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Cobalamin and Folate Deficiency and their Relation to Activated Protein C Resistance as Risk Factors for Thrombosis in Diabetic Patients

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ABSTRACT

Diabetic patients with macro and micro-vascular arterial disease are likely to have deficiencies of cobalamin and folate with higher levels of homocysteine that may represent a risk factor for atherothrombosis independent of their glycemic control. Diabetes mellitus is also associated with activated protein C (APC) resistance especially in the presence of proteinuria which may represent an aggravating factor.

This work aimed at studying APC resistance, serum B_{12}, serum and red cell folate in a diabetic population and compare these parameters to those of age-sex matched non-diabetic controls. Patients and controls were subjected to thorough clinical examination, history taking and routine laboratory investigations.

Significant differences in APC ratio, serum B_{12} and folate between patients and controls as well as between diabetics with and those without vascular complications were noted. There were also a significant positive correlation between APC ratio and serum B_{12} and folate, and a significant negative correlation between APC ratio and proteinuria or microalbumiuria, in the diabetic population. This proves the significance of APC resistance as well as cobalamin and folate deficiency as risk factors for thrombosis in diabetic patients.

Key Words: *Diabetes - Vascular - Homocysteine - Folate - Vitamin B_{12} - Activated protein C resistance.*

INTRODUCTION

Homocysteine (Hcy) has emerged as a new risk factor for both arterial and venous thrombosis **[1]**. Recent studies demonstrate that a 5μmol/l increase in serum Hcy, i.e. 1 SD from the mean value is associated with a significant increased relative risk for the development of coronary heart disease (CHD) by 2.2 or more **[2]**. The risk/odds are even greater with peripheral arterial disease **[3]** and in cerebrovascular disorders **[4]**.

The metabolism of Hcy involves many enzymes that may be partly absent necessitating the presence of large amounts of cofactors in order to clear the toxic substance from the body. The cofactors involved are namely vitamin B_{12} (cobalamin), folic acid and vitamin B_6 **[5]**. Recently, it was demonstrated that subtle deficiencies of vitamin B_{12} may be associated with increased risk of thrombosis **[6]**. The issue of folic acid is not less important as it was demonstrated in three different studies that the third of the population with the lowest folate have a 69% increased risk of thrombosis compared to the third with the highest levels **[7]** and that for each 100μg of folate given daily more the risk of CHD is reduced by 5% **[8]**. Vitamin B6 is also important contributing factor **[9]**. In cases of acquired hyperhomocysteinemia, levels of B_{12}, and serum and red cell folate inversely correlate linearly with Hcy levels **[10]**.

The mechanism through which Hcy induces its toxic effect on the vessels and on the coagulation cascade involves inhibition of the docking of factor V to its inhibitor protein C, interference with antithrombin III enhancement by its cofactor heparin sulfate and many other routes of action **[11]**.

Diabetes mellitus, is associated with macro and microvascular complications and is known to be coupled by resistance to activated protein C (APC) especially in the presence of proteinur-

ia **[12]**. High levels of Hcy have been also reported among diabetics **[13]**. So the aim of this work is to study APC resistance, serum B_{12} and serum and RBCs folate as risk factors for thrombosis in a diabetic population, and comparing these parameters to age-sex matched non-diabetic control subjects.

PATIENTS AND METHODS

This study was conducted between May 2000 and December 2002 in the Internal Medicine Department, Clinical Hematology Unit, Kasr Al Aini Hospital, Cairo University.

The study enrolled eighty subjects (24 males and 56 females) including 60 diabetic patients and 20 normal healthy non-diabetic controls.

All diabetic patients and control subjects were subjected to full history taking, thorough clinical examination and laboratory investigations. Complete urine analysis, quantitative 24 hrs urinary proteins and test for microalbuminuria for patients and controls with no gross proteinuria were done. Routine hematological investigations and blood chemistry were also done. Finally, all patients and controls were subjected to the following specific laboratory investigations:

1- Determination of serum B_{12} using chemiluminescence technique.

2- Determination of serum and red blood cell folate using chemiluminescence technique.

Estimation of activated protein C resistance using clotting technique.

Exclusion criteria:

- All patients and controls were not receiving any vitamin supplementation.
- Patients with renal failure were excluded.
- Patients with impaired liver functions, liver cirrhosis and liver cell failure were also excluded.
- All studied subjects had normal basal coagulation profile and no one was receiving anticoagulants.

The diabetic patients were subdivided according to the presence of vascular complications into three groups each of 20 patients:

Group I: Included patients with diabetes in the absence of micro and macrovascular complications (4 males and 16 females) with mean age ± SD = 52.8±11.05 years.

Group II: Included patients with evident microvascular and no macrovascular complications (8 males and 12 females) with mean age ± SD = 50.8 ±12.56 years.

Group III: Included patients with macrovascular disease (6 males and 14 females) with mean age ± SD = 53.85±11.64 years.

The 20 normal healthy non-diabetic controls were 6 males and 14 females with mean age ± SD = 51.95±14.46 years.

RESULTS

Table (1) Mean ± SD of serum B_{12}, serum and RBCs folate levels and APC ratio in the controls and the 3 groups of patients.

Table (1): Shows Mean ± SD of serum B_{12}, serum and RBCs folate levels and APC ratio in the controls and the 3 groups of patients.

	Serum B_{12} pg/ml	S. folate ng/ml	RBCs folate ng/ml	APC ratio
Group I	377.6±192.6	8±2.9	361.8±174.9	2.54±1.09
Group II	316.9±197.5	7.4±2.8	355.8±113.7	1.94±0.69
Group III	239±107.1	6.3±1.5	304.8±149.3	1.83±0.59
Control	618.9±264.9	12.2±5.7	443.9±127.5	2.84±0.57

Table (2): Shows *p* values of mean serum B_{12}, serum and RBCs folate levels and APC ratio between various diabetic groups and controls.

	Serum B_{12}			Serum folate			RBCs folate			APC ratio		
	I	II	III	I	II	III	I	II	III	I	II	III
Group I	0.15	0.005	0.003	0.24	0.01	0.005	0.4	0.15	0.08	0.24	0.02	0.07
Group II	–	0.05	0.001	–	0.07	0.003	–	0.09	0.02	–	0.1	0.01
Group III	–	–	4	–	–	0.0001	–	–	0.003	–	–	0.0004

There was a high significant difference in mean values of serum B_{12} between control subjects and group I patients, a very high significant difference between control subjects and group II patients, a high significant difference between group I patients and group III patients and a significant difference between group II patients and group III patients as shown in Fig. (1) and Table (2).

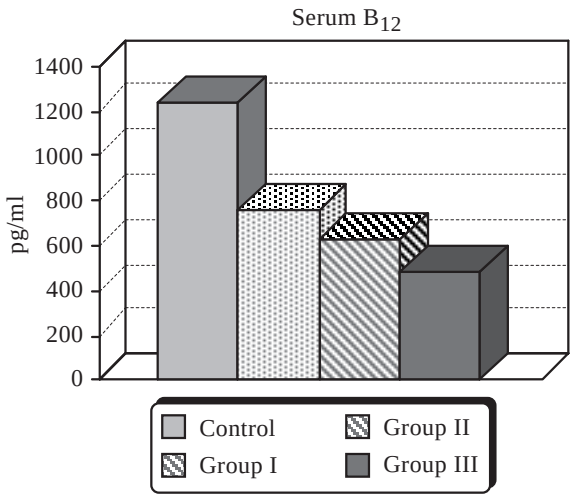

Fig. (1): Mean values of serum B_{12} in various diabetic groups and controls.

There was a high significant difference in mean values of serum folate between control subjects and group I patients, a high significant difference between control subjects and group II patients, a very high significant difference between control subjects and group III patients and a high significant difference between group I patients and group III patients as shown in Fig. (2) and Table (2).

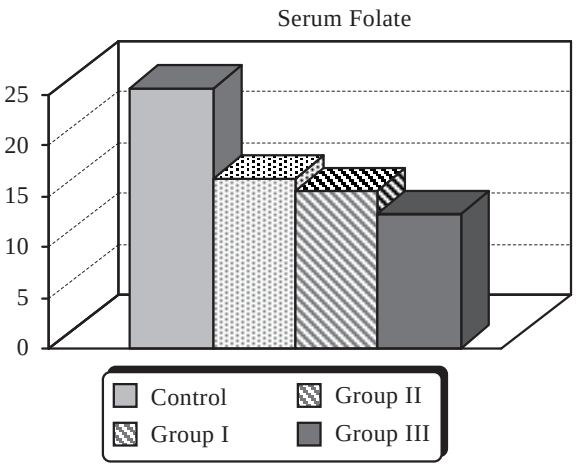

Fig. (2): Mean values of serum folate in ng/ml in various diabetic groups and controls.

There was a significant difference in mean values of RBCs folate between control subjects and group II patients and a high significant difference between control subjects and group III patients as shown in Fig. (3) and Table (2).

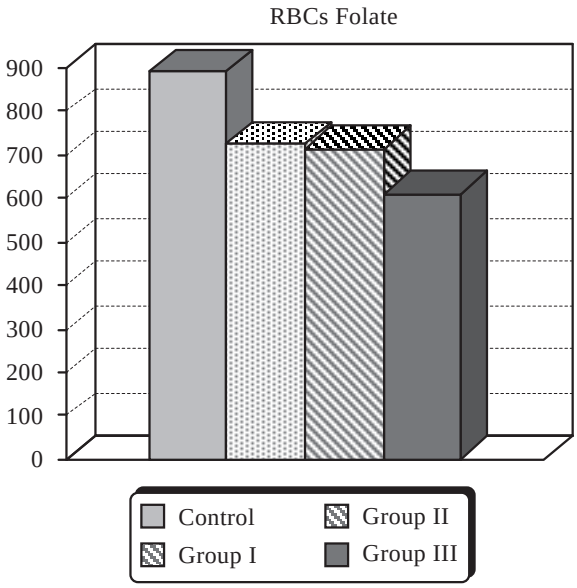

Fig. (3): Mean values of RBCs folate in ng/ml in various diabetic groups and controls.

There was a high significant difference in APC ratios between control subjects and group II patients, a very high significant difference between control subjects and group III patients and a significant difference between group I and group III patients as shown in Figs. (4,5) and Table (2).

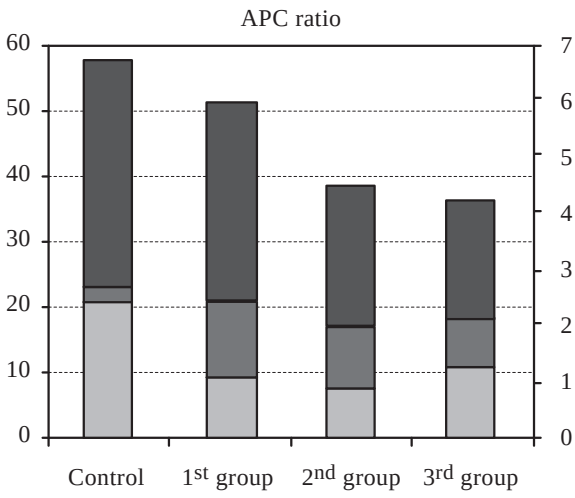

Fig. (4): Mean values ± SD of APC ratio in various diabetic groups and controls.

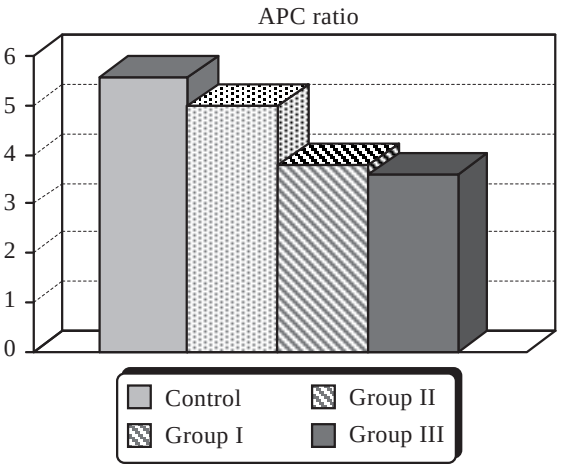

Fig. (5): Parametric mean values of APC ratio in various diabetic groups and controls.

A significant positive correlation between serum B_{12} and APC ratio was found in group I (r=0.56) and group III (r=0.55) patients as illustrated in Figs. (6,7) respectively.

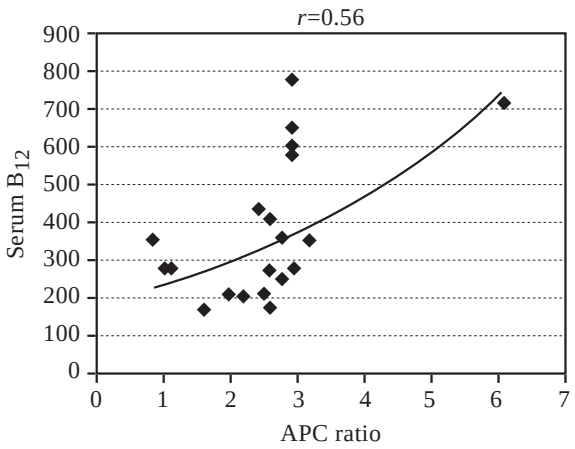

Fig. (6): Correlation between serum B_{12} in pg/ml and APC ratio in group I patients.

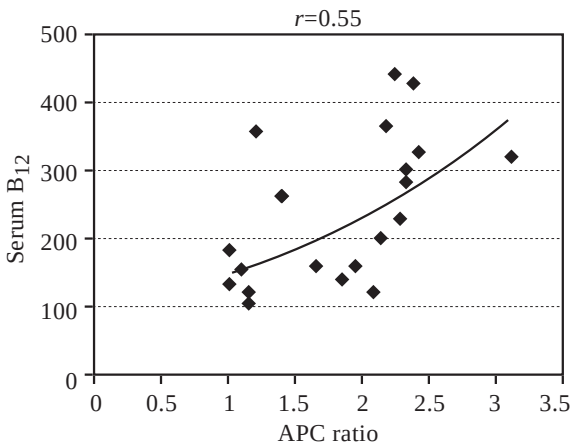

Fig. (7): Correlation between serum B_{12} in pg/ml and APC ratio in group III patients.

A positive correlation between serum folate and APC ratio was found in group I (r=0.41) and a significant positive correlation was found in group III (r=0.49) patients as illustrated in Figs. (8,9) respectively.

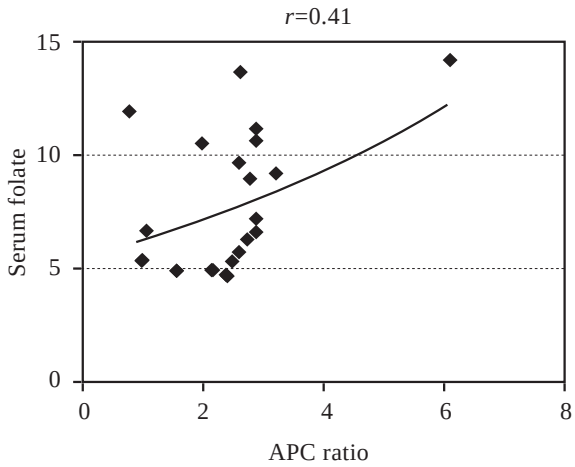

Fig. (8): Correlation between serum folate in ng/ml and APC ratio in group I patients.

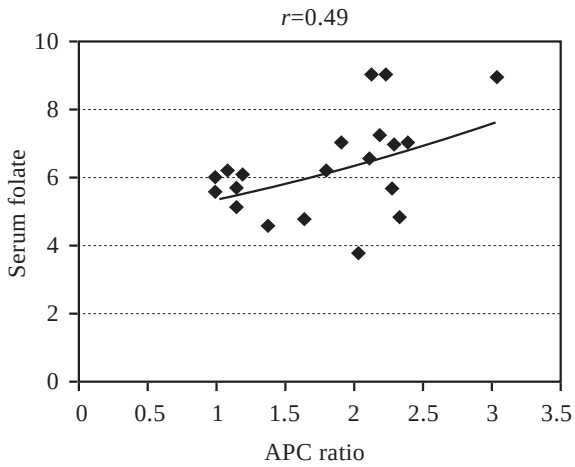

Fig. (9): Correlation between serum folate in ng/ml and APC ratio in group III patients.

A significant positive correlation between serum folate and RBCs folate was found in group I (r=0.62) and group II (r=0.51) patients as illustrated in Figs. (10,11) respectively.

A significant negative correlation between proteinuria and APC ratio was found in group II (r=-0.65) patients a significant negative correlation between microalbuminuria and APC ratio was found in group I (r=-0.45) and group III (r=-0.61) patients.

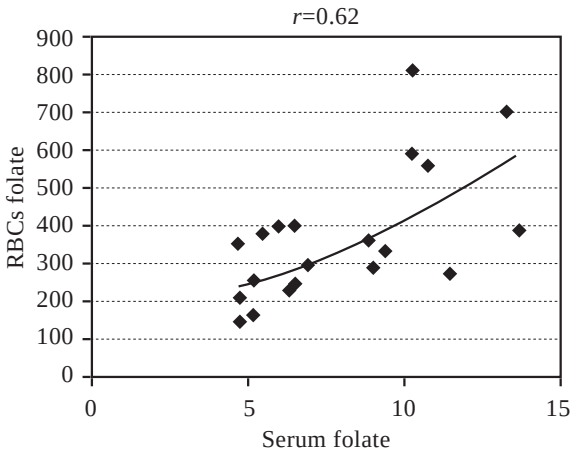

Fig. (10): Correlation between serum folate in ng/ml and RBCs folate in ng/ml in group I patients.

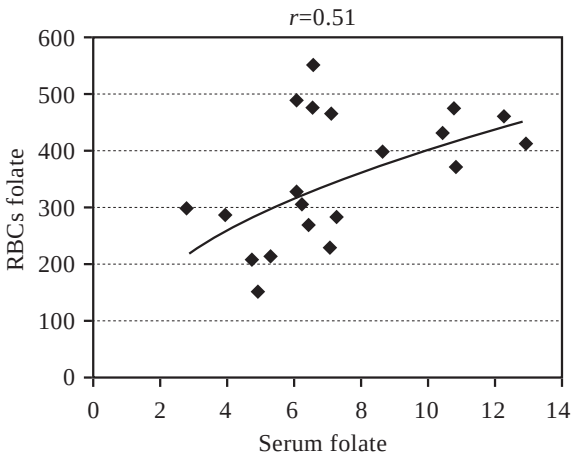

Fig. (11): Correlation between serum folate in ng/ml and RBCs folate in ng/ml in group II patients.

DISCUSSION

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is also associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels **[14]**.

In diabetes there is premature development and accelerated progression of macrovascular atherothrombotic disease. The coronary, cerebral, and peripheral arteries are the vessels mainly affected. Cardiovascular disease accounts for up to 80% of deaths in patients with diabetes, with approximately 75% of these deaths occurring as a result of ischemic heart disease **[15]**. Microvascular abnormalities and dysfunction are a systemic disease in diabetes. Clinically, diabetic microangiopathy leads to retinopathy and glomerular dysfunction and contributes to neuropathy **[16]**.

In the recent years, the potential significance of APC resistance occurring in the absence of known mutations of the factor V gene has been recognized i.e. acquired APC resistance, suggesting that APC resistance is a common mechanism whereby a number of risk factors for arterial and venous thrombosis promote thrombogenesis **[17]**. APC resistance appears to be an independent marker of a prothrombotic phenotype and the activated partial thromboplastin time (APTT-) based test is the most widely applicable acquired APC resistance screening tool **[17]**.

Esmon et al., expected that deficiencies in the protein C pathway would potentiate arterial thrombosis **[18]**. Patients with homozygous protein C or protein S deficiency usually exhibit neonatal purpura fulminans similar to the microvascular thrombosis seen in certain forms of septic shock **[19]**. In 1998, Sampram and colleagues were the first to report an increased risk of peripheral artery disease in patients with APC resistance but no gene mutation **[20]**. The Bruneck study extended these findings to other types of vascular disease and atherosclerosis in general. It revealed that poor response to APC is a prominent risk predictor of advanced atherosclerosis and arterial disease **[21]**. The study was the first to demonstrate a linear dose-response, independent, relation between decreasing APC ratio and increasing risk of advanced atherosclerosis **[21]**.

In our study there was a high significant difference in mean values of APC ratio between control subjects and group II patients; diabetics with evident microvascular complications, a very high significant difference between control subjects and group III patients; diabetics with evident macrovascular complications and a significant difference between group I and group III patients i.e. between diabetics without evident vascular complications and those with evident macrovascular complications, indicating the role of acquired deficiency in protein C pathway in diabetic vascular disease.

These results were in contrary to those reported by Biondi et al., where PC activity was

not different between diabetic patients and controls, being even higher, although not significant, in type 1 patients than in type 2 patients and controls **[22]**. Krugluger et al., also reported that PC level was significantly increased in the studied diabetic population with no significant changes in total or free protein S. They speculated that the increase in PC is part of a compensatory response to enhanced levels of procoagulants and that the highly significant increase of the APC ratio in diabetic patients compared with healthy subjects indicates an improved anticoagulant protection, which may help to counterbalance or moderate coagulant activation in these patients **[23]**.

However, our results were concordant with Hooper and Evatt who suggested that acquired deficiency in the protein C pathway is frequently a consequence of diabetes **[24]**, as well as with Gruden et al., who reported that APC ratio was significantly lower in diabetics than in the control subjects suggesting that the final steps of the protein C pathway could be abnormal in diabetes **[25]**.

In 1984, both Mogensen **[26]** and Jarrett et al. **[27]** independently reported that microalbuminuria was a marker of risk for development of cardiovascular disease (CVD) responsible for the increased mortality in diabetic patients. They concluded that microalbuminuria predicts cardiovascular and all-cause mortality. The Steno group has theorized that microalbuminuria represents a generalized vascular hyperpermeable state, wherein a decrease of the positive charges on the glomerular basement membrane allows leakage of albumin, and that similar changes in blood vessels elsewhere in the body allow potentially atherogenic lipoproteins to penetrate into the vessel walls, causing structural and functional damage to the endothelial cell barrier **[28]**. A decrease in the density of heparansulfate proteoglycans in glomerular basement membranes and in coronary vessels of diabetic individuals may also cause a disruption of structural integrity, which could result in widespread vascular involvement. Other proposed mechanisms responsible for the generalized endothelial dysfunction include abnormalities of lipid metabolism, cation membrane transport, coagulation factors, or toxic free radical generation **[28]**.

This work aimed at studying the correlation between APC resistance and proteinuria / microalbuminuria in the diabetic population. There was a significant negative correlation between proteinuria and APC ratio (r=-0.65) in group II patients; diabetics with evident microvascular complications.

Our study also showed a significant negative correlation between microalbuminuria and APC ratio in group I patients; diabetics without evident vascular complications (r=-0.45), as well as in group III patients; diabetics with evident macrovascular complications (r=-0.61).

Microalbuminuria is a marker of risk for development of CVD. That microalbuminuria predicts cardiovascular and all-cause mortality, has been confirmed in a number of studies in both type 1 and type 2 diabetes and even in nondiabetic individuals **[26]**.

The association of APC resistance and albuminuria found in our study cannot be interpreted by the findings reported in Sala et al., who on studying patients with nephrotic syndrome due to various causes including diabetic nephropathy found that urinary protein C (PC) antigen levels were above the normal range in 62% of the patients. PC in urine showed a positive correlation with antithrombin-III antigen excretion. However, average PC antigen in plasma was also significantly increased with respect to controls. PC in plasma was not correlated with either PC in urine or proteinuria **[29]**. They suggested that with albuminuria PC behaves like other vitamin K-dependent factors whose levels, probably due to increased synthesis in the liver, in plasma are normal or increased in spite of urinary leakage **[30]**.

Cosio and colleagues also reported a significantly elevated plasma concentrations of PC, its cofactor protein S (PS) and prothrombin in patients with severe proteinuria. They also suggested a generalized elevation in vitamin K-dependent protein synthesis in patients with proteinuria and the elevated PC levels may represent a protective mechanism for the hypercoagulable state in patients with proteinuria **[31]**.

However, our results can be interpreted by the reporting in Vigano D'Angelo et al., who observed low free PS levels in patients with nephrotic syndrome despite having elevated levels of total PS antigen. They were able to

demonstrate a urinary loss of free PS and increased C4b-binding protein leading to a shift from free to bound PS, thereby resulting in a pronounced decrease in free PS. The lowered free PS levels in nephrotic syndrome is attributed to urinary loss of low-molecular-weight free PS and to increased high-molecular-weight C4b-binding protein bound to PS, resulting in a hypercoagulable state **[32]**.

The association of APC resistance and albuminuria found in our study is concordant with the fact that hyperhomocysteinemia in diabetes, leading to an acquired APC resistance, is positively associated with the presence of albuminuria. Increases in fasting Hcy in diabetic patients are associated with increased albumin excretion rate. Hcy elevation is in the stages of incipient and overt nephropathy in the absence of renal failure **[33]**.

In our study there was a significant positive correlation between serum B_{12} and APC ratio in group I patients; diabetics without evident vascular complications (r=0.56), as well as in group III patients; diabetics with evident macrovascular complications (r=0.55).

A significant positive correlation between serum folate and APC ratio was found in group III patients; diabetics with evident macrovascular complications (r=0.49). A positive correlation between serum folate and APC ratio was also found in group I patients (r=0.41), however not statistically significant.

The inverse correlation between APC resistance and both vitamin levels was evident in group III patients. This inverse correlation could be attributed to hyperhomocysteinemia resulting from Cbl and folate deficiency which is a cause of acquired APC resistance.

Homocysteine is a sulfur-containing amino acid formed as an intermediate step in the metabolism of methionine, an essential amino acid abundant in animal protein. Metabolism of Hcy through the transsulfuration or remethylation pathways involves enzymes necessitating Cbl, folate and vitamin B_6 as cofactors. Abnormalities of these pathways, as a result of nutrient deficiencies may result in the accumulation of Hcy **[34]**.

Mechanisms through which Hcy induces its toxic effect on the vessels and on the coagulation cascade include interference with the antithrombotic and fibrinolytic mechanisms of the endothelium, reduced bioavailability of endothelium-derived nitric oxide, reduced glutathione and glutathione peroxidase the major intracellular buffers, affection of the function of other endothelial anticoagulant mechanisms, such as those of heparin-like glycosaminoglycans and ATIII interactions **[35]**. It also decreases endothelial binding sites for t-PA. Hcy influences proliferation of vascular smooth muscle cells and collagen deposition in the growing atherosclerotic plaque and enhances platelet aggregation **[36]**. Homocysteinemia is believed to be a cause for acquired APC resistance.

Yeromenko et al., on reviewing the literature dealing with the relationship between diabetes mellitus, B vitamins and Hcy concluded that low plasma B vitamins results in hyperhomocysteinemia in patients with diabetes mellitus **[37]**. Pavia et al found a negative correlation between total Hcy and serum folate (p<0.001) and Cbl (p<0.05) in 91 patients with type 1 diabetes **[38]**.

The association of hyperhomocysteinemia and diabetes was confirmed by many studies. Hofmann et al., presented evidence that ~35% of people with type 1 diabetes whom they studied had elevated plasma Hcy levels. In most subjects, levels were elevated in the fasting state; however, in a small subgroup, methionine loading was necessary to bring out an elevated level. Individuals with elevated plasma Hcy levels had a significantly greater prevalence of microvascular as well as macrovascular disease. Hcy may produce endothelial damage in vessels exposed to advanced glycation end products and, by this mechanism, could contribute to microvascular damage **[39]**. Araki et al., found that the levels of total Hcy in plasma were significantly higher in diabetic patients with macroangiopathy (10.8±3.8nmol/ml) than in those without macroangiopathy (8.3±3.1 mmol/ml, p<0.001) or non-diabetic subjects (7.5±2.1nmol/ml, p<0.001). The high levels of plasma Hcy were significantly associated with the presence of diabetic macroangiopathy (p=0.01) **[40]**. Pavia et al., found that in patients with type 2 diabetes, especially when signs of nephropathy or macroangiopathy coexist, hyperhomocysteinemia is a usual finding **[41]**. Modest elevations in non-fasting plasma total

Hcy were associated with all-cause mortality in population-based cohorts in Framingham **[42]**.

In our study there was a high significant difference in mean values of serum B_{12} between control subjects and group I patients; diabetics without evident vascular complications and a very high significant difference between control subjects and group II patients; diabetics with evident microvascular complications.

These findings denotes the relation between diabetes and Cbl deficiency which may be attributed to the increased loss of water soluble vitamins due to polyuria associated with diabetes mellitus and also due to increased tissue demands.

There was also a high significant difference in mean values of serum B_{12} between group I patients and group III patients i.e. diabetics without evident vascular complications and diabetics with evident macrovascular complications and a significant difference between group II patients and group III patients i.e. diabetics with evident microvascular complications and diabetics with evident macrovascular complications. These findings signify the relation between Cbl deficiency and vascular complications which is due to the consequent hyperhomocysteinemia and thus acquired APC resistance.

There was also a high significant difference in mean values of serum folate between control subjects and group I patients; diabetics without evident vascular complications, a high significant difference between control subjects and group II patients; diabetics with evident microvascular complications and a very high significant difference between control subjects and group III patients; diabetics with evident macrovascular complications.

These findings denotes the association between folate deficiency and diabetes mellitus as manifested by the high significant difference in serum folate between control subjects and all the 3 diabetic groups. This can be attributed to the increased loss of water soluble vitamins due to polyuria associated with diabetes mellitus and also due to increased tissue demands. Havivi et al on comparing the status of various vitamins in plasma of diabetic patients to those of age and sex matched healthy subjects found that vitamin deficiencies is common in the diabetic patients. The plasma concentration of folic acid and pyridoxine were found to be decreased in the diabetic patients in comparison to the healthy subjects. However, in diabetics the mean plasma concentrations of the vitamins were within the range of normal values **[43]**.

There was also a high significant difference in serum folate between group I and group III patients i.e. diabetics without evident vascular complications and diabetics with evident macrovascular complications. This finding signifies the relation between folate deficiency and vascular complications which is due to the consequent hyperhomocysteinemia and thus acquired APC resistance.

The significant difference in mean values of RBCs folate between control subjects and group II patients; diabetics with evident microvascular complications and the high significant difference between control subjects and group III patients; diabetics with evident macrovascular complications indicates that folate deficiency in the diabetic patients is not due to a recent change in their diet. RBCs folate level is more reflective of true body folate stores and correlates closely to that in the liver **[44]**. There was a significant positive correlation between serum folate and RBCs folate in group I (r=0.62) and group II (r=0.51) patients.

The absence of absolute Cbl or folate deficiency in our diabetic patients can be attributed to the habitual routine intake of B vitamins by diabetic patients.

In conclusions, this work reveals the role of APC resistance in diabetes mellitus as an important risk factor involved in the development of diabetic micro- and macrovascular complications adding to the prothrombotic state associated with diabetes. The state of hypercoagulability is present in diabetes even before the development of evident vascular complications. It also reveals the role of proteinuria and microalbuminuria in the development of APC resistance in diabetic nephropathy as well as in diabetics with incipient nephropathy.

The presence of APC resistance was directly associated with Cbl and folate deficiency found in diabetes mellitus pointing to the role of their deficiency in the development of diabetic vascular complications.

Measuring serum B_{12}, serum and RBCs folate and serum Hcy in diabetic patients and supplementing the individuals with low vitamin level or hyperhomocysteinemia with vitamin B_{12} and folate aiming at the prevention of diabetic vascular complications.

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