age.

Age (years)	NT	Patients without abnormal plantar pressure distribution (%)	Patients with abnormal plantar pressure distribution (%)
→40	36	5 (13.9)	31 (86.1)
41-50	51	3 (5.9)	48 (94.1)
51-60	233	15 (6.4)	218 (93.6)
61-70	333	28 (8.4)	305 (91.6)
71-80	269	22 (8.2)	247 (91.8)
81-90	52	7 (13.6)	45 (86.5)
NT	974	80	894
P		0.44	

A P value of <0.005 is considered statistically significant. Data are presented as number (percentage). NT: total number of patients. APD: abnormal plantar pressure distribution.

mentioned in the literature [71]. Considering the natural course of the disease, it could be that abnormal pressure distribution can be found before peripheral neuropathy detected by routine tests. Dinh and Veves [61] in their review also summarised that increase of peak pressures within the forefoot could be the first observations in the absence of any detectable signs or symptoms of neuropathy which is the consequence of the progressive nature of the disease.

We also connected the different loading points with gender, BMI, and age. Generally, as in the previous study [72], the authors of this one also did not find an association of gender to the plantar pressure distribution. The only difference was that while the pathologies within the forefoot were found in a similar number of males and females, the APD for the lateral part of the foot and midfoot was found more often in females. Hills et al. [72] also demonstrated an increase in pressure under the midfoot for obese females as compared to obese males. For females, a slightly higher BMI was demonstrated in our study so the authors can only speculate that an increased body mass generates overload in these locations.

The result for the relationship between APD for forefoot and BMI was unexpected. The more frequent occurrence of APD in this location in people with a lower BMI indicates the participation of factors other than weight in the formation of forefoot overload. For midfoot and the lateral part of the foot, the APD was found more frequently for BMI equal or higher than 35 kg/m^2, as we expected. Despite the connection between the BMI and the value of the plantar pressure [3], our findings suggest that in APD, forefoot identification, BMI as the most important factor, should not be considered. People with BMI lower than 1st degree (according to the WHO description) of the obesity seem to be at the higher risk for forefoot overload.

This work was not intended to propose a new classification of pressure distribution on the sole (pressure mapping) but to assess the prevalence of the various regions of the highest peak plantar pressure with indication of the abnormal location of this pressure beneath the plantar surface (without defining its value) represented by colour mapping, in everyday practice. Such visual assessment is simple and understandable for both the primary care physician and the patient [56], which facilitates its use in everyday practice. The impact of pressure pattern on foot ulcer location will be mandatory in the future to determine whether there are correlations between this two.

In the study, we do not refer to healthy population, because such a population was not examined in the DFC. For this reason, to define the potentially incorrect pressure location on the sole, we referred to tripod theory based on Taha et al.'s [2] observation.

The lack of our study is that the evaluation of the neuropathy in the Diabetic Foot Centre was based on local recommendation and not on, e.g., Michigan Neuropathy Screening Instrument (MNSI). However, as a research tool in the mentioned centre, foot inspection, vibration sensation, monofilament testing, and questionnaire for symptoms were used. These tools were similar to the MNSI as well as mentioned in reporting standards prepared by Jeffcoate and coauthors [31].

Because of the retrospective nature of the study, unfortunately, it was not possible to carry out this analysis for the different types and duration of DM due to the lack of complete data in the history of the disease coming from the DFC. This is why for such a big number of patients, we took into consideration only the parameters of interest which were available for all consulted patients within the mentioned period. The baseline characteristics of the study population appear to be typical for subjects with type 2 diabetes (due to BMI and

age), and the results could change for different types of DM. The disease duration may be connected with higher peak pressure within the feet due to the plantar contact area narrowing (shown in dynamic evaluation) [73]. Mayfield et al. [74] found that age and duration of diabetes are connected with ulcers and amputations so it cannot be ruled out that both could also influence the pressure distribution. However, we should recognize that in type 2 DM, the known duration of the disease is only approximate, so diabetes duration is a quite frequent problem which biases study results. In the study, we demonstrate that the age of patients with DM does not affect the presence of APD. This indicates a need to consider APD testing regardless of patient age.

The limitations of our study, in addition to the aforementioned, are mainly related to a semiquantitative analysis of the pressure map, the retrospective nature of the study, and the nature of the centre. Although the authors involved patients from only one centre of a large urban area, the available data seem to be representative of the entire diabetic population. The strength of this study is its large size and uniform assessment of the neuropathy even if not strictly relating to MNSI. As we analysed the information from barefoot print, it also should be taken into consideration that this analysis does not provide us with information about the interaction between foot and footwear. It is mean that potentially "healthy" people may also have problems if they wear unsuitable shoes.

Relieving pressure points and avoiding callus formation are still the basic goal in patient care. The ability to visualize the focal pressures under the foot as easy-to-red, colour-coded diagrams can facilitate patient training and education [56]. This simple low-cost static pressure analysis also provides the clinician with information about a possible intervention, e.g., the surgical or application of the insoles [75]. Although there is no clear evidence that off-loading is important in the prevention of primary foot ulcers in diabetic patients, as highlighted in the Cavanagh and Bus review [76], in everyday practice, it is unethical to avoid actions that are aimed at improving the pressure distribution on the sole. In the absence of a clinical gold standard, the current approach in the choice of simple pressure map analysis remains an important part of the patient care. Because the lack of standard practices on this field may limit clinical use, so further validity and reliability of the colour intensity measure of plantar pressure is required.

5. Conclusions

The prevalence of the abnormal plantar pressure distribution when applied, the tripod theory was high in this analysis. The most common location of the highest plantar pressure was the central part of the forefoot. Female gender and BMI ≥ 35 predispose to the lateral part and midfoot abnormal pressure distribution, whereas other, unsearchable factors are responsible for APD of the forefoot. Connections between calluses or deformation after months/years of duration of asymptomatic APD need to be identified.

Because the prevalence of the abnormal plantar pressure distribution among patients with diabetes is high and dynamic measurements are much more time consuming and expensive, the simple colourful print analysis should be recognized as a helpful tool in identifying invisible pathology on the sole in each patient. There is no simple relationship between the clinical-available variables and APD so such analysis can help practitioners to choose the appropriate prophylaxis.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Additional Points

Key Messages. (1) The prevalence of the abnormal regions for the highest pressure on the sole, among patients with diabetes, is very high. (2) The simple colourful print analysis should be recognised as a helpful tool in identifying invisible pathology on the sole in each patient. (3) The standard physical examination (inspection) and tests (vibration, monofilament tests) could be not sufficient in everyday practice to avoid foot ulceration.

Ethical Approval

This study was approved by the Medical University Commission of Bioethics in Wroclaw (specific agreement number KB-434/2012).

Conflicts of Interest

The authors declare that there is no conflict of interest associated with this manuscript.

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Research Article

Factors Affecting Cardiovascular Risk in Children, Adolescents, and Young Adults with Type 1 Diabetes

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Cardiovascular risk and obesity are becoming major health issues among individuals with type 1 diabetes (T1D). The aim of this study was to evaluate cardiovascular risk factors and obesity in youth with T1D in Lithuania. *Methods*. 883 patients under 25 years of age with T1D for at least 6 months were investigated. Anthropometric parameters, blood pressure, and microvascular complications were evaluated, and the lipid profile and HbA1c were determined for all patients. *Results*. Study subjects' mean HbA1c was $8.5 \pm 2\%$; 19.5% were overweight and 3.6% obese. Hypertension and dyslipidemia were diagnosed in 29.8% and 62.6% of participants, respectively. HbA1c concentration was directly related to levels of total cholesterol ($r = 0.274$, $p < 0.001$), LDL ($r = 0.271$, $p < 0.001$), and triglycerides ($r = 0.407$, $p < 0.001$) and inversely associated with levels of HDL ($r = 0.117$, $p = 0.001$). Prevalence of dyslipidemia increased with duration of diabetes ($p < 0.05$). Hypertension was more prevalent in overweight and obese compared to normal-weight patients (40.6 and 65.6 vs. 25.6%, respectively, $p < 0.001$). Frequency of microvascular complications was higher among patients with dyslipidemia (27.2 vs. 18.8%, $p = 0.005$) and among those with hypertension (25.9 vs. 23.2%, $p < 0.001$). *Conclusion*. The frequency of cardiovascular risk factors is high in youth with T1D and associated with diabetes duration, obesity, and metabolic control.

1. Introduction

The main cause of death in European countries is cardiovascular diseases (CVD) [1]. Several studies showed that atherosclerosis presents more frequently in people with diabetes. This is usually explained by persistently elevated glucose levels in the blood [2, 3].

CVD tend to present at a younger age in patients with diabetes than in the general population [4]. The SEARCH for Diabetes in Youth Study showed that significant complications severely affect the quality of life of diabetics early in their life [5]. Therefore, adolescence and young adulthood are the best times for actions to lower cardiovascular risk [6].

The other growing issue in type 1 diabetes (T1D) patients is obesity, which aggravates the risk of hypertension and dyslipidemia [7].

The aim of our study was to analyze the risk factors for CVD in children and young adults under the age of 25 years with T1D in Lithuania.

2. Materials and Methods

2.1. Subjects. The presented cohort included 883 subjects from the recent joint Lithuanian-Swiss project "Genetic Diabetes in Lithuania," which had 1209 subjects overall, covering all children and 70% of young adult (under the age 25) patients with T1D in Lithuania, described previously [8].

This analysis included patients with established diabetes longer than 6 months and treated with insulin: 590 of them (66.8%) were from 1- to 17-year-old children and adolescents, and 293 are young adults (33.2%) from 18 to 25 years old. Subjects with diabetes duration less than 6 months were not included in this analysis due to the weight fluctuation and metabolic instability that are usually seen at the onset and initial therapy of diabetes [9]. None of the study participants were taking any medication affecting body composition, blood pressure, or renal function.

At the time of involvement in the project, data about age, duration of T1D, insulin delivery method (pump/injection), and total daily insulin dose (U/kg/d) were collected and clinical and laboratory assessments done. The participants were consulted by a single ophthalmologist for evaluation of diabetic retinopathy and a pediatric or adult neurologist for assessment of diabetic neuropathy.

The Lithuanian Bioethics Committee granted the approval for this biomedical research (No. BE-2-5). Subject information forms and informed consent forms were signed by each participant or official representative.

2.2. Clinical Assessment and Examination. Anthropometric parameters were measured by clinical nurses at the Endocrinology Department of the Hospital of Lithuanian University of Health Sciences. At this department, the Harpenden Stadiometer (Holtain, Crymych, UK) is used for height measurement. Patients' height was measured to the nearest ±0.1 cm three times, then the average was estimated for analyses. seca 700 medical scales (seca GmbH & Co. KG) were used for weight in kg, with precision of 0.1 kg.

For body mass index (BMI), the equation weight in kg/recumbent length or standing height in m^2 was used.

For participants under 19 years, a BMI z-score was evaluated according to age and gender using the references of the World Health Organization (WHO); for participants aged 19 to 25 years, a BMI z-score was evaluated according to the references of WHO for 19-year-old individuals, assuming that their linear growth is over.

For all participants, normal weight was defined as BMI ranging from -2 standard deviations (SD) to less or equal to +1 SD (which corresponds to BMI 25 kg/m^2 at 19 years); overweight was defined as BMI less than +2 SD (which corresponds to BMI 30 kg/m^2 at 19 years), obese as BMI>+2 SD, and underweight as BMI < -2 SD.

A medical measuring tape was used for waist and hip measurements. The approximate middle point between the lower margin of the last palpable rib and the top of the iliac crest was measured, to the nearest ±0.1 cm, for waist circumference [10]. Hip circumference was measured at the widest point of the buttocks, to the nearest ±0.1 cm.

In analyses, the waist-hip ratio (waist circumference (cm)/hip circumference (cm)) and waist-to-height ratio (WtHR) (waist circumference (cm)/height (cm)) were used. The waist-hip ratio ≥ 0.90 cm for men and ≥0.85 cm for women were considered significantly increased [10]. WtHR < 0.5 cm was considered as optimal [11].

Arterial blood pressure for children and adults was measured after sitting in silence for 5 min using an oscillometric sphygmomanometer in the left arm with appropriate cuff size. For children, "The Fourth Report from the National High Blood Pressure Education Program (NHBPEP) Working Group on Children and Adolescents" guidelines was used to classify measurements of arterial blood pressure: "Normal BP was defined as systolic blood pressure (SBP) and diastolic blood pressure (DBP) less than the 90th percentile for sex, age, and height. Hypertension was defined as average SBP or DBP that was greater than or equal to the 95th percentile for sex, age, and height on at least three separate occasions" [12].

Measurements of arterial blood pressure in adults were classified according to the American Heart Association (AHA) guidelines: "Normal blood pressure defined as SBP <120 mmHg and DBP <80 mmHg, hypertension beginning at 140/90 mmHg and higher" [13].

2.3. Laboratory Analyses. Glycosylated hemoglobin (HbA1c) and lipid profiles were measured by the UniCel DxC 800 Synchron system (Beckman Coulter, USA). The normal cutoff values of HbA1c were 4-6% (20 mmol/mol-42 mmol/mol). International Society for Pediatric and Adolescent Diabetes (ISPAD) guidelines were used to define optimal metabolic control when HbA1c < 7.5% (58 mmol/mol) for children and adolescents and <7% for young adults with reference to American Diabetes Association guidelines [14, 15].

Normal values for low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), and triglycerides (Tg) were defined as <2.6 mmol/l, >1.1 mmol/l, and <1.7 mmol/l, respectively [14, 16]. Normal values for total cholesterol were defined as <5.2 mmol/l for patients ≥16 yrs and <5.5 mmol/l for children under 16 yrs. If at least one lipid value was abnormal, dyslipidemia was considered to be present.

2.4. Evaluation of Microvascular Diabetes Complications. All participants were screened for microvascular diabetes complications at the same Endocrinology Department. A single diabetes ophthalmologist and an adult/pediatric neurologist consulted with all diabetes patients for the presence of retinopathy and neuropathy, respectively. Diabetic retinopathy was identified from stereoscopic fundal examination. Sensations for vibration, pressure, and temperature were evaluated for each patient, and all of them were surveyed with the Michigan Neuropathy Screening Questionnaire. If ≥2 of these tests were abnormal, peripheral neuropathy was diagnosed [8, 17, 18].

For diabetic kidney damage, a 24-hour urine albumin excretion rate (AER) was evaluated. AER < 30 mg/24 h was defined as normal, 30-300 mg/24 h—microalbuminuria, and >300 mg/24 h—macroalbuminuria [19].

2.5. Statistical Analyses. The IBM SPSS Statistics Base version 22.0 was used for statistical analysis of the data. In case of normal data distribution, Student's 2-tailed t test, χ^2 statistics, and parametric one-way ANOVA were used. For nonnormally distributed data, the Mann-Whitney U test was used, and for ordinal data, Kruskal-Wallis one-way ANOVA was used. For estimation of trends, linear regression models were used. For testing the hypothesis about relationships between dichotomous-dependent variables and continuous predictors, binary logistic regression analysis was carried out. p values <0.05 were considered as statistically significant. All p values were two-sided.

3. Results

3.1. General Characteristics and Weight Status. Of the 883 subjects enrolled for the current analysis, 49.2% ($n = 434$) were males. The mean age of study subjects was 16.2 ± 5.6 yrs. The average diabetes duration was 6.7 ± 4.8 yrs (0.5-24.73, median 5.6 yrs). The distribution of patients by duration of T1D was as follows: 0.5-4 years 45.4% ($n = 401$), 5-9 years 30.9% ($n = 273$), 10-14 years 17.9% ($n = 158$), 15-19 years 4.3% ($n = 38$), and ≥20 years 1.5% ($n = 13$).

The mean BMI z-score in the whole cohort was 0.29 ± 0.99. 75.8% of study subjects ($n = 666$) were of normal weight, 19.5% ($n = 171$) overweight, 3.6% ($n = 32$) obese, and 1.1% ($n = 10$) underweight. The distribution of weight status among different age groups is shown in Figure 1 ($p > 0.05$). 20.5% of females and 18.3% of males were overweight ($p > 0.05$), and 3.3% of females and 3.9% of males were obese ($p > 0.05$).

Clinical characteristics according to weight group are shown in Table 1. Hypertension was more frequent among overweight and obese patients than among normal weight patients (40.6% and 65.6% vs. 25.6%, respectively, $p < 0.05$).

21.8% ($n = 192$) of patients had WtHR higher than 0.5. As expected, overweight and obese individuals had higher WtHR than normal-weight subjects (0.49 ± 0.04 and 0.55 ± 0.06 vs. 0.44 ± 0.15, respectively, $p < 0.001$). However, no significant differences in the waist-hip ratio were found comparing different-weight-status participants.

3.2. Glycemic and Metabolic Control. Study subjects' mean HbA1c was 8.5 ± 2% (69.2 ± 2 mmol/mol). 32.7% ($n = 289$) of patients had optimal glycemic control. The best glycemic control was recorded in the group of youngest patients: patients aged 1-4 yrs and 5-9 yrs had significantly lower HbA1c compared to patients aged 10-14 yrs, 15-19 yrs, and ≥20 yrs (7.3 ± 1% and 7.5 ± 1.2% vs. 8.5 ± 1.9%, 8.9 ± 2.1%, and 8.6 ± 1.9%, respectively, $p < 0.05$). In all age groups, females had significantly higher HbA1c than males, except in the youngest 1-4 yrs of age group (Table 2).

The average insulin dose was 0.83 ± 0.3 U/kg/d for the whole cohort. Adjusted for diabetes duration, patients with optimal glycemic control had a lower insulin dose compared to patients with suboptimal HbA1c (0.76 ± 0.31 U/kg/d vs. 0.87 ± 0.29 U/kg/d, respectively, $p < 0.001$). In the whole cohort, 30.2% of patients were on insulin pumps. HbA1c of

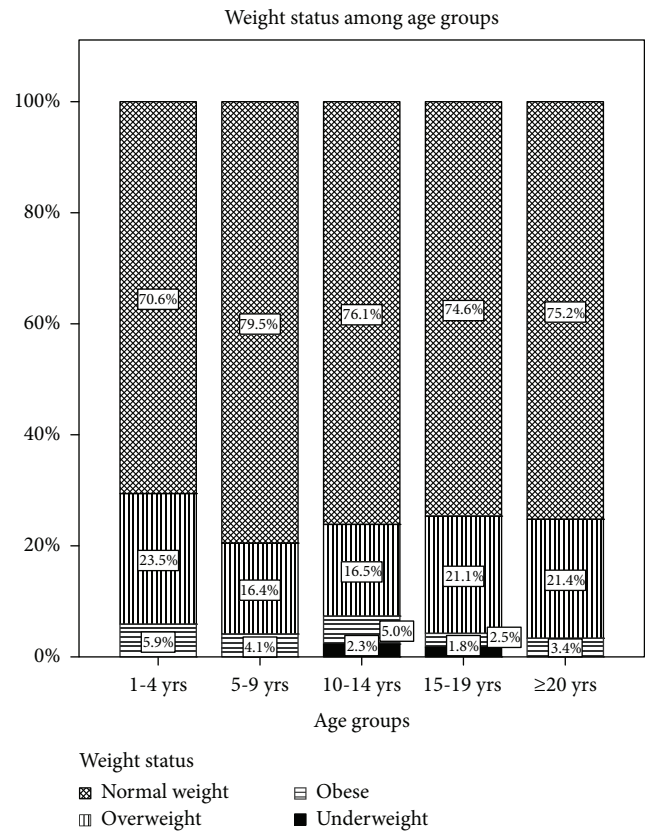

Figure 1: Normal weight, overweight, obese, and underweight frequency among different age groups.

patients treated with insulin pumps and multiple daily injections (MDI) was 8.5 ± 2% and 8.4 ± 1.8%, respectively, $p > 0.05$; also, there was a similar proportion of subjects with optimal glycemic control in insulin pump users or those on MDI (29.4% and 30.6%, respectively, $p > 0.05$).

Dyslipidemia was found in 62.6% ($n = 552$) of the total cohort. Increased total cholesterol was found in 28.2% ($n = 249$) of patients, increased LDL in 54.9% ($n = 484$), increased Tg in 9.2% ($n = 81$), and decreased HDL in 13.5% ($n = 119$) of patients. The frequency of dyslipidemia was similar among all age groups: 50% ($n = 8$) among patients aged 1-4 years, 52% ($n = 62$)—5-9 years, 64.7% ($n = 141$)—10-14 years, 64.4% ($n = 183$)—15-19 years, and 64.7% ($n = 156$)—≥20 years.

Dyslipidemia was more frequent in the group with poor glycemic control compared to that with optimal glycemic control (66.9% vs. 53.6%, respectively, $p < 0.001$). Also, the HbA1c level was significantly higher in patients with dyslipidemia compared to patients with a normal lipid profile (8.8 ± 2.1% vs. 8 ± 1.7%, respectively, $p < 0.001$). A significantly higher prevalence of dyslipidemia was found in patients with WtHR exceeding 0.5 compared to subjects whose WtHR was less than 0.5 (73.4% vs. 26.6%, respectively, $p < 0.001$).

Significant direct correlations between the levels of HbA1c and total cholesterol, LDL, and Tg and reverse correlation between the levels of HbA1c and HDL were found (Figure 2).

Table 1: Clinical characteristics according to weight group.

	Weight group			
	Normal weight % (N)	Overweight % (N)	Obese % (N)	Underweight % (N)
All	75.8 (666)	19.5 (171)	3.6 (32)	1.1 (10)
Using insulin pumps	30.6 (204)	30.4 (52)	25 (8)	0
Optimal glycemic control	34.5 (230)	27.5 (47)	21.9 (7)	30 (3)
WtHR ≥ 0.5	11.1 (74)	50.9 (87)*	84.4 (27)*	10 (1)
Dyslipidemia	61.4 (408)	66.7 (114)	68.8 (22)	60 (6)
Hypertension	25.6 (168)	40.6 (69)*	65.6 (21)*	50 (5)
Microvascular complications				
Retinopathy	11 (72)	11.7 (20)	6.3 (2)	0
Neuropathy	9.6 (63)	17 (29)*	12.5 (4)	10 (1)
Elevated AER	10.2 (66)	8.5 (14)	9.4 (3)	0

HbA1c: glycosylated hemoglobin; WtHR: waist-to-height ratio; AER: albumin excretion rate. *$p < 0.05$, compared to the normal-weight group.

Table 2: HbA1c levels between genders in different age groups.

Gender Age group	Males Mean HbA1c (%)	Females Mean HbA1c (%)
1-4 yrs	7.3 ± 1.2	7.2 ± 0.5
5-9 yrs	7.3 ± 1.1*	7.8 ± 1.3*
10-14 yrs	8.2 ± 1.7*	8.7 ± 2*
15-19 yrs	8.6 ± 2.1*	9.2 ± 2.2*
≥20 yrs	8.4 ± 1.9*	8.7 ± 2*

HbA1c: glycosylated hemoglobin. *$p < 0.001$ comparing HbA1c between males and females for the same age group.

A logistic regression was carried out to assess the predictors for the likelihood that the patient would or would not have dyslipidemia. Binary variable "Dyslipidemia" was coded as "1" if present and "0" if absent. The full model containing continuous predictors, HbA1c levels and WtHR, was statistically significant, $\chi^2 = 39.473$, $p < 0.001$, indicating that the model was able to differentiate between patients with and without dyslipidemia. Analysis of predictors on the probability of dyslipidemia is shown in Table 3.

The logistic regression model could be expressed as

$$\text{Probability}(\text{Dyslipidemia}) = \frac{1}{1 + e^{-z}},$$
$$z = -3.525 + 0.207 * X_1 + 5.113 * X_2, \quad (1)$$

where e is the base of the natural logarithm, X_1 is HbA1c, and X_2 is WtHR.

Overall model predictions were successful in 62.1%. Binary logistic regression indicated that HbA1c and WtHR are significant predictors of likelihood of dyslipidemia. Cook's distances for the model ranged from a minimum of 0.0058 to a maximum of 0.0907. The maximum values of DFBETA for HbA1c and WtHR were 0.003 and 0.2, respectively.

Impairment of all lipid fractions' metabolism and glycemic control was dependent on diabetes duration. The linear regression models are presented in Figure 3, showing levels of HbA1c, total cholesterol, LDL cholesterol, and Tg directly, and HDL cholesterol concentrations negatively related with duration of diabetes.

In linear regression models, controlled for diabetes duration and glycemic control, BMI z-scores were weakly but significantly negatively associated with HDL ($r = -0.095$, $p = 0.005$) and directly related to LDL and Tg concentrations ($r = 0.086$ and $p = 0.011$ and $r = 0.088$ and $p = 0.009$, respectively).

3.3. Blood Pressure and Microvascular Complications. Hypertension was diagnosed in 29.8% ($n = 263$) of patients. It was more frequent among males compared to females (34.5% vs. 26.1%, $p = 0.007$). Hypertension was diagnosed more often in children (<18 yrs) than in adults (18-25 yrs) (36.1% vs. 18%, respectively, $p < 0.001$). Controlled for age and gender, the direct relationship between SBP, DBP, and BMI z-scores was found ($r = 0.227$ and $p < 0.001$ and $r = 0.139$ and $p < 0.001$, respectively).

In the whole cohort, 212 (24%) subjects were diagnosed with at least one microvascular complication. Retinopathy was diagnosed in 10.8% ($n = 94$), neuropathy in 11.5% ($n = 97$), and elevated AER in 9.4% ($n = 83$) of participants.

Glycemic control, duration of diabetes, and microvascular complications were significantly related. Hypertension was more frequent among patients with elevated AER. Prevalence of dyslipidemia was higher among patients with neuropathy. Comparison of patients according to the presence of diabetes complications is shown in Table 4.

Patients with an altered lipid profile had higher frequency of at least one microvascular complication compared to patients with normal levels of lipids (27.2 vs. 18.8%, respectively, $p = 0.005$); the same trend was found in patients with hypertension vs. patients with normal BP (25.9 vs. 23.2%, respectively, $p < 0.001$).

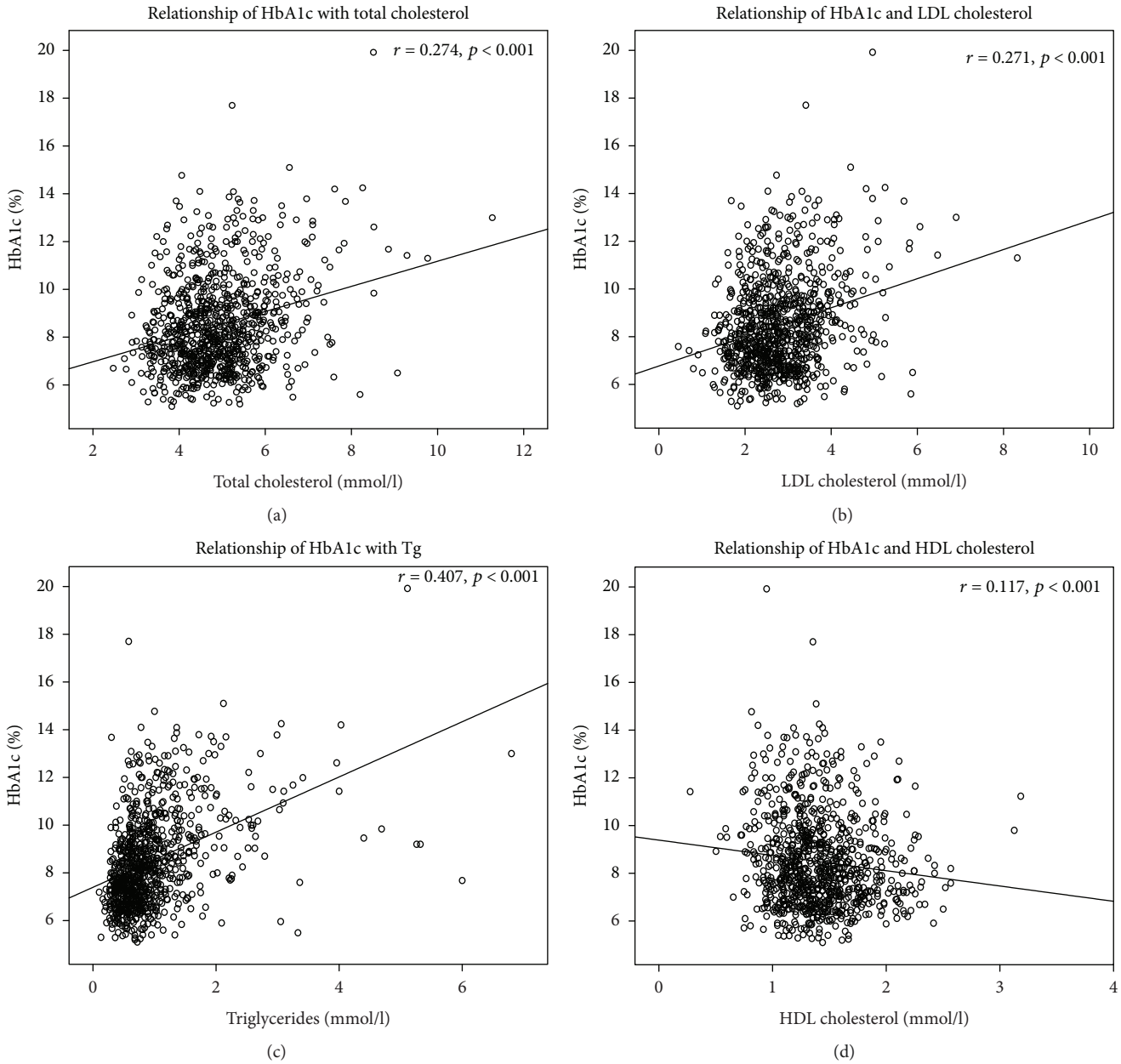

Figure 2: Correlations between levels of glycosylated hemoglobin (HbA1c) and lipids: (a) relationship of HbA1c and total cholesterol, (b) relationship of HbA1c and low density-lipoprotein cholesterol (LDL), (c) relationship of HbA1c and triglycerides (Tg), and (d) relationship of HbA1c and high density-lipoprotein cholesterol (HDL).

Table 3: Logistic regression: analysis of predictors on "Dyslipidemia".

Predictor	β	SE β	Wald's χ^2	p	OR	95% CI	
						Lower	Upper
Constant	-3.525	0.813	18.793	<0.001	0.029	NA	
HbA1c	0.207	0.042	24.632	<0.001	1.229	1.133	1.334
WtHR	5.113	1.674	9.327	0.002	166.153	6.244	4421.299

HbA1c: glycosylated hemoglobin; WtHR: waist-to-height ratio; β: coefficients estimated from the data; SE: standard error; OR: odds ratio; CI: confidence interval; NA: not available. Cox and Snell $R^2 = 0.046$; Nagelkerke $R^2 = 0.063$.

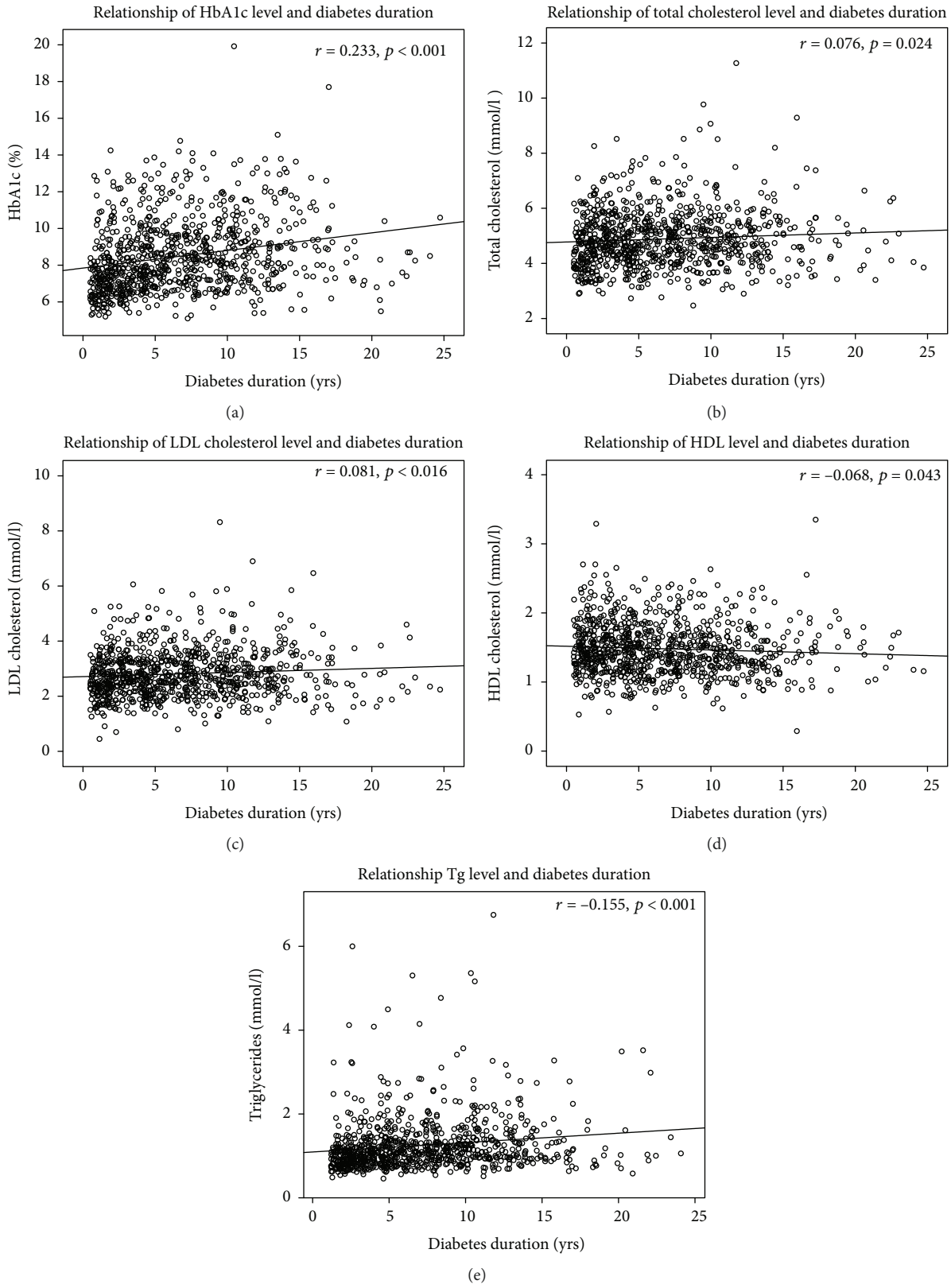

Figure 3: Correlations between diabetes and metabolic control parameters: (a) relationship of diabetes duration and glycosylated hemoglobin (HbA1c), (b) relationship of diabetes duration and total cholesterol, (c) relationship of diabetes duration and low density-lipoprotein cholesterol (LDL), (d) relationship of diabetes duration and high density-lipoprotein cholesterol (HDL), and (e) relationship of diabetes duration and triglycerides (Tg).

Table 4: Clinical characteristics according to presence of diabetic microvascular complications.

	Retinopathy		Neuropathy		AER	
	Absent	Present	Absent	Present	Normal	Elevated
Duration of DM (yrs)	6 ± 4.2	12.8 ± 4.4	6.2 ± 4.5	11 ± 4.7	6.4 ± 4.6	9.3 ± 5.7
	$p < 0.001$		$p < 0.001$		$p < 0.001$	
HbA1c (%)	8.3 ± 1.8	10 ± 2.3	8.4 ± 1.9	9.3 ± 2.3	8.4 ± 1.9	9.5 ± 2.4
	$p < 0.001$		$p < 0.001$		$p < 0.001$	
% of participants with dyslipidemia	61.9	70.2	61.4	74.2	62.1	69.9
	$p = 0.117$		$p = 0.014$		$p = 0.164$	
% of participants with hypertension	31.2	23.7	30.4	30.2	29.2	41.5
	$p = 0.083$		$p = 0.9$		$p = 0.031$	

DM: diabetes mellitus; HbA1c: glycosylated hemoglobin; AER: albumin excretion rate.

4. Discussion

Here, we present population-based study results for metabolic control, obesity, and hypertension in young people under the age of 25 with T1D treated by intensive insulin therapy, whose diabetes duration was more than 6 months.

The principal finding of our study is an unusually high frequency of dyslipidemia among pediatric and young adult patients with T1D compared to previously published data from other studies reporting dyslipidemia in 3.8% to 30.3% of subjects with T1D [7, 20]. These striking differences in the prevalence of dyslipidemia might partly be explained by different cutoff levels of lipids used in different studies. In some reports, ADA and National Cholesterol Education Program recommendations were used, defining dyslipidemia as TG level > 1.7 mmol/l, LDL cholesterol level > 3.36 mmol/l, HDL cholesterol level < 1.03 mmol/l, or total cholesterol > 5.17 mmol/l [20]. In other studies, dyslipidemia was defined as physician-diagnosed and recorded in medical documentation [7]. In our study, we used the ISPAD guidelines for the definition of optimal lipid levels [14]. Therefore, the use of different guidelines and normative data is certainly affecting the reported frequency of dyslipidemia in patients with T1D, and the comparisons between such studies are limited.

Interestingly, dyslipidemia in our study was present with similar frequency in all age groups and was directly related to diabetes duration and worse glycemic control that were independent factors influencing the occurrence of dyslipidemia in our cohort. Only after adjustment for diabetes duration and glycemic control was lipid fraction concentrations found to have a weak association with adiposity expressed in BMI z-scores. However, in our study, we reported slightly lower frequency of weight problems among young type 1 diabetics compared to that in recent studies from USA and Europe [7, 21]. Therefore, the overweight and obesity could not explain high rates of dyslipidemia in our cohort. On the contrary, the frequency of overweight youth among T1D patients seems to increase over time, as in the present study it was found to be higher (19.5%) than what was reported in 2013 in patients with T1D in Lithuania (13.4%) [22]. Furthermore, overweight and obese patients in our study had worse glycemic control and higher frequency of hypertension compared to normal-weight subjects, which is compatible with the results reported in other studies highlighting obesity among diabetic patients becoming a considerable health problem, affecting both adults and children [7, 21].

We found a significantly higher waist-to-height ratio in overweight and obese groups and in those with at least one lipid fraction out of the normal values. The waist-to-height ratio appeared to be a more sensitive parameter in defining risk of obesity and dyslipidemia than the waist-hip ratio, traditionally used in an adult population in the definition of central obesity. Several studies showed that the waist-to-height ratio is a better indicator than BMI and the waist-hip ratio for evaluating obesity and predicting risks for diabetes, hypertension, and CVD [11, 23]. The cutoff of 0.5 for the waist-to-height ratio was proved to be optimal in detecting abdominal obesity, metabolic syndrome, and the associated health risks [23].

We reported the mean HbA1c of 8.5 ± 2% in the whole cohort, which is markedly higher than the recommended optimal glycemic control. We found that young females have worse glycemic control than males, which is consistent with other studies' results [7, 24]. Of all age groups, patients aged 15-19 yrs have the worst glycemic control, probably because of adjustments in the endocrine system, and increased independence in diabetes care during adolescence makes achieving optimal HbA1c really difficult [25]. We observed that dyslipidemia was directly related to HbA1c levels. A positive correlation was found between the level of HbA1c and that of total cholesterol, LDL cholesterol, and Tg. These findings are in agreement with data from previous studies and provide further evidence that good glycemic control in young age would have a positive impact on lipid metabolism [26, 27].

Our analysis showed that levels of HbA1c and all lipid fraction concentrations were significantly related to the duration of diabetes. The link between T1D duration and microvascular complications and other CVD risk factors has been discussed by other authors [20, 28]. Previously published data from a study in Lithuanian T1D patients showed positive associations between the duration of diabetes and

levels of total cholesterol, LDL cholesterol, and triglycerides [22]. Diabetic nephropathy was reported as the most common diabetic complication, with longer diabetes duration having a significant impact for the development of microalbuminuria [20].

Dyslipidemia in T1D has been shown to be associated with the early development of cardiac and vascular abnormalities [6]. Cholesterol is well known to be one of the key players in the process of atherosclerosis [29]. Epidemiological studies in diabetic patients have shown that increased LDL and decreased HDL cholesterol levels are associated with an increased cardiovascular risk [30]. In accordance with other studies, increased LDL cholesterol levels were the most prevalent and hypertriglyceridemia the least prevalent lipid abnormality in our cohort of young patients with T1D [31]. High levels of triglycerides were shown to be accompanied by a high rate of microangiopathic alterations [31]. Since poor glycemic control can result in increased levels of triglycerides and LDL and decreased HDL cholesterol levels, optimization of glycemic control is essential in controlling lipid levels [30].

Physical activity, smoking, alcohol consumption, and dietary habits were not included in our analysis, constituting one of the limitations of this study; however, studies show the importance of these factors for CVD risk in T1D [6]. Smoking was reported to be a significant player in the progression of atherosclerotic changes of arteries in the Pittsburgh Epidemiology of Diabetes Complications Study [32]. It is known that physical activity positively affects BP, lipid profile, and weight; therefore, it is necessary to decrease physical inactivity in T1D patients [6, 32].

Even though ADA and AHA have clinical recommendations for preventing dyslipidemia in youth with diabetes, there is still lack of clinical trial data on treatment efficacy and safety of dyslipidemia in these patients [33]. Recent data reviews suggest that dyslipidemia is one of the potentially modifiable CVD risk factors; therefore, there is a need for clinical trials to examine the safety and efficacy of lipid-lowering drugs and their impact on future health outcomes [33, 34].

In our cohort, we found a higher incidence of hypertension (29.8%), especially in children, compared to other authors [7, 23]. This high frequency of hypertensive patients might possibly be explained by unrecognized "white-coat" hypertension, whose prevalence in population-based studies was found to be up to 29.2%, and it is suggested that white-coat hypertension could be present in about one-third of subjects with high blood pressure [35]. This type of hypertension may be identified using BP monitoring at home, elucidating the frequency of real hypertension.

We found a significant correlation between SBP, DBP, and BMI z-scores. Our results support findings from other studies that weight management is one of the principal strategies to lower the risk of CVD in T1D patients. In the future, it is expected that metformin would also bring some benefits for obese youth with T1D [36, 37].

We report here similar frequency of microvascular complications among young T1D patients, compared to recently published data [20]. Higher frequency of hypertension was found in patients with elevated AER. Hypertension is one of the key risk factors for the development of nephropathy, and management of BP is essential in reducing the risk of kidney damage [20].

We discussed several limitations of our study. We did not perform BP monitoring at home, which would have elucidated the real prevalence of hypertensive and prehypertensive patients. Furthermore, in our cohort, we did not assess apolipoprotein B concentrations and carotid artery intima-media thickness, which are both significant predictors of CVD risk [38].

Finally, the findings of our study highlight that the management of T1D should be multifaceted and most importantly include glycemic control, weight management, and dyslipidemia treatment.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The abstract "Prevalence of Cardiovascular Risk Factors and Obesity in Youth with Type 1 Diabetes in Lithuania" was presented at the 55th Annual ESPE conference on 10-12 Sep 2016 (Nr. 86 P-P2-277). The research project "Genetic Diabetes in Lithuania" was carried out during 2012-2016. The article "The Course of Diabetes in Children, Adolescents and Young Adults: does the Autoimmunity Status Matter" by Verkauskiene et al. (BMC Endocrine Disorders (2016)16:61) described the whole cohort of the project and focused on β-cell autoimmunity and diabetes course according to the presence of pancreas antibodies. In this article, we also compared data with the data from "The Risk of Early Cardiovascular Disease in Lithuanian Diabetic Children and Adolescents: a Type 1 Diabetes Register Database Based Study" by Dobrovolskiene et al. (Diabetes Res Clin Pract. 2013;100(1):119-25), which analyzed cardiovascular risk in youth with T1D in Lithuania and was conducted at the same research center.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

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Research Article

Association between Metformin Use and Coronary Artery Calcification in Type 2 Diabetic Patients

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Objectives. Type 2 diabetes mellitus (T2DM) is associated with coronary artery calcification (CAC) which is an independent risk factor for cardiovascular events. Metformin is the first-line antidiabetic medication. We aimed to investigate the association between metformin use and CAC. *Methods*. We included 369 patients with T2DM in this cross-sectional study. CAC scores, clinical characteristics, and antidiabetic drug prescription information of the patients were acquired. Baseline parameters were balanced for metformin and nonmetformin users using the propensity score matching (PSM) strategy. *Results*. Among the 369 subjects who met our inclusion criteria, 288 subjects were included for further analysis after PSM. Metformin prescription rather than other antidiabetic medications was related to lower CAC scores (OR [95% CI] = 0.55 [0.34–0.90]; $P = 0.018$). Further multivariable logistic regression analysis demonstrated that metformin was negatively associated with CAC severity (OR [95% CI] = 0.58 [0.34–0.99]; $P = 0.048$), which was independent of age, BMI, eGFR, gender, cigarette smoking, duration of diabetes, hypertension, statin prescription, and number of nonmetformin antidiabetic agents. A subgroup analysis revealed a significant association between metformin and CAC scores in smokers (OR [95% CI] = 0.38 [0.16–0.93]; $P = 0.035$), but the association was not observed in never-smokers (OR [95% CI] = 0.72 [0.34–1.51]; $P = 0.383$). *Conclusions*. Metformin usage was independently associated with lower CAC scores in T2DM patients. The negative correlation between CAC scores and metformin was most prominent in patients with a history of cigarette smoking.

1. Introduction

During the past decades, compelling evidence has demonstrated that coronary artery calcification (CAC) is an independent risk factor of cardiovascular events. Moreover, CAC is a challenge for percutaneous coronary intervention (PCI) and is linked with increased post-PCI events. However, there is no clinically proved therapy for vascular calcification [1]. Type 2 diabetes mellitus (T2DM) is deemed as a coronary artery disease (CAD) equivalent [2], which doubles or even triples the CAD incidence [3]. Moreover, T2DM patients tend to suffer from more calcified and diffuse coronary artery lesions, while having a blunted appreciation of ischemic episodes [4].

Imaging by computed tomography (CT) reveals that T2DM-affected individuals have extensive calcification of their vascular beds, which is reported as the CAC scores, reflecting significant cardiovascular disease burden [5, 6]. The CAC

score has independent added value beyond traditional risk factors in predicting the outcome of major cardiovascular events, especially in asymptomatic patients [7]. In patients with an intermediate Framingham risk score, CAC scores less than 99, between 100 and 399, and more than 400 are related to 0.4%, 1.3%, and 2.4% of annual CAD death, respectively [8].

As the first-line antidiabetic therapy, recent studies indicate that metformin has highlighted effect on alleviating vascular calcification. We and other groups reported that metformin prevents vascular calcification via AMP-activated protein kinase (AMPK) activation [9, 10], and we identify that metformin prevents atherosclerotic calcification in mice [10]. Moreover, recent clinical data showed that metformin prescription was independently associated with a decreased level of lower-limb arterial calcification [11]. Thus, it is reasonable to hypothesize that metformin therapy may be associated with lower levels of CAC severity in T2DM patients. We therefore performed a cross-sectional study in a population of asymptomatic T2DM patients to evaluate the association between metformin use and CAC scores.

2. Materials and Methods

2.1. Study Design. This is a cross-sectional study conducted among in-hospital T2DM patients who underwent coronary artery CT for preoperative screening between June 1st, 2016, and May 31st, 2017, in the Second Affiliated Hospital, Zhejiang University School of Medicine. Those patients were candidates for noncardiac surgery including hip/knee replacement, lumbar surgery, radical resection of pulmonary carcinoma, and cerebral artery aneurysm intervention. Main inclusion criteria were (1) diagnosed as T2DM and took antidiabetic drugs regularly for at least 3 months and (2) antidiabetic drug prescription remained unchanged for the last 3 months. Main exclusion criteria were (1) a history of CAD or PCI or coronary artery bypass grafting or clinical presentation of CAD like chest pain and shortness of breath; (2) type 1 diabetes mellitus; and (3) glomerular filtration rate ≤ 30 mL/min measured by the CKD-EPI equation [12]. The study was conducted on the grounds of the Declaration of Helsinki and approved by the Ethics Committee of the Second Affiliated Hospital, Zhejiang University School of Medicine.

2.2. CAC Quantification. Coronary artery CT images were acquired with Siemens SOMATOM definition flash CT. A radiologist who was blinded to patients reread all CT scans and reassessed their CAC scores. CAC scores were calculated with the Agatston method [13], i.e., by multiplying the area with a density above 130 Hounsfield units (Hu) by a factor reflecting the maximum attenuation.

2.3. Data Collection. We collected fasting blood data within 7 days before the coronary artery CT scan, in avoidance of the interference of contrast-induced nephropathy. This work was accomplished by one clinician, who was blinded to patients' medical history. Hemoglobin A1c (HbA1c), serum creatinine, fasting glucose, calcium, phosphate, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG) results were collected. The CKD-EPI equation was adopted for estimating the glomerular filtration rate (eGFR).

2.4. Statistical Analyses. Data were presented as means ± standard deviation (SD) or median (25th and 75th percentiles) for continuous variables, as appropriate. Data were expressed as the number (percentage) for qualitative variables. Data were categorized into metformin and nonmetformin users. Major imbalances were found in age, BMI, history of hypertension, eGFR, and glucosidase inhibitor usage between metformin and nonmetformin users. Those imbalanced factors were used to perform propensity score matching (PSM) for patients in two groups. A multivariable logistic regression model including these variables was applied. Matching was performed using the nearest neighbor matching, with a default caliper of 0.1. The CAC score = 100 Agatston units was set as the cutoff point in accordance with the risk classification in ACCF/AHA 2007. The χ^2 test was used to compare categorical variables, and Student's t-test or the Mann–Whitney test was used for continuous variables between the two groups. The association between antidiabetic drugs and CAC scores was analyzed by univariate logistic regression. To assess the independence of this association, we performed a multivariable logistic regression that included both variables identified in univariate analyses and relevant clinical variables or demographic factors (age, BMI, gender, eGFR, duration of diabetes and hypertension, and cigarette smoking). Furthermore, we did a subgroup analysis among male and female patients, which demonstrated that there was no gender difference. Another subgroup analysis among patients with or without a history of smoking was processed. Multivariable logistic regression with the same adjustments was used in the subgroup analysis. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 22.0 (IBM, Armonk, New York).

3. Results

3.1. Demographic and Clinical Characteristics. Among the 656 subjects, we excluded 287 subjects who did not meet our inclusion criteria. The remaining 369 subjects were divided into the metformin group ($n = 150$) and the nonmetformin group ($n = 219$). The median CAC score in the metformin group was 8.05 (0-124.6), and the score was almost octupled in the nonmetformin group (61.6 (0-319.6), $P = 0.005$) (Figure 1). Major imbalances were spotted in age ($P < 0.001$), BMI ($P = 0.005$), history of hypertension ($P = 0.011$), eGFR ($P < 0.001$), and glucosidase inhibitor usage ($P = 0.012$) between metformin and nonmetformin users. We performed PSM of the imbalanced factors and excluded another 81 unmatched subjects. Demographic and clinical characteristics of metformin and nonmetformin users before and after PSM were presented in Table 1, and the flow chart of exclusion was presented in Supplemental Figure 1. The median CAC score in the metformin group after PSM was 8.05 (0-124.6), and the score in the nonmetformin group after PSM was 37.00 (0.00, 220.50), but P value is marginal (0.097) (Table 1, Figure 1).

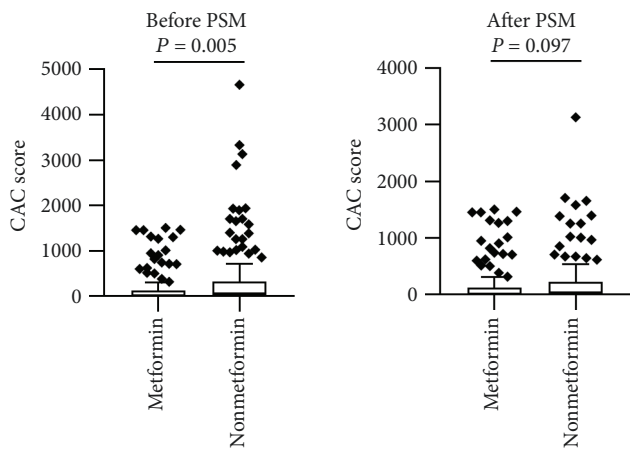

Figure 1: The comparison of CAC scores between patients treated with or without metformin. The Mann–Whitney test was used for the comparison between patients treated with or without metformin. The median CAC scores were 8.05 (0, 124.6) and 61.6 (0, 319.6), respectively ($P = 0.005$), before propensity score matching (PSM). And median CAC scores were 8.05 (0, 124.6) and 37.00 (0.00, 220.50) ($P = 0.097$) after matching.

In accordance with the risk classification in ACCF/AHA 2007, we categorized the subjects as CAC < 100 and CAC ≥ 100 to clarify the baseline differences between subjects with mild and moderate calcification (Table 2). Subjects in the higher CAC score group were significantly older and had longer history of hypertension. The higher CAC score was also related to lower eGFR. There were no significant intergroup differences for BMI, gender, smoking status, or relevant laboratory index including serum calcium, phosphate, fasting glucose, LDL-C, HDL-C, or TG. The data of HbA1c of 114 subjects were missing, but there was no intergroup difference in the remaining 255 subjects. Another interesting finding was that a higher percentage of subjects underwent statins or antiplatelet therapies, like aspirin and clopidogrel in the group with higher CAC scores ($P < 0.05$). The associations remained unchanged after PSM.

Notably, the CAC scores of 146 subjects were 0. In order to investigate the characteristics of subjects with CAC = 0, we classified subjects into CAC = 0, 0 < CAC < 100, and CAC ≥ 100 (Supplemental Table 1). Metformin was used in 46.58%, 49.37%, and 30.07% subjects in CAC = 0, 0 < CAC < 100, and CAC ≥ 100 groups, respectively. The intergroup difference was also significant ($P = 0.004$).

3.2. Association between Antidiabetic Therapy and CAC Severity. As per Table 2, metformin usage had a significant difference between CAC < 100 and ≥100 groups. Interestingly, 47.3% of subjects were treated with metformin in the CAC < 100 group, while the proportion was 30.1% in the ≥100 group, indicating that metformin usage corresponded to the lower CAC scores. Subjects in the groups of CAC < 100 and ≥100, respectively, took 1.16 ± 0.69 and 1.26 ± 0.76 kinds of nonmetformin antidiabetic drugs, which were not significantly different between the two groups ($P = 0.230$). The estimation association remained unchanged after PSM (Table 2).

Univariate logistic regression was further applied, with CAC score = 100 as the cutoff point. The result confirmed that metformin prescription was related to lower CAC scores (OR [95% CI] = 0.55 [0.34–0.90]; $P = 0.018$). In contrast, the usage of other antidiabetic medicines did not affect the CAC scores (Figure 2, Supplemental Table 2).

3.3. Independent Association between Metformin and CAC Severity. Univariate logistic regression analysis was adopted to recognize factors that were statistically significant between CAC < 100 and ≥100. Among all the clinical characteristics, we identified eGFR ($P < 0.001$), metformin ($P = 0.018$), statins ($P = 0.009$), and antiplatelet drug prescription ($P = 0.005$) as predictive factors. Since combinations of medication might impact the outcome, we also took the number of nonmetformin antidiabetic drugs into consideration. Those factors together with demographic characteristics including age, BMI, male gender, smoking, and duration of diabetes and hypertension were reevaluated in a multivariable logistic regression analysis. The result indicated that metformin was still significantly associated with CAC severity (OR [95% CI] = 0.58 [0.35–0.96]; $P = 0.035$) (Figure 3, Supplemental Table 3). The association between metformin and CAC scores was consistent after PSM (OR [95% CI] = 0.58 [0.34–0.99]; $P = 0.048$, Figure 3, Supplemental Table 3).

3.4. Association between Metformin and CAC Severity in Smokers or Never-Smokers. Cigarette smoking was positively related to CAC scores (after PSM, OR [95% CI] = 2.08 [1.01-4.24]; $P = 0.046$). The interaction between smoking and metformin use was not significant ($P = 0.371$). Since smoking was the only modifiable factor that was associated with CAC scores, we decided to take a further look at this factor. One hundred and thirty-five subjects with a history of cigarette smoking were included. Notably, in our study, all smokers were male. In order to rule out gender difference, we first did a sensitivity analysis by looking at the association between metformin and CAC scores in both genders. A CAC score = 100 was set as the cutoff point in the analysis. After adjustment of the predictive factors and demographic characteristics, metformin was not related to CAC in male (OR [95% CI] = 0.64 [0.34–1.20]; $P = 0.162$) and female (OR [95% CI] = 0.42 [0.17–1.03]; $P = 0.059$) subgroups.

We proceeded to perform subgroup analysis in subjects with or without a history of smoking. Multivariable logistic regression revealed a significant association between metformin and CAC scores in smokers (OR [95% CI] = 0.38 [0.16–0.93]; $P = 0.035$) (Figure 4(a), Supplemental Table 4), but the association diminished in never-smokers (OR [95% CI] = 0.72 [0.34–1.51]; $P = 0.383$) (Figure 4(b), Supplemental Table 5).

4. Discussion

In the present study, we investigated the association of metformin on CAC severity in T2DM populations. The major finding is that metformin is linked to a lower level of

Table 1: The characteristics of included patients according to metformin usage.

Variables	Metformin usage (before PSM)			Metformin usage (after PSM)		
	Metformin ($n = 150$)	Nonmetformin ($n = 219$)	P value	Metformin ($n = 150$)	Nonmetformin ($n = 138$)	P value
Age	65.04 ± 9.47	70.03 ± 10.12	<0.001	65.04 ± 9.47	66.91 ± 9.69	0.100
BMI	25.20 ± 3.36	24.18 ± 3.52	0.005	25.20 ± 3.36	24.69 ± 3.41	0.206
Male gender	96 (64.00%)	132 (60.27%)	0.469	96 (64.00%)	85 (61.59%)	0.673
Smoking	61 (40.67%)	74 (33.79%)	0.178	61 (40.67%)	52 (37.68%)	0.604
DM duration	10.00 (4.75, 15.00)	8.00 (3.00, 15.00)	0.119	10.00 (4.75, 15.00)	7.00 (3.00, 15.00)	0.039
HTN	93 (62.00%)	163 (74.43%)	0.011	93 (62.00%)	98 (71.01%)	0.106
HTN duration	6.00 (0.00, 15.00)	10.00 (0.00, 20.00)	0.605	6.00 (0.00, 15.00)	5.25 (0.00, 15.00)	0.513
HbA1c (%)	7.50 (6.70, 8.15)	7.30 (6.78, 8.43)	0.990	7.50 (6.70, 8.15)	7.30 (6.80, 8.48)	0.988
eGFR (mL/min/1.73 m^2)	96.53 (90.49, 105.50)	92.02 (81.63, 99.23)	<0.001	96.53 (90.49, 105.50)	94.82 (85.60, 101.10)	0.037
FBG (mmol/L)	6.64 (5.52, 8.57)	7.11 (5.76, 8.70)	0.480	6.64 (5.52, 8.57)	7.04 (5.66, 8.94)	0.395
Ca (mmol/L)	2.28 ± 0.16	2.25 ± 0.12	0.020	2.29 ± 0.16	2.26 ± 0.13	0.098
P (mmol/L)	1.15 ± 0.19	1.13 ± 0.20	0.429	1.15 ± 0.19	1.13 ± 0.20	0.361
LDL-C (mmol/L)	2.25 (1.61, 2.75)	2.30 (1.72, 2.89)	0.141	2.25 (1.61, 2.75)	2.48 (1.75, 2.99)	0.171
HDL-C (mmol/L)	1.05 (0.89, 1.28)	1.09 (0.91, 1.27)	0.835	1.05 (0.89, 1.28)	1.13 (0.93, 1.29)	0.153
TG (mmol/L)	1.46 (1.01, 2.15)	1.39 (0.98, 1.89)	0.219	1.46 (1.01, 2.15)	1.56 (0.99, 1.92)	0.900
Sulfonylureas	50 (33.33%)	86 (39.27%)	0.246	50 (33.33%)	56 (40.58%)	0.203
Glinides	28 (18.67%)	27 (12.33%)	0.093	28 (18.67%)	20 (14.49%)	0.429
GI	49 (32.67%)	100 (45.66%)	0.012	49 (32.67%)	53 (38.41%)	0.309
TZD	4 (2.67%)	12 (5.48%)	0.193	4 (2.67%)	8 (5.80%)	0.302
DPP4 inhibitor	2 (1.33%)	5 (2.28%)	0.511	2 (1.33%)	2 (1.45%)	0.675
Insulin	26 (17.33%)	54 (24.65%)	0.094	26 (17.33%)	31 (24.65%)	0.345
Statins	54 (36.00%)	62 (28.31%)	0.118	54 (36.00%)	43 (22.46%)	0.385
Antiplatelets	47 (31.33%)	70 (31.96%)	0.898	47 (31.33%)	43 (31.16%)	0.975
CAC scores	8.05 (0, 124.6)	61.6 (0, 319.6)	0.005	8.05 (0, 124.6)	37.00 (0.00, 220.50)	0.097

BMI: body mass index; DM: diabetes mellitus; GI: glucosidase inhibitors; HTN: hypertension; eGFR: estimated glomerular filtration rate; FBG: fasting blood glucose; Ca: calcium; P: phosphorus; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; TG: triglyceride; DPP4: dipeptidyl peptidase-4; TZD: thiazolidinediones; PSM: propensity score match.

CAC scores in T2DM patients, especially in those with a history of cigarette smoking.

Metformin has been established for its unshakeable status in T2DM therapy as it reduces the morbidity and mortality of macrovascular complications [14]. Long-term metformin therapy is related to a 33% reduction in myocardial infarction [15]. In the CAMERA study, investigators also explored the effect of metformin on nondiabetic CAD. Even though metformin reduced the HbA1c level and improved insulin resistance, there was no difference in carotid intima-media thickness (cIMT) progression between the metformin and placebo groups [16]. However, the result was confounded by the small sample size and the use of statins, which made the window of opportunity for cIMT improvement rather small. We believe that the undergoing large randomized clinical trial GLINT might bring good news on the use of metformin in CAD patients without T2DM.

So far, the mechanism of metformin in CAD still needs to be explored. A randomized placebo-controlled trial conducted in 50 HIV-infected subjects with metabolic syndrome demonstrated that metformin reduces CAC progression [17], but the validity of the result in general diabetic population was hampered by the small sample size and HIV infection status in the study. Moreover, a recent study indicated that metformin treatment had protective effect on coronary atherosclerosis in male prediabetic population, but the result was inconsistent in female subjects [6]. However, in the present study, we found that there was no gender difference in the association between metformin and CAC. This might be explained by the difference in participants' age. In the former study, the average of female subjects was 49.1 ± 9.3, including 38% premenopausal, while the average age of female was 69.3 ± 9.8 in our study. It would be interesting to further investigate the association of metformin and CAC in premenopausal diabetic patients.

As mentioned, the current antidiabetic regimen was defined as drugs that were taken regularly without modification in prescription for at least 3 months. It was of interest that metformin rather than other antidiabetic agents was associated with lower CAC scores in our study. Since we did not have the information of antidiabetic medications they

Table 2: The characteristics of included patients according to CAC scores.

Variables	CAC scores (before PSM)			CAC scores (after PSM)		
	Score < 100 (n = 226)	Score ≥ 100 (n = 143)	P value	Score < 100 (n = 187)	Score ≥ 100 (n = 101)	P value
Age	65.65 ± 9.65	71.73 ± 9.83	<0.001	64.15 ± 9.25	69.24 ± 9.41	<0.001
BMI	24.57 ± 3.47	24.63 ± 3.53	0.886	24.96 ± 3.47	24.18 ± 3.52	0.987
Male gender	143 (63.27%)	85 (59.44)	0.460	119 (63.64%)	62 (61.39%)	0.706
Smoking	77 (34.07%)	58 (40.56)	0.207	68 (36.36%)	45 (44.55%)	0.174
DM duration	9.50 (4.00, 5.00)	10.00 (3.00,15.00)	0.814	9.00 (4.00, 15.00)	10.00 (3.00, 15.00)	0.993
HTN	149 (65.93%)	107 (74.83)	0.071	119 (63.64%)	72 (71.29%)	0.190
HTN duration	5.00 (0.00, 15.00)	10.00 (0.00, 20.00)	0.002	5.00 (0.00, 15.00)	8.00 (0.00, 15.00)	0.087
HbA1c (%)	7.4 (6.7, 8.3)	7.4 (6.8, 8.2)	0.790	7.40 (6.70-8.25)	7.45 (6.90-8.20)	0.456
eGFR (mL/min/1.73 m^2)	95.70 (88.02, 104.88)	91.75 (81.47, 98.46)	<0.001	97.40 (89.74, 105.80)	92.46 (85.55, 99.03)	<0.001
FBG (mmol/L)	7.04 (5.91, 8.90)	6.82 (5.44, 8.39)	0.220	7.00 (5.93-8.94)	6.82 (5.38-8.35)	0.205
Ca (mmol/L)	2.26 ± 0.13	2.26 ± 0.16	0.963	2.27 ± 0.12	2.27 ± 0.18	0.924
P (mmol/L)	1.13 ± 0.19	1.16 ± 0.21	0.151	1.13 ± 0.19	1.15 ± 0.21	0.399
LDL-C (mmol/L)	2.27 (1.70, 2.85)	2.25 (1.60, 2.82)	0.603	2.23 (1.71-2.88)	2.27 (1.58-2.97)	0.848
HDL-C (mmol/L)	1.09 (0.81, 1.28)	1.03 (0.89, 1.27)	0.267	1.11 (0.92-1.29)	1.03 (0.88-1.27)	0.158
TG (mmol/L)	1.41 (0.99, 2.04)	1.39 (0.97, 1.88)	0.497	1.50 (1.00-2.06)	1.56 (0.98-2.00)	0.995
Metformin	107 (47.34%)	43 (30.07%)	0.001	107 (57.22%)	43 (42.57%)	0.018
Sulfonylureas	82 (36.28%)	54 (37.76%)	0.774	68 (36.36%)	38 (37.62%)	0.832
Glinides	33 (14.60%)	22 (15.38%)	0.837	31 (16.58%)	17 (16.83%)	0.956
GI	85 (37.61%)	64 (44.76%)	0.173	65 (34.76%)	37 (36.63%)	0.751
TZD	10 (4.42%)	6 (4.29%)	0.916	7 (3.74%)	5 (4.95%)	0.857
DPP4 inhibitor	5 (2.21%)	2 (1.40%)	0.711	4 (2.14%)	0 (0.00%)	0.341
Insulin	47 (20.80%)	33 (23.07%)	0.605	36 (19.25%)	21 (20.79%)	0.754
Statins	62 (27.43%)	54 (37.76%)	0.037	53 (28.34%)	44 (43.56%)	0.009
Antiplatelets	59 (26.11%)	58 (40.56%)	0.004	48 (25.67%)	42 (41.58%)	0.005

BMI: body mass index; DM: diabetes mellitus; GI: glucosidase inhibitors; HTN: hypertension; eGFR: estimated glomerular filtration rate; FBG: fasting blood glucose; Ca: calcium; P: phosphorus; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; TG: triglyceride; DPP4: dipeptidyl peptidase-4; TZD: thiazolidinediones; PSM: propensity score match.

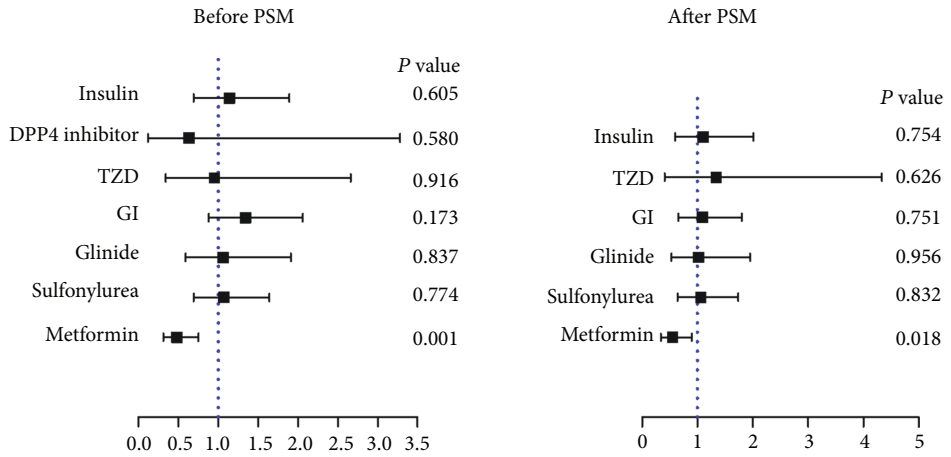

Figure 2: The association between metformin and other antidiabetic medicines and CAC scores. Metformin was negatively related to the CAC score before (OR [95% CI] = 0.48 [0.31–0.75]; $P = 0.001$) and after propensity score matching (OR [95% CI] = 0.55 [0.34–0.90]; $P = 0.018$). BMI: body mass index; DM: diabetes mellitus; HTN: hypertension; eGFR: estimated glomerular filtration rate; NMA: nonmetformin antidiabetic agents; PSM: propensity score match. Since the DPP4 inhibitor user was 0 among subjects with CAC ≥ 100 after propensity score matching, we omitted this data accordingly.

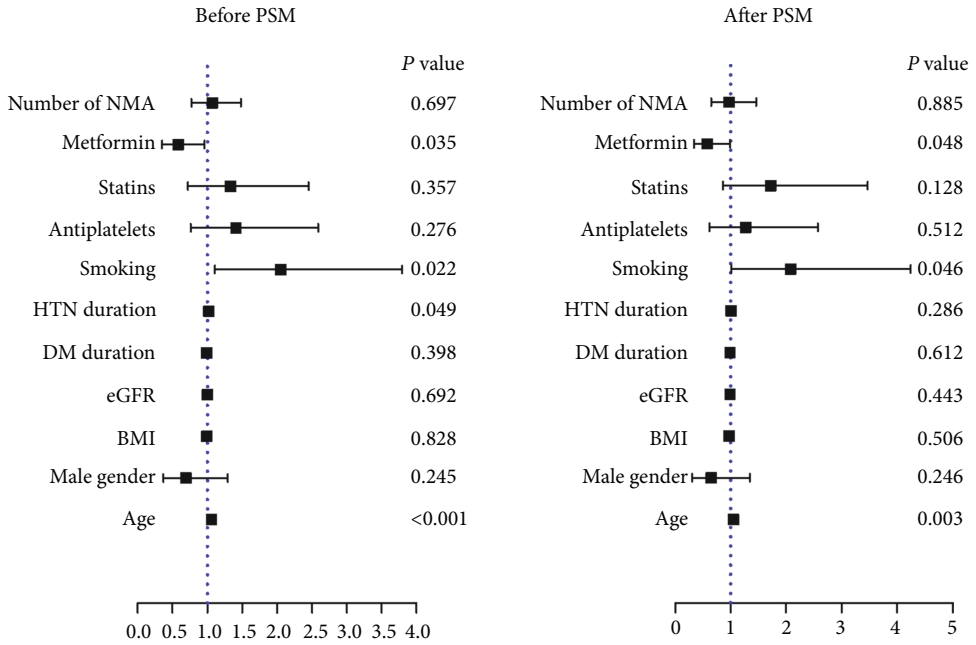

Figure 3: The independent association of metformin and other factors with CAC scores. The multivariable logistic regression analysis demonstrated that metformin was negatively associated with CAC severity before (OR [95% CI] = 0.58 [0.35–0.96]; $P = 0.035$) and after propensity score matching (OR [95% CI] = 0.58 [0.34–0.99]; $P = 0.048$), while smoking (OR [95% CI] = 2.08 [1.01–4.24]; $P = 0.046$) and age (OR [95% CI] = 1.05 [1.02–1.09]; $P = 0.003$) were positively associated with CAC severity. BMI: body mass index; DM: diabetes mellitus; HTN: hypertension; eGFR: estimated glomerular filtration rate; NMA: nonmetformin antidiabetic agents; PSM: propensity score match.

received in the past, subjects who discontinued metformin before the study gave rise to time-related bias. Yet, this bias tightened the association between metformin and CAC scores. It was because some patients in the nonmetformin group had metformin before the study, which would weaken the difference in the CAC scores between the metformin and nonmetformin groups in the study. A number of clinical studies had shown that metformin had cardiovascular protective effects independent of glucose-lowering effects [6, 18]. Since the CAC score is a well-accepted risk factor for cardiovascular events, the preventive effect of metformin on CAD, may at least partially, attributes to its role in reducing CAC.

The mechanisms by which metformin might result in lower CAC are still not fully understood. Our group revealed that metformin prevents atherosclerotic calcification via the activation of AMPK and subsequent Runx2 degradation [10]. Cao, et al. reported that metformin upregulated endothelial nitric oxide synthase (eNOS) in vascular smooth muscle cells to prevent calcification [9]. However, other mechanisms may also be involved, such as proautophagy, antioxidative stress, and anti-inflammation via both AMPK-dependent and AMPK-independent pathways [19].

It is noteworthy that metformin is profoundly linked with lower CAC scores in smoking subjects in the light of our study. Cigarette smoking is a well-established risk factor that is modifiable for CAD. The Heinz Nixdorf Recall study reported that smoking was also positively associated with CAC scores [20]. In another large retrospective cohort study in T2DM patients, researchers found that metformin is associated with lower risk of CAD and mortality in current and former smokers [21]. Smoking releases numerous toxic chemicals and free radicals, exposing the coronary artery to excess oxidative stress, while vascular calcification is also attributed to oxidative stress [22]. Metformin may exhibit its beneficial role in CAC due to its antioxidative stress properties [23] as well as its role in upregulating some components of the antioxidant defense system [24]. Nevertheless, the specific underlying mechanism is yet to be elucidated.

The limitations of our study include (1) the cross-sectional design, (2) involvement of relatively small numbers of subjects, (3) the missing data of HbA1c that hampered the interpretation of blood glucose control over the CAC severity, and (4) inability to exclude other interfering factors like lifestyle intervention. A prospective randomized controlled trial is needed to overcome these limitations. It would be interesting to examine the type and dose of antidiabetic medications, the CAC scores, and if possible, the change in the CAC scores.

To sum up, our results reveal that metformin prescription rather than other antidiabetic agents is negatively and independently associated with CAC severity in T2DM patients. We also witnessed a markedly negative association of metformin and CAC scores in smokers. The findings emphasize the use of metformin in all T2DM populations, especially those patients with a history of smoking.

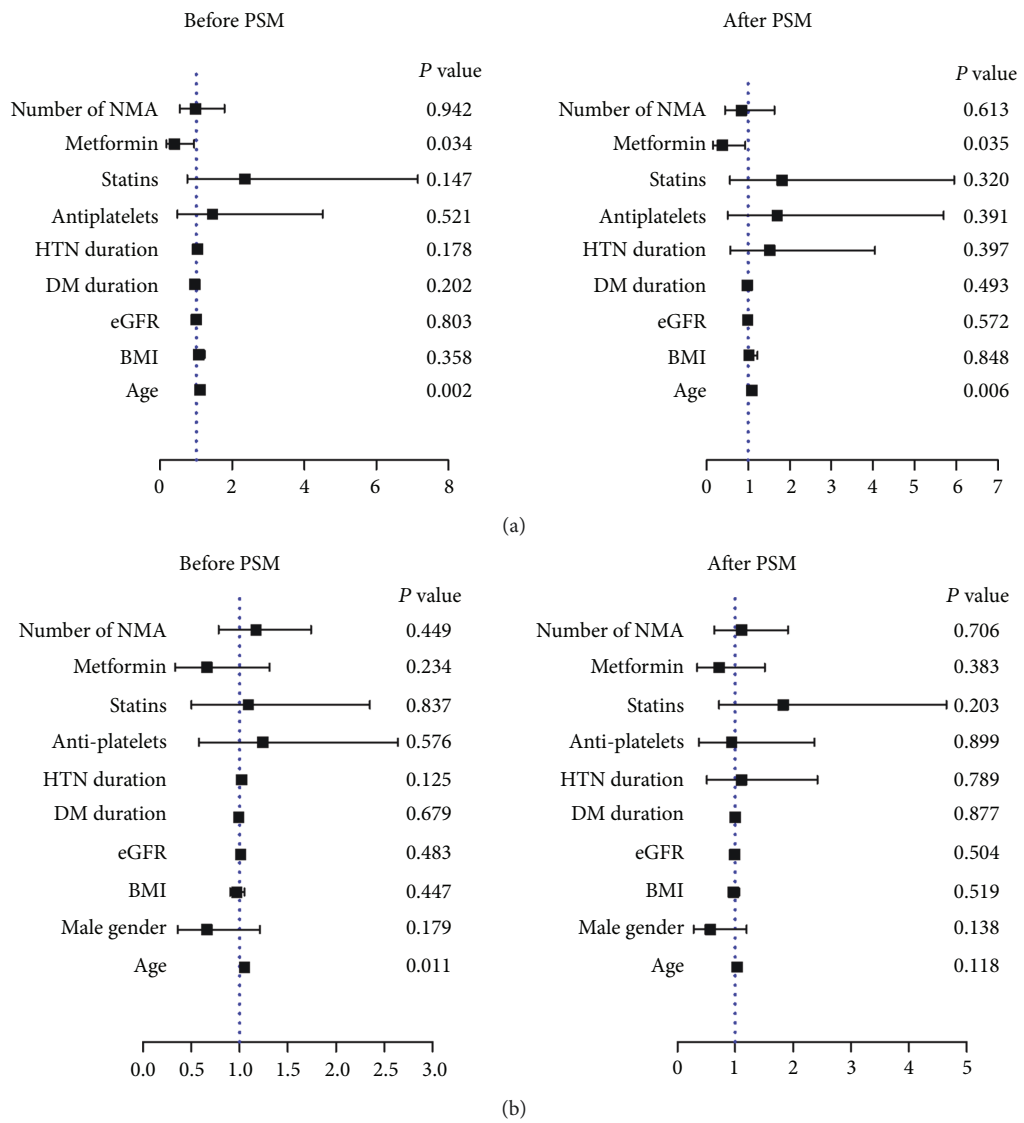

Figure 4: The relationship of CAC scores and metformin usage in smokers and never-smokers. Multivariable logistic regression revealed a significant association between metformin and CAC scores in male smokers before (OR [95% CI] = 0.40 [0.17–0.94]; $P = 0.034$) and after propensity score matching (PSM, OR [95% CI] = 0.38 [0.16–0.93]; $P = 0.035$) (a), but the association was not observed in never-smokers (OR [95% CI] = 0.72 [0.34–1.51]; $P = 0.383$) (b). BMI: body mass index; DM: diabetes mellitus; HTN: hypertension; eGFR: estimated glomerular filtration rate; NMA: nonmetformin antidiabetic agents.

Data Availability

The clinical data used to support the findings of this study are restricted by the Ethics Committee of the Second Affiliated Hospital, Zhejiang University School of Medicine, in order to protect patient privacy. Data are available from the corresponding author for researchers who meet the criteria for access to confidential data.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

Yi Lu and Yidong Wang contributed equally.

Acknowledgments

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Supplementary Materials

Supplemental Figure 1: the flow chart of the patient inclusion and exclusion. Supplemental Table 1: the characteristics of included patients according to CAC scores. Supplemental Table 2: the association between metformin and other

antidiabetic medicine and CAC scores. Supplemental Table 3: the independent association of metformin and other factors with CAC scores. Supplemental Table 4: the subgroup analysis in patients with a history of smoking. Supplemental Table 5: the subgroup analysis in patients without a history of smoking. *(Supplementary Materials)*

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Research Article

Impact of Coronary Artery Disease and Diabetes Mellitus on the Long-Term Follow-Up in Patients after Retrograde Recanalization of the Femoropopliteal Arterial Region

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The most relevant comorbidities in patients with peripheral artery disease (PAD) are coronary artery disease (CAD) and diabetes mellitus (DM). However, data of long-term follow-up of patients with chronic total occlusion (CTO) are scarce. The aim of the study was to assess the impact of CAD and DM on long-term follow-up patients after superficial femoral artery (SFA) CTO retrograde recanalization. In this study, eighty-six patients with PAD with diagnosed CTO in the femoropopliteal region and at least one unsuccessful attempt of antegrade recanalization were enrolled in 2 clinical centers. Mean time of follow-up in all patients was 47.5 months (±40 months). Patients were divided into two groups depending on the presence of CAD (CAD group: $n = 45$ vs. non-CAD group: $n = 41$) and DM (DM group: $n = 50$ vs. non-DM group: $n = 36$). In long-term follow-up, major adverse peripheral events (MAPE) occurred in 66.6% of patients with CAD vs. 36.5% of patients without CAD and in 50% of patients with DM vs. 55% of non-DM subjects. There were no statistical differences in peripheral endpoints in both groups. However, there was a statistically significant difference in all-cause mortality: in the DM group, there were 6 deaths (12%) (P value = 0.038). To conclude, patients after retrograde recanalization, with coexisting CTO and DM, are at higher risk of death in long-term follow-up.

1. Introduction

Coexistence of peripheral artery disease (PAD), coronary artery disease (CAD), and cerebrovascular disease causes almost 2 million deaths in Europe each year [1]. There are an increasing number of patients with PAD, who have increased mortality rate and higher risk of cardiovascular events; however, PAD is still underdiagnosed [2]. It is worth pointing out that PAD is an independent risk factor for increased cardiovascular mortality [1]. The most relevant comorbidities in patients with PAD are CAD and diabetes mellitus (DM) [3]. Patients with diagnosed DM are characterized by higher frequency of PAD occurrence [4], and DM itself accelerates the progression of atherosclerosis. Recent studies have shown the impact of glycemia control and severity of CAD on patency of peripheral arteries in patients with PAD and poorer outcomes in long-term follow-up [5]. Chronic total occlusion (CTO) affects around 50% of patients with PAD. Patients with CTO in the femoropopliteal region require complex treatment of PAD and

comorbidities [1]. The most challenging cases in patients with CTO are those with unsuccessful antegrade recanalization (around 10-15%) [6]. Data of long-term follow-up of patients with CTO are scarce. The aim of the study was to assess the influence of CAD and DM on the long-term follow-up in patients after superficial femoral artery (SFA) CTO retrograde recanalization.

2. Material and Methods

We included 86 patients, enrolled in two centers, with symptomatic PAD and diagnosed chronic total occlusion in the artery of the lower extremity, after at least one attempt of unsuccessful antegrade recanalization. CTO lesions were verified in angiography of the lower extremities' vessels. Patients were screened for most relevant comorbidities and risk factors. Before the procedure, all patients were evaluated according to the Rutherford/Fontaine scale and the ankle-brachial index (ABI) measurements were performed. All patients were qualified for retrograde recanalization, after previous unsuccessful antegrade crossing, and registry description was previously published [7].

The procedure of retrograde recanalization was performed according to the local protocol. Puncture was performed under the guidance of ultrasound or fluoroscopy. All patients required 2 approaches: proximal, mostly in the femoral common artery, and distal, in the distal part of SFA or the proximal part of the popliteal artery. A few patients required procedures also in arteries below the knee.

After the index procedure, patients were administrated dual antiplatelet therapy (75 mg of aspirin, 75 mg of clopidogrel) for 1-3 months, low molecular weight heparin for 1 month, and high dose of statin.

Two separated analyses were performed based on the presence of CAD and DM. Patients were divided into 2 groups: patients with diagnosed CAD and without CAD. CAD was defined as previous acute coronary syndrome, history of percutaneous coronary intervention, bypass grafting (CABG), or lesions in coronary arteries ≥ 50% stenosis evaluated in coronary angiography.

In the second analysis, patients were divided into 2 groups based on the presence of DM—patients with diagnosed DM and without DM. Patients were enrolled to the DM group regardless of the type of treatment (diet, oral medications, and insulin) and duration of DM. Patients with newly diagnosed DM during index hospitalization were excluded from analysis.

All patients were screened during clinical follow-up for major adverse cardiac and cardiovascular events (MACCE), major adverse peripheral events (MAPE), and all-cause mortality.

MACCE was defined as acute coronary artery syndrome, coronary artery intervention, coronary bypass grafting, and stroke/transient ischemic attack (TIA).

MAPE was defined as target vessel reintervention, nontarget vessel reintervention, and amputation.

All patients were screened for all-cause deaths: cardiac and noncardiac.

Regression analysis was performed to find independent predictors for cardiac events, cerebrovascular events, peripheral events, and all-cause death in patients after CTO retrograde recanalization in the femoropopliteal region.

2.1. Statistical Analysis. Results are presented as the number of patients (percentage) or mean value ± standard deviation (SD)/median value or interquartile range (IQR) where applicable. Differences between groups were tested using the Chi-square test and Fisher's exact test for dichotomous variables and the Mann-Whitney U test for continuous variables. The Kaplan-Meier method was used to assess the difference in mortality during follow-up between patients. Additionally, univariate Cox regression analysis was performed. In all tests, P value of <0.05 was considered statistically significant.

3. Results

Mean time of long-term follow-up in all patients was 47.5 months (±40 months). Patients were divided into two groups depending on the presence of CAD (CAD: $n = 45$ vs. non-CAD: $n = 41$) and DM (DM: $n = 50$ vs. non-DM: $n = 36$). In group DM vs. non-DM, the average age was similar. In both groups, most of the patients were male. Diabetic patients were treated with insulin, oral hypoglycemic medications, or diet only. Details of medical history are presented in Table 1. In long-term follow-up, MAPE was observed in 50% of patients with DM and 55.5% of patients without DM.

Target vessel revascularization, nontarget vessel revascularization, stroke, acute coronary syndrome, and unsuccessful revascularization were similar in both groups (Table 2). The observed number of amputations in the DM group was twofold higher than that in the group without DM, but it was not statistically significant. The all-cause mortality rate was higher in the DM group (12% vs. none, $P = 0.038$, Figure 1). In Cox regression analysis, DM was an independent risk factor for death ($P = 0.0147$).

In the CAD/no-CAD cohort, the number of patients in both groups was similar. The mean age of patients were 66.7 (±11.8) in the CAD group and 75 years (±14.1) in the non-CAD group. Both groups were comparable in terms of the presence of comorbidities. All demographics and medical history are presented in Table 1.

In long-term follow-up, reinterventions in the target vessel were observed in 27% of the CAD group and in 6% of the non-CAD group. All endpoints are presented in Table 2. MAPE occurred in 66.6% of patients with CAD and in 36.5% of patients without CAD. There was no significant difference in all endpoints in both groups (Table 3 and Figure 2).

4. Discussion

PAD is an atherosclerotic disease and becomes an increasing problem in elderly population. PAD alone is associated with a higher rate of mortality and morbidity, including cerebrovascular events, as compared to the mortality and morbidity rate of patients without PAD [2, 8, 9]. Moreover, PAD shares

Table 1: Demographics and medical history of patients in the DM/non-DM group and CAD/non-CAD group.

	All patients ($n = 86$)	DM ($n = 50$)	Non-DM ($n = 36$)	P value	CAD ($n = 45$)	Non-CAD ($n = 41$)	P value
Mean age, years (±SD)	65 (±9)	65 (±8.9)	62 (±10.1)	0.57	67 (±11.8)	75 (±14.1)	0.19
Sex (male, n, %)	58 (67.4)	33 (60)	25 (69)	0.51	28 (62)	29 (70)	0.39
BMI (kg/m^2) (±SD)	29 (±5.9)	31	26	0.4	28	30	0.51
Hypertension (n, %)	75 (87)	45 (90)	30 (83)	0.55	40 (88.8)	35 (85)	0.64
DM (n, %)	50 (58.1)						0.8
(i) Insulin		21 (42)			12 (26.6)	10 (24.4)	0.9
(ii) Oral		28 (56)			14 (31)	13 (31)	0.95
(iii) Diet only		1 (2)			1 (2)	0	
(iv) Non-DM		0	36 (100)		18 (40)	18 (43.9)	0.87
CAD (n, %)	45 (52.3)	28 (56)	17 (47)	0.56	45 (100)	0	
COPD (n, %)	11 (12.8)	7 (14)	4 (11)	0.3	7 (15.5)	4 (9)	0.42
Hypercholesterolemia (n, %)	52 (60.5)	27 (54)	25 (69)	0.1	27 (60)	25 (62)	0.79
Previous stroke/TIA (n, %)	8 (9.3)	5 (10)	3 (8)	0.85	4 (8)	4 (9)	0.89
Smoking (n, %)	38 (44.2)	21 (42)	17 (47)	0.54	18 (40)	20 (48.7)	0.24

BMI: body mass index; CAD: coronary artery disease; COPD: chronic obstructive pulmonary disease; DM: diabetes mellitus; SD: standard deviation: TIA: transient ischemic attack.

Table 2: Major adverse cardiac and cerebrovascular events (MACCE) and other events after SFA CTO revascularization stratified by DM presence.

	All patients ($n = 86$)	DM ($n = 50$)	Non-DM ($n = 36$)	P value
TVR (n, %)	18 (20.9)	9 (18)	9 (25)	0.4
Non-TVR (n, %)	23 (26.7)	13 (26)	10 (27.8)	0.8
Amputation (n, %)	4 (4.6)	3 (6)	1 (2.8)	0.8
Stroke (n, %)	0	0	0	
ACS (n, %)	3 (3.5)	2 (4)	1 (2.8)	0.8
All-cause mortality (n, %)	6 (6.9)	6 (12)	0	0.038
Unsuccessful revascularization (n, %)	5 (5.8)	2 (4)	3 (8.3)	0.6

ACS: acute coronary syndrome; CTO: chronic total occlusion; SFA: superficial femoral artery; non-TVR: nontarget vessel revascularization; TVR: target vessel revascularization.

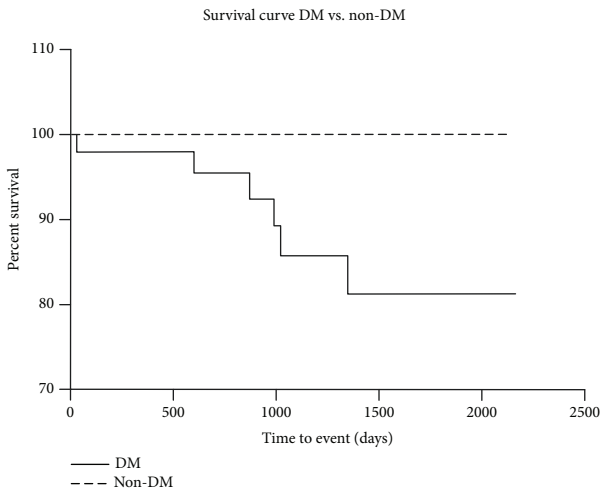

Figure 1: Kaplan-Meier survival curve in group DM vs. non-DM. DM: diabetes mellitus.

similar risk factors with CAD [10, 11] and patients with comorbid PAD and CAD reach worse outcomes in follow-up than patients with CAD alone [12, 13].

Around 50% of patients with diagnosed symptomatic PAD suffer from CTO [6]. Despite the development of numerous endovascular techniques, CTO lesions in the femoropopliteal region are still challenging for endovascular treatment and those patients required complex treatment. Retrograde recanalization of SFA CTO in patients with at least one unsuccessful antegrade crossing is a safe and effective option with a patency rate at 88.2% after one year [14] and an amputation rate 4.7% in long-term follow-up [7]. The mortality rate in follow-up varies between 6.9% and 23% [7, 15], and the patency rate of the target vessel in critical limb ischemia is up to 42% [16]. However, additional data from long-term follow-up after retrograde recanalization is still limited.

In our study, we confirmed that DM has a relevant impact on long-term outcomes in patients after retrograde revascularization of the femoropopliteal region. Cooccurrence of PAD and DM reaches more than 30% [17]. In

Table 3: Major adverse cardiac and cerebrovascular events (MACCE) and other events after SFA CTO recanalization stratified by CAD presence.

	All patients ($n = 86$)	CAD ($n = 45$)	Non-CAD ($n = 41$)	P value
TVR (n,%)	18 (20.9)	12 (27)	6 (14.6)	0.2
Non-TVR (n,%)	23 (26.7)	15 (33)	8 (19.5)	0.2
Amputation (n,%)	4 (4.6)	3 (6.6)	1 (2.4)	0.36
Stroke (n,%)	0	0	0	
ASC (n,%)	3 (3.5)	1 (2.2)	2 (4.9)	0.6
All-cause mortality (n,%)	6 (6.9)	4 (8.9)	2 (4.9)	0.68
Unsuccessful revascularization (n,%)	5 (5.8)	2 (4.4)	3 (7.3)	0.7

ACS: acute coronary syndrome; CTO: chronic total occlusion; SFA: superficial femoral artery; non-TVR: nontarget vessel revascularization; TVR: target vessel revascularization.

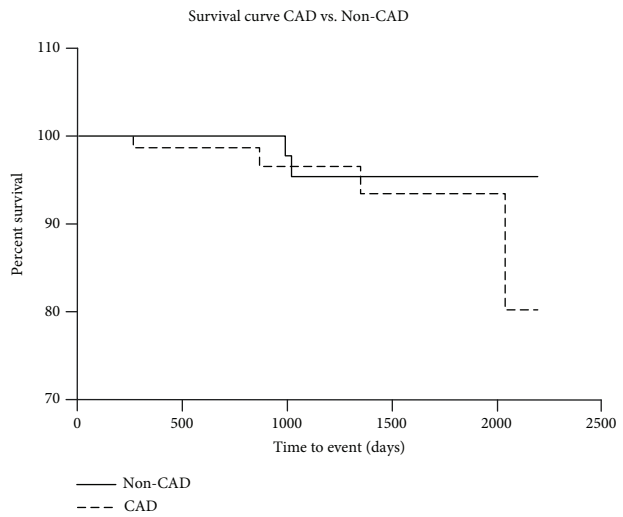

Figure 2: Kaplan-Meier survival curve in group CAD vs. non-CAD. CAD: coronary artery disease.

patients with coexisting PAD and DM, there is greater severity of PAD, which can be explained by the higher rate of amputation in this population (DM 41.4% vs. non-DM 11.5%) [18]. This trend can be seen also in our study, where the amputation rate was twice lower in the non-DM group. Additionally, the mortality rate in DM patients is twice higher (51.7% vs. 25.6%) than that in non-DM patients [18]. These findings were confirmed by Reiber et al. who pointed out that 5-year mortality in diabetic patients with PAD reaches 50% and overall amputation risk is up to 20% [19]. Our results stay in accordance with previously published data where the mortality rate among patients with coexisting PAD and DM is higher and statistically relevant (12% vs. 0, P value = 0.038). Moreover, Jude et al. reported that in a diabetic deceased group of patients, there was a trend toward higher prevalence of CTO in arteries [18].

According to the study by Leibson et al., coexistence of PAD and DM increases risk of death 1.67 times compared to PAD alone and 1.55 times compared to DM alone [2].

On the other hand, Weis-Müller et al. suggested that at 1-, 3-, and 5-year of follow-up, limb salvage rates were not influenced by DM or CAD and 1-, 3-, and 5-year survival was uninfluenced by DM alone, though CAD reduced life expectancy [20].

In patients with PAD, the prevalence of CAD reaches between 46 and 71% [21, 22].

Previous studies point out that PAD is associated with higher rate of cardiovascular adverse events (5.35% vs. 4.52%) [23] and higher rate of total mortality in long-term observation (47.8% vs. 36.4%) than CAD [13]. In analysis of patients who underwent vascular surgery, postoperative and long-term outcomes are determined by CAD [24, 25]. In a group of patients with lower limb reconstruction procedures, the main causes of 30-day mortality were cardiovascular events and infections [25]. Results from our study show higher frequency of amputations and mortality rate in the group of patients with diagnosed CAD. It is also worth pointing out that patients with CAD are characterized by a twofold rise in the reintervention rate in the previous treated lesion. Although results are not statistically significant, it shows a trend of increased tendency of adverse events, including MAPE, in long-term follow-up.

What is more, recent studies show comparison differences between reducing risk factors in patients with CAD and PAD. Patients with CAD are more intensively treated for atherosclerotic risk factors than PAD patients [25, 26]. Administration of hypolipemic drugs was higher in patients with hypercholesterolemia and CAD than in patients with high cholesterol level and PAD (58% vs. 46%) [26].

Our study confirmed that most of the results from studies designed for the general population of patients with PAD can be extrapolated to a subgroup of patients after retrograde recanalization of the femoropopliteal region. Coexistence of DM in those patients is correlated with higher all-cause mortality rate in long-term follow-up, although the amputation rate and TVR revascularization were uninfluenced by DM or CAD. However, the expected impact of CAD on MACCE and MAPE was not confirmed in this study. The study group included a small number of selected patients with CTO and unsuccessful antegrade recanalization. Patients with CAD were on average 10 years younger than the non-CAD group, which could have influenced the results. What is more, the study was planned as a retrospective registry which may be considered limitation; for example, data about duration of DM and level of glycemia control were not collected.

5. Conclusion

Patients, after retrograde femoropopliteal recanalization, with coexisting CTO and DM, are at higher risk of death than non-DM patients. Despite our previous study, we do not confirm the impact of CAD on the long-term follow-up in patients with PAD. However, complex treatment of those patients should be focused on intensively reducing atherosclerotic risk factors.

Data Availability

All external data used in this paper are listed in References.

Conflicts of Interest

All authors listed meet the authorship criteria according to the latest guidelines of the International Committee of Medical Journal Editors. The authors declare that there is no conflict of interest.

Authors' Contributions

All authors agree with the contents of the manuscript.

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Research Article

Factors Associated with Health-Related Quality of Life among Jordanian Patients with Diabetic Foot Ulcer

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Objective. This study is aimed at determining factors associated with the quality of life among Jordanian diabetic patients with foot ulcers. *Methods*. 144 consecutive patients with diabetic foot ulcers aged ≥ 18 years who were attending the diabetic foot clinic at a diabetes-specialized center were included in this study. Health-related quality of life was assessed using two self-administered questionnaires: Diabetic Foot Scale-Short Form (DFS-SF) and Short Form-8 (SF-8). *Results*. Patients with diabetic foot ulcer had low mean DFS-SF score and low mean scores on physical and mental component summary scales (PCS8 and MCS8). Males had significantly higher DFS-SF score indicating better health-related quality of life than females (P value 0.038). A patient with stressful life events had significantly lower health-related quality of life using DFS-SF scale and SF-8 summary scales. Patients with peripheral vascular disease (PVD) and patients with obesity had lower DFS-SF and PCS8 quality of life. *Conclusion*. Patients with diabetic foot ulcer had low quality of life. Female gender, obesity, presence of PVD, and stressful life events were the most important factors associated with lower quality of life in patients with diabetic foot ulcer.

1. Introduction

Diabetic foot ulcers have substantial economic burden on health care systems [1]. It is estimated that 15% of all diabetic patients will develop a foot ulcer during the course of their lifetime [2]. Diabetic foot ulcers progress to major amputation in 14% to 24% of patients [3]. The five-year mortality rate is also high, reaching 50-68% among patients who undergo major lower limb amputation [4–6]. Additionally, diabetic foot ulcers markedly increase the morbidity in patients with diabetes, leading to an increase in the number of outpatient appointments and emergency room visits as well as hospitalization days with greater risks of osteomyelitis and amputation [7–10].

Diabetic foot ulcers negatively affects patients' perceived Health-Related Quality of Life (HRQoL) due to decreased mobility and consequently the ability to perform daily activities and increasing dependence on others [11, 12]. Moreover, the perceived stress linked to wound healing or reulceration and the fear of foot amputation both increase the negative mood and lead to sleep disturbance in patients with diabetic foot ulcers [13]. Reduction of quality of life in such patients not only affects the outcome of treatment but also increases health care expenditures as a result of the frequent referring to physicians and clinical care settings [14]. Psychological comorbidity such as depression confers additional risks on diabetic patients resulting in poorer outcomes and poorer self-care. Depression in type 2 diabetes had been shown to be associated with twice the rate of first diabetic foot ulcer over 4 years of follow-up period and higher rates of amputation [15]. Moreover, depression in patients developing the first diabetic foot ulcer is associated with twofold increase of mortality over 5 years [16].

Although the impact of diabetic foot ulcers on HRQoL was studied in many countries, there is scarcity of data in Jordan on the impact of diabetes complications including

diabetic foot ulcers on HRQoL. The degree by which diabetic foot ulcers impairs the quality of life is population specific. Therefore, our study was conducted to determine the impact of diabetic foot ulcers on patients' HRQoL and determine its associated factors among Jordanian patients with diabetes.

2. Methods

2.1. Study Design. A cross-sectional study was conducted among 144 patients with diabetic foot ulcers who attended the National Center for Diabetes, Endocrinology and Genetics (NCDEG) in Jordan. Patients were included in the study if their age was ≥18 years. Patients who attended the clinic twice or more during the study period of three months were interviewed during their first attendance. Pregnant or lactating women and patients with history of stroke, cancer, or mental retardation were excluded. Patients who met the inclusion criteria had been invited to participate in the study after explaining the study and its goal. All participants who agreed to participate in this study had signed the informed consent. The study was approved by the ethics committee at NCDEG.

2.2. Data Collection. A self-administered questionnaire was used to collect data on sociodemographic and clinical characteristics. Findings from the physical assessment were recorded in the questionnaire including presence of neuropathy symptoms, presence of peripheral vascular disease (PVD), site of the ulcer, number of ulcers, recurrence of the ulcer, duration of the ulcer, presence of previous amputation, and ulcer classification grade 1, 2, 3, 4, or 5 according to the Wagner classification [17]. Other relevant data were abstracted from the medical records including diabetes complications and comorbidity, anthropometric, and biomedical data.

2.3. Physical Assessment. Vascular assessment was determined by palpating dorsalis pedis and posterior tibial pulses, presence of intermittent claudication, and assessment of clinical signs and symptoms of ischemia (loss of hair, shine skin, pale skin, and skin temperature). Neurological assessment was performed for detecting the presence of neuropathic symptoms such as numbness, tingling pain, and burning sensation. Musculoskeletal assessment was performed for detecting the presence of previous amputation. Ulcer assessment included ulcer site, recurrence of the ulcer, ulcer duration, number of ulcers, and ulcer classification grade 1, 2, 3, 4, or 5 according to the Wagner classification [17].

2.4. Diabetic Foot Scale (DFS-SF). The DFS is a descriptive system, which provides a comprehensive measurement of the impact of diabetic foot ulcers on patients' quality of life. The DFS consists of 29 items comprising six subscales. The six domains are leisure (enjoying life), physical health, daily activities' dependence, negative emotions, concern about wound, and wound care. After we took the permissions for use of DFS-SF from Mapi Research Trust (MRT), the questionnaire was translated into Arabic using forward-backward method. The DFS-SF subscale scores were computed based on scoring conventions published elsewhere [18]. Items were aggregated within each six subscales and then transformed to a score from 0 to 100, with higher scores indicating better quality of life for each subscale. The final version was pilot-tested among 24 patients and the necessary changes had been made. The internal consistency of subscales (Cronbach's alpha) ranged from 0.74 to 0.83. The instrument demonstrated good constructional validity when correlated with the SF-8.

2.5. SF-8 Health Survey. The SF-8 was developed to replicate the SF-36 version 2 with one question for each health domain. The eight domains are vitality, physical functioning, bodily pain, general health perceptions, physical role functioning, emotional role functioning, social role functioning, and mental health. Each SF-8 single-item scale and the SF-8 summary measures were scored on the same norm-based metrics as the SF-36 scales and summary measures [19]. The Arabic version which has been translated and culturally adapted in Lebanon was used [20].

2.6. Measurements and Definitions. Anthropometric measurements, including weight, height, and waist circumference, were measured while the subjects were wearing light clothing and no shoes. Body mass index (BMI) was expressed as the quotient between weight (kg) and height in meter squared. Patients were classified according to BMI following the recommendation of the World Health Organization as adopted by the American Diabetes Association [21]. Readings of systolic and diastolic blood pressures were taken while the subjects were seated and the arm was kept at the heart level, after at least five minutes of rest, using a standardized mercury sphygmomanometer. High blood pressure was defined as systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 80 mmHg or if the patient was already on antihypertensive drugs [22]. Diabetes mellitus was diagnosed if the patient had a FPG ≥ 126 mg/dL (7.0 mmol/L) in two occasions or if the patient had a random plasma glucose ≥ 200 mg/dL (11.1 mmol/L) in the presence of classical symptoms of hyperglycemia or if he or she had HbA1c ≥ 6.5%. Moreover, diabetes was considered to be controlled if the patient had HbA1c < 7.0% according to the American Diabetes Association (ADA) 2011 guidelines [22]. Metabolic abnormalities were defined according to the American Diabetes Association 2011 [22]. Smoking was classified into three categories according to WHO guidelines 1998 [23, 24].

Retinopathy was diagnosed if it was documented by either the ophthalmologist or the treating physician in the medical records or if the patient had received laser treatment. Neuropathy was diagnosed if there was any of the following symptoms (numbness, tingling, or pain in the toes, feet, legs, hands, arms, and fingers) in patient's medical records or if the patient had done Nerve Conduction Study (NCS) which proves the presence of diabetic neuropathy or if the patient was receiving treatment for the above condition.

Stressful life event during the last year was assessed by life events, described as death, divorce, marital separation, illness, personal injury, imprisonment, dismissal from work, and retirement. Lower limb ischemia was defined as absent

Table 1: Sociodemographic and clinical characteristics of the study participants ($N = 144$).

Variable	No. (%)
Age (year)	
<50	28 (19.4)
50-60	64 (44.4)
>60	52 (36.1)
Gender	
Female	42 (29.2)
Male	102 (70.8)
Marital status	
Married	120 (83.3)
Not married	24 (16.7)
Educational status	
≤high school	82 (56.9)
>high school	62 (43.1)
Health insurance	
Insured	113 (78.5)
Uninsured	31 (21.5)
Occupational status	
Employed	36 (25.0)
Unemployed	108 (75.0)
Total family monthly income (JD)	
≤500	84 (58.3)
>500	60 (41.7)
Smoking	
Nonsmoker	110 (76.4)
Smoker	34 (23.6)
Stressful events in the last year	
Yes	91 (63.2)
No	53 (36.8)
Type of diabetes mellitus	
Type 1 DM	11 (7.6)
Type 2 DM	133 (92.4)
Duration of diabetes mellitus (years)	
≤10	43 (29.9)
>10	101 (70.1)
Type of treatment	
Insulin therapy	16 (11.1)
Oral	19 (13.2)
Insulin and oral	109 (75.7)
Control of diabetes	
Controlled	23 (16.0)
Uncontrolled	121 (84.0)
Hypertension	
Yes	108 (75.0)
No	36 (25.0)
Dyslipidemia	
Yes	112 (77.8)
No	32 (22.2)
Peripheral neuropathy	
Yes	142 (98.6)
No	2 (1.4)
Peripheral vascular disease	
Yes	42 (29.2)
No	102 (70.8)
Retinopathy	
Yes	80 (55.6)
No	64 (44.4)
Coronary artery disease	
Yes	43 (29.9)
No	101 (70.1)
Body mass index****	
Obese	83 (57.6)
Nonobese	58 (40.3)

posterior tibial artery pulses with or without symptoms and signs of PVD or absent dorsalis pedis pulses with at least one symptom or sign indicating PVD. These symptoms and signs include intermittent claudication, edema, mottled skin, loss of hair, cold feet, and cyanotic feet. Osteomyelitis was diagnosed as physician diagnosis of osteomyelitis, which is based on radiological findings and or positive probe-to-bone test. Minor amputation refers to any amputation performed below the level of the ankle (forefoot, midfoot, or hindfoot). Major amputation refers to any amputation performed above the level of the ankle (below the knee or above the knee).

2.7. Statistical Analysis. Data was analyzed using the statistical program for social sciences (SPSS) version 16. Descriptive statistics used the means and standard deviations (SD) for continuous variables and used the frequency distribution for categorical variables. One-way Analysis Of Variance (ANOVA) was used to analyze the differences among group means. Multivariate Analysis of Variance was used to examine the net effect for each of the independent variable on quality of life scales and subscales. A P value of ≤0.05 is considered significant.

3. Results

3.1. Participants' Characteristics. A total of 144 participants aged between 24 and 90 years with a mean age (SD) of 56.8 (11.0) were included in the study. The sociodemographic, anthropometric, and clinical characteristics of the study population are presented in Tables 1 and 2.

3.2. Quality of Life and Subscales. The overall average score of DFS-SF was 42.1 (17.0). Table 3 shows the mean (SD) scores of the six subscales of DFS-SF. The mean scores were 36.7 (20.1) for leisure/enjoying life, 44.2 (22.6) for physical health, 48.2 (25.7) for dependency/daily life, 43.5 (24.6) for negative emotions, 32.7 (24.2) for worried about ulcer, 46.1 (27.8) for

Table 2: Foot ulcer characteristics among diabetic patients under treatment in the National Center for Diabetes, Endocrinology and Genetics ($N = 144$).

Variable	No. (%)
Duration of foot ulcer	
<1 month	56 (38.9)
1-3 months	37 (25.7)
>3 months	51 (35.4)
Number of foot ulcers	
1 ulcer	112 (77.8)
≥2 ulcers	32 (22.2)
Offloading device	
None	37 (25.7)
Felted foam padding	47 (32.6)
Half shoe	19 (13.2)
Removable cast walker	34 (23.6)
Total contact cast	7 (4.9)
Site of foot ulcer	
Forefoot	115 (79.9)
Midfoot	16 (11.1)
Hindfoot	13 (9.0)
Wagner classification of foot ulcer	
Grade 1	42 (29.2)
Grade 2	57 (39.6)
≥Grade 3	45 (31.2)
Soft tissue infection	
Yes	71 (49.3)
No	73 (50.7)
Osteomyelitis	
Yes	52 (36.1)
No	92 (63.9)
Recurrence of ulcer	
Yes	71 (49.3)
No	73 (50.7)
Charcot foot	
Yes	19 (13.2)
No	125 (86.8)

bothered by ulcer care, 39.3 (9.9) for physical component summary-8, and 41.9 (11.1) for mental component summary-8. The summary scores showed a lower physical component summary score than mental component summary score. Table 4 shows the mean scores of DFS-SF, PCS8, and MCS8 according to sociodemographic, clinical, and diabetic foot characteristics. Male gender, >high school level of education, no stressful events in the last year, not having PVD, absence of soft issue infection, lower Wagner classification grade, and normal body weight were significantly associated with higher DFS-SF scores, indicating better quality of life.

3.3. Factors Associated with the Quality of Life of Patients with Diabetic Foot Ulcer. Table 5 shows the multivariate analysis of factors associated with the quality of life scales. In the multivariate analysis, the only factors that remained significantly associated with the quality of life were gender, stressful events, PVD, and BMI. Males had significantly higher DFS-SF score indicating better health-related quality of life than females (P value 0.038). Patient with stressful life events had significantly lower health-related quality of life using DFS-SF scale and SF-8 summary scales. Patients with PVD and patients with obesity had lower DFS-SF and PCS8 quality of life.

Table 3: Mean scores for the Diabetic Foot Scale-Short Form and its subscales and the two summaries of Short Form-8 (physical and mental component summaries) for the quality of life of diabetic foot ulcer patients.

Variables	QoL mean (SD)	CI (95%)
Leisure/enjoying life	36.7 (20.1)	33.4–40.0
Physical health	44.2 (22.6)	40.5–47.9
Dependency/daily life	48.2 (25.7)	44.0–52.4
Negative emotions	43.5 (24.6)	39.4–47.5
Worried about ulcer	32.7 (24.2)	28.7–36.7
Bothered by ulcer care	46.1 (27.8)	41.5–50.7
DFS-SF score	42.1 (17.0)	39.3–44.9
PCS8	39.3 (9.9)	37.6–40.9
MCS8	41.9 (11.1)	40.0–43.7

Table 6 shows the multivariate analysis of factors associated with the quality of life subscales. Females scored significantly lower than males on the physical health and negative emotions DSF-SF subscales than men. Patients who had an educational level of more than high school were more worried about ulcer. Those with family income more than 500 JDs scored higher on physical health subscale. Scores in the most of DFS-SF subscales were lower in patients who had stressful life events in the last year. Patients who did not have ischemic foot ulcer had a better health-related quality of life on dependency/daily life and worried about ulcer subscales. Presence of retinopathy was associated with poor quality of life on leisure/enjoying life as well as dependency subscales. Patients with obesity scored significantly lower on bothered by ulcer care subscale.

4. Discussion

In this study, diabetic patients with foot ulcers had low DFS-SF, PCS8, and MCS8 scores. Diabetic foot ulcers have been shown to have a high impact on the quality of life. Ashford's study reported that families of diabetic foot ulcers patients were unable to do certain procedures, which led to family-related problems. Such problems included wound dressing, moderate mobility reduction shopping, and taking a shower and had a negative impact on patients' quality of life [25]. Goodridge et al. showed that patients with diabetic foot ulcers had a poorer physical quality of life than patients with unhealed ulcers [26]. Recent US and UK studies showed that diabetic foot ulcers adversely affect the quality of life of patients [27, 28].

Table 4: Mean scores of Diabetic Foot Scale-Short Form and the two component summary of Short form-8 according to sociodemographic, clinical, and diabetic foot characteristics.

Clinical variables	DFS-SF mean (SD)	PCS8 mean (SD)	MCS8 mean (SD)
Gender			
Male	44.9 (17.5)	40.4 (9.8)	42.8 (11.5)
Female	35.4 (14.0)	36.5 (9.9)	39.7 (9.9)
P value	0.002*	0.034*	0.126
Educational status			
≤high school	39.0 (15.4)	37.3 (9.5)	39.9 (10.7)
>high school	46.2 (18.3)	41.9 (10.0)	44.4 (11.2)
P value	0.011*	0.006*	0.017*
Occupational status			
Employed	45.0 (18.4)	42.1 (9.1)	43.4 (11.7)
Unemployed	41.1 (16.5)	38.3 (10.1)	41.4 (10.9)
P value	0.242	0.049*	0.351
Stressful events in the last year			
Yes	36.9 (14.5)	37.4 (9.7)	39.7 (11.0)
No	51.0 (17.6)	42.5 (9.5)	45.5 (10.4)
P value	0.000*	0.003*	0.003*
Duration of foot ulcer (month)			
<1	45.8 (17.3)	42.1 (10.0)	44.0 (10.6)
1-3	39.5 (13.6)	37.4 (10.0)	40.2 (10.1)
>3	39.9 (18.5)	37.5 (9.2)	40.7 (12.1)
P value	0.116	0.022*	0.170
PVD			
Yes	35.8 (13.2)	35.9 (8.9)	40.1 (10.7)
No	44.7 (17.8)	40.7 (10.1)	42.6 (11.2)
P value	0.004*	0.009*	0.235
Offloading device			
None	43.9 (17.9)	41.1 (10.5)	40.2 (10.8)
Felted foam padding	47.2 (18.4)	40.0 (10.1)	45.0 (11.0)
Half shoe	40.7 (13.4)	39.1 (9.8)	40.5 (10.9)
Removable cast walker	36.4 (15.0)	37.8 (9.4)	40.2 (12.0)
Total contact cast	30.3 (5.7)	32.9 (7.0)	40.9 (6.2)
P value	0.016*	0.280	0.223
Infection			
Yes	39.1 (15.9)	38.6 (10.3)	41.7 (10.7)
No	45.1 (17.7)	40.0 (9.6)	42.0 (11.5)
P value	0.035*	0.400	0.886
Amputation			
Yes/minor	39.6 (17.5)	39.0 (8.4)	38.8 (11.6)
No	43.3 (16.7)	39.4 (10.6)	43.3 (10.6)
P value	0.227	0.812	0.023*
Wagner classification			
Grade 1	48.3 (18.4)	41.0 (10.2)	44.7 (10.1)
Grade 2	40.3 (15.6)	39.6 (9.9)	40.8 (10.9)
≥Grade 3	38.6 (16.2)	37.3 (9.6)	41.6 (12.0)
P value	0.016*	0.226	0.149
Type of diabetes			
Type 1	45.7 (20.4)	40.3 (10.1)	48.5 (13.6)
Type 2	41.8 (16.8)	39.2 (10.0)	41.3 (10.8)
P value	0.470	0.734	0.037*
BMI			
Nonobese	46.3 (17.1)	41.6 (9.7)	43.5 (11.1)
Obese	39.5 (16.4)	37.8 (9.9)	40.8 (11.2)
P value	0.018*	0.026*	0.159

*P value < 0.05.

Table 5: Multivariate analysis of factors associated with quality of life.

Variables	Quality of life		
	DFS-SF	SF-8	
		PCS8	MCS8
Gender			
Male	44.9 (17.5)	40.4 (9.8)	42.8 (11.5)
Female	35.4 (14.0)	36.5 (9.9)	39.7 (9.9)
P value	0.038*	0.146	0.306
Stressful life events			
Yes	36.9 (14.5)	37.4 (9.7)	39.7 (11.0)
No	51.0 (17.6)	42.5 (9.5)	45.5 (10.4)
P value	0.000*	0.013*	0.006*
PVD			
Yes	35.8 (13.2)	35.9 (8.9)	40.1 (10.7)
No	44.7 (17.8)	40.7 (10.1)	42.6 (11.2)
P value	0.004*	0.016*	0.147
BMI			
Nonobese	46.3 (17.1)	41.6 (9.7)	43.5 (11.1)
Obese	39.5 (16.4)	37.8 (9.9)	40.8 (11.2)
P value	0.024*	0.036*	0.695

*P value < 0.05.

Our data showed that females had significantly lower health-related quality of life than males. Women are likely to be more concerned about their health conditions and their impact on family environment than men, particularly among housewives. In agreement with our finding, most previous studies had shown that males had better health than females. Lebanese women had a lower quality of life than Lebanese men [20]. Canadian men had markedly higher scores than women in all SF-36 Health Survey domains [29]. Similarly, US men fared better than women in all SF-36 domains [30]. Except for the general health domain, British male scores were also higher than females [31]. Other studies also showed that women had a poorer quality of life [32].

PVD and diabetes often entail neuropathy, foot ulcer, increased risk of developing gangrene, ischemia, and amputation to lower extremities. Impaired lower extremity

Table 6: Multivariate analysis of factors associated with quality of life subscales.

Clinical variables	Leisure/enjoying life mean (SD)	Physical health mean (SD)	Dependency/daily life mean (SD)	Negative emotion mean (SD)	Worried about ulcer mean (SD)	Bothered by ulcer care mean (SD)
Gender						
Male	38.8 (20.6)	48.3 (22.1)	50.5 (26.6)	41.4 (24.5)	47.8 (28.0)	47.1 (27.6)
Female	31.4 (18.0)	34.3 (20.9)	42.6 (22.7)	28.8 (16.5)	33.0 (20.8)	43.8 (28.5)
P value	0.187	0.023*	0.705	0.015*	0.796	0.937
Educational status						
≤high school	36.8 (20.5)	39.1 (21.2)	45.1 (23.7)	39.0 (22.0)	27.5 (20.7)	45.3 (27.0)
>high school	36.5 (19.7)	50.9 (22.8)	52.3 (27.8)	49.4 (26.7)	39.5 (26.9)	47.2 (29.0)
P value	0.110	0.181	0.769	0.338	0.024*	0.554
Family income						
≤500	36.9 (18.4)	40.1 (22.6)	46.6 (23.8)	40.6 (23.9)	30.7 (22.1)	46.1 (28.0)
>500	36.3 (22.4)	50.0 (21.4)	50.4 (28.3)	47.5 (25.1)	35.5 (26.9)	46.1 (27.7)
P value	0.842	0.033*	0.472	0.332	0.562	0.909
Stressful events						
Yes	32.0 (19.2)	37.1 (21.5)	42.7 (24.0)	38.0 (22.2)	29.5 (21.8)	41.4 (27.2)
No	44.7 (19.1)	56.3 (19.2)	57.5 (26.1)	52.8 (25.9)	38.2 (27.2)	54.1 (27.2)
P value	0.000*	0.000*	0.001*	0.009*	0.261	0.008*
PVD						
Yes	33.8 (17.1)	40.6 (18.0)	37.1 (21.3)	38.2 (22.8)	24.7 (21.4)	38.4 (26.3)
No	37.8 (21.2)	45.7 (24.1)	52.7 (26.1)	45.6 (25.1)	36.0 (24.6)	49.3 (27.9)
P value	0.460	0.573	0.005*	0.088	0.018*	0.118
Retinopathy						
Yes	34.1 (17.7)	43.4 (21.1)	42.6 (24.3)	42.2 (24.7)	32.9 (24.2)	44.7 (26.5)
No	39.9 (22.5)	45.2 (24.5)	55.2 (25.9)	45.1 (24.6)	32.4 (24.5)	47.9 (29.4)
P value	0.031*	0.327	0.007*	0.605	0.323	0.886
BMI						
Nonobese	39.7 (17.7)	47.8 (23.7)	52.5 (27.5)	47.4 (23.7)	35.9 (24.1)	54.0 (27.6)
Obese	34.4 (21.5)	41.8 (22.0)	46.0 (24.2)	40.7 (24.7)	30.9 (24.5)	41.5 (26.9)
P value	0.401	0.346	0.652	0.275	0.421	0.022*

*P value < 0.05.

functioning is considered an important predictor of future disability and may lead to poorer quality of life [33–35]. In agreement with previous studies, patients with PVD had lower quality of life than patients without PVD; Dolan et al. found that subjects with PAD and diabetes have poorer lower extremity function than those with PAD alone [33]; Siersma et al. also reported that factors such as limb threatening ischemia, inability to stand or walk independently, and ulcer size were the most important contributors to health-related quality of life [34]. In addition, Lloyd et al. also proved that PVD in diabetic patients was significantly associated with lower physical and social functioning scale scores [35].

Our data also showed that obese diabetic patients with foot ulcers had significantly lower quality of life than nonobese diabetic patients with foot ulcers. Consistent with our result, Redekop et al.'s study also showed that obesity was related to poorer quality of life in T2DM patients [36].

Our study showed that patients with stressful life events scored lower than those without stressful life events on health-related quality of life. Stressful life events, linked to wound healing, will eventually mark an increase in the negative mood and result in improper sleep patterns [37]. Recently, many studies have illustrated the mechanism of stress in slowing the healing rate of acute and chronic ulcers, which leads to long-term ulcer care and this creates further burden, pressure, and low quality of life.

5. Conclusion

Patients with diabetic foot ulcer had low quality of life. Female gender, obesity, presence of PVD, and stressful life events were the most important factors associated with lower quality of life in patients with diabetic foot ulcer. Further studies are needed to assess all variables that may impact the quality of life in patients with diabetes in general and diabetic foot ulcer in particular.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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