Association of Serum Folate and Homocysteine Parameters in Diabetic Patients with and Without Foot Ulcer

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ABSTRACT

Objective: To assess the association of serum folate and homocysteine parameters in diabetic patients with and without foot ulcers.

Study Design: A comparative study.

Place and Duration of the Study: Department of Physiology, Baqai Medical University, Karachi, Pakistan, from June 2023 to 2024.

Methodology: Participants were divided into three groups. Group I was healthy control, Group II had diabetic patients without foot ulcers, and Group III had diabetic patients with foot ulcers. Individuals with history of insulin-dependent diabetes and gestational diabetes mellitus (DM) were excluded. ELISA method was used to estimate serum folate and homocysteine. Pearson's Chi-square test / Fisher's exact test was used for the association and the Kruskal-Wallis test was used to compare the median of non-normally distributed parameters after checking the assumption of normality using the Shapiro-Wilk's test. Post-hoc analysis was done using the Dunnett's T3 test for significant parameters.

Results: The median of age, BMI, serum folate, FBS, HbA1c, and homocysteine levels were significantly different across all three groups ($p < 0.05$). The Dunnett's T3 test for multiple comparisons showed insignificant differences for Folate, HbA1c, and FBS between diabetes and Diabetes with Foot ulcer samples ($p > 0.05$). Spearman's rank correlation showed significant positive association between HbA1c and homocysteine ($r = 0.65$, $p = 0.001$).

Conclusion: HbA1c and blood homocysteine levels were correlated significantly among three groups. This indicates that HbA1c and serum homocysteine levels may influence foot ulcer pathogenesis in individuals with Type II DM.

Key Words: *Diabetes mellitus, Insulin, Foot ulcer, Fasting blood sugar, Folate, Homocysteine*.

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INTRODUCTION

Type-II Diabetes mellitus (T2DM) is characterised by elevated blood plasma sugar levels (hyperglycaemia), which occur due to insulin deficiency, insulin resistance, or the onset of metabolic syndrome.[1] Currently, 537 million people aged 20 to 79 years have Type II Diabetes. By 2030, this number is expected to rise to 643 million, which equates to one in ten adults. This implies that almost three new cases of DM will be diagnosed every ten seconds.

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It is estimated that by 2045 the number of cases of DM will increase by over 783 million globally.[2] According to data from the International Diabetes Federation (IDF), there were 6.9 million people in Pakistan aged 20 to 79 years living with diabetes in 2003. Projections indicate that this number could rise to 11.5 million by 2025, potentially making Pakistan the country with the highest number of diabetics over the next decade.[3,4]

Approximately 15% of over 150 million diabetics worldwide will develop diabetic foot ulcers at a certain time, as is to be expected.[5] Diabetic foot ulcers (DFUs) are a chronic microvascular disorder associated with DM. DFUs are defined by the presence of lesions affecting the deeper tissues of the lower limbs with secondary bacterial infection, which is caused by poorly controlled sugar in diabetic patients, neuropathies, and peripheral arterial diseases. Currently, in Pakistan, 12.6% is the prevalence of DFUs.[6] DFUs will occur in the lifetimes of 19 to 34% of the estimated 537 million diabetics globally. Diabetes-

related foot ulcers can lead to a decline in functional status or decline in quality of life, repeated infections or osteomyelitis, repeated hospital admissions, amputation of the lower limbs without trauma, and even death.[7] Nutrient deficiencies are among the major modifiable determinants of DFUs associated with the development and restoration process in Type-II diabetic patients.[8] Vitamin B9, commonly referred to as folate or folic acid, is a vitamin that dissolves in water and is a part of various biochemical processes in the human body, especially decreasing the plasma homocysteine and decreasing insulin resistance in obese diabetic patients. Glycaemic diseases can arise from homocysteinaemia caused by a deficiency of folate.[9] Homocysteine is a sulfur-containing amino acid, which produces endothelial dysfunction. It is characterised by platelet aggregation, vasoconstriction, inflammation, and thrombosis by exerting an effect on the bioavailability of the nitric oxide, by enhancing the oxidative degradation of nitric oxide (NO) and by an increase in asymmetric dimethylarginine (ADMA), an inherent inhibitor of endogenous nitric oxide synthase (eNOS), also makes diabetic people more susceptible to vascular atherosclerosis.[10] People with T2DM are at risk of DFUs and neuropathy has been found to rise by 10% for every micromole increase in fasting plasma homocysteine levels.[11] The lowering effect of folate directly decreases the homocysteine in serum and lowers the risk of DFU development in Type II diabetic patients by increasing the availability of NO.[12] By enhancing NO synthesis and enabling the coupling of endothelial NO synthase, folic acid, and its active metabolite 5-methyl tetrahydrofolate play a crucial role in raising the bioavailability of NO. In addition, these compounds are effective in superoxide radicals scavenging directly. Improved wound healing in diabetic patients may result from the preservation or augmentation of normal endothelial function brought about by folic acid's increase in NO bio-availability.[13] As there is currently no research on Type II diabetic people in Pakistan who have or do not have diabetic foot ulcers. Therefore, this study was designed to assess the association of serum folate and homocysteine parameters in diabetic patients with and without foot ulcers.

METHODOLOGY

A comparative cross-sectional design was used for this investigation, conducted in the Department of Physiology, Baqai Medical University, Karachi, Pakistan, in partnership with the Baqai Institute of Diabetes and Endocrinology. A purposive sampling approach was utilised to recruit the study population from June 2023 to 2024, following the endorsement from the Ethics Committee of the University, which issued permission for the research on 10th February 2023, and on 12th July 2023, the Board of Advance Studies and Research of Medical University approved the research synopsis. The calculation of the sample size was performed with the aid of Open-Epi online software.[14] A calculated sample size of 138 was determined using the frequency of methyltetrahydrofolate reductase in a population withconfidence level: 95% power and 1% level of significance.[15] The 138 participants divided into three groups (46 in each group): Group I (normal and healthy individuals), Group II (Type II Diabetics without foot ulcers), and Group III (diabetic patients with foot ulcers). This study focused on diagnosed cases of T2DM in both genders, all with duration of diabetes lasting over five years. The participants were divided into the following categories: Individuals with diabetic foot ulcers (which could be ischaemic or neuropathic), 46 individuals with diabetes but without foot ulcers, 46 individuals with diabetes who had foot ulcers, and 46 healthy controls (diagnosed through HbA1c and fasting blood glucose tests). All participants voluntarily agreed to take part in this research, and submitted written informed consent before the start of data collection. Individuals with Type I DM, a history of gestational DM, and those with deep venous thrombosis and ulcers were excluded.

A detailed medical history was gathered utilising a predefined proforma. An extensive physical and medical examination was conducted, which encompassed the demographic profile. Body mass index (BMI) was calculated using anthropometric data including height, weight, and blood pressure assessment. Ten ml of blood was collected after an overnight fast (only water was permitted). Six ml of serum was used to measure fasting blood glucose (FBG), haemoglobin A1c (HbA1c), folic acid, and homocysteine levels. The blood serum was separated immediately and stored for subsequent evaluation. FBG was analysed by Indiko thermoscientific by Briogene, and HbA1c was determined by high-performance liquid chromatography. Folic acid levels and serum homocysteine concentrations were assessed through the application of an enzyme-linked immunosorbent assay using CALBIOTECH and BT Lab, respectively. The reference ranges established in the research laboratory were serum folate levels between 2.6 and 12.7 ng/ml, homocysteine levels between 3.7 and 13.9 µmol/L, fasting blood glucose at 70 mg/dL (3.9 mmol/L) to 100 mg/dL (5.6 mmol/L), and the HbA1c level between 4% and 5.6%.

Data were stored and analysed using the IBM-SPSS version 23.0. Counts and percentages were reported for gender, occupation, BMI levels, family history of diabetes, smoking history, and medicine history (OHD / Insulin). The occupation or profession, diabetes in the family, and medicine history ($p < 0.05$) significantly correlated within the control, diabetic, and ulcer groups, according to Pearson's Chi-square test, and the Fisher's exact test was applied to variables with low counts. Normality of age (years), BMI (kg/m^2), systolic blood pressure (mmHg), diastolic blood pressure (mmHg), folate, haemoglobin A1c (HbA1c), fasting blood sugar (mg/dl), and homocysteine was tested using Shapiro-Wilk's test. Data were found non-normally distributed ($p < 0.05$); therefore, median with 25th and 75th percentiles were reported. Comparison of the median across three studied groups was made using the Kruskal-Wallis test and Post-hoc comparison for significant parameters was done using the Dunnett's T3 test. Non-parametric Spearman's rank correlation was used to study the correlation of HbA1c and homocysteine, p-values less than 0.05 were considered statistically significant.

RESULTS

The association of baseline characteristics among the studied groups was evaluated. The factors of occupation, family history of diabetes, and medicine history showed significant association with the patients group, as indicated by Pearson's Chi-square test ($p < 0.05$). Additionally, Fisher's exact test revealed a significant association with medicine history ($p = 0.001$). In this study, the results indicated that gender, BMI, and smoking history did not give any significant association with a studied group of patients ($p > 0.05$, Table I).

Median of age, BMI, SBP, and DBP were compared across three studied groups, Kruskal-Wallis test showed a significant difference in the median age and BMI of samples ($p < 0.05$). There was no significant difference in median of SBP and DBP ($p > 0.05$, Table I).

Median of Folate, HbA1c, FBS, and homocysteine were compared across groups; the Kruskal-Wallis test showed a significant difference in the median of these parameters across groups ($p < 0.05$, Table I).

Dunnett's T3 test showed that diabetic and DFU samples had a significantly higher age as compared to the control group ($p < 0.01$). For BMI, samples with DFUs had significantly lower BMI as compared to the diabetes and control group samples ($p < 0.05$, Table II).

Table I: Baseline features and biochemical parameters of the studied population (n = 138).

Characteristics		Groups			p-value
		Normal individuals (n = 46) N (%), M (IQR)	Diabetic patients (n = 46) N (%), M (IQR)	DFUs (n = 46) N (%), M (IQR)	
Gender	Male	23 (50%)	23 (50.0%)	28 (60.9)	0.48
	Female	23 (50.0%)	23 (50.0%)	18 (39.1%)	
Occupation	Unemployed	6 (13.0%)	12 (26.1%)	8 (17.4%)	0.03*
	Employed	32 (69.6%)	18 (39.1%)	21 (45.7%)	
	Housewife	8 (17.4%)	16 (34.8%)	17 (37.0%)	
BMI Levels	Normal Weight	11 (25.0%)	10 (22.7%)	14 (35.0%)	0.33
	Overweight	11 (25.0%)	10 (22.7%)	13 (32.5%)	
	Obese	22 (50.0%)	24 (54.5%)	13 (32.5%)	
Family history of diabetes	Yes	22 (47.8%)	32 (69.6%)	20 (43.5%)	0.02*
	No	24 (52.2%)	14 (30.4%)	26 (56.5%)	
Smoking history (current)£	Yes	3 (6.5%)	11 (23.9%)	8 (17.4%)	0.06
	No	43 (93.5%)	35 (76.1%)	38 (82.6%)	
Medicine history (OHD/insulin)£	Yes	0 (0.0%)	42 (91.3%)	45 (97.8%)	0.001*
	No	46 (100.0%)	4 (8.7%)	1 (2.2%)	
Age (years)		37 (42.5-29.5)	52 (62-43)	52.5 (59-48)	0.001*
BMI (kg/m^2)		27.8 (33.22-24.17)	29.13 (32.1-24.55)	26.42 (28.12-23.47)	0.041*
SBP (mmHg)		120 (120-120)	120 (130-110)	120 (130-120)	0.23
DBP (mmHg)		80 (80-80)	80 (80-80)	80 (80-80)	0.23
Folate		13.63 (18.18-8.78)	1.78 (2.19-1.11)	1.38 (1.55-1.02)	0.001*
HbA1c		5.4 (5.6-5.2)	8.45 (10.1-7.2)	9.8 (10.9-7.6)	0.001*
FBS (mg/dl)		86 (91-80)	149.5 (200-119)	118 (184-92)	0.001*
Homocysteine		7.46 (9.03-6.03)	126.93 (139.53-107.28)	180.33 (199.26-152.29)	0.001*

*£: p-value was obtained by using Fisher's exact test otherwise Pearson's Chi-square test, p <0.05 considered as statistically significant, *p <0.05 was considered statistically significant using Kruskal-Wallis test.*

Table II: Multiple comparisons of age, BMI, FBS, HbA1c, folate, and homocysteine between the groups.

Variable(s)	Groups		Mean difference	S.E	95% Confidence interval		p-value
					Lower bound	Upper bound	
Age (years)	Control	Diabetic	-14.3	2.6	-20.6	-8.0	0.001*
	Control	DFUs	-16.5	1.9	-21.2	-11.7	0.001*
	Diabetic	DFUs	-2.1	2.4	-7.9	3.7	0.75
BMI (Kg/m^2)	Control	Diabetic	-0.2	1.2	-3.1	2.8	0.99
	Control	DFUs	2.6	1.1	0.1	5.2	0.04*
	Diabetic	DFUs	2.8	1.1	0.2	5.4	0.03*
FBS (mg/dl)	Control	Diabetic	-79.5	11.4	-107.6	-51.5	0.001*
	Control	DFUs	-67.5	14.2	-102.6	-32.4	0.001*
	Diabetic	DFUs	12.0	18.0	-31.7	55.8	0.877
HbA1c	Control	Diabetic	-3.2	0.3	-4.0	-2.5	0.001*
	Control	DFUs	-4.1	0.3	-4.9	-3.3	0.001*
	Diabetic	DFUs	-0.9	0.4	-1.9	0.2	0.142
Folate	Control	Diabetic	11.7	0.8	9.6	13.8	0.001*
	Control	DFUs	12.0	0.8	9.9	14.1	0.001*
	Diabetic	DFUs	0.3	0.1	0.1	0.6	0.012*
Homocysteine	Control	Diabetic	-116.7	2.6	-123.0	-110.4	0.001*
	Control	DFUs	-171.7	6.2	-187.1	-156.3	0.001*
	Diabetic	DFUs	-55.0	6.7	-71.4	-38.5	0.001*

**p <0.05 was considered statistically significant using Dunnett's T3.*

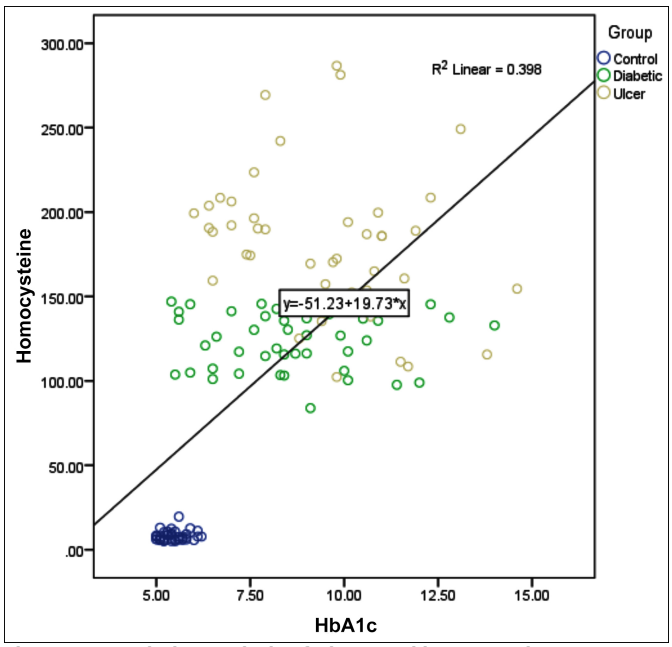

Figure 1: Correlation analysis of HbA1c and homocysteine.

Post-hoc analysis using Dunnett's T3 for FBS, HbA1c, folate, and homocysteine showed that samples from both diabetic groups had significantly higher levels of FBS, HbA1c, and homocysteine compared to the control group samples ($p < 0.05$), whereas significantly lower folate levels in comparison of the control group samples ($p < 0.05$). However, no significant differences in FBS, HbA1c, or folate were observed between diabetic and DFU groups ($p > 0.05$) (Table II, Figure 1).

Scatter plot showed a 65% significant positive correlation between HbA1c and homocysteine, 0.38 is not a correlation coefficient, it is a coefficient of determination (R-square) based on correlation value, correlation was 0.65 and found significant using a test of correlation with a p-value less than 0.05.

DISCUSSION

DFUs are a serious and painful consequence of diabetes, often proving to be difficult to manage effectively. These ulcers develop after a break in the skin tissue, especially in big toes with balls of feet, and can be associated with underlying bones or osteomyelitis. The foot ulcer can affect Type II diabetic patients, but keen observation with cleaning feet can prevent this complication.[16] Studies suggest that between one-third and one-fifth of individuals suffering from DM will develop a chronic non-healing wound, including diabetic foot ulcers, throughout their lifetime. The rate at which these ulcers recur is notably high, with 40% of patients facing a recurrence in the first year and 65% within five years. Patients with diabetes have several risk factors for developing foot ulcers, such as major (neuropathic 35%, ischaemic 15%, and neuro-ischaemic 50%) and minor (microvascular, biochemical abnormalities, infections, oxidative stress, duration of DM, uncontrolled hyperglycaemia, and nutritional deficiencies), so few diabetic patients are at higher risk than others.[17]

The findings of this study indicated that the mean age and BMI for each of the three groups varied significantly ($p < 0.05$, Table I). An Indian study showed similar results to the present findings, which also established that diabetes-related foot ulcers are independently predicted by BMI.[18] Individuals' age is thought to be a significant Diabetes-related foot ulcer risk factor, with those more than 50 years more susceptible, and connected to the severity of ulcers in people with diabetes.[19]

There were notable variations ($p < 0.05$) in HbA1c across the three groups of research participants. (Table II). In Turkiye, the study's showed similar results to this study's findings, it has been observed that there is a positive association between HbA1c and DFUs. The level of HbA1c is recognised as a distinct potential factor for DFU development.[20] This study revealed that folate levels did not show substantial variations across the three groups ($p > 0.05$). In line with the present results, an Indian study revealed no substantial variations in folate levels among the three groups ($p > 0.05$).[21] The lack of significant differences in folate levels across the groups may imply that folate is not a crucial element in the growth or development of DFUs.

In this study, significant variations for homocysteine were observed across the three groups ($p < 0.05$). Gonzalez *et al*. also observed a significant association ($p < 0.05$) of plasma homocysteine level with foot ulcers in Type II diabetic patients.[22] This observation showed similar results to the present study.

The elevated homocysteine level or hyperhomocysteinaemia has significant effects on the onset and progression of several diabetes-related problems such as neuropathy, peripheral vascular disease, and foot ulceration.[22]

A comprehensive understanding of the various diabetes-related risk factors for DFUs in patients necessitates focusing on the critical roles of prevention and early detection. Integrating multidisciplinary care and following established guidelines is key to alleviating the morbidity and disparities related to DFUs. Moreover, more studies are required to assess the therapeutic possibilities associated with folate supplementation and interventions that target the reduction of homocysteine levels in the context of DFU manage-ment. HbA1c and blood homocysteine levels were correlated significantly among the three groups. This suggests that HbA1c and serum homocysteine levels may play a significant role in the development of DFUs in Type II diabetic patients. These factors should be considered when assessing ulcer risk and developing comprehensive diabetes management plans addressing glycaemic control (HbA1c) and vascular health (homocysteine concentration). This

holistic approach could ultimately lead to better outcomes, early prevention, and improved quality of life (QOL) for individuals with T2DM.

CONCLUSION

HbA1c and blood homocysteine levels were correlated significantly among the three groups. This indicates that HbA1c and serum homocysteine levels may influence foot ulcer pathogenesis in individuals with T2DM.

ETHICAL APPROVAL:
The Ethics Committee of the Baqai Medical University granted ethical permission under the ethical guidelines for human research and experimentation.

PATIENTS' CONSENT:
Written informed consent was obtained from the participants included in this study.

COMPETING INTEREST:
The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:
SUA: Conception, writing, reviewing, editing, and principal investigation.
QA: Conception and supervision.
IA: Conception and co-supervision.
ZM: Data collection and curation.
AF: Conception and data analysing.
MKA: Critical reviewing.
All authors approved the final version of the manuscript to be published.

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