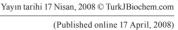## Research Article (Araştırma Makalesi)

# Inverse Correlation of Low Vitamin $B_{12}$, Folic Acid and Homocysteine Levels in Diabetic Retinopathy

[Diyabetik Retinopatide Düşük Vitamin $B_{12}$, Folik Asit ve Homosistein Düzeylerinin Ters İlişkisi]

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**ABSTRACT**

**Purpose:** Evaluation of total plasma homocysteine, serum vitamin $B_{12}$ and folic acid levels in patients with diabetic retinopathy.

**Materials and Methods:** Fifty patients having insulin dependent diabetes mellitus were included and subdivided into 3 groups: Diabetics without retinopathy (n=10), with background retinopathy (n=20), and proliferative retinopathy (n=20). Ten normal subjects served as controls. Plasma total homocysteine levels were measured using HPLC. Serum vitamin $B_{12}$ and folic acid levels were carried out using radioimmunoassay technique.

**Results:** There was a statistically significant increase of plasma total homocysteine in diabetics without retinopathy compared to controls, as well as decrease in serum levels of both serum folate and $B_{12}$. There was a statistically significant decrease of serum $B_{12}$ and folic acid levels and a significant increase of plasma total homocysteine level in diabetics with retinopathy in comparison to those without retinopathy. There was no association between the different degrees of retinopathy studied and plasma total homocysteine concentrations, nor serum $B_{12}$ and folic acid concentrations.

**Conclusion:** Diabetic patients with retinopathy are most prone to develop an increase in plasma total homocysteine which may be caused by a deficiency of both blood folate and vitamin $B_{12}$ concentrations. This finding directs physicians to the benefit of recommending the use of those vitamins as replacement therapy in diabetics to prevent future atherogenic processes and diabetic complications due to hyperhomocysteinemia.

**Key Words:** Homocysteine, Vitamin $B_{12}$, Folic acid, Diabetic retinopathy, Type I diabetes.

**ÖZET**

**Amaç:** Diyabetik retinopatili hastalarda total plazma homosistein, serum vitamin $B_{12}$ ve folik asit düzeylerini incelemektir.

**Materyel ve Metod:** İnsüline bağımlı diabetes mellituslu elli hasta 3 gruba ayırıldı. Retinopatisi olmayanlar (n=10), "background" retinopatililer (n=20), proliferatif retinopatililer (n=20). On normal kişi kontrol olarak kullanıldı. Plazma total homosistein düzeyleri HPLC ile ölçüldü. Serum vitamin $B_{12}$ ve folik asit düzeyleri radioimmunoassay yöntemi ile çalışıldı.

**Bulgular:** Retinopatisi olmaya diabetlilerin plazma total homosistein düzeylerinde kontrole göre anlamlı artış, serum folat ve $B_{12}$ düzeylerinde azalış gözlendi. Retinopatili diyabet hastalarının retinopatisi olmayan diyabetlilere göre serum $B_{12}$ ve folik asit düzeylerinde anlamlı azalış, plazma total homosistein düzeylerinde ise anlamlı artış bulundu. Çalışmada plazma retinopati derecesi ile total homosistein, serum $B_{12}$ ve folik asit düzeyleri arasında bir ilişki bulunmadı.

**Sonuç:** Retinopatisi olan diabetli hastalar, kan folat ve vitamin $B_{12}$ düzeylerindeki azalmaya bağlı olarak plazma total homosistein artışı geliştirmeye eğilimlidirler. Bu bulgular, klinikte hiperhomosisteinemi nedeniyle gelişen aterojenik oluşumlar gibi diyabetik komplikasyonların önlenmesinde bu vitaminlerden yararlanabileceğini gösterir.

**Anahtar Kelimeler:** Homosistein, Vitamin $B_{12}$, Folik asit, diyabetik retinopati, Tip I diabetes

# INTRODUCTION

Progressive microangiophathy is characteristic of the diabetic state. Retinal, renal, neural, myocardial and peripheral vascular diseases are a long list of diabetic complications (1).

Diabetic retinopathy is the leading cause of blindness among diabetic patients (2). A previous study (3) considered elevated total plasma homocysteine (tHcy) as an independent risk factor for atherosclerotic disease in subjects with normal glucose tolerance. Recent literatures (4,5) have found elevated plasma tHcy levels in older diabetics which were associated with accelerated atherosclerosis, osteopenia and Alzheimer's disease.

Other studies (6) attributed homocysteine to be involved in a complex and dynamic way in vascular injury and repair, hence contributing to the development of diabetic microangiophathy.

Homocysteine is the demethylated derivative of methionine (7) and can be metabolized by two pathways, either catabolyzed by the trans-sulphuration pathway to cysteine, or remethylated to methionine, mainly by the folate and vitamin $B_{12}$-dependent enzyme methionine synthase. Homocysteine accumulates when the activity of methionine synthase decreases. Plasma levels of homocysteine have been shown to be elevated in patients with cobalamine and folate deficiency (8-14).

The aim of this work was to evaluate the total plasma homocysteine (tHcy), serum vitamin $B_{12}$ and folic acid levels in diabetic patients in relation to different stages of diabetic retinopathy.

# MATERIAL AND METHODS

## Subjects

This study was conducted with the Research Institute of Ophthalmology (diabetic clinic). Fifty patients with IDDM were included in the study and subdivided into 3 groups: Group (1) diabetics without retinopathy (n=10); Group (2) diabetics with background retinopathy (n=20); Group (3) diabetics with proliferative retinopathy (n=20). The ages of these diabetic patients ranged from 20-45 years (mean=37.5 years). Ten normal subjects served as controls, their ages ranged from 25-40 years (mean=32.5 years).

All patients and controls were subjected to detailed clinical history and examination, ECG tracing. All subjects had detailed ophthalmologic examination including, visual acuity (VA) corrected and uncorrected using Landolt's VA chart, slit lamp examination using the Haag striet model, intraocular pressure (IOP) measurement using the Goldmann applanation tonometer, fundus examination after full pupillary dilatation with patient seated at the slit lamp using the (+) 90D lens, fundus photography and fluorescein angiography to determine the stage of diabetic retinopathy.

## Sample collection

Blood samples for determination of plasma tHcy were collected after an overnight fast in evacuated tubes containing EDTA and were centrifuged. Plasma was stored at -70$^o$C till analysis of tHcy. Serum was prepared, to estimate levels of vit. $B_{12}$ and folate and for estimation of other biochemical parameters (urea, TG, HDL-c, etc.).

## Laboratory Analysis

Routine chemistry tests, kidney function (serum creatinine and creatinine clearance test), detection of 24 hour urine protein, complete lipid profile (cholesterol, triacylglycerol (TG), low density lipoprotein cholesterol (LDL-c), high density lipoprotein cholesterol (HDL-c)) and post-prandial blood sugar were performed on a Beckman $CX_4$ autoanalyzer (USA) with Beckman kits (15).

Glycosylated hemoglobin ($HbA_1C$) was assessed by the kit provided from Human (Germany) (16), using a fast ion-exchange resin separation method in which whole blood is mixed with a lysis reagent containing a detergent and borate ions. Elimination of the labile Schiff's base is thus achieved during hemolysis. The hemolysate is then mixed for 5 minutes with a weakly binding cation exchange resin. During this time, $HbA_0$ binds to the resin. A special resin separator is used to remove the resin from the supernatant fluid which contains the $HbA_1$. The glycohemoglobin percentage of total hemoglobin is determined by measuring the absorbance of the glycohemoglobin and of the total hemoglobin fraction at 415 nm or 405 nm in comparison with a standard glycohemoglobin preparation carried through the test procedure.

Total plasma homocysteine level (μmol/L) was determined using reversed-phase high pressure liquid chromatography (HPLC). The method was adopted from that of Ubbink et al. (17) on the basis of the chemical description provided by Araki and Sako (18), which was built on labeling of plasma homocysteine with a thiol-specific fluorogenic reagent (SBD-F), followed by HPLC and fluorescence detection. The addition of internal standard mercaptopropionylglycine was done according to Vester and Rasmussen (19). Separation and quantification were performed with GBC system, (Australia) (pump LC 1150, fluorescence detector LC 1255) equipped with phenomenex (USA, UK) phenosphere ODS analytical column (150 mm x 4.6 mm ID, 5 μm particle size). The fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm. Mobile phase was 0.1 mol/L potassium dihydrogen phosphate buffer (pH 2.1) containing 4 % acetonitrile at a flow rate of 2 ml/minute. The peak of homocysteine was identified according to the retention time of the standard. The concentration was automatically calculated by dividing the ratio between the area of the homocysteine peak and the mercaptopropionylglycine peak.

Determination of serum vitamin $B_{12}$ (pg/ml) and folic acid (ng/ml) levels using dual count radioassay kit for the simultaneous quantitative determination of vitamin $B_{12}$ ([57]Co) and folate (^{125}I) provided by the Diagnostic Products Corporation (DPC) (Los Angeles, CA, USA) (20). In competitive protein binding, the binder should

have an equal affinity for the calibrator and the substance which is present in the patient's sample. The unlabeled vitamin $B_{12}$ or folate competes with its labeled species for the number of available binding sites on its specific binder, thus reducing the amount of labeled vitamin $B_{12}$ or folate bound. Therefore, the level of radioactivity bound is inversely related to the concentration in patient's sample or calibrator. In this kit, levels of vitamin $B_{12}$ and folate are determined simultaneously in a single tube. The two tracers, ($^{57}Co$) for vitamin $B_{12}$ and ($^{125}I$) for folate, produce energies at levels which can be easily separated by a two-channel counter.

## Exclusion criteria
Subjects with any other major systemic disease like hypertension or any cardiac disease, nephropathy or neuropathy and any other ocular disease were excluded form the study.

## Statistical analysis
Analysis of data was done via SPSS package version 9 (statistical package social science). Different tests were applied. Mean and standard deviation were used for data description. T-tests for dependent and independent variables were used. Kruskal Wallis ANOVA was done to compare mean ranks of different parameters for more than two groups, followed by an LSD test for significance (P-value<0.05 is considered significant) (21).

## RESULTS
Results of this study are illustrated in tables 1 and 2. A statistically significant increase of plasma tHcy in diabetics without retinopathy compared to controls (P<0.01) as well as decreased serum levels of both serum folate and vitamin $B_{12}$ (P<0.01) was observed (Table 1). There was a statistically significant increase of plasma tHcy levels and statistically significant decrease of serum $B_{12}$ and folic acid levels in diabetics with retinopathy compared to those without (P<0.01) (Table 1). There was no association between the different stages of retinopathy studied and plasma tHcy concentrations nor serum $B_{12}$ and folic acid concentrations (Table 2). Lipid profile of diabetic patients was normal. Also, they were normo-tensives and controlled. Kidney function tests (serum creatinine and creatinine clearance tests) were within the normal levels in all studied groups.

## DISCUSSION
The exact mechanisms by which homocysteine causes atherogenesis is unclear, but there is increasing evidence that it may be mediated through generation of reactive oxygen species such as superoxide anions and hydrogen peroxide (22). Experimental evidence has shown that the generated hydrogen peroxide causes direct endothelial cell damage, exposing the underlying matrix which promotes platelet aggregation (23,24).
Part of the abnormal endothelial cell response includes reduction in cellular nitric oxide levels. Nitric oxide protects against the toxic effects of homocysteine (25-28). Homocysteine alters the antithrombotic function of endothelial cells by activating factors V, X and XII. These factors prevent protein C activation (29,30) inhibit expression of thrombomodulin (31), suppress heparin sulfate expression (32) and decrease tissue plasminogen activator activity (33), all of which result

**Table 1.** Mean plasma levels of tHcy, serum vitamin $B_{12}$ and folic acid levels in all groups

|  | Controls | Diabetics with retinopathy (n = 40) | Diabetics without retinopathy (n = 10) | P value |
|---|---|---|---|---|
| Mean plasma tHcy (µmol/L) | 8.21 ± 9.25 a | 441.42 ± 108.95 b | 219.82 ± 8.28 c | <0.01 * |
| Mean serum vit. $B_{12}$ (pg/ml) | 605.80 ± 111.18 a | 117.00 ± 29.34 b | 199.14 ± 28.45 c | <0.01 * |
| Mean serum folic acid (ng/ml) | 12.01 ± 0.92 a | 2.48 ± 1.01 b | 6.42 ± 0.78 c | <0.01 * |

- Groups with different letters are with statistical significant differences

- The ratio of tHcy levels between controls and diabetics with retinopathy was 0.019; between controls and diabetics without retinopathy was 0.037.

- Conversion of µg/ml or mg/L to (µmol/L) was done by dividing to 0.1352.

**Table 2.** Mean plasma levels of tHcy, serum vitamin $B_{12}$ and folic acid in different stages of diabetic retinopathy

|  | Background diabetic retinopathy (n= 20) | Proliferative diabetic retinopathy (n= 20) |
|---|---|---|
| Mean plasma tHcy (µmol/L) | 441.42 ± 108.95 | 441.35 ± 104.51 |
| Mean serum vit. $B_{12}$ (pg/ml) | 117.00 ± 29.34 | 117.01 ± 29.30 |
| Mean serum folic acid (ng/ml) | 2.48 ± 1.01 | 2.48 ± 1.00 |

in a prothrombotic state. Homocysteine also promotes smooth muscle proliferation. It accelerates (34,35) lipid peroxidation (36,37), oxidation of low density lipoproteins (38) and elevation of triacylglycerol levels (39), which further contribute to the development of atheromas and vascular thrombosis. There is experimental evidence that homocysteine can accelerate the development of atherosclerotic lesions by initiating a cascade of inflammatory pathways mediated by tissue factors and receptors resulting in glycation end products (40).

However, little is known about the extent at which a marginal vitamin deficiency contributes to mild hyperhomocysteinemia and vascular disease. Plasma concentrations and dietary intake of folate, vitamin $B_6$ and vitamin $B_{12}$ relate inversely to the tHcy level, it was demonstrated that inadequate folate intake was the main determinant of homocysteine related increase of carotid artery wall thickening (41). Daily folate and vitamin $B_{12}$ supplements of 500 µg or less can reduce tHcy levels by up to 30 % (41). The addition of vitamin $B_{12}$ (normal serum level is 140-820 pg/ml) at an oral dose of 400 µg/day or more has been associated with plasma tHcy lowering effect and protection against the development of subacute combined degeneration of spinal cord (41). Treatment with vitamin $B_6$ has not shown the same effect on plasma tHcy levels.

Association between retinal vascular disease and homocysteine was studied by other authors (42), who concluded that elevated tHcy is an independent risk factor for retinal vascular occlusive disease and that lowering this level by administration of folate, vitamin $B_{12}$ and vitamin $B_6$ can improve prognosis.

The results of this work yielded a statistically significant increase in plasma tHcy in diabetics (with and without retinopathy) compared to controls as well as decreased serum levels of both serum folate and $B_{12}$.

The increased plasma tHcy concentrations in those patients may partly be caused by the decrease in blood folate concentration. It is known that several types of chronic inflammatory disorders result in lower blood folate (43) (normal whole blood folic acid 165-760 mg/ml). It is possible that the diabetic process itself in these patients also has such an influence on blood folate.

On the other hand, there was a statistically significant decrease of serum $B_{12}$ and folic acid levels and a significant increase of plasma tHcy level in diabetics with retinopathy in comparison to those without.

Upon studying diabetic retinopathy, Vaccaro O et al. (6) found significant increase in tHcy levels in patients with diabetic retinopathy while other studies (44,45) found insignificant levels. A possible relation between diet, hyperhomocysteinemia and arteriosclerosis has been suggested (46). However, little is known about the extent to which a marginal vitamin deficiency contributes to mild hyperhomocysteinemia and vascular disease. It was demonstrated that inadequate folate intake was

the main determinant of homocysteine related increase of carotid artery wall thickening (41). Furthermore, other studies (47) have reported that low serum folate concentration, but not serum cobalamine concentration, is associated with coronary artery disease. Recent work (48) has shown that low serum folate concentrations are associated with an increased risk of fatal coronary disease. It is probable that the association between folate and vascular disease can be explained mainly by folate regulation of plasma tHcy concentration.

As we can see, despite the basic fact that hyperhomocysteinemia is a risk factor for diabetic vascular complications, yet the results are contradictory. This may be explained by an important fact that plasma tHcy can be affected by both glomerular hyper-and hypofiltration (which are two conditions not present in our patients as their kidney functions were normal) that can respectively decrease and increase tHcy levels.

Lastly, correlation of homocysteine plasma level with lipid profile of our diabetic patients yielded non significant results. This is contradictory to those found by other authors (3,45) who found increased tHcy in dyslipidemic patients. This may be explained by the finding that our patients were both normo-tensives and normolipedimics.

The results of this work have shown no association between different stages of retinopathy and plasma tHcy concentrations in any occasion.

## Conclusion

The present study showed that diabetic patients with retinopathy are most prone to developing increased plasma tHcy which may be caused by a deficiency of both blood folate and vitamin $B_{12}$ concentrations. To minimize the risk of diabetic complications due to hyperhomocysteinemia a replacement therapy of these vitamins is recommended.

We recommend further research on larger numbers of patients affected by different stages of retinopathy and study of the importance of vitamin $B_6$ in diabetic retinopathy.

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