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Pre-diabetes and diabetic neuropathy are associated with low serum levels of interleukin-9

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Abstract

Background Interleukin-9 (IL-9) is a cytokine that has recently been proposed to be associated with type 2 diabetes mellitus (T2DM) risk, but the role it plays in the development of pre-diabetes (PD) and diabetic neuropathy (DN) is unknown. Therefore, this study analyzed serum IL-9 levels in individuals with PD (n = 89), T2DM patients without DN (n = 66), T2DM patients with DN (n = 21), and non-diabetic controls (n = 84) using an ELISA kit**.**

Results Serum IL-9 levels (median and interquartile range) were significantly lower in the PD (18.9 [12.6–22.1] pg/mL; probability [*p*] < 0.001) and T2DM (19.4 [16.3–28.0] pg/mL; $p = 0.04$) groups than in the control group (20.8 [19.4–25.8] pg/mL). Patients with DN also showed lower levels of IL-9 than patients without DN, but the *p* value was not significant (19.4 [12.5–22.7] vs. 20.6 [17.1–28.1] pg/mL; $p = 0.13$). IL-9 showed better diagnostic performance in PD and T2DM with DN than in T2DM without DN (area under the curve: 0.699 and 0.702 vs. 0.567, respectively). Moreover, lower levels of IL-9 were significantly associated with PD and DN risks (odds ratio = 0.86 and 0.85, respectively).

Conclusions Serum IL-9 levels were significantly decreased in individuals with PD and patients with T2DM compared with HC. The decrease in IL-9 levels in T2DM patients was more pronounced in patients with DN than in patients without DN. Therefore, low levels of IL-9 can be considered as a potential biomarker associated with an increased risk of PD and DN.

Keywords Pre-diabetes, Type 2 diabetes mellitus, Diabetic neuropathy, Interleukin-9, Receiver operating characteristic curve, Odds ratio

1 Background

The metabolic disorder type 2 diabetes mellitus (T2DM) is a public health issue of global concern. In 2017, the global prevalence of T2DM was approximately 6000 cases per 100,000 population and the disease burden is projected to reach 7000 individuals per 100,000 population by 2030 [1]. Moreover, in addition to being associated with a high mortality rate (more than 1 million deaths annually), T2DM is a major cause of various clinical complications such as retinopathy, neuropathy, and nephropathy [2].

Chronic hyperglycemia is the hallmark of T2DM, and primarily results from impaired insulin production by

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pancreatic beta-cells, as well as the inability of tissues to respond adequately to insulin [3]. These insulin-related functional defects occur in T2DM patients as a result of the interaction of several predisposing factors, including genetic, environmental, and metabolic risk factors. Among these factors are lifestyle, family history, ethnicity, psychosocial status, smoking, obesity, cardiovascular disease, dyslipidemia, hypertension, aging, and physical inactivity [4]. Compelling evidence suggests that these interactions are associated with dysregulated immunity and an enhanced inflammatory response, which are key pathophysiologic phenomena that increase the risk of developing T2DM and its complication [5]. Observational and experimental investigations have disclosed that T2DM patients exhibit a common feature of low-grade inflammation, the level of which correlates with disease-related features such as obesity, hyperglycemia, and insulin resistance [6].

The key regulators of inflammatory responses and mediators of cellular interactions are cytokines, which act through pro-inflammatory and anti-inflammatory effects. The process of inflammation is mediated by pro-inflammatory cytokines, while resolution of inflammation is associated with anti-inflammatory cytokines [7]. In T2DM, cytokines are indicated as major mediators of inflammation, and there is clear evidence to suggest that adipokines (leptin, adiponectin, and resistin), hepatokines (hepassocin, fibroblast growth factor 21, and fetuins A and B), pro-inflammatory cytokines (tumor necrosis factor-alpha [TNF-α], interleukin [IL]-1beta, and IL-6), myokines (IL-13, IL-15, and irisin), and osteokines (osteocalcin, osteopontin, lipocalin 2, and sclerostin) are significantly linked to abnormal glucose metabolism, insulin resistance, and consequently T2DM [8].

Among the cytokines that have recently attracted attention in inflammatory conditions is IL-9, and suggestive evidence has revealed a role for this cytokine in the regulation of inflammatory responses [9]. IL-9 was first discovered in 1988 as a cytokine produced by T helper (h)2 cells, but other cellular resources have recently been described. It has been found that IL-9 is also produced by Th17 cell, T regulatory cell, natural killer T cell, and type 2 innate lymphoid cell [10]. In addition, IL-9 is specifically produced by the recently discovered CD4 + T cell, the Th9 cell. Therefore, IL-9 is considered a signature cytokine for this cell [11]. Early studies recognized the pleotropic effects of IL-9 and its impact on the pathogenesis of asthma and bronchial hyperactivity, as well as immunity against parasites [12]. Elevated levels of IL-9 were reported in the serum and sputum of patients with asthma, and were negatively associated with the percentage of apoptotic eosinophils [13, 14]. It has also been suggested that IL-9 can induce airway eosinophilia by regulating the IL-5 response and potentiating the IL-5-mediated maturation of eosinophil precursors [15]. However, recent studies have renewed interest in IL-9 and have linked this cytokine to systemic inflammation in some human inflammatory conditions such as psoriasis, alcoholic liver injury, multiple sclerosis, and doxorubicin-induced cardiotoxicity. Dysregulated expression of IL-9 has been reported in these immune-mediated diseases, and its association with pathogenesis has been proposed [16–19]. Additional studies have also indicated that IL-9 may play an etio-pathological role in T2DM, although with conflicting results. It has been shown that IL-9 levels were significantly lower in patients with T2DM than in individuals with normal glucose tolerance or patients with diabetic kidney disease [20]. Another study reported inconsistent findings and IL-9 levels were significantly elevated in T2DM patients compared to a control group [21]. A further study linked IL-9 to depression in patients with T2DM [22]. However, the association of IL-9 with pre-diabetes (an intermediate state of hyperglycemia that precedes T2DM) and complications of T2DM such as retinopathy and neuropathy is poorly understood.

In this study, serum IL-9 levels were analyzed in individuals with pre-diabetes (PD) and patients with T2DM. Diabetic neuropathy (DN) was also considered in the analysis. The correlation of IL-9 with some clinical and laboratory indicators of diabetes was also evaluated. Data in this regard are either limited or unavailable, and this study may develop concepts for a better understanding of the potential prognostic significance of IL-9 in predicting the risks of PD, T2DM, and DN.

2 Methods

2.1 Study groups

Three groups were enrolled in a cross-sectional, case–control study from November 2021 to June 2022. The first group included 89 individuals with PD (47.2% male and 52.8% female). They were apparently healthy individuals who visited Primary Health Care Centers for laboratory tests of general health. The second group included 87 T2DM patients (51.7% male and 48.3% female) who were on oral hypoglycemic medication. The included patients were 18 years of age and older and had non-insulin dependent T2DM. Patients with T1DM, gestational diabetes, chronic diseases, and cancer were excluded. Twenty-one T2DM patients (24.1%) developed DN (42.9% males and 57.1% females). DN was diagnosed by a consultant endocrinologist using the modified TCNS system (Toronto Clinical Neuropathy Scoring) [23]. The suggested TCNS classification is 0–5 points (no DN), 6–8 points (mild DN), 9–12 point (moderate DN), and 13–19 points (severe DN). In the current study, DN patients

were classified as mild DN (TCNS: 6–8 points). The third group included 84 healthy individuals from blood donors and university employees who did not suffer from chronic diseases (HC group; 50.0% male and 50.0% female).

Body mass index (BMI) was calculated for each participant in addition to examining lipid profile parameters. To determine the glycemic status of the participants, the American Diabetes Association (ADA) guidelines were followed. In this context, two laboratory assays were performed, which included fasting plasma glucose (FPG) and glycated hemoglobin (HbA1c). According to these guidelines, FPG was < 100, 100–125, and ≥ 126 mg/dL, and HbA1c was < 5.7, 5.7–6.4, and ≥ 6.5% in HC, PD, and T2DM, respectively [24].

2.2 IL-9 immunoassay

Serum IL-9 levels were measured according to the principles of an enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Cat. No.: E-EL-H0180). The instructions of the manufacturer were followed (Elabscience Biotechnology Inc., USA). IL-9 levels were calculated according to a plotted standard curve (concentration range: 0–1000 pg/mL). The kit sensitivity was 9.38 pg/mL.

2.3 Statistical analyses

The normal distribution of continuous variables was analyzed using Kolmogorov–Smirnov test. These variables followed a nonparametric pattern. Therefore, median with interquartile range (IQR: 25–75%) was used to express these variables. Significance was assessed with Mann–Whitney U test or Kruskal–Wallis test. Receiver operating characteristic (ROC) curve analysis was adopted to explore the diagnostic performance by estimating the area under the curve (AUC) and its 95% confidence interval (CI). Multinomial logistic regression analysis was performed to evaluate the association with disease risk by calculating the odds ratio (OR) and 95% CI. The correlation coefficient (r_s) between variables was determined using Spearman's rank-order correlation test. A probability (p) less than 0.05 was considered significant. IBM SPSS Statistics version 25 (Armonk, NY: IBM Corp) was used to perform statistical analyses and GraphPad Prism version 9.4.1 (San Diego, CA, USA) was used to plot the figures.

3 Results

3.1 Baseline characteristics and laboratory data

All data were given as median and IQR. Age was significantly higher in the PD (49 [41–55] years; $p = 0.017$) and T2DM (54 [44–61]; $p < 0.001$) groups than in the HC group (41 [34–55] years), while the difference was not significant between the PD and T2DM groups ($p = 0.105$). BMI was significantly elevated in the PD (31.1 [29.3–33.7] kg/cm^2; $p < 0.001$) and T2DM (29.5 [27.2–33.4] kg/cm^2; $p < 0.001$) groups compared to the HC group (26.7 [24.6–28.6] kg/m^2). BMI was also significantly elevated in individuals with PD compared to T2DM patients ($p = 0.04$). FPG and HbA1c levels in the PD, T2DM, and HC groups followed the reference ranges set by the ADA standards [24], and thus the glycemic status in each group was ascertained. Regarding lipid profile parameters, in general, their levels were significantly increased in the PD and T2DM groups compared to the HC group with a few exceptions as shown in Table 1.

3.2 IL-9 levels

IL-9 showed significantly lower serum levels (median and IQR) in the PD (18.9 [12.6–22.1] pg/mL; $p < 0.001$) and T2DM (19.4 [16.3–28.0] pg/mL; $p = 0.04$) groups than in the HC group (20.8 [19.4–25.8] pg/mL). These levels were also lower in individuals with PD than in patients with T2DM but the difference was close to significant ($p = 0.051$). IL-9 tended to show lower levels in males than in females in the PD, T2DM, and HC groups, but the differences were not significant. T2DM patients with DN also showed lower levels of IL-9 than patients without DN, but the difference did not reach a significant level (19.4 [12.5–22.7] vs. 20.6 [17.1–28.1] pg/mL; $p = 0.13$) (Fig. 1). ROC curve analysis of IL-9 was conducted in PD, T2DM without DN, and T2DM with DN versus HC. As indicated by the AUC value, IL-9 showed better diagnostic performance in PD and T2DM with DN compared to T2DM without DN (AUC: 0.699 and 0.702 vs. 0.567, respectively) (Fig. 2). Crude and age-, gender-, and BMI-adjusted multinomial logistic regression analysis indicated the significance of IL-9 in the risks of PD, T2DM without DN, and T2DM with DN. The adjusted OR was 0.86 for PD (95% CI 0.81–0.92; $p < 0.001$), 0.94 for T2DM without DN (95% CI 0.88–0.99; $p = 0.024$), and 0.85 for T2DM with DN (95% CI 0.77–0.94; $p = 0.001$) (Table 2).

3.3 Correlation coefficients relating IL-9 and diabetes variables

A bivariate non-parametric correlation analysis (Spearman's rank-order correlation test) between IL-9 levels and diabetes variables (age, BMI, FPG, HbA1c, and lipid profile parameters) was performed in each group studied (PD, T2DM, and HC). The analysis revealed that IL-9 was negatively correlated with FPG in PD ($r_s = -0.219$; $p = 0.039$), T2DM ($r_s = -0.224$; $p = 0.037$), and HC ($r_s = -0.216$; $p = 0.049$). In addition, IL-9 showed a positive correlation with BMI ($r_s = 0.267$; $p = 0.012$) and a negative correlation with HbA1c ($r_s = -0.226$; $p = 0.035$) in T2DM. Among the HC group, IL-9 showed a negative

Table 1 Baseline characteristics and laboratory data of study groups

Characteristic	Median (IQR; 25–75)			p_1-value (PD vs. HC)	p_2-value (T2DM vs. HC)	p_3-value (PD vs. T2DM)
	PD; n = 89	T2DM; n = 87	HC; n = 84			
Age; year	49 (41–55)	54 (44–61)	41 (34–55)	**0.017***	**< 0.001*****	0.105^{ns}
BMI; kg/cm^2	31.1 (29.3–33.7)	29.5 (27.2–33.4)	26.7 (24.6–28.6)	**< 0.001*****	**< 0.001*****	**0.04***
FPG; mg/dL	108.3 (104.3–115.0)	154.8 (132.1–192.0)	80.2 (77.1–85.7)	**< 0.001*****	**< 0.001*****	**< 0.001*****
HbA1c; %	5.9 (5.8–6.2)	7.2 (6.9–8.1)	4.6 (4.2–4.8)	**< 0.001*****	**< 0.001*****	**< 0.001*****
TC; mg/dL	179.3 (160.5–222.5)	168.3 (142.0–227.2)	176.6 (168.3–188.9)	0.063^{ns}	0.499^{ns}	0.169^{ns}
TG; mg/dL	150.5 113.0–192.6)	153.1 (87.5–187.2)	96.8 (74.0–101.6)	**< 0.001*****	**< 0.001*****	**0.019***
HDL; mg/dL	49.4 (39.4–59.6)	51.5 (45.5–57.4)	40.1 (38.5–55.2)	0.095^{ns}	**0.001****	0.299^{ns}
LDL; mg/dL	102.6 (84.2–125.0)	87.1 (66.5–171.5)	111.7 103.0–120.6)	0.225^{ns}	**0.02***	1.0^{ns}
VLDL; mg/dL	30.6 (23.0–38.7)	30.6 (18.6–38.5)	19.2 (14.7–20.4)	**< 0.001*****	**< 0.001*****	0.185^{ns}

IQR interquartile range; *PD* pre-diabetes; *DM* diabetes mellitus; *DN* diabetic neuropathy; *HC* healthy controls; *BMI* body mass index; *FPG* fasting plasma glucose; *Hb1Ac* glycated hemoglobin; *TC* total cholesterol; *TG* triglycerides; *HDL* high-density lipoprotein; *LDL* low-density lipoprotein; *VLDL* very low-density lipoprotein; *p*; Dunn's multiple comparisons test adjusted probability (significant *p* value is indicated in bold; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; *ns* not significant). Significance was assessed using Kruskal–Wallis test

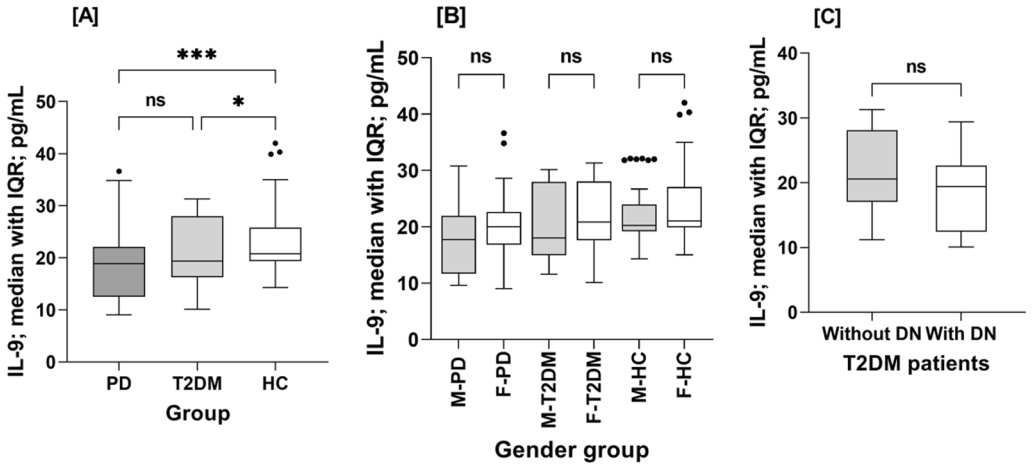

Fig. 1 Box and whisker plot (Tukey method) of serum interleulin-9 (IL-9) levels in: **A** individuals with pre-diabetes (PD; n = 89), patients with type 2 diabetes mellitus (T2DM; n = 87), and healthy controls (HC; n = 84), **B** PD, T2DM, and HC classified by gender (M: Male, F: Female), and **C** T2DM patients classified by diabetic neuropathy (DN). The horizontal line inside the boxes indicates the median. Whiskers indicate interquartile range (25–75%). Black circles indicate outliers. Significance was detected using Mann–Whitney U test (*$p < 0.05$;*** $p < 0.001$; ns: not significant). Serum IL-9 levels were significantly lower in PD (18.9 [IQR: 12.6–22.1] pg/mL; $p < 0.001$) and T2DM (19.4 [IQR: 16.3–28.0] pg/mL; $p = 0.04$) than in HC (20.8 [IQR: 19.4–25.8] pg/mL). Serum IL-9 levels were also lower in PD than in T2DM but the difference was not significant ($p = 0.051$). Serum IL-9 tended to show lower levels in males than in females in PD, T2DM, and HC, but the differences were not significant. Patients with DN also showed lower levels of IL-9 compared to patients without DN, but the difference was not significant (19.4 [IQR: 12.5–22.7] vs. 20.6 [IQR: 17.1–28.1] pg/mL; $p = 0.13$)

correlation with triglycerides ($r_s = -0.539$; $p < 0.001$) and very low-density lipoproteins ($r_s = -0.483$; $p < 0.001$) (Fig. 3).

4 Discussion

T2DM is a metabolic syndrome with a common feature of a low-grade systemic inflammation. The disease is distinguished by up-regulated plasma/serum

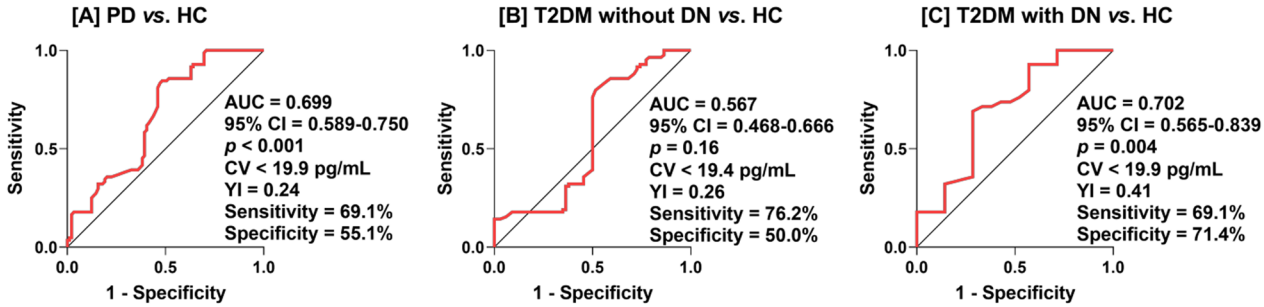

Fig. 2 Receiver operating characteristic (ROC) curve analysis of interleukin-9 (IL-9) in: **A** pre-diabetes (PD; n = 89), **B** type 2 diabetes mellitus without diabetic neuropathy; DN (n = 66), and **C** T2DM with DN (n = 21) versus (vs.) healthy controls (HC; n = 84). Area under the curve (AUC), 95% confidence interval (CI), probability (*p*), cut-off value (CV), sensitivity, and specificity are shown. The CV was adjusted with Youden index (YI). As indicated by the AUC value, IL-9 showed better diagnostic performance in PD and T2DM with DN compared to T2DM without DN (AUC: 0.699 and 0.702 vs. 0.567, respectively)

Table 2 Crude and adjusted multinomial logistic regression analysis of interleukin-9 in and pre-diabetes and type 2 diabetes mellitus (without and with diabetic neuropathy)

Group[a]	Crude analysis		Adjusted analysis[b]	
	OR (95% CI)	*p* value	OR (95% CI)	*p* value
PD; n = 89	0.90 (0.85–0.94)	**< 0.001*** **	0.86 (0.81–0.92)	**< 0.001*** **
DM-NDN; n = 66	0.97 (0.92–1.02)	0.225^{ns}	0.94 (0.88–0.99)	**0.024***
DM-DN; n = 21	0.89 (0.82–0.97)	**0.008****	0.85 (0.77–0.94)	**0.001****

PD pre-diabetes; *DM-NDN* type 2 diabetes mellitus without diabetic neuropathy; *DM-DN* type 2 diabetes mellitus with diabetic neuropathy; *OR* odds ratio; *CI* confidence interval; *p* two-tailed probability (significant *p* value is indicated in bold; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; *ns* not significant)

[a] The reference category was healthy controls

[b] The analysis was adjusted for age, gender, and body mass index

Variable	IL-9					
	PD; n = 89		T2DM; n = 87		HC; n = 84	
	r_s	*p*-value	r_s	*p*-value	r_s	*p*-value
Age	0.036	0.735^{ns}	-0.21	0.051^{ns}	0.04	0.721^{ns}
BMI	-0.136	0.205^{ns}	0.267	0.012*	0.108	0.327^{ns}
FPG	-0.219	0.039*	-0.224	0.037*	-0.216	0.049*
HbA1c	0.168	0.115^{ns}	-0.226	0.035*	0.14	0.20^{ns}
TC	0.096	0.369^{ns}	-0.093	0.39^{ns}	0.202	0.065^{ns}
TG	0.118	0.27^{ns}	0.155	0.152^{ns}	-0.539	< 0.001***
HDL	-0.057	0.598^{ns}	-0.013	0.902^{ns}	0.19	0.083^{ns}
LDL	-0.079	0.461^{ns}	-0.078	0.475^{ns}	0.087	0.431^{ns}
VLDL	0.118	0.271^{ns}	0.125	0.25^{ns}	-0.483	< 0.001***

Fig. 3 Spearman's rank correlation analysis of interleukin-9 with other variables among study groups. Red indicates a significant positive correlation. Blue indicates a significant negative correlation. Green indicates no significant correlation. IL-9: Interleukin-9; PD: Pre-diabetes; T2DM: Type 2 diabetes mellitus; HC: Healthy controls; r_s: Spearman's correlation coefficient; *p*: Two-tailed probability (significant *p* value is indicated in bold; *$p < 0.05$; ***$p < 0.001$; ns: not significant); BMI: Body mass index; FPG: Fasting plasma glucose; Hb1Ac: Glycated hemoglobin; TC: Total cholesterol; TG: Triglycerides; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; VLDL: Very low-density lipoprotein

levels of TNF-α, IL-1, IL-6 and IL-17A (pro-inflammatory cytokines) and other indicators of inflammation (for instance, C-reactive protein) [25]. Recent studies have indicated a role for IL-9 in regulating the inflammatory response in several inflammatory conditions [16, 17, 26]. These studies led us to hypothesize that IL-9 may also be involved in the regulation of inflammation in diabetes. Serum IL-9 levels were measured in three groups of diabetes (PD and T2DM with and without DN) and compared to non-diabetic HC. The main focus was on PD and DN, because IL-9 has not been explored in these two groups of diabetes. As shown in the results, serum IL-9 levels were significantly decreased in PD and T2DM (particularly T2DM with DN) compared with HC. However, the significance of IL-9 as a biomarker was better in PD and T2DM with DN than in T2DM without DN. On the other hand, multinomial logistic regression analysis demonstrated that decreased levels of IL-9 were associated with susceptibility to diabetes, particularly in PD and T2DM with DN. In addition, IL-9 tended to show lower levels in females than in males in the PD, T2DM, and HC groups. This may indicate that IL-9 is influenced by sex but there is no data to support or refute this finding and further studies are needed.

As revealed by observational and experimental studies, IL-9 is a pleotropic cytokine potentiated to perform

pro-inflammatory and anti-inflammatory functions. A pro-inflammatory function associated with IL-9 has been demonstrated in a number of inflammatory and autoimmune conditions such as systemic lupus erythematosus, respiratory allergies, inflammatory arthritis and others. In most of these diseases, up-regulated expression of IL-9 mRNA/protein was observed, and association with disease severity/activity was also indicated [10, 11, 17, 19, 26]. However, another study reported that IL-9 attenuates the inflammatory response by reducing the production of serum IL-6 and TNF-α (pro-inflammatory factors) in a mouse model of alcohol-induced liver injury. Besides, the study indicated that macrophage-derived IL-9 was associated with decreased apoptosis in hepatocytes [16]. Further studies have linked IL-9 with induction of immune tolerance [27–29]. In this context, IL-9 was indicted to enhance the inhibitory functions of FoxP3 + natural T-regulatory (Treg) cells, and defective signaling of IL-9 could impair the suppressive activity of these cells and exacerbate inflammation [27]. Serum IL-9 levels were also significantly increased in those who had a stable liver transplant and had not experienced organ rejection episodes over eight years. Thus, it has been proposed that IL-9 can induce tolerance in liver transplantation [28]. Additional data indicated that trophoblast-derived IL-9 enhanced the production of Th2 cytokines, such as IL-4 and IL-10, thus shifting the Th1/Th2 ratio to the Th2 type. Also, naïve CD4 + T cells exposed to IL-9 showed up-regulated expression of FoxP3 and enhanced secretion of TGF-β and IL-10 (Treg cytokines), while pro-inflammatory Th17 cells were inhibited [29].

With regard to T2DM, although IL-9 has not been well investigated, studies have reported inconsistent findings. The first study was conducted in 2015 and revealed that IL-9 levels were lower in T2DM patients than in subjects with normal glucose tolerance or T2DM patients with kidney disease [20]. On the contrary, another study showed that IL-9 levels were elevated in T2DM patients who experienced depression compared to T2DM patients who did not have depression or HC [22]. An additional study indicated that IL-9 levels were also elevated in patients with T2DM compared to HC. Besides, a single nucleotide polymorphism of *IL9* showed an association with T2DM risk [21]. In contrast to these studies, with the exception of Vasanthakumar's study [20], the present study showed that IL-9 levels were decreased in PD individuals and T2DM patients compared to HC. Regardless of these conflicting findings, IL-9 may be indirectly related to the etiology and/or pathogenesis of T2DM, because it has been pointed out that IL-9 can activate two insulin receptor substrate (IRS) proteins, IRS1and IRS2 [10]. IRS1 and IRS2 are essential molecules that mediate insulin signaling and play a recognized role in maintaining basic physiological and cellular functions. Both proteins exert a distinct role in coordinating the signals from insulin and insulin-like growth factor receptor tyrosine kinases with those from nutrients and pro-inflammatory cytokines [30]. Importantly, dysregulated expression of IRS1 and IRS2 has been linked to insulin resistance and obesity; both well recognized risk factors for diabetes [31, 32]. In addition, IRS1 and IRS2 may have a role in regulating the functions of pancreatic beta-cells [33]. It has been demonstrated that up-regulated expression of *Irs2* (the gene encoding IRS2) in beta-cells was associated with a promotion of glucose tolerance in mice and a prevention of diabetes in *Irs2*-knowkdown and obese mice. Besides, IRS2 protected beta-cells from streptozotocin-induced destruction and improved the function of these cells upon transplantation. Therefore, it has been proposed to consider up-regulation of *Irs2* in beta-cells as a therapeutic strategy in diabetes [34]. Based on these findings, understanding the functional relationship between IL-9 and IRS proteins, particularly in PD and DN, may shed light on the mechanistic clues behind the occurrence of PD and DN.

IL-9 tended to show lower levels in patients who suffered from DN. OR estimates indicated that lower levels of IL-9 were generally associated with the risk of developing diabetes, particularly of PD and DN. With regard to DN, recent indirect evidence indicated that IL-9 has a protective effect against podocyte injury in mice with experimentally induced nephropathy [35]. Therefore, IL-9 may similarly protect against DN and decreased levels of this cytokine may be associated with the development of this type of diabetic complication. DN is one of the most common complications associated with T2DM worldwide and causes high rates of disability and mortality with few effective therapeutic options. Besides, the etiological mechanisms and molecular events of DN are still not fully understood [36]. However, accumulating evidence indicates that systemic inflammation mediated by pro-inflammatory cytokines and other inflammatory markers plays an essential role in the development of DN [37]. Various immune cells were detected in the damaged nerves of DN patients such as CD4 + T cells, CD8 + T cells, and macrophages [38]. Besides, the ratio of neutrophils to lymphocytes, an indicator of systemic inflammation, has been linked to the etiology of DN [39]. It has also been shown that pro-inflammatory cytokines are involved in the pathogenesis of DN. In fact, it has been proposed that analysis of plasma levels of these cytokines may predict the incidence of DN. In this context, C-reactive protein, TNF-α, IL-1 receptor antagonist and IL-6 were shown to be associated with incident DN [37, 40]. As IL-9 is a cytokine involved in the regulation of pro-inflammatory cytokines [18], it may also play a

role in the pathogenesis of DN. In multiple sclerosis, it has been demonstrated that IL-9 expression in patients' cerebrospinal fluid was inversely correlated with neurodegeneration, disability, and inflammatory activity [19]. These results may also indicate a link between IL-9 and DN but further studies are warranted to understand this issue in T2DM.

In this study, we also sought to understand the pattern of relationships between IL-9 and diabetes variables (age, BMI, FPG, HbA1c, TC, TG, HDL, LDL, and VLDL) in PD, T2DM, and HC. The three studied groups shared a negative correlation between IL-9 and FPG. This observation may indicate negative effects of hyperglycemia on IL-9 levels. In addition, IL-9 showed a negative correlation with HbA1c in T2DM, while a positive correlation was found with BMI. In the HC group, IL-9 showed a negative correlation with triglycerides and very low-density lipoproteins. This observation may be important as it links two parameters of lipid profile and IL-9 in healthy individuals, but needs further examination to validate it.

4.1 Limitations

The study encountered three limitations. The small number of DN cases was the first limitation. Second, insulin resistance was not evaluated. Third, we could not ascertain the patients' physical activities.

5 Conclusions

Serum IL-9 levels were significantly decreased in individuals with PD and patients with T2DM compared with HC. The decrease in IL-9 levels in T2DM patients was more pronounced in patients with DN than in patients without DN. Therefore, low levels of IL-9 can be considered as a potential biomarker associated with an increased risk of PD and DN.

Abbreviations

AUC	Area under the curve
BMI	Body mass index
CI	Confidence interval
DN	Diabetic neuropathy
FPG	Fasting plasma glucose
Hb1Ac	Glycated hemoglobin
HC	Healthy controls
HDL	High-density lipoprotein
IL	Interleukin
IQR	Interquartile range
LDL	Low-density lipoprotein
OR	Odds ratio
p	Probability
PD	Pre-diabetes
ROC	Receiver operating characteristic
r_s	Spearman's correlation coefficient
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TG	Triglycerides
Th	T helper
VLDL	Very low-density lipoprotein

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NFK, AHA, INS, YDS, SWN, and OHJ contributed to laboratory work, data handling, writing and revising the manuscript. AHA managed data, carried out statistical analyses and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Written consent was provided by all participants and the Ethics Committee of the College of Science, University of Baghdad approved the study protocol (approval reference: CSEC/0122/0021).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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