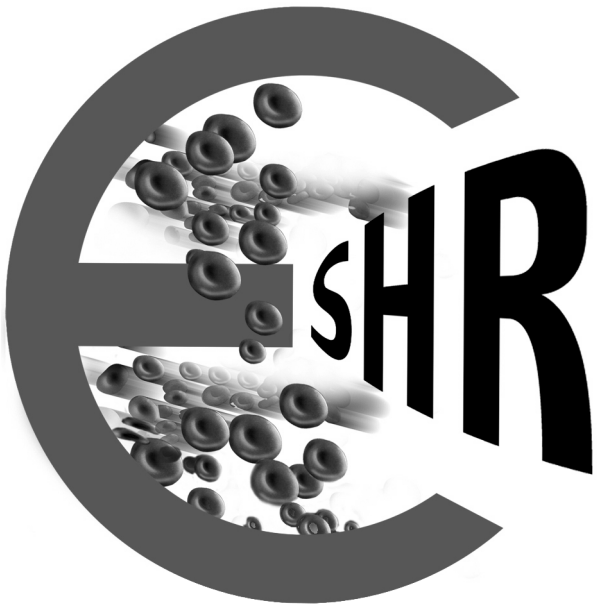
The Journal of the Egyptian Society of Haematology & Research



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A Study of Bone Marrow Levels of CD44, IL3 and IL6 in Acute Leukemia and Non-Hodgkin's Lymphoma at Diagnosis and During Remission

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ABSTRACT

The aim of this study was to clarify the diagnostic and prognostic aspect of bone marrow microenvironment elements in Acute leukemia and Non-Hodgkin's lymphoma. In this study, bone marrow plasma soluble CD44 (sCD44) and cytokines IL3 and IL6 were measured by enzyme-linked immunosorbent assay in 20 NHL and 40 Acute leukemia patients at diagnosis and after remission, and 10 bone marrow donors for transplantation as control. All the cases were subjected to clinical assessment, laboratory tests including bone marrow aspiration, immunophenotyping, cytochemistry and trephine biopsy.

Results: The BM sCD44 mean level was significantly elevated in patients with NHL (426.9±146ng/ml) and Acute leukemia (938.5±203.17ng/ml) reaching 2 folds the mean in NHL, while it decreased to near normal levels after remission with no statistical difference when compared to control group. In NHL patients, BM sCD44 >280ng/mL were associated with 2.8 and 4.7 folds higher frequencies of BM invasion and B-symptoms respectively at diagnosis. In NHL, the mean values of BM cytokines (IL-6 and IL3) were significantly increased in: Patients with B-symptoms, in high grade lymphoma, BM involvement and high IPI. Also, in acute leukemia, the mean values of BM cytokines (IL-6 and IL3) were found to be elevated at diagnosis and decreased to near normal during remissions.

Conclusions: It was concluded that sCD44 is useful in evaluating NHL and Acute leukemia disease activity, extent and response to treatment and can be used as a prognostic marker in these patients. Also, it was concluded that BM IL3 and IL6 are good indicators of disease activity and regression, so they can be considered good diagnostic and prognostic factors.

Key Words: *BM microenvironment - Extracellular matrix ECM - CD44 - IL-3, IL-6 - NHL.*

INTRODUCTION

The bone marrow microenvironment supports growth and differentiation of normal hematopoietic cells and can contribute to malignant growth. Since malignant cells localize and accumulate in bone marrow, it is important to understand the influence of the bone marrow microenvironment not only on the growth of the malignant cells, but also on the therapeutic response of malignant cells **[1]**.

Studies have demonstrated that haematopoiesis depends not only on specific cytokines (secreted by stromal cells) but also by adhesion molecules, that allow different cell types to adhere stably to one another or to ECM **[2]**.

In the hematopoietic system, adhesion and migration are essential activities for normal development and function of hematopoietic cells **[3]**.

The lymphocyte homing receptor, CD44, is a polymorphic glycoprotein with a molecular mass ranging from 85 to 250 kda. It represents a family of glycoproteins encoded by a single gene on the short arm of chromosome 11p13 that contains 20 exons **[4]**. Ten additional exons can be alternatively introduced into a common splice site in different combinations creating various splice variants designated as CD44v **[5,6]**. The standard form (commonly referred to CD44H or CD44S) of the molecule lacks all variant exons and is expressed on cells of hemopoietic and mesodermal origin **[7]**.

Soluble CD44 has been detected in serum, lymph, arthritic synovial fluid, and bronchoalveolar lavage. Malignant disease and immune activation and inflammation are often associated with increased plasma levels of sCD44, whereas

immunodeficiency correlates with low plasma levels of sCD44 **[8]**.

A high serum sCD44 level and/or tumour cells expression at diagnosis is associated with prognostic criteria and/or unfavourable outcome in childhood lymphoblastic leukemia/lymphoma and not in acute myeloid leukemia **[9]**. Also a high serum level of sCD44 was reported to be correlated with a poor outcome of aggressive NHL suggesting that it could be a useful prognostic marker **[10]**.

Cytokines are glycoproteins that regulate all the important biological processes including cell growth, cell activation, inflammation, immunity, tissues repair, fibrosis and morphogenesis. Cytokines that influence hematopoiesis can be divided into three categories; direct acting e.g. IL3, IL6, GM-CSF on multipotent progenitors, indirect acting e.g. IL1 and TNF on stromal cells and miscellaneous e.g IL8, IL9, IL10, IL11 and IL12 **[11]**.

This study is conducted to investigate the role of sCD44, IL3 and IL6 in bone marrow microenvironment in acute leukemia and Non Hodgkin's lymphoma at diagnosis and remission after treatment, and to study their use as prognostic markers for disease activity and effect of therapy on BMM.

MATERIAL AND METHODS

Materials:

Study samples were divided into two groups of patients at diagnosis and during remission and controls. They were divided as follows:

Group (Ia): 40 patients with de novo diagnosis of acute leukemia (24 males and 16 females) with a mean age 35 years (range 8-70). Patients of this group were subdivided according to FAB Classification into: (a) ALL (6 cases L_1 and 9 cases L_2) (b) AML (4 cases M_1-7 cases M_2-3 cases M_3-4 cases M_4-3 cases M_5 and - 4 cases M_7).

Group (Ib): Group Ia (35 out of 40 patients) during remission, after induction course of chemotherapy (21 males and 14 females), age range 8-60 years. 5 cases died during follow up (2 cases M_7 & 3 cases M_3).

Group (IIa): 20 patients with de novo diagnosis of Non-Hodgkin's lymphoma (13 males and 7 females with a mean age 50 years (range 20-72). Patients of this group were subdivided after immunophenotyping into B-NHL (16/20) and T-NHL (4/20).

Group (IIb): The same 20 patients of Group IIa during remission.

Group (III) Control group: 10 healthy subjects (8 males and 2 females) with mean age 29 years (range 20-38 years), selected from bone marrow transplantation donors. All the cases were selected from National Cancer Institute and Nasser Institute from 2002 to 2003.

Methods:

Sample collection: Peripheral blood samples were collected for routine lab investigations. (CBC, ESR, LFTs and KFTs). Serum samples were separated and used for the determination of LDH and B2 microglobulin (for NHL cases).

Bone marrow aspiration: This was done for all patients and controls by the standard technique **[12]**. Diagnosis was done based on morphology and cytochemistry. The remaining aspirate was transferred into a tube containing heparin and centrifuged at 1000xg/ for 10min and plasma was divided into sterile aliquots and stored at -70°C till use.

Immunophenotyping: This was done for all cases of Acute leukemia and NHL by flow cytometry using fluorescent labeled antibodies according to Landy and Muirhead 1989 **[13]**.

Bone marrow trephine biopsy:

BM biopsy was done to fulfill the diagnosis whenever indicated (done only for 10 cases of ALL, 9 cases of AML and all cases of NHL) to study the stromal cells and for staging of NHL, using the standard technique according to Williams and Nicholson, 1963 **[14]**.

Investigations:

1- Serum LDH: Was determined by the kinetic assay on the Beckman-Synchron CX R Systems Chemistry Information (LD-P) kit by Handerson, 1995 **[15]**.

2- Serum B2 microglobulin: Was determined by an immunometric enzyme immunoassay EISA for the quantitative determination in human serum, plasma or urine **[16]**. The kit produced by ORGEN Tec. Diagnostila GM-BH, (Mainz, Germany). The amount of color

is directly proportional to the concentration of Beta-2 microglobulin present in the original sample.

3- Assay of IL-3: Was performed in bone marrow plasma by ELISA technique for quantitative determination of human IL-3 by Fishman, 1990 **[17]** (manufactured by BioSource International, California, USA). IL3 is expressed in (pg/ml).

4- Assay of IL6: Was measured in bone marrow plasma by a two step sandwich ELISA technique manufactured by Diaclone Research, 2000 **[18]**, (FRANCE) for in-vitro quantitative determination. IL6 is expressed in (pg/ml).

5- Assay of CD44: Was measured in bone marrow plasma by a two step sandwich ELISA technique manufactured by Diaclone Research 2000 **[18]**, (FRANCE) for in-vitro quantitative determination. CD44 is expressed in (ng/ml).

All assays were performed as per the manufacture's instructions. Each sample was assayed in duplicate.

Statistical analysis: **[19]**

The collected data were tabulated and statistically analyzed (Minitab SPSS statistical software version, 1998) :12-1. For quantitative data, the range, mean and standard deviation were calculated.

The difference between two means was statistically analyzed using the students (*t*) test. Paired *t* test was performed to test mean values at diagnosis and at remission.

Chi-square as a non parametric test was used to assess the statistical significance of associations among categorical variables when assumptions for its application were fulfilled.

Pearson correlation coefficient (*r*) was used to assess the statistical significance of correlation among normally distributed quantitative variables the value of *r* ranges from –1 to +1, if the value of *r* positive then the correlation is positive, whereas negative values of *r* indicate inverse or negative correlation.

Difference, associations and correlations were considered significant when the *p*-value of the corresponding test is less than or equal to 0.05.

RESULTS

Characteristic and clinical data of patients and controls:

In Group Ia (Acute leukemia patients): Pallor was the most common finding present in 80% of cases, 56% of cases were presented by fever, 48% were presented by purperic rashes & 32% were presented by ecchymosis. Splenomegaly was felt in 60% of cases hepatomegaly in 44% of cases and lymphadenopathy in 60% of cases.

In Group IIa (NHL patients): 60% presented by axillary lymphadenopathy, followed by cervical and inguinal lymph nodes enlargement in 45% and 40% respectively. 86% presented by weight loss while 50% presented by fever and night sweat. Bone marrow infiltration was detected in 75%. Splenomegaly and hepatomegaly were present in 90% and 75% of patients respectively. CNS invasion was present in 10% of cases.

Laboratory investigations:

Hematological tests were performed for all cases. In Group Ia patients at diagnosis; 82% represented by anaemia and leucocytosis, while 20% represented by leucopenia and 90% represented by thrombocytopenia. In Group IIa patients at diagnosis; 70% presented by anaemia, 35% by leucocytosis and thrombocytopenia in 40%.

Immunophenotyping: Evaluation of the following markers was done for all cases: CD45, CD34, HLA DR, CD10, CD19, CD20, CD21 CD22, CD23, IgM, SIg Kappa, SIg Lambda, FMC7, CD1, CD2, CD3, CD5, CD7, CD4 and CD8, CD13, CD33, CD14, CD15, MPO, CD41, and CD61.

I- Acute leukemia cases were diagnosed as follows:

A- *Acute lymphoblastic leukemia:* Precursor B-ALL (CD19, CD20, CD22 and cytoplasmic μ positive) in 4/40 (10%) cases L1 and 4/40 (10%) cases L2. Common ALL (CD10, CD19, HLA DR, CD34 and CD22 positive) in 2/40 (5%) cases L1 and 5/40 (12.5%) cases L2.

B- *Acute myeloid leukemia:* CD 13+ve, CD33+ve, MPO+ve in 4/40 cases M1 (10%) and 7/40 (17.5%) cases M 2, 3/40 (7.5%) cases M 3 also HLA DR+ve, 4/40 (10%)

cases M 4 and 3/40 (7.5%) cases M 5 also CD14+ve, CD15, CD34+ve, HLA DR+ve and 4/40 (10%) cases M 7 CD41 +ve and CD61 +ve.

II- *NHL cases were divided into:*

A- B-cell Lymphoma which constituted 80% of patients:

Follicular cell lymphoma (FCL) 8/20 cases (40%) were CD10, FMC7, CD22, SIg Light chain +ve. Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) 3/20 cases (15%) were CD19+ve, CD5+ve, CD23+ve and SIg Light chain dim, FMC7 and CD22 negative. Diffuse large cell lymphoma 5/20 cases (25%) were CD 19+ve, CD20+ve and CD21+ve, FMC7 with positive expression of SIg Kappa (3/5 cases) and SIg Lambda (2/5 cases) (mature B cell lymphoma).

B- T-cell lymphoma constituted 20% of patients:

Early T-cell phenotype in 2/20 cases (10%) were CD2+ve, CD5+ve, CD7+ve cytoplasmic CD3+ve with CD4-ve and CD8-ve.

Intermediate T-cell phenotype in 2/20 cases (10%) were CD1, CD2+ve, CD5+ve, CD7+ve, cytoplasmic CD3+ve with CD4 and CD8 coexpression.

NHL cases were further divided according to the grade of disease into: Low grade lymphoma 11 cases (55%), Intermediate grade lymphoma 5 cases (25%) and High-grade lymphoma 4 cases (20%).

I- *Assay of serum level of LDH:*

Fig. (1) shows the serum levels of LDH (U/L) in the studied groups: The mean level of LDH in acute leukemic patients at diagnosis (1250±879) was found to be significantly increased as compared to the mean level in controls.

During remission, the mean level of LDH (541±196.47) was less than its level at diagnosis but it still higher than its level in controls. A comparison of mean serum LDH level between ALL and AML at diagnosis and during remission: Revealed a nonsignificant difference.

In Group IIa (NHL at diagnosis), mean level of LDH was (570.65±161.84U/L), while it decreased (at remission) in Group IIb, where it was (282.80±66.36 U/L), close to that of control group (278±51.22). There was a statistical significant increase at diagnosis when compared to control group (p>0.001) and at remission (p<0.001).

II- *Assay of serum B2 microglobulin only in NHL patients:*

The mean value of serum B2 microglobulin showed a statistically significant increase in Group IIa (NHL at diagnosis) when compared to control group (p<0.001), while Group IIb showed a statistically nonsignificant increase when compared to control group (p>0.05).

III- *Assay of bone marrow level of IL3 (pg/ml):*

In Fig. (2) the mean level of BM IL3 (pg/ml) in Acute leukemic patients at diagnosis Group Ia (360.6±45.7) was found to be statistically significantly increased as compared to the mean level in controls (28.4±10.5) i.e Group Ia was nearly 13 folds higher frequencies of Group III (p<0.001).

During remission of acute leukemia, Group Ib, the mean level of BM IL3 (185±26.35) was 50% less than its level at diagnosis but still higher than in controls i.e. group Ib was 6.5 folds of group III (p<0.001). A comparison in mean level of BM IL3 level between ALL and AML at diagnosis and during remission revealed a nonsignificant statistical difference (p>0.05).

In NHL Group IIa, the mean level of BM IL3 (95.85±49.34) pg/ml at diagnosis, showed a statistically significant increase when compared to the mean level during remission (Group IIb) and controls (Group III) (p<0.001) and (p<0.001), respectively. In the meantime, the mean level of BM IL3 in Group IIb showed a nonsignificant statistical difference when compared to control Group III (p>0.05).

IV- *Assay of bone marrow level of IL6 (pg/ml):*

In Fig. (3), the mean level of BM IL6 in acute leukemic patients at diagnosis Group Ia (59.4±12.5) was found to be statistically significantly increased as compared to the mean level in controls (18.6±6.3) i.e. Group Ia was 3 folds higher than control. During remission, in Group Ib, the mean level of BM IL6 (28.2±5.19) was less than that at diagnosis but still higher than controls.

A comparison in mean levels of BM IL6 level between ALL and AML at diagnosis and during remission showed a nonsignificant statistical difference.

In NHL Group, the mean level of BM IL6 showed a statistically significant increase in Group IIa at diagnosis (49±19.3) when compared to Group IIb during remission (20.1±7.83) and control ($p<0.001$) and ($p<0.001$) respectively. While the mean level of Group IIb showed a nonsignificant statistical difference when compared to control group ($p>0.05$).

V- *Assay of bone marrow level of CD44s (ng/ml):*

Fig. (4) shows the BM levels of CD44s (ng/l) in Acute leukemia and NHL. The mean level of CD44s in acute leukemic patients at diagnosis (938.5±203.17) was found to be statistically significantly increased as compared to the mean level in controls (194.6±61.55) i.e. Group Ia was 5 folds higher frequencies of group III.

During remission in Group Ib, the mean level of CD44s (238.7±45) was less than its level at diagnosis but it was still higher than its level in controls i.e. Group Ib was higher than control group. A comparison of CD44s level between ALL and AML at diagnosis and during remission showed a nonsignificant statistical difference.

In NHL group, the mean level of BM CD44s was statistically significantly increased in Group IIa at diagnosis (426.9±146) when compared to control group and during remission in Group IIb (205.2±70.2) ($p<0.001$) and ($p<0.001$) respectively. While during remission, there was a nonsignificant statistical difference when compared to control group ($p>0.05$).

A coefficient correlation study was done in Acute Leukemia Group Ia at diagnosis between the studied levels of BM IL3, BM IL6, BM CD44s, and HB level, LDH, total leukocyte count, % of blasts in peripheral blood and in bone marrow.

There were significant positive correlations between BM CD44s levels and total leucocytic count, % blasts in both peripheral blood and BM ($p<0.05$). There were significant positive correlations between BM IL3 and BM IL6 with TLC and LDH respectively. There were nonsignificant correlations between levels of BM IL3 as well as BM IL6 and rest of the studied parameters ($p>0.05$) (Table 2).

A correlation matrix was done between the studied parameters in NHL at diagnosis Group IIa revealing the following:

There was a positive correlation between BM IL-6 and IL3, Serum LDH and B2 microglobulin with percentage of blasts in peripheral blood and bone marrow and IPI (International NHL Prognostic index).

BM CD44s level showed a significant positive correlation with each of BM IL-6, LDH, B2 microglobulin, Hb level, total leucocytic count and % of blast cells in peripheral and bone marrow ($p<0.05$).

The relation between the different cytokines and symptoms & signs of BM and CNS invasion in NHL cases was studied showing the following:

In Group IIa the symptoms and signs of BM invasion were present in 13/17 (76.5%) of NHL cases with a BM plasma level of IL-3 above >50pg/ml (highest level in control group), and in 2/3 cases (66.7%) with BM IL3 less than <50pg/ml, a non significant statistical difference ($p>0.05$).

The symptoms of CNS invasion were present in only 2/17 (11.76) of cases with BM IL-3 >50pg/ml. While no symptoms of CNS invasion were present in NHL cases who had BM IL-3 ≤50pg/ml 3/3 (100%), and 15/17 (88.24%) with BM IL3 >50pg/ml.

As for IL6, the symptoms and signs of BM invasion were present in 14/17 (82.3%) NHL cases at diagnosis with a BM plasma level of IL-6 above >28pg/ml, (highest level in control group), and in only 1/3 cases (33.3%) with BM IL6 less than ≤28pg/ml, a nonsignificant statistical difference ($p>0.05$).

The symptoms of CNS invasion were present in only 2/17 (11.76) of cases with BM IL-6 >28pg/ml. In the meantime, no symptoms of CNS invasion were present in NHL cases, who had BM IL-6 ≤28pg/ml 3/3 (100%) and in 15/17 (88.24%) with BM IL6 >28pg/ml, a statistical nonsignificant difference.

The relation between BM adhesion molecule sCD44 and symptoms & signs of bone marrow and CNS invasion in NHL was studied revealing the following:

In Group IIa, 82.35% of patients who had a BM sCD44 level >280ng/ml (highest level in control group), suffered bone marrow invasion compared with 33.3% of those who had BM sCD44 level ≤280ng/ml, the difference was not statistically significant (p>0.05).

The symptoms of CNS invasion in relation to BM CD44 showed the same results as those for BM IL6.

The mean value of BM IL6 and CD44 showed a highly significant statistical increase in patients with extranodal site >1 than those patients with extra nodal sites ≤1 at diagnosis and remission with a p value (p<0.001).

The mean values of BM cytokines (IL6 and IL3), serum LDH and B2 microglobulin were increased in patients with high grade lymphoma than those with low/intermediate grade lymphoma at diagnosis and after remission with no statistical significance (p>0.05).

In the meantime, the mean value of BM CD44 was statistically significantly increased with high grade lymphoma than those with low/intermediate grade lymphoma at diagnosis and after remission (p<0.05). The mean values of BM cytokines (IL3 and IL6) were more increased in patients with BM invasion than those without BM invasion at diagnosis and remission with no statistical significant difference (p>0.05) for each.

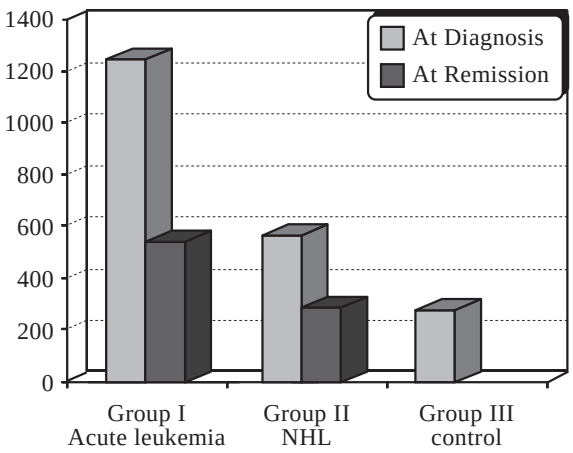

Fig. (1): Comparison between Serum LDH levels in Acute Leukemia and NHL at diagnosis and remission.

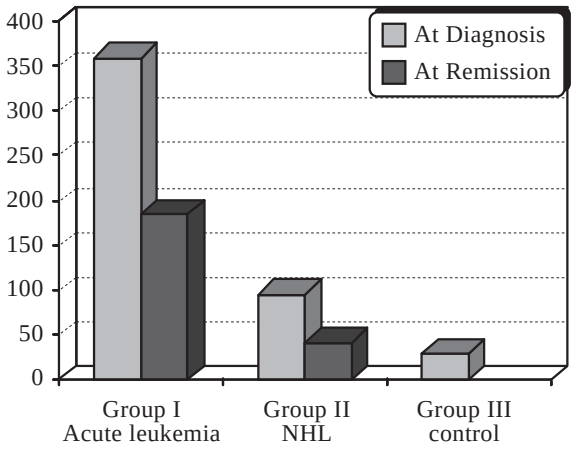

Fig. (2): Comparison between BM IL-3 in Acute Leukemia and NHL at diagnosis and remission.

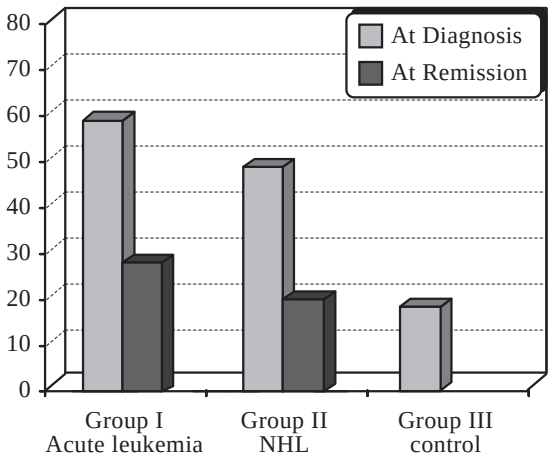

Fig. (3): Comparison between BM IL-6 in Acute Leukemia and NHL at diagnosis and remission.

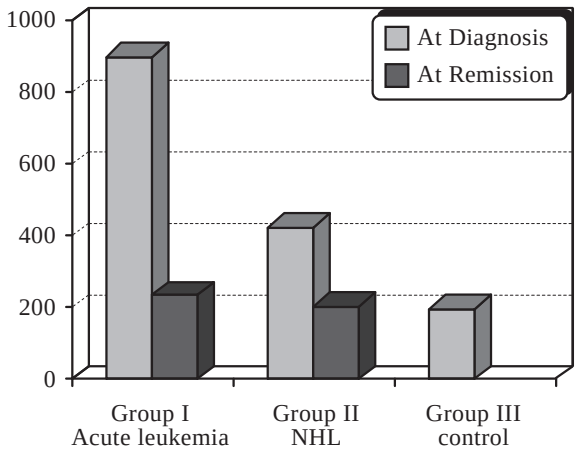

Fig. (4): Comparison between BM SCD44 in Acute Leukemia and NHL at diagnosis and remission.

Table (1): Classification of studied cases.

Group	No. of Patients	Diagnosis
Group Ia	40	De novo Acute leukemia patients before receiving chemotherapy
Group Ib	35	Same Acute leukemia patients during remission
Group IIa	20	De novo NHL patients before receiving chemotherapy
Group IIb	20	Same NHL patients during remission
Group III	10	Donors of bone marrow transplantation as controls

Table (2): Correlation coefficient (*r*) of Acute Leukemia at diagnosis Group Ia.

	HB	TLC	% PB BLASTS	% BM BLASTS	LDH
LDH:					
r	–0.032	0.174	–0.032	0.090	0.371*
p	0.846	0.284	0.847	0.607	0.018
CD44:					
r	0.003	0.770*	0.750*	0.670*	–0.032
p	0.987	0.000	0.001	0.001	0.845
IL3:					
r	–0.184	0.405*	0.158	–0.193	-0.044
p	0.255	0.009	0.332	0.265	0.788
IL6:					
r	–0.744*	0.003	0.046	0.050	0.279*
p	0.001	0.987	0.777	0.773	0.018

*: Significant. *r*: Pearson correlation.

Table (3): Correlation coefficient (*r*) of NHL at diagnosis Group IIa.

Variables	LDH		IL6		sCD44		B2 microglobulin		IL-3	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
IL6	0.666	0.001*								
S-CD44	0.744	0.001*	0.505	0.023*						
B2-microglobulin	0.654	0.002*	0.168	0.479	0.59	0.006*				
IL3	0.781	0.001*	0.465	0.039*	0.804	0.001*	0.66	0.002*		
Hb	–0.714	0.001*	–0.746	0.001*	0.860	0.001*	–0.772	0.001*	–0.745	0.001*
TLC	0.516	0.020*	0.581	0.007*	0.459	0.042*			0.631	0.003*
% Blasts in PB	0.715	0.001*	0.759	0.001*	0.757	0.001*	0.485	0.007*	0.701	0.001*
% of Blasts in B.M	0.671	0.001	0.520	0.019	0.621	0.003	0.239	0.310	0.675	0.001
ESR 1st hour	0.864	0.001*	0.727	0.001*	0.828	0.001*	0.828	0.001*	0.833	0.001*
ESR 2nd hour	0.837	0.001*	0.699	0.001*	0.819	0.001*	0.823	0.001*	0.822	0.001*
IPI	0.523	0.018*	0.590	0.006*	0.692	0.001*	0.593	0.006*	0.621	0.003*

*: Significant. *r*: Pearson correlation.

DISCUSSION

Hematological malignancies including acute leukemia and NHL are clonal disorders resulting from the neoplastic transformation of progenitor cells. Similar to their normal counterparts transformed hematological progenitor cells remain dependent on signals from the microenvironment for survival and proliferation during their malignant progression. These cells can also induce reversible changes in the marrow stroma that will further faster the development of malignant cells. This dynamic reciprocal interaction between the microenvironment and the malignant hematological cells continues throughout disease progression **[20]**.

The aim of the present work is to study the bone marrow microenvironment (BMM) elements in Acute leukemia and NHL patients at diagnosis (before treatment) and after complete remission to evaluate the impact of chemotherapy on these patients. For this purpose we estimated BM cytokines (IL3 and IL-6), BM adhesion molecule (sCD44) in 40 Acute Leukemia and 20 NHL patients at diagnosis and in remission with 10 apparently healthy bone marrow donors as a control group.

BM aspiration and Trephine biopsy were performed for all patients at diagnosis and after remission for evaluation of BM, also to examine infiltration of BM by lymphoma cells, its types, and degree to be compared with during remission.

Descriptive analysis of our patients showed male predominance (62%) in comparison to females who represented (38%) with male to female ratio 1.63. Mean age of patients in this study was 35 years in acute leukemia group and 50 years in NHL group.

The pathological examination revealed that 85% of cases had B-cell lymphoma and 15% had T-cell lymphoma, Among B-cell lymphoma cases, 35% were follicular lymphoma, 30% were diffuse large cell lymphoma and 15% were B-CLL, while all cases of T-cell lymphoma were of Lymphoblast lymphoma type.

Cytokines are a master element of BMM, they are glycoproteins that regulate proliferation, differentiation, maturation and survival of hematopoietic stem cells & progenitor cells **[21]**. Tumor necrosis factor (TNFα), interleukin 3 (IL3) and interleukin 6 (IL6) are cytokines produced by cells of the immune system after immunologic stimuli and are vital to normal immunity, they act in a network of factors directing both immune and malignant response.

IL3 appears to have effects predominantly on stem and progenitor cells and minimal effect on more mature cells **[22]**.

IL6 plays a role in mediation of inflammation and immune response. It enhances the formation of multilineage blast cells colonies in vitro and to support the formation of lineage restricted cell types. IL6 also, has an effect on thrombopoiesis **[23]**.

In the present study, it was found that the BM levels of IL3 and IL6 were significantly higher in acute leukemic patients at diagnosis than healthy controls. Their levels (IL3 and IL6) during remission were noticeably lowered but still slightly more than normal controls.

Also in NHL patients, the BM IL-6 level was increased in (80%) at diagnosis above the upper limit of control group (28pg/ml). The mean value of BM IL-6 was statistically significantly increased in NHL in patients at diagnosis when compared to control group. While after remission, it was more decreased than its level at diagnosis with a statistically significant difference, but still to be more than that level in control group with no stastically significant difference ($p>0.05$).

A high level of IL3, in sera from acute leukemia patients, was reported after comparing its level to healthy subjects and it was lowered towards normal after achieving complete remission **[24]**. All the findings were in agreement with other authors as **[25,26]**.

In NHL group, it was found that 16 patients (80%) had a bone marrow IL-3 level more than reference level at diagnosis. The mean value of BM IL-3 was statistically significantly increased at diagnosis when compared to control group, while after remission, it was more decreased than its level at diagnosis with a statistically significant difference, but still more than its level in control group with no statistically significant difference ($p>0.05$).

In the present study the mean value of BM IL-3 was higher in NHL patients with B symp-

toms (at diagnosis) than without with a statistically significant difference ($p<0.05$). Also the mean values of BM IL-3 were higher in patients with extra nodal sites than those with extra nodal site ≤1 at diagnosis and after remission with no significant difference at diagnosis ($p>0.05$). It was, also, increased in patients with BM involvement than those without BM involvement at diagnosis and after remission with a statistical significance difference ($p<0.05$).

The mean value of BM IL-3 was increased in high grade lymphoma than low/intermediate grade lymphoma with no statistical significant difference ($p>0.05$). Also it was increased in patients with high IPI than those with intermediate/high, intermediate/low and low IPI patients at diagnosis and after remission with statistical significance difference ($p<0.05$).

The results of this study showed that there were positive correlations between BM IL-3 and each of IL-6, sCD44, serum LDH and B2 microglobulin, IPI, total leukocytic count, and % of blast cells in peripheral blood, while it showed negative correlation with Hb level.

The mean value of BM IL-6 was increased in patients with B-symptoms at diagnosis with a statistical significant difference ($p<0.05$). BM IL-6 >28pg/ml was associated with 5.45 folds higher frequencies of B-symptoms than those patients with BM IL-6 <28pg/ml at diagnosis with no statistical significant difference ($p>0.05$). This is in agreement with others who reported that IL-6 was increased in patients with B-symptoms than those without B - symptoms **[27]**.

Also the mean values of BM IL-6 were increased in patients with extranodal sites than those with extranodal site ≤1 at diagnosis and in patients with BM involvement than those without BM involvement at diagnosis and after remission with no statistical significance difference ($p>0.05$) for each.

Also, patients with BM IL-6 >28pg/ml at diagnosis were associated with 1.5 fold higher frequencies of BM invasion than those with IL-6 ≤28pg/ml. Patients with BM IL-6 >28pg/ml at diagnosis were associated with 2.83 fold higher frequencies of BM invasion than those patients had BM IL-6 ≤28pg/ml.

In the present study the mean value of BM IL-6 in patients with T-cell lymphoma is more than that in patients with B-cell lymphoma at diagnosis and after remission showing a statistical significant difference ($p<0.05$).

The result of this study showed that there were significant positive correlation between IL-6 and each of serum BM IL-3, sCD44, serum LDH, IPI, total leukocytic count and % of peripheral blast sales while it shows strong negative correlation with Hb level. This in agreement with Fayed et al., 1998 **[28]**.

As regard CD44s in the present study: The estimated BM levels of CD44 in acute leukemic patients (both ALL & AML) at diagnosis were found to be significantly higher than their levels in controls ($p<0.05$). Also, in complete remission (C.R.) CD44s levels were significantly lowered than their levels at diagnosis ($p<0.05$), and not significantly different from controls ($p>0.05$).

There was no statistical significant difference in CD44 levels between ALL and AML cases neither at diagnosis ($p>0.05$) nor during remission ($p>0.05$).

In agreement with our study, Zitterman et al., 2001 **[29]** found that acute leukemic patients, before starting their treatment, had four folds higher level of serum CD44 than normal controls involved in the study. Also, Gadhoum et al., 2003 **[30]** found that BM levels of CD44 were significantly elevated in acute leukemic patients at diagnosis than controls.

It was found that 16 patients (80%) in NHL patients at presentation showed BM sCD44 more than the upper limit level of control (280ng/ml). The mean value of BM sCD44 was statistically significantly increased in NHL patients at diagnosis when compared to control group, while after remission, it was more decreased than its level of diagnosis with a statistically significant difference, but still more than that level in control group with no statistically significant difference ($p>0.05$).

This is agreement with Ristamaki et al., 1997 **[31]** who explained that sCD44 level changes in parallel with treatment response in malignant lymphoma and that the origin of soluble sCD44 probably originates from the tumor cell, the strong association between SCD44 levels and stage change of SCD44 parallels with treatment response.

The result of this study showed that the mean value of BM sCD44 in patients with B symptoms is much more than in patients without B-symptoms at diagnosis and after remission and there was high statistical significant difference between them ($p<0.05$).

Also CD44s was increased in NHL patients with BM involvement than those without BM involvement at diagnosis with statistical significant difference, NHL patients with BM CD44s level >280ng/mL were associated with 4.7 and 2.8 folds higher frequencies of B-symptoms and bone marrow invasion respectively at diagnosis extranodal site than those ≤280ng/mL.

The result of this present study showed that there was significant increase in BM sCD44 level in patients with high grade lymphoma than low/intermediate lymphomas if compared with control or compared to each other at diagnosis and after remission ($p<0.05$).

The mean value BM CD44s was significantly increased in patients with extranodal sites >1 than those who had extra nodal sites ≤1 at diagnosis and after remission ($p<0.05$). Also the mean value of BM CD44s was significantly increased in patients with high IPI in comparison to patients with intermediate/high, intermediate/low and low IPI score at diagnosis and after remission ($p<0.05$).

This is in agreement with Ponta et al., 2003 **[32]** who explained that sCD44 molecule is shed from the cell surface and released in some solid tumors and is correlated with poor outcome and could be a useful prognostic marker for some hematological malignancies as aggressive lymphoma. Also Niitsu and Ljima, 2002 **[10]** reported that a high level of sCD44 was correlated with a poor outcome of aggressive NHL suggesting that sCD44 levels could be a useful prognostic marker for aggressive lymphoma.

These results confirmed previous studies that concluded that a high serum sCD44 level and/or tumor tissue expression at diagnosis is associated with poor prognostic criteria and/or unfavorable outcome in childhood leukemias and lymphomas **[9]**.

It was concluded from this study that evaluation of BMM elements including (IL3, IL6 and sCD44) in Acute leukemia and NHL patients, can be useful in evaluating disease activity, extent and response to treatment and they can be used as prognostic markers in these patients.

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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).

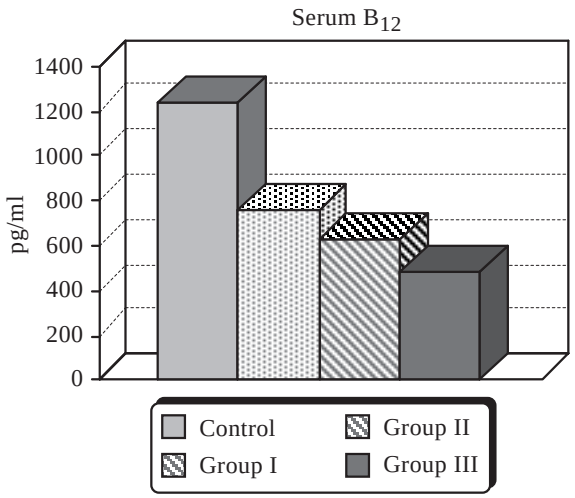

Fig. (1): Mean values of serum B_{12} in various diabetic groups and controls.

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).

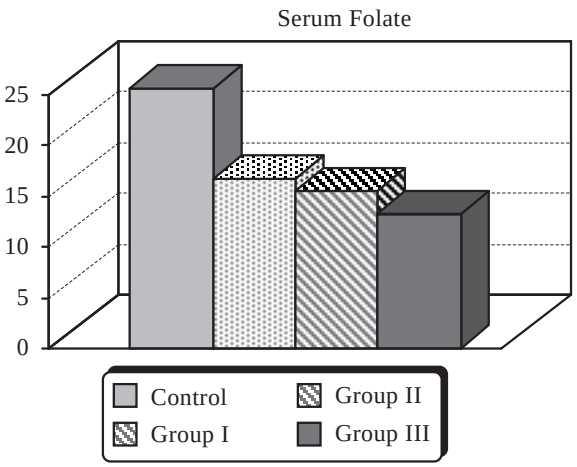

Fig. (2): Mean values of serum folate in ng/ml in various diabetic groups and controls.

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).

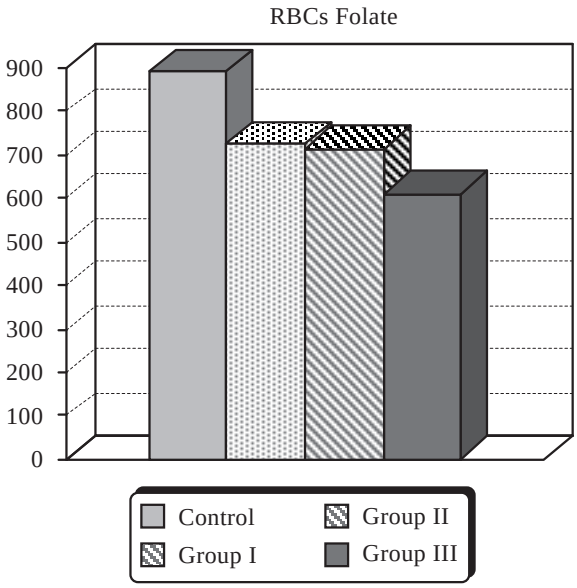

Fig. (3): Mean values of RBCs folate in ng/ml in various diabetic groups and controls.

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).

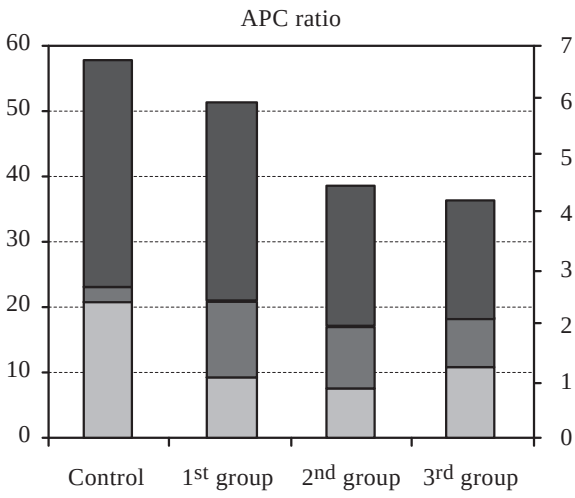

Fig. (4): Mean values ± SD of APC ratio in various diabetic groups and controls.

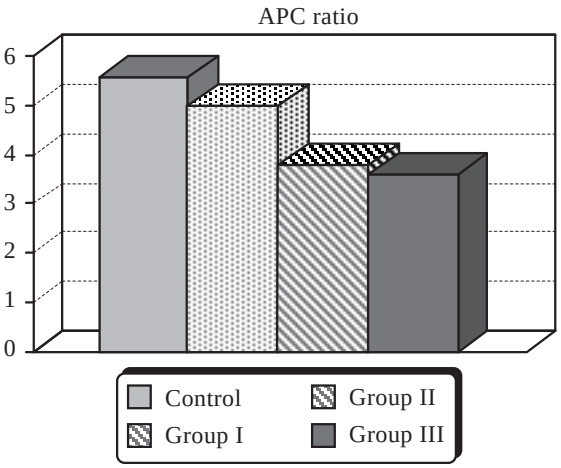

Fig. (5): Parametric mean values of APC ratio in various diabetic groups and controls.

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.

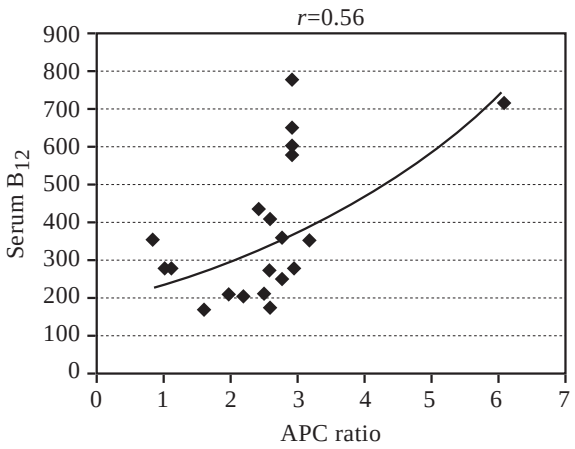

Fig. (6): Correlation between serum B_{12} in pg/ml and APC ratio in group I patients.

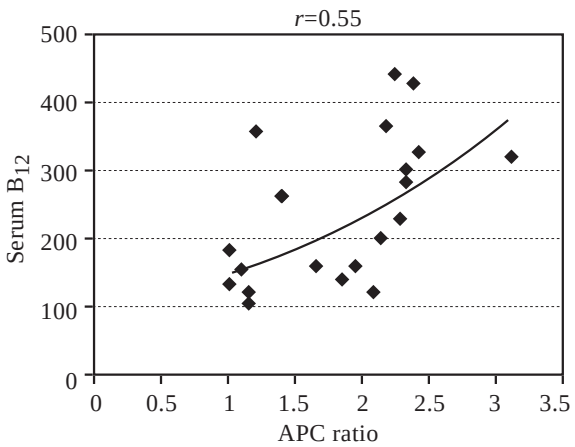

Fig. (7): Correlation between serum B_{12} in pg/ml and APC ratio in group III patients.

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.

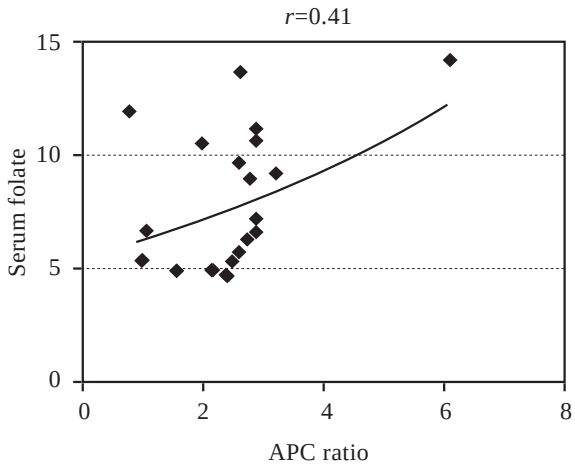

Fig. (8): Correlation between serum folate in ng/ml and APC ratio in group I patients.

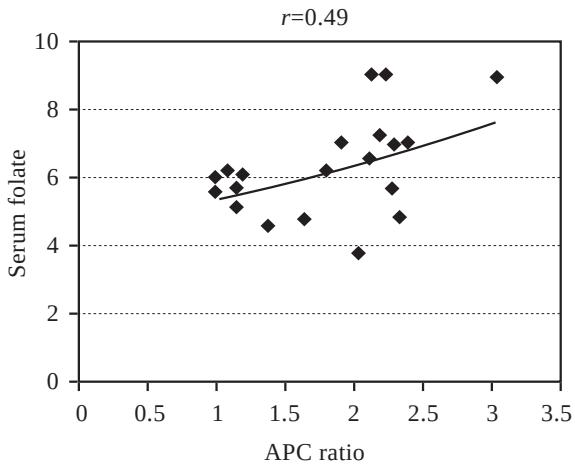

Fig. (9): Correlation between serum folate in ng/ml and APC ratio in group III patients.

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.

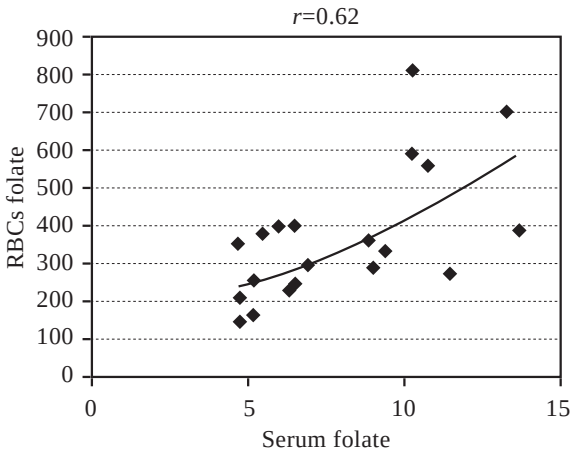

Fig. (10): Correlation between serum folate in ng/ml and RBCs folate in ng/ml in group I patients.

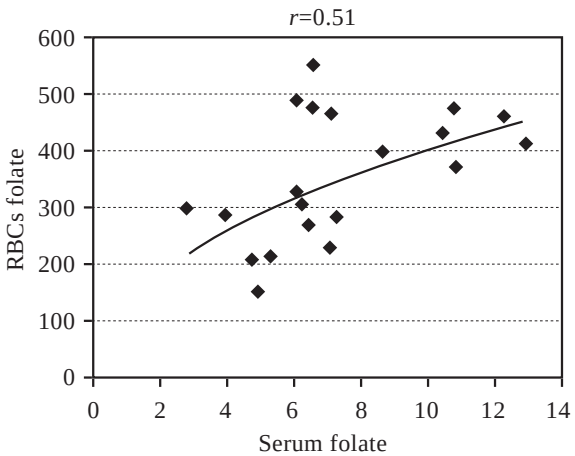

Fig. (11): Correlation between serum folate in ng/ml and RBCs folate in ng/ml in group II patients.

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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Chronic B-Lymphocytic Leukaemia: Immunoregulatory Molecules, Antigen-Experienced B-Cells and Nurse-Like Cells

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ABSTRACT

Introduction: Chronic lymphocytic leukemia (CLL) is characterized by accumulation of a single clone of $CD5^+$ B cells in the peripheral blood and bone marrow. B-CLL cells are usually arrested in the G0/G1 phase of the cell cycle and therefore their accumulation in vivo appears to result from the inhibition of apoptosis rather than increased proliferation.

Patients and Methods: The study included 40 patients of newly diagnosed CLL as well as 10 age- and sex-matched normal volunteers were taken as controls. Both patients and controls were evaluated for the expression of CD40, CD80, CD86, CD27, CD25, CD14, CD25 and CXCR4. Correlation of these markers with clinical features known to influence prognosis in CLL and their impact on response to chemotherapy were evaluated.

Results: Our results revealed that CD40 was positive in 39/40 (97.5%) of the CLL group and was comparable to the control. CD80 was positive in 6/40 (15%) of the CLL patients with no difference in its expression between the CLL and the control group. CD86 was detected in none of our CLL patients. CD86 was significantly lower in CLL group than in the control group (p<0.001). By Roc curve analysis we found that CD86 could be used to predict favourable response to treatment in CLL patients at a cut off value of 3.5%, (p=0.008). Thirty eight of 40 patients (95%) were positive for the memory cell marker CD27, implying that all B-CLL cells resemble antigen-experienced and "memory" B lymphocyte. Twenty two of 40 patients (52.5%) were positive for the lymphocyte activation marker CD25 with a significantly higher expression among CLL compared to control (p.value <0.001). CD25 significantly correlated with favourable response to chemotherapy (p.value <0.001), and higher overall survival (p=0.007). Thirty six of 40 patients (90%) were positive for CXCR4, with a range of 14-98% and a mean of 53.8±22.5% and significantly four-fold greater than control group (p<0.001). CXCR4 showed a positive correlation with BM lymphocyte % (p=0.003 r=0.459) No correlation was found between over expression of CXCR4 and response to chemotherapy. CD14 (a marker of nurse-like cells which are a subset of monocytes) was negative in all CLL patients at a cut off value of 20% with no significant difference among CLL patients and the control group. Our results revealed that CD14 at a statistical cut off value of 6% could be used to predict poor response to treatment, with 94% sensitivity and 100% specificity (p.value <0.001) and prognosis of CLL patients with short over all survival for patients with high CD14 expression (p.value <0.001).

In conclusion: CD86 is essential for the function of any APC so B-CLL cells are similar to anergic B cells that have a reduced ability to process and present antigen to the T-helper cells. This defective may be one of the pathogenic mechanisms of CLL and a major cause of why the body fails to clear the B-CLL cells via immunological means. Over expression of CXCR4 on CLL cells may increase the avidity of malignant B cells to hematopoietic and possibly also to lymphoid tissue.

Key Words: *B-CLL - Antigen-experienced B-Cells - Nurse-like cells.*

INTRODUCTION

Chronic lymphocytic leukemia (CLL) usually presents as a slowly progressive lymphoproliferative disease characterized by accumulation of a clonal $CD5^+$ B cells in the peripheral blood and bone marrow with frequent involvement of lymph nodes and spleen **[1]**.

B-CLL cells are usually arrested in the G0/G1 phase of the cell cycle and therefore their accumulation in vivo appears to result from the inhibition of apoptosis rather than increased proliferation **[2]**.

Resistance to apoptosis is not an intrinsic property of B-CLL cells. In fact, when they are cultured in vitro, they usually die after a short term incubation, suggesting the existence, in vivo, of a survival-promoting microenvinment **[3]**.

B-CLL cells over express CXCR4, the receptor for stromal derived factor-1 (SDF-1), which contributes to their tropism to bone marrow and lymphoid system where interaction with bone marrow stromal cells and nurse-like cells (NLCs) protect them from spontaneous apoptosis **[4]**.

CD40 is a molecuole of the family of tumour necrosis factor receptor (TNFR), which is expressed throughtout B-cell development and is implicated in cell survival and differentiation. Its physiological ligand is, CD40L (CD154) a member of TNF family. CD40/CD40L interaction stimulates B-cells, dendritic cells and monocytes to proliferate, differentiate, up-regulate co-stimulatory molecules and increase antigen presentation **[5]**.

Finally, the CD40 on CLL cells can down-regulate CD40 on activated T-cells protecting the cells from their cytolytic effect, and this may contribute to the T-cell dysfunction seen in this disease **[5]**.

CD80 and CD86 molecules deliver co-stimulatory signals to T-cells and serve as counter-receptors that transduce distinct signal to the antigen presenting cell (APC) upon engagement by CD28 or CTLA-4 **[5]**.

B-CLL cells from all cases express the classical activation markers CD23, CD25, CD69 and CD71. Furthermore, the increased density of expression of CD5 and CD27 (which is typically a marker of memory B-cells) is in line with the activation hypothesis, since the level of both of these markers can be up-regulated upon cellular activation **[5]**.

The expression of costimulatory and immunoregulatory molecules in CLL patients potentially influences the clinical progression and drug response in these patients **[5]**.

The nurse-like cells (NLCs), which are a subset of peripheral blood $CD14^+$ mononuclear cells that develop into large round, adherent cells when grown in the presence of CLL cells, produce survival factors, including SDF-1, for the leukemia cells **[6]**. These NLCs protect CLL cells from undergoing spontaneous or drug-induced apoptosis by enhancing the expression of antiapoptotic proteins of the bcl-2 family **[7]**.

Patients with CLL may have greater numbers of circulating NLC progenitor cells. CLL cells may elaborate factors, such as transforming growth factor-beta (TGF-ß) that could support survival of NLC in vitro **[8]** and a potent differentiation factor for stromal cells **[9]**.

The freshly isolated $CD14^+$ splenocytes from patients with CLL expressed significantly higher levels of $CD68^+$ than did the $CD14^+$ splenocytes from patients without lymphoproliferative disease. Moreover, when the mononuclear splenocytes of CLL patients were cultured, after 1 to 2 days of culture, cells that had a morphology similar to that of the NLCs and that differentiated from $CD14^+$ blood cells after 11 to 14 days in vitro were identified **[7]**.

Therefore, the relative number and activity of such stromal elements and NLCs might be a limiting factor governing tumor progression particularly during early stages of disease when the interdependency of leukemia cells with accessory cells seems most apparent **[7]**.

The knowledge that B-CLL is the outcome of many different molecular defects may allow the development of chemotherapies, immunotherapies and gene therapies targeting that specific defect **[10]**.

Aim of the work:

The aim of this study is to asses the expression of CD40, CD80, CD86, CD27, CD25, CD14 and CXCR4 and their correlation with clinical features known to influence progression in CLL patients (age, Hb level, WBCs, PB lymlphocyte, absolute lymphocyte count, platelet count and BM lymphocytes) as well as their impact on response to chemotherapy.

PATIENTS AND METHODS

A- Patients:

The present study was carried out on forty patients with de novo chronic lymphocytic leukemia (CLL), who had not received any treatment attending the outpatient clinic of the medical oncology department National Cancer

Institute, Cairo University during the period between December 2002 and May 2005. The patients were 22 males (55%) and 18 females (45%) with a male: female ratio of 1.2: 1.0. Their ages ranged from 36 to 71 years with a mean ± SD of 57.6±8.35 and a median of 56.5.

In addition, 10 age- and sex-matched normal volunteers were taken as controls. They were 6 males and 4 females, with a male: female ratio 1.5: 1.0 and an age range of 40-70 years with a mean ± SD of 55.7±8.3 and a median of 56.0.

Patients were subjected to thorough history taking, full clinical examination (particularly for hepatomegaly, splenomegaly and lymphadenopathy), morphology and immunophenotyping.

Patients as well as the controls were evaluated for the expression of CD40, CD80, CD86, CD27, CD25, CD14 and CXCR4. These were CD80 FITC, CD40 PE, CD86 PE, CD27 PE, CD25 PE, and CXCR4 PE. All monoclonal antibodies used were purchased from Dako A/S Denmark. All marker studies were done on flow cytometry (Partec III) and results were expressed as the percentage of cells showing positive expression.

The erythrocyte lysing reagent was FACS lysing solution B D Bioscience Cat. No. 349202.

B- Methods:

Procedure of the techinque for immunophenotyping:

1- Fifty UL of the anticoagulated EDTA blood containing 10^4 cells were transferred to each of test tubes.
2- Five UL of each monoclonal antibody were added to its corresponding tube and mixed gently.
3- The first tube was a control one where no monoclonal antibody was added and with which the machine was adjusted to obtain the basic histogram showing the main cell populations and to adjust the auto fluorescence region.
4- Tubes were incubated in the dark for 30 minutes.
5- Two ml of erythrocyte lysing reagent were added to each tubes and mixed gently and left for 5 minutes.
6- Tubes were then centrifuged at 1500rpm for 5 minutes and supernatant aspirated.
7- Three ml of 0.01mol/L PBS were added to each tube and vortexed gently and step 6 was repeated.
8- The cell button was resupended in 0.5ml 0.01mol/L PBS and analyzed on the flowcytometer.

Flowcytometric analysis and immunophenotyping:

After warming up the argon laser (488nm) for 30 minutes, the full alignment procedures were performed using the standared immunocheck aligment flurospheres for adjusting forward scatter, side scatter and photomultiplier tubes for an orange and red adjustements.

The proper protocol for each monoclonal was loaded and used for interpretation of each.

The control sample was the first tube to be introduced to the machine, to show the cell populations in the basic histogram and to adjust autoflurescence region. Then, the other tubes were introduced sequentially.

Five thousand events (cells) were passed in front of the laser for each case for each mononclonal antibody. Lymphocytes were then selectively gated for the expression of our studied markers. Cells having high both forward angle light scatter (FSC) and side angle light scatter (SCC) corresponding to nurse-like cells (NLCs) were selected and analyzed for the expression of CD14 **[11]**.

Interpretation of results:

The number of cells expressing the receptor will emit fluoresce signals which will be summated and multiplied in the photomultiplier tubes. These data will be shown as single and double coloured frequency histograms.

For each sample, the expression of each of the studied markers was defined as positive when it is present on more than or equal to 20% of cells **[3]**.

Statistical methods:

SPSS (Statistical Package for Social Sciences) version 12.0 was used for data analysis. Mean and standard deviation are descriptive

values for quantitative data. Student *t* test was used for comparing means of two independent groups and Kruskal Wallis ANOVA (analysis of variance) for comparing means of more than 2 independents groups. Chi-square and Fisher-exact tests compared independent proportions.

ROC analysis helped to choose the best cut-off points for quantitative parameters to be used either as diagnostic or prognostic variables. Kaplan-Meier method estimated probability of overall and disease free survival. Long rank test was used for comparing survival curves. *p* value is significant at 0.05 level.

RESULTS

The present study included fourty patients with newly diagnosed CLL, who presented to the National Cancer Institute, Cairo University during the period between Dec. 2002 and May 2005. Ten age-and sex-matched normal volunteers were taken as a control gruoup.

All patients were of the B-cell type and all showed weak positivity for surface immunoglobulin (SIg) of either kappa or lambda.

Expression of the studied markers:

Thirty nine patients (97.5%) were positive for CD40 with a range of 18-87% showing no statistically significant difference as compared to the control. Six patients (15%) were positive for CD80 with a range of 0-74% showing no statistically significant difference as compared to the control. All patients were positive for CD86 with a range of 0-10 showing a statistically significant lower expression in the CLL group as compared to the control ($p<0.001$). Thirty eight patients (95%) were positive for CD27 with a range of 7-82 showing no statistically significant difference as compared to the control group. Twenty one patients (52.5%) were positive for CD25 with a range of 0-86 showing a statistically significant higher expression as compared to the control group ($p<0.001$). CXCR4 was positive in 36 patients (90.0%) with a range of 14-98 showing a statistically significant higher expression as compared to the control group ($p<0.001$). Finally, CD14 was expressed in none of the CLL patients with a range of 0-18 showing no statistically significant difference as compared to the control group (Table 1).

Table (1): The studied markers in 40 CLL patients before treatment and controls.

Parameter	CLL Frequency	CLL Range	CLL Percentage	CLL Mean ± SD	Control Mean ± SD	*p*. value
CD40	39	18-87	97.5	55.9±17.4	61.2±24.0	0.44
CD80	6	0-74	15	9.9±16.7	10.9±6.1	0.08
CD86	0	0-10	0	3.9±2.4	41.6±20.8	<0.001**
CD27	38	7-82	95	54.6±18.7	49.7±20.7	0.47
CD25	21	0-68	52.5	19.5±13.4	4.4±1.4	<0.001**
CXCR4	36	14-98	90	53.8±22.5	12.6±6.6	<0.001**
CD14	0	0-18	0	6.0±4.9	7.6±2.5	0.14

** Highly statistically significant.

Relation between the studied markers and response to chemotherapy:

There was no statistically significant difference in expression of CD40, CD80, CD27 and CXCR4 and response to chemotherapy. However, there was a statistically significant higher expression of both CD86 and CD25 for patients who entered complete remission (CR) as compared to those with either partial remission (PR), or stable disease (SD)/progressive disease (PD) ($p= 0.02$, <0.001 respectively). Also, there was a statistically significant lower expression of CD14 for patients who entered CR as compared to those with either PR or SD/PD ($p=$ <0.001) (Table 2).

Table (2): Relation between the studied markers and response to chemotherapy.

		CR (22)	PR (11)	SD/PD (7)	*p*. value
CD40	<20	1	–	–	
	≥20	21	11	7	0.66
CD80	<20	19	8	7	
	≥20	3	3	–	0.28
CD86	<3.5	6	8	5	0.02*
	≥3.5	16	3	2	
CD27	<20	–	2	–	
	≥20	22	9	7	0.10
CD25	<20	2	10	7	
	≥20	20	1	–	<0.001**
CXCR4	<20	4	–	–	
	≥20	18	11	7	0.19
CD14	<6	22	2	–	
	≥6	–	9	7	<0.001**

* Statistically significant. ** Highly statistically significant.

Studied markers as predictive factors:

By Roc curve analysis we found that CD86 could be used as a prognostic factor to predict response of the patients; as at a value of 3.5% the sensitivity was 72.7% and the specificity was 72.2% with the area under the curve is equal to 0.745 and *p*.value 0.008 (Fig. 1).

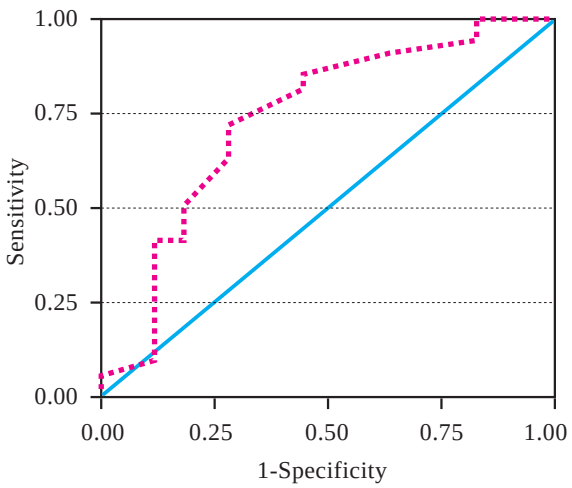

Fig. (1): Roc curve analysis for CD86 in CLL patients before treatment.
Area: 0.745 & *p*. value 0.008

CD25 could be used as a prognostic factors to predict response of the patients; as at a value of 20%, the sensitivity was 90% and the specificity was 95% with the area under the curve is equal to 0.956 and *p*.value <0.001 (Fig. 2).

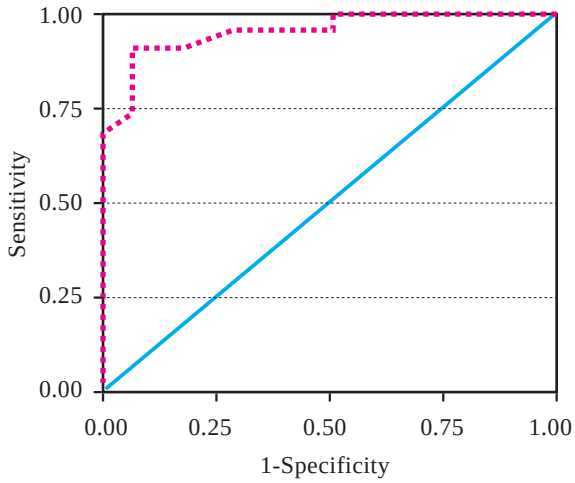

Fig. (2): Roc curve analysis for CD25 in CLL patients before treatment.
Area: 0.956 & *p*. value <0.001.

CD14 could be used as a prognostic factors to predict response of the patients; as at a value of 6%, the sensitivity was 94% and the specificity was 100% with area under the curve is equal to 0.997 and *p*.value <0.001 (Fig. 3).

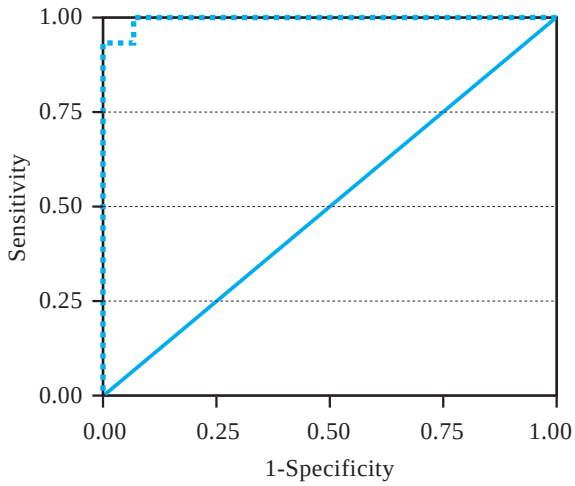

Fig. (3): Roc curve analysis for CD14 in CLL patients before treatment.
Area: 0.997& *p*. value <0.001

Correlation study:

Correlation study revealed no statistically significant correlation for CD40, CD80, CD86, or CD27 with either age, Hb level, TLC, PB lymphocyte, ALC, platelet count, or BM lymphocytes. However, CD25 showed a statistically significant correlation with TLC and ALC (r=–0.417, p=0.007 and r=–0.381, p=0.015

respectively), CXCR4 with BM lymphocytes (r=0.459, p=0.003) and CD14 with Hb level (r=–0.321, p=0.043), TLC (r=0.589, p=0.000), PB lymphocyte (r=0.339, p=0.032), ALC (r=0.589, p=0.000), platelet count (r=–0.327, p=0.040) and BM lymphocytes (r=0.340, p=0.040) (Table 3).

Survival analysis:

Survival analysis revealed a significantly higher overall survival for patients with low CD14 expression (p<0.001) (Fig. 4) as well as a significantly higher overall survival in CLL patients with higher CD25 expression (p 0.007) (Fig. 5).

Table (3): Correlation between the expression of studied markers and prognostic variables of CLL patients.

	CD40	CD80	CD86	CD27	CD25	CXCR4	CD14
Age:							
r	-0.003	–0.004	0.114	–0.183	0.074	–0.097	0.213
p	0.987	0.982	0.484	0.258	0.651	0.553	0.187
Hb level:							
r	–0.188	0.041	0.249	0.159	0.094	0.173	–0.321*
p	0.245	0.802	0.122	0.327	0.564	0.285	0.043
TLC:							
r	0.101	0.023	–0.262	0.075	–0.417**	0.171	0.589**
p	0.533	0.890	0.102	0.644	0.007	0.292	0.000
PB Lymph:							
r	0.159	0.011	0.067	-0.106	0.089	0.068	0.339*
p	0.326	0.944	0.680	0.515	0.586	0.678	0.032
ALC:							
r	0.116	0.006	–0.252	0.061	–0.381*	0.171	0.589**
p	0.477	0.973	0.117	0.709	0.015	0.292	0.000
Plt:							
r	-0.048	–0.142	0.191	0.186	0.231	–0.159	–0.327*
p	0.769	0.382	0.237	0.250	0.151	0.326	0.040
BM Lymph:							
r	0.205	–0.103	-0.234	0.014	–0.296	0.459**	0.340*
p	0.204	0.527	0.146	0.931	0.064	0.003	0.040

* Correlation is significant at 0.05 level (2-tailed).
** Correlation is highly significant at the 0.01 level (2-tailed).

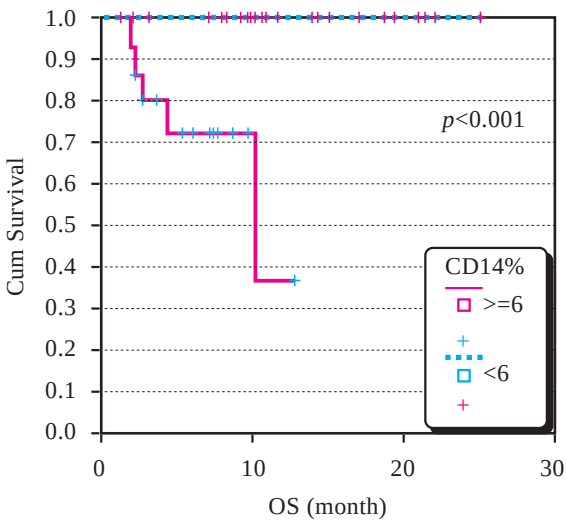

Fig. (4): Overall survival of CLL patients regarding the expression of CD14.

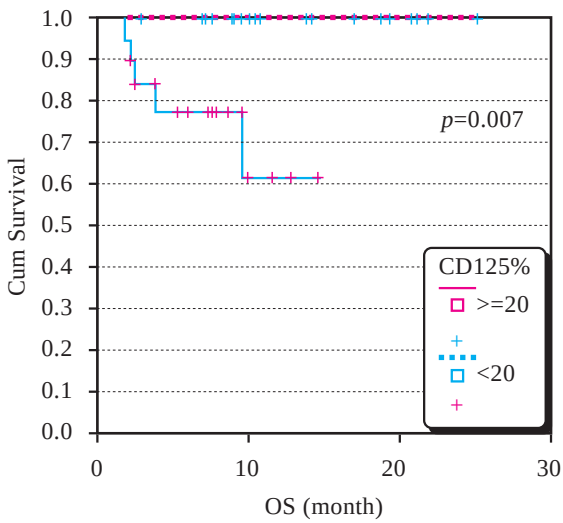

Fig. (5): Overall survival of CLL patients regarding the expression of CD25.

DISCUSSION

Chronic lymphocytic leukemia (CLL) is a unique lymphoproliferative disorder characterized by accumulation of a single clone of $CD5^+$ B cells which did not retain the capacity to differentiate into functionally mature cells, and accumulate in the peripheral blood and bone marrow with frequent involvement of lymph nodes and spleen **[1]**.

There is currently much evidence demonstrating that both pharmacological and cellular mechanisms contribute to whether a patient with CLL is cured or not and leukemic cells are rarely eradicated with chemotherapy. Even when an initial response is observed secondary progression is the rule **[12]**.

The study included 40 patients of newly diagnosed CLL and 10 age- and sex-matched controls. Both patients and control were studied for the expression of CD40, CD80, CD86, CD27, CD25 and CXCR4. Correlation of these markers with clinical features known to influence prognosis in CLL as well as their impact on response to therapy were also evaluated.

In our work, thirty nine of 40 patients (97.5%) were CD40 postive. This was in agreement with both Hulkkonen et al. **[5]** and Younes et al. **[13]**.

Similarily, Orsini et al. **[14]** found higher levels of CD40 on the surface of CLL cells leading to down-regulation of CD40L on activated T cells, thus protecting the leukemic cells from their cytotoxic effect and this may contribute to T-cell dysfunction seen in this disease.

Also, Romano et al. **[15]** reported that CLL cells express CD40 and release CD40L leading to its elevated level in the plasma of these patients. The released CD40L may induce growth, survival and proliferation of neoplastic B-CLL cells and stimulate non-malignant B cells to produce autoantibodies. This may be the mechanism for the autoimmune phenomenon in CLL.

Six of our forty CLL patients (15%) were positive for CD80. This was in agreement with both Hulkkonen et al. **[5]** and Tsukado et al. **[16]** who showed a heterogeneous pattern of expression of CD80 in B-cell cells with most cases being negative.

In our work, no difference was found between B-CLL and normal control group regarding the expression of CD80. This was in agreement with Dai et al. **[17]** who reported no significant difference between B-CLL group and normal control group either in the B7.1 (CD80) expression or in co-expression of B7.1 and B7.2.

In our study, none of our patients expressed CD86. This was in agreement with Freedman et al. **[18]**, Dozzi et al. **[19]** and Von Berwelt-Bailden et al. **[20]** as all found that CLL cells, in common with other B-cell malignancies, fail to express, or express only weakly, adhesion molecules especially CD86. CD86 is essential for the function of any APC and so B-CLL cells are similar to anergic B cells that have a reduced ability to process and present antigen to the T-helper cells. Treatment of both normal and malignant B cells by activation via CD40 results in up regulation of B7 (CD80, CD86) expression and generation of professional autologous APC.

In our work, we found a significantly lower expression of CD86 in the B-CLL group as compared to the normal control group (p value <0.001).This was in agreement with Van den Hove et al. **[21]**. This defective expression of CD86 may be one of the pathogenic mechanisms of CLL and a major cause of why the body fails to clear the B-CLL cells via immunological mechanisms. T cell unresponsiveness towards autologous B-CLL cells may be, at least in part, attributed to inadequate costimulatory capacity of this tumor.

However, our results regarding expression of CD80 & CD86 on CLL cells were not in agreement with Trentin et al. **[22]** who found that CD40 was expressed on all B cells (normal and malignant), CD27 & CD70 were expressed on tumor B cells and not on normal B cells, CD80 was distributed on all neoplastic cells at an intermediate density and CD86 was present at a low density. This probably indicates that B CLL cells are equipped with different co stimulatory molecules.

In our study we reported by Roc curve analysis that CD86 could be used to predict response to treatment in CLL patients at a cut off value of 3.5%, with 72.8% sensitivity and 72.2% specificity (p value 0.008), where most patients with high CD86 had a good response to treatment and a favorable prognosis.

In this work, 38 out of 40 patients (95%) were positive for CD27. This was in agreement with Hulkkonen et al. **[5]** and Klein et al. **[23]**. This finding of CD27, a memory cell marker, implies that all B-CLL cells resemble antigen-experienced and "memory" B lymphocyte and differ from both $CD5^+$ naive B-cell or follicle center cells. So, the origin of CLL cells appears to be the memory B cell, regardless of whether or not there was mutation of IgV gene.

On the other hand, only one report of Sembries et al. **[24]**, showed a reduced expression of CD27 in CLL along with different costimulatory molecules.

In our work, we found 22 of 40 patients (52.5%) to be positive for CD25. This was in agreement with Hulkkonen et al. **[5]**, and Gattei et al. **[25]**. The latter divided 123 well characterized B-CLL patients into at least three subgroups, one of them included 62 cases (50.4%) over expressing CD25, CD55, CD62L, CD54 and CD49c and 80% of this subgroup were of mutated IgVH cases.

In our results, we found a significantly higher expression of the lymphocyte activation marker CD25 among CLL compared to control (*p*.value <0.001). Similar results were reported by both Damle et al. **[26]** and Sellitto et al. **[27]**.

In our study, the expression of CD25 significantly correlated with response to chemotherapy (*p*.value <0.001), with most CD25 positive patients showing good response and achieving complete remission. Our results were in agreement with Damle et al. **[26]**. Also, CD25 could be used to predict overall survival, with a significantly higher over all survival in patients with higher CD25 expression (*p*.value 0.007). The same results were reported by Gattei et al. **[25]**. Therefore, CD25 overexpression may be used as an additional prognostic factor to identify good prognosis B-CLL patients.

In this work 36/40 patients (90%) were positive for CXCR4, with a range of 14-98% and a mean of 53.8±22.5% and significantly four-fold greater than control group (*p*.value <0.001). This was in agreement with Möhle et al. **[28]**. Also, Barretina et al. **[29]** reported a five-fold over expression of CXCR4 on the cells of their CLL patients as well as lowered plasma level of SDF-1 as compared to control group. These results suggest that the CXCR4/SDF-1 system appears to be important for tissue localization and increased survival of B-CLL cells.

In our work, we found a positive correlation between the expression of CXCR4 on B-CLL cells and BM lymphocyte % (*p*.value 0.003) and this was in accordance with Burger et al. **[30]** suggesting that over expression of CXCR4 on CLL cells may increase the avidity of malignant B cells to hematopoietic and possibly also to lymphoid tissue, eventually leading to bone marrow infiltration and suppression of hematopoiesis as well as lymph node and spleen enlargement.

Also, Burger et al. **[31]** found that the growth and survival of B-CLL cells are favored by interaction between CLL cells and non tumoral accessory cells. Marrow stromal cells and NLCs constitutively secret $CXCL_{12}$(SDF-1), the ligand for CXCR4, thereby attracting and resuscitating B-CLL cells from apoptosis in a contact dependent fashion.

In our work, we found no correlation between over expression of CXCR4 and response to chemotherapy. Our results go hand in hand with those of Barretina et al. **[29]**.

In this work, we found all patients to be negative for the expression of CD14 at a cut off value of 20% and no significant difference was found among CLL patients and the control group. Our results were in agreement with those of Polliack et al. **[32]** and Tassies et al. **[33]** who stated that CD14 was negative in all CLL patients.

On the other hand, two earlier studies, Molica et al. **[34]** and Pinto et al. **[35]** reported a high CD14 antigen frequency in their B-CLL patients than their control group with a direct correlation between CD14 expression and advanced clinical stages.

In our study, we found a significant correlation between the expression of CD14 at a statistical cut off value of 6%, corresponding to NLCs, and poor response to treatment and advanced disease as shown by high TLC, increased ALC, low hemoglobin level, decreased platelet count and high lymphocyte percentage in both bone marrow and peripheral blood (p=0.000, p=0.000, p<0.001, p=0.043, p=0.040, p=0.04, and p=0.032 respectively).

Our results go hand in hand with that of Pinto et al. **[35]** who reported a direct correlation between CD14 expression and advanced disease, diffuse bone marrow infiltration and bad prognosis.

From our study, we conclude that CD14 at a cut of value of 6% could be used to predict poor response to treatment, with 94% sensitivity and 100% specificity (*p*.value <0.001) and prognosis of CLL patients with short over all survival for patients with high CD14 expression (*p*.value <0.001).

A similar conclusion was drawn by Callea et al. **[36]**, as they detected a cut off of 5% for positivity of CD14 and this was considered as an independent significant factor for the prediction of over all survival. They also suggested the inclusion of CD14 in the B-CLL immunological panel because of its ability to discriminate between groups with different prognoses which may need more appropriate treatments.

Conclusion:

- CD40 was detected in 97.5% of our patients. Its expression on the surface of B-CLL cells may lead to down regulation of CD40L on activated T-cells, thus protecting the leukaemic cells from the cytotoxic effect of T-cells and this may contribute to T-cell dysfunction seen in this disease.
- CD80 was detected in 15% of CLL patients with no difference in its expression between the CLL and the control group.
- B-CLL cells failed to express the adhesion molecule CD86 which is essential for the function of any APC and so B-CLL cells are similar to anergic B lymphocytes that have a reduced capacity to process and present antigens to T- helper lymphocytes. This may be a major cause of why the body fails to clear the CLL cells via immunological mean.
- CD86 could be used to predict the response to treatment in CLL patients at a statistical cut-off value of 3.5%.
- The expression of CD27, a marker of memory B-cells, on the surface of B-CLL cells reflects that B-CLL cells resemble antigen- experienced and memory B lymphocytes and differ from both $CD5^+$ naïve B-cells or follicle center cells. So, the origin of CLL cells appears to be the memory B cell.
- The B-CLL cells express the functional chemokine receptor CXCR4, allowoing B-CLL cells to actively migrate to BM and to lymphoid tissue where interaction of B-CLL cells with stromal cells and Nurse-like cells occurs.
- CD14 could be used at a statistical cut off value of 6% to predict response to treatment, prognosis and overall survival in CLL patients.
- CD25 could be used at a statistical cut-off value of 20% as a prognostic marker to predict good response to chemotherapy and overall survival in B-CLL patients.

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Study of β-Thalassemia Mutations Using the Polymerase Chain Reaction-Amplification Refractory Mutation System and Direct DNA Sequencing Techniques in a Group of Egyptian Thalassemia Patients

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ABSTRACT

The aim of this study was the molecular characterization of β-thalassemia (thal) mutations in a group of 95 Egyptian thalassemic patients from Fayoum in Upper Egypt, Cairo, Alexandria and Tanta in Lower Egypt and the Nile Delta. To identify these anomalies the polymerase chain reaction-amplification refractory mutation system (PCR-ARMS) was used, complemented by direct DNA sequencing for uncharacterized cases.

In 80 of the 95 patients, the b-thal mutation was detected by PCR-ARMS. The most common allele encountered in our study was IVS-I-6 (T→C) (36.3%); the second most common mutation was IVS-I-110 (G→A) (25.8%). In addition, we report three homozygous cases for the promoter region –87 (C→G) allele with a frequency of 3.2%. DNA sequencing of uncharacterized cases (14 cases, 15 alleles) revealed six cases (six alleles) of codon 27 (G→T), and three cases (three alleles) of the IVS-II-848 (C→A) mutation. Codon 37 (G→A) in the homozygous state was found in one patient with positive consanguinity. The frameshift codon 5 (–CT) mutation was detected in two of our uncharacterized cases. The codon 15 (TGG→TGA) mutations was detected in one patient (one allele, 0.5%). All studied cases were fully characterized by this strategy.

Screening for β-thalassemic mutations using ARMS-PCR for the seven most frequent alleles in Egypt succeeded in determining the β-globin genotype in 84.2% of our patients (91.6% of the expected alleles). To improve the efficiency of routine screening, the PCR-ARMS mutation panel should be updated to include the reported rare alleles. Direct DNA sequencing is an additional way to allow a full characterization of β-thal patient in Egyptian population.

Key Words: *β-Thalassemia mutations - DNA sequencing - β-Thalassemia in Egypt.*

INTRODUCTION

β-Thalassemia (thal) is a group of inherited disorders characterized by a reduced β-globin chains synthesis. Around 7% of the world's population is affected by the disease which is highly prevalent in tropical and subtropical regions including the Mediterranean, Southeast Asia and Southern China **[1,2]**. At the molecular level, more than 190 mutations affecting the β-globin gene are associated with this disease **[2]**. However, the spectrum and frequency of these mutations vary among different populations. Immigration plays a major role in both the distribution and the extent of mutation variations within each country **[3]**. Previous studies done for the molecular characterization of β-thal in Egypt have screened the most common Mediterranean mutations with recovery of a varying percentage of uncharacterized alleles **[4,5]**.

The aim of this study was the molecular characterization of β-thal mutations in a group of Egyptian thalassemic patients originating from Fayoum in Upper Egypt, Cairo, Alexandria and Tanta in Lower Egypt and the Nile Delta, using a polymerase chain reaction-amplification refractory mutation system (PCR-ARMS) technique fitted for a panel of common Mediterranean b alleles and complemented by DNA se-

quencing of the β-globin gene(s) to find out the accuracy of such a technique in our population and to identify new alleles.

SUBJECTS AND METHODS

Our study was conducted on 95 thalassemic patients, 52 patients from the New Children's Hospital Haematology Unit at Cairo University, 22 patients from the Pediatric Hematology Unit at Tanta University, 12 patients from the Haematology Unit at Alexandria University and four from the Pediatric Outpatient Clinic at Fayoum University. (Author: is this correct?).

All the thalassemic patients had their clinical history recorded including their parents' relationship (consanguinity), frequency of blood transfusions and history of splenectomy. Prior to sampling for DNA analysis, patients or their parents gave written consent in agreement with regulation.

Laboratory Investigations:

Routine hematological investigations included a complete blood count CBC, reticulocyte count and hemoglobin (Hb) electrophoresis using cellulose acetate membranes (Helena Laboratories, Beaumont, TX, USA) in Tris-EDTA borate buffer, pH 8.4.

Molecular Investigations:

DNA was extracted from 2mL EDTA anticoagulated whole blood using QIAamp DNA Blood Mini Kit from Qiagen (Hilden, Germany) according to manufacturer's instructions. We used PCR ARMS for each mutation in the following manner: The patient's sample was amplified using a set of three primers fitted to amplify either the wild type or mutated allele as described by Newton et al. **[6]**. Seven common Mediterranean mutations were detected [IVS-I-110 (G→A), IVS-I-6 (T→C), IVS-I-1 (G→A), IVS-II-1 (G→A), IVS-II-745 (C→G), –87 (C→G) and codon 39 (C→T)] **[4,5,7,8]**. All uncharacterized alleles were identified using direct sequencing using the following protocol **[9]**. The whole β-globin gene was amplified in two fragments using the following primers **[10,11]**: fragment A: 21: RSA/β 5'-AGA CAT AAT TTA TTA GCA TGC ATG-3' (forward); 22: 5'-ACA TCA AGG GTC CCA TAG AC-3' (reverse); fragment B: 16: 5'-CAG TCA AGG CTG AGA GAT GCA GGA-3' (reverse); 30: 5'- ACC TCA CCC TGT GGA GCC AC-3' (forward) and internal sequencing primers. In total, 100μL reaction volume was treated according to the following conditions: Initial denaturation was done at 95ºC for 4min., then 35 cycles of 1min. at 94ºC, 2min. at 58ºC, and finally, 2min. at 72ºC, followed by extension for 10min. at 72ºC using a 9600 Thermal Cycler (Perkin Elmer Life Science Corporation?, Boston, MA, USA). The PCR product was then purified using the QIA quick PCR purification kit (Qiagen) prior to cycle sequencing reaction using an ABI PRISM™ 310 automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA).

The sequencing primers design **[10,11]**. Forward primers: P30→5'-ACC TCA CCC TGT GGA GG AC-3'; Exit B-F→5'-CTA GCA ACC TCA AAC AGA CAC-3'; IntB-F→5'-CT GAG GAG AAG TCT GCC GTT-3'; Seq. F→5'-CAT GAG TGT GGA AGT CTC AG-3'; P15-F→5'-ATG ATA CAA TGT ATC ATG CCT CTT TGC ACC ATT CTA-3'; P13→5'-TGG ATT CTG CCT AAT AAA A C-3'; Seq. H-F→5'-AAG GCT GGA TTA TTC TGA GT-3'. Reverse primers: Int. B-R→5'-TCC GAC GAC CAC CAG ATG-3'; Ext. B-R→5'-G AAC CTG GGT CTC CAA GAA ACT-3'; P22→5'-CA GAT ACC CTG GGA ACT ACA-3'; P20→5'-TCA AAT CTT ACC CTT TGT CTG CTT-3'; P20→5'-TCA AAT CTT ACC CTT TGT CTG CTT-3'; Seq. G-R→5'-GAG ACT CTA TGT AAT TCA TTG-3'; Seq. C-R→5'-TGT GTC AGA CGG ATC ATG TA-3'; Seq. B-R→5'-CGA TTA TCG TCG ATG TTA GG-3'; Seq. A-R→5'-ACC ACA CCG ATT ACG GGA CC-3'; Seq. K-R→5'-ACG GAT ACG GAA TAA GTA GG-3'; P16-R→5'-AGG AAC GTA GAG AGT CGG AAC TGA G-3'.

RESULTS

The patients' clinical data and molecular results are indicated in Tables (1,2), respectively. The PCR-ARMS analyses succeeded in identifying 75% of the expected thalassemic alleles. The 15 partially or entirely (one patient) uncharacterized patients were identified using direct DNA sequencing. The results of the sequencing step are given in Table (2). At the end of the sequencing step all thalassemic alleles were characterized. Allele frequency of the β-thal mutations found in this study is indicated in Table (3).

Table (1): Summary of the clinical data.

Age in months	Mean: 95.25±63.74; median 72 months
Male to female ratio	1.3:1
Positive consanguinity	51.9%
Frequency of blood transfusion per year	11.88±6.16
Splenomegaly	73.7%
Splenectomy	25.2%

Table (2): Results of β-Thalassemia genotypes using PCR-ARMS and seven common mediterranean mutations complemented by DNA sequencing.

Genotype using PCR-ARMS	n	%
IVS-I-6(T→C)/IVS-I-6(T→C)	23	24.20
IVS-I-110(G→A)/IVS-I-110(G→A)	16	16.80
IVS-I-1(G→A)/IVS-I-1(G→A)	13	13.60
IVS-I-110(G→A)/IVS-I-6(T→C)	6	6.30
IVS-II-745(C→G)/IVS-II-745(C→G)	4	4.20
–87(C→G)/–87(CÆG)	3	3.20
IVS-I-6(T→C)/IVS-I-1(G→A)	5	5.30
IVS-I-6(T→C)/IVS-II-1(G→A)	4	4.20
IVS-I-110(G→A)/IVS-I-1(G→A)	3	3.20
IVS-I-1(G→A)/IVS-II-745(C→G)	1	1.05
IVS-II-745(C→G)/codon 39(C→T)	2	2.10
Totally uncharacterized patients	1	1.05
Partially uncharacterized patients	14	14.7
Genotype of Uncharacterized Cases at the End of DNA Sequencing Results		
Codon 37(G→A)/codon 37(G→A)	1	1.05
IVS-I-110(G→A)/codon 27(G→T)	3	3.20
IVS-I-6(T→C/codon 27(G→T)	2	2.10
IVS-I-110(G→A)/Hb S [b6(A3)Glu→Val, GAG→GTG]	1	1.05
IVS-I-6(T→C)/Hb S [b6(A3)Glu→Val, GAG→GTG]	1	1.05
IVS-I-110(G→A)/IVS-II-848(C→A)	2	2.10
IVS-I-1(G→A)/codon 27(G→T)	1	1.05
IVS-I-110(G→A)/frameshift codon 5(–CT)	2	2.10
IVS-I-6(T→C)/codon 15(TGG→TGA)	1	1.05
IVS-II-745(C→G)/IVS-II-848(C→A)	1	1.05
Totally uncharacterized patients	0	0.00
Partially uncharacterized patients	0	0.00

Table (3): Allel frequency of β-Thalassemia mutations.

Mutation	n	Allele frequency (%)
IVS-I-6 (T→C)	66	36.3
IVS-I-110 (G→A)	49	25.8
IVS-I-1 (G→A)	39	19.0
IVS-II-745 (C→G)	12	6.4
Codon 27 (G→T) [Hb Knossos, β27(B9)Ala→Ser, GCC→TCC]	6	3.2
–87 (C→G)	6	3.2
IVS-II-848 (C→A)	3	1.6
Frameshift codon 5 (–CT)	2	1.0
Codon 39 (C→T)	2	1.0
Codon 37 (G→A)	2	1.0
Codon 15 (TGG→TGA)	1	0.5

DISCUSSION

The molecular basis of β-thal were extensively studied in the Mediterranean region and Arab countries allowing the set-up of mutation detection protocols fitted for the common mutations present in these various regions. In Egypt, most protocols used a PCR-ARMS method for detecting seven common β-thal mutations **[4,5,7,8]**. However, uncharacterized cases may represent up to 23%. Reported percentages of uncharacterized cases were 13.8% **[12]**, 11.8% **[13]** and 23% **[8]**. In our study, coverage of the PCR-ARMS technique was 91.6% alleles (175/190 expected alleles). We were able to find the genotype of 80/95 (84.2%) of the studied cases, with only 15.8% of partially characterized cases (14/95 cases) and one completely uncharacterized case. Using direct DNA sequencing as a second step, we characterized all 16 missing alleles.

The frequency of the β-thal mutations reported in this study was more or less similar to the limited number of previous reports about b-thal mutations in Egypt **[4,5,8,12]**. However, a few exceptions do exist. The most common allele encountered in our study was IVS-I-6 (T→C) with a frequency of 42 cases and 66 alleles (36.3%). This is a relatively higher frequency when compared to several other studies carried out on Egyptian patients. Waye et al. **[13]** reported a frequency of 13.6%, Weatherall and Clegg **[1]** reported a frequency of 15.1%. On the other hand, the IVS-I-110 (G→A) mutation is considered to be the most common mutation by most studies, with frequencies up to 41% **[4]**; this mutation was the second most common one (25.8%) found in our study. In addition, we reported three homozygous cases for the promotor region –87 (C→G) allele with a frequency of 3.2%. In the study by Hussein et al. **[4]**, a frequency of 1.4% was reported for this allele, while Weatherall and Clegg **[1]** reported this mutation to occur with a frequency of 0.8%.

Using direct DNA sequencing **[9]** for undetected cases, we were able to find some rare β-globin gene mutations. Six cases (six alleles) showed a mutation at codon 27 (G→T) [Hb Knossos, b27(B9)Ala→Ser, GCC→TCC], three cases (three alleles) were found to have the IVSII-848 (C→A) mutation, and one case displayed the rare codon 37 (G→A) mutation. This

mutation changes the codon 37 (TGG), coding for tryptophan, into TGA which is a stop codon, thus terminating translation **[14]**. Codon 37 (G→A) was found in one homozygous case (two alleles); this patient was an offspring of a consanguineous marriage, thus explaining the homozygosity for this rare allele. The frameshift codon 5 (–CT) mutation was detected in two patients. The last mutation, codon 15 (TGG→TGA), was detected in one patient (one allele, 0.5%). To the best of our knowledge, this mutation has not been reported in Egypt before this study. At the end of the direct DNA sequencing step no β-globin alleles were uncharacterized as two patients were found to be compound heterozygotes for Hb S [β6(A3) Glu→Val, GAG→GTG] and a β-thal mutation.

In conclusion, mutation panels used for routine PCR-ARMS screening for β-thal mutations should be updated to include the reported rare alleles in different studies. Complementing PCR-ARMS with direct DNA sequencing can minimize the possibility of uncharacterized cases, and thus enhance molecular characterization studies of β-thal.

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Assessment of Risk Factors for Recurrence of Deep Vein Thrombosis in a Cohort of 125 Patients

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ABSTRACT

A combined prospective and retrospective descriptive analysis of a cohort of patients with recurrent lower limb DVT was conducted. The study included 125 patients studied for a series of risk factors including clinical evidence of Behcet's disease, internanal malignancy and laboratory evidence of APC resistance, Prothrombin gene mutation G>A 20210 by PCR, Protein C, S and Antithrombin. Ancticardiolipins aCL, Homocysteine or evidence of Paroxysmal nocturnal haemoglobinuria or a myeloproliferative disorder.

In our study, 51.2% of patients with recurrent DVT had an idiopathic aetiology, 26.6% had a single endogenous risk factor, 13.6% had two endogenous risk factors, 8.8% had both endogenous and exogenous risk factors and 5.6% had an exogenous risk factor.

Statistical analysis was done to difference in relation to site, bilaterality, recurrence and patency of the affected vessels. A correlation was statistically significant between the site and recurrence (p=0.05) as well as between incomplete patency and recurrence (p=0.000).

Key Words: *Risk factor - DVT.*

INTRODUCTION

Deep vein thrombosis (DVT) is a common disease, with an annual incidence in the general population of approximately 1 per 1000. It is defined as a partial or complete occlusion of a deep vein by thrombus. DVT is an important complication of several inherited and acquired disorders, but may also occur spontaneously. The clinically important problems associated with venous thromboembolism (VTE) are death from pulmonary embolism (PE), morbidity resulting from the acute event, recurrent thromboembolic events and the post-thrombotic syndrome. Prevention of recurrent VTE and PE is the main reason for accurate diagnosis and adequate treatment **[1]**.

The risk of recurrent thromboembolism after the discontinuation of anticoagulant therapy is highly dependent on patient-specific risk factors. Patients who have thrombosis in the absence of known risk factors (i.e., who have idiopathic venous thrombosis) or in association with persistent risk factors (such as cancer and thrombophilia) are at higher risk of recurrence than patients with thrombosis associated with time-limited, reversible risk factors **[2]**.

The pathophysiology of vein thrombosis involves three interrelated factors “Virchow’s triad”: Damage to the vessel wall, slowing down of the blood flow and increase in blood coagulability. The first two components of Virchow’s triad, in most instances, represent acquired conditions but blood hypercoagulability has both intrinsic and extrinsic causes **[3]**.

Several conditions enhance the chances of thrombosis. Atheromatous vessels, myeloproliferative disorders, polycythaemia, paroxysmal nocturnal haemoglobinuria (PNH), associated malignancies, Behcet syndrome, abnormalities of lipid profile, antiphospholipid syndrome, oral contraceptives and prolonged bed rest are some conditions attributed to cause thrombosis **[4]**.

These states not only predispose apparently healthy people to thrombosis, but are also likely to trigger thrombosis in people with inherited

thrombophilic abnormalities. The well - established inherited prothrombotic abnormalities are deficiencies of antithrombin III (ATIII), protein C (PC), protein S (PS) as well as factor V Leiden and prothrombin gene mutation (G20210A) **[5]**. Hyperhomocysteinaemia, dysfibrinogenaemia, abnormalities of the fibrinolytic system and increased plasma levels of coagulation factors VIII, IX and XI are additional prothrombotic risk factors **[5]**.

Co-existing thrombophilic abnormalities increase the risk of thrombosis. Compound heterozygotes with both factor V Leiden and prothrombin gene mutation are relatively common. The risk of thrombosis may be further increased by the co-existence of antiphospholipid antibodies or hyperhomocysteinaemia **[6]**.

Similarly; other hereditary or acquired abnormalities may co-exist substantially increasing the risk of venous thrombosis. For example, it has been shown that women who have the prothrombin gene mutation increase the risk of developing a DVT by about 16 times by using oestrogen-containing oral contraceptives. Hormone replacement therapy also increases the risk of DVT by 2 to 4 times in females with the prothrombin 20210 mutation **[7]**.

Study objectives:

To determine the prevalence of endogenous and exogenous risk factors for venous thrombosis in patients with recurrent lower limb DVT and to evaluate the risk of recurrence in patients with incomplete patency.

MATERIAL AND METHODS

A combined prospective and retrospective descriptive analysis of a cohort of patients with recurrent lower limb (LL) DVT was conducted. The study included 125 patients referred for evaluation of thrombosis from August 1999 to December 2005. All patients had recurrent LL DVT (second episode) proved by Doppler.

Detailed history was taken to exclude reversible precipitating causes like prolonged bed rest, diabetes mellitus, prolonged use of oral contraceptives, atherosclerosis, cardiovascular or congenital heart disease, surgical procedures and preceding infections. Hence, the study predominantly included only those patients who needed further evaluation for the cause of recurrent thrombosis.

Detailed epidemiological and clinical data were obtained. All patients were subjected to meticulous clinical examination and routine laboratory investigations. In addition, specific laboratory tests were performed in an attempt to identify the cause of thrombophilia. The latter included testing for PC, PS and ATIII deficiencies, anticardiolipin antibodies (aCL), prothrombin gene mutation and activated PC resistance (APCR).

The patients' plasmas were tested for PC and PS deficiencies by enzyme-linked immunosorbent assay (ELISA) using Corgenix kit; while ATIII deficiency was tested by colorimetric analysis using kits supplied by Organon Tekinka. The patients' sera were used to test for aCL IgG antibodies by using the commercially available Orgentek ELISA kits.

Testing for prothrombin gene mutation was performed by DNA polymerase chain reaction (PCR). Although a definitive diagnosis of factor V Leiden can be done by PCR, yet the relatively simple plasma screening test for APCR was used instead.

Patients were categorized on the basis of tests' results as being with or without thrombophilia. Those without thrombophilia were further classified as having idiopathic or secondary DVT. All patients received therapeutic doses of clexane and oral anticoagulants.

Patients were followed up to document the incidence of symptomatic recurrent DVT or PE. They were educated about the main signs and symptoms of recurrent VTE and received a card with the telephone numbers of the thrombosis clinic. They were instructed to return to the study center if they noted clinical manifestations suggestive of recurrent venous thrombosis (edema, redness, tenderness, pain, or swelling) in either leg or suggestive of PE (dyspnea, chest pain, or tachycardia).

Patients were also seen at the time of ultrasonographic assessments and were contacted at least twice yearly to ascertain whether signs and symptoms had occurred in which case they were invited to come to the study center for additional diagnostic procedures.

Recurrent DVT was diagnosed by compression ultrasonography. Patients with suspected PE had ventilation-perfusion lung scanning.

RESULTS

The studied group included 125 patients with recurrent LL DVT. They were 52 females and 73 males. Their age ranged between 14 and 75 years with a mean ± SD of 47.2±13.1 years.

An endogenous risk factor for thrombophilia was found in 54 patients representing 43.2% of the entire studied group. Twenty six patients (20.8%) had a single risk factor while 17 patients (13.6%) had two risk factors of thrombophilia. Both an exogenous and an endogenous risk factor were detected in 11 patients (8.8%) and seven patients had a secondary cause for thrombosis (myeloproliferative, malignancy and PNH). The different aetiologies are shown in Table (1).

Table (1): The distribution of patients according to the risk factors (n=125).

Risk Factor	n	%
APCR	18	14.4
Prothrombin G20210A mutation	3 (2 homozygous and 1 heterozygous)	2.4
ATIII deficiency	6	4.8
PC deficiency	7	5.6
PS deficiency	3	2.4
Behcet	11	8.8
Hyperhomocysteinaemia	2	1.6
aCL	16	14.4
PNH	3	2.4
Myeloproliferative disorders	6	4.8
Malignancy	9	7.2

The detected risk factors varied widely with the highest incidence (14.4%) in patients having APCR and aCL (18 and 16 cases respectively). The lowest incidence (1.6%) was in patients with hyperhomocysteinaemia (2 cases).

No definite aetiology could be detected in 64 patients (51.2%), *p* value was not significant. Clinically detectable pulmonary emboli occurred among the LL DVT patients. Three patients had minor post-phlebitic symptoms and 8 patients experienced recurrent DVT. The descriptive statistics of the study subjects is shown in Table (2).

Patients were divided into 5 groups according to the aetiology of LL DVT:

1- Group 1: Included patients with one endogenous risk factor.

2- Group 2: Included patients with two endogenous risk factors.

3- Group 3: Included patients with both exogenous and endogenous risk factors.

4- Group 4: Included patients with exogenous risk factor.

5- Group 5: Included patients with idiopathic aetiology.

Table (2): The descriptive statistics of the study subjects (n=125).

Variable	n	%
Group:		
1	26	20.8
2	17	13.6
3	11	8.8
4	7	5.6
5	64	51.2
Site:		
Peripheral	105	84
Ilio-femoral	20	16
Bilaterality:		
Unilateral	115	92
Bilateral	10	8
Recurrence:		
No	117	93.6
Yes	8	6.4
Patency:		
Complete	107	85.6
Incomplete	18	14.4

Statistical analysis was done to detect any correlation and if there is statistical difference between the different groups in relation to site, bilaterality, recurrence and patency of the affected vessels.

Significant statistical differences were found in the rate of recurrence in group 2, patency in group 3, site in group 4 and all the previous variables in group 5 when compared to other groups.

Group 1 with one risk factor showed no significant correlation with recurrence, patency or the site when compared to other groups [Table (3)].

Table (3): Chi square to test for statistical differences between group 1 (n=26) and other aetiologies.

	One risk factor		Other subjects		*p**
	n	%	n	%	
Site:					
Peripheral	23	18.4	82	56.6	0.49
Ilio-femoral	3	24	17	13.6	
Bilaterality:					
Unilateral	25	20	90	72	0.38
Bilateral	1	0.8	9	7.2	
Recurrence:					
No	26	20.8	91	72.8	0.13
Yes	0	0	8	6.4	
Patency:					
Complete	21	16.8	86	68.8	0.43
Incomplete	5	4	13	10.4	

* *p* value <0.05 is considered statistically significant.

Group 2 with two thrombophilic risk factors showed significant correlation with recurrence *p* value =0.000, but no significant correlation with patency or the site when compared to other groups [Table (4)].

Table (4): Chi square to test for statistical differences between group 2 (n=17) and other aetiologies.

	Two risk factor		Other subjects		*p**
	n	%	n	%	
Site:					
Peripheral	12	9.6	93	74.4	0.15
Ilio-femoral	5	4	15	12	
Bilaterality:					
Unilateral	16	12.8	99	79.2	0.73
Bilateral	1	0.8	9	7.2	
Recurrence:					
No	11	8.8	106	84.4	0.000
Yes	6	4.8	2	1.6	
Patency:					
Complete	13	10.4	94	75.2	0.25
Incomplete	4	3.2	14	11.2	

* *p* value <0.05 is considered statistically significant.

Group 3 with both exogenous and endogenous risk factors showed significant correlation with incomplete patency, *p* value =0.000, but no significant correlation was found with recurrence or the site when compared to other groups [Table (5)].

Table (5): Chi square to test for statistical differences between group 3 (n=11) and other aetiologies.

	Exogenous and endogenous risk factors		Other subjects		*p**
	n	%	n	%	
Site:					
Peripheral	8	6.4	97	77.6	0.29
Ilio-femoral	3	2.4	17	13.6	
Bilaterality:					
Unilateral	11	8.8	104	83.2	0.31
Bilateral	0	0	10	8	
Recurrence:					
No	9	7.2	108	86.4	0.09
Yes	2	1.6	6	4.8	
Patency:					
Complete	5	4	102	81.6	0.000
Incomplete	6	4.8	12	9.6	

* *p* value <0.05 is considered statistically significant.

Group 4 with endogenous risk factor showed significant correlation with ilio-femoral affection, *p* value =0.04, but no significant correlation with the other parameters [Table (6)].

Table (6): Chi square to test for statistical differences between group 4 (n=9) and other aetiologies.

	Exogenous risk factors		Other subjects		*p**
	n	%	n	%	
Site:					
Peripheral	4	3.2	101	80.8	0.04
Ilio-femoral	3	2.4	17	13.6	
Bilaterality:					
Unilateral	6	4.8	109	87.2	0.53
Bilateral	1	0.8	9	7.2	
Recurrence:					
No	7	5.6	110	88	0.48
Yes	0	0	8	6.4	
Patency:					
Complete	5	4	102	81.6	0.27
Incomplete	2	1.6	16	12.8	

* *p* value <0.05 is considered statistically significant.

Group 5 with idiopathic aetiology showed significant correlation with incomplete patency, recurrence and ilio-femoral affection, *p* value =0.000, 0.003 and 0.04 respectively, but no significant correlation with bilaterality when compared to other groups [Table (7)].

Table (7): Chi square to test for statistical differences between group 5 (n=64) and other aetiologies.

	Idiopathic aetiology		Other subjects		*p**
	n	%	n	%	
Site:					
Peripheral	58	46.4	47	37.6	0.04
Ilio-femoral	6	4.8	14	11.2	
Bilaterality:					
Unilateral	57	45.6	58	46.4	0.21
Bilateral	7	5.6	3	2.4	
Recurrence:					
No	64	51.2	53	42.4	0.003
Yes	0	0	8	6.4	
Patency:					
Complete	63	50.4	44	35.2	0.000
Incomplete	1	0.8	17	13.6	

* *p* value <0.05 is considered statistically significant.

The association between the site of thrombosis, bilaterality, recurrence and patency was tested by Chi square and the results are shown in Tables (8,9,10) respectively.

Table (8): The association between the site of thrombosis and other variables.

	Peripheral		Ilio-femoral		*p**
	n	%	n	%	
Bilaterality:					
Unilateral	95	76	20	16	0.15
Bilateral	10	8	0	0	
Recurrence:					
No	100	80	17	13.6	0.05
Yes	5	4	3	2.4	
Patency:					
Complete	92	73.6	15	12	0.14
Incomplete	13	10.4	5	4	

* *p* value <0.05 is considered statistically significant.

Table (9): The association between bilaterality of thrombosis and other variables.

	Unilateral		Bilateral		*p**
	n	%	n	%	
Recurrence:					
No	107	85.6	10	8	0.4
Yes	8	6.4	0	0	
Patency:					
Complete	97	77.6	10	8	0.18
Incomplete	18	14.4	0	0	

* *p* value <0.05 is considered statistically significant.

Table (10): The association between recurrence of thrombosis and other variables.

	No recurrence		Recurrence		*p**
	n	%	n	%	
Patency:					
Complete	104	83.2	3	2.4	0.000
Incomplete	13	10.4	5	4	

* *p* value <0.05 is considered statistically significant.

A correlation was statistically significant between the site and recurrence (p=0.05) as well as between incomplete patency and recurrence (p=0.000).

DISCUSSION

Prospective studies in asymptomatic carriers of inherited thrombophilic defects have shown rather low annual incidence of VTE (between 0.2 and 2% patient-years), which however, is 2-20 times higher than in non-carriers **[9]**.

In the last few years, questions have been raised on whether these thrombophilic conditions are also responsible for an increased risk of recurrent VTE. It is indeed generally expected that carriers are more prone to develop recurrent thrombotic events than non-carriers. This has been shown to be the case for conditions like ATIII, PC and PS deficiencies, mild hyperhomocysteinaemia, increased factor VIII levels, and antiphospholipid antibodies. Surprisingly, there is still some debate on whether heterozygous FV Leiden mutation and G20210A prothrombin variant, which are the most common causes of thrombophilia, are associated with an increased risk of VTE, as some studies are in favour of and others against this association. Homozygous or double heterozygous carriers of either defect, however, appear to be consistently exposed to a higher risk of recurrent VTE **[5]**.

In our study, 51.2% of patients with recurrent DVT had an idiopathic aetiology, 26.6% had a single endogenous risk factor, 13.6% had two endogenous risk factors, 8.8% had both endogenous and exogenous risk factors and 5.6% had an exogenous risk factor.

The detected risk factors varied widely with the highest incidence (14.4%) in patients having APCR and aCL (18 and 16 cases respectively). The lowest incidence (1.6%) was in patients with hyperhomocysteinaemia (2 cases).

Prandoni et al., reported an increased risk of recurrent VTE in patients who showed ultrasound findings compatible with persistent residual thrombus after a first episode of proximal-vein thrombosis compared with those who did not. Interestingly, in a multivariate analysis including thrombophilic abnormalities, persistent residual thrombus appeared to be an independent risk factor for recurrent VTE. This is in accordance with our study in which a statistically significant correlation was found between patency and recurrence.

It is well established that patients who are deficient in one of the natural coagulation inhibitors (i.e., AT-III, PC, or PS) have a markedly increased risk for VTE. Retrospective studies revealed an increased risk for thromboembolism in the inhibitor-deficient compared with the inhibitor-nondeficient individuals. In our study, ATIII deficiency was found in 6 patients, PC deficiency in 7 patients and PS deficiency in 3 patients.

In almost 50% of patients with recurrent DVT, decrease of at least one plasma coagulation inhibitor (ATIII, PC, and PS) level was observed in a study by Swiatkiewicz et al. **[11]**. Also, among 30 patients with ATIII, PC, and PS deficiencies (2, 21 and 7 cases respectively) studied by Lefrancois et al., ten of the 30 cases have had recurrent venous thrombosis at the time of bed rest, trauma, surgery, pregnancy, postpartum or during oral contraceptive treatment. Spontaneous DVT occurred in 3 cases. Seventeen patients had remained asymptomatic till then **[12]**.

In a study by Pabinger et al. **[13]** the probability of developing thrombosis in patients with hereditary AT III, PC and PS deficiency was high (80-90% by the fifth to sixth decade of life). Although a significant difference among the three deficiency states with regard to age at the first thrombotic event was not detected in males, females with ATIII deficiency developed thrombosis significantly earlier in life compared with females with PC or PS deficiency. This difference is due to the extremely high thrombotic risk associated with pregnancy and oral contraceptive use in ATIII-deficient females.

Interestingly, these results in patients with AT-III, PC, or PS deficiency are different from those of patients with resistance to APC, since Svensson and Dahlbäck **[14]** reported only a 30% risk of thrombosis at age 60 for APC-resistant individuals. Hereditary AT-III, PC and PS deficiencies seem to be stronger risk factors for thrombosis than is APC R. However, in our study 18 patients (14.4%) showed APCR while 16 patients (12.8%) collectively had AT-III, PC and PS deficiency.

Many investigators have reported that factor V Leiden is a stronger risk factor for DVT than PE. Prothrombin gene mutation is associated with DVT in the lower extremities alone or when complicated by PE, but it is not associated with isolated PE. Carriers of inherited thrombophilic risk factors were less frequently found among patients with PE alone. Also, carriers of two inherited thrombophilic defects were more frequent among patients with DVT only than among those suffering from isolated PE **[15,16,17]**.

Margaglione et al. **[15]** stated that in patients from different ethnic groups, factor V Leiden has been found in up to 20% and the prothrombin gene mutation in up to 14% of cases of unselected patients with DVT. Double heterozygotes showed higher risk of VTE **[18]**.

When patients with a known alternative risk factor for thrombosis (factor V Leiden mutation or deficiency of ATIII, PC or PS) were excluded, the G20210 variant was found to increase the risk for venous thrombosis by approximately 5 fold **[19]**. Varga and Moll **[20]** also agreed that having a heterozygous prothrombin mutation increases the risk of developing a first DVT by about 2 to 3 times the background. Having homozygous prothrombin mutations increases the risk further, but is not yet known how much the risk is increased.

Surprisingly, De Stefano et al. **[21]** found that patients with the prothrombin mutation had a risk for spontaneous recurrent VTE similar to that of patients with normal genotype. The circumstances of the first event (spontaneous or secondary) did not produce any substantial variation in the risk of recurrence.

Some investigators reported that the risk of recurrent DVT is similar among carriers of factor V Leiden and patients without this mutation. They did not corroborate a stronger association of factor V Leiden in DVT than PE or

in older than in younger participants. Thus, for them, whether factor V Leiden is associated with recurrent events is somewhat controversial **[22,23]**.

In our study, recurrence of VTE was not statistically significant between the idiopathic cases and those with a definite aetiology 51.2 and 49.8% respectively. However, group 2 with two endogenous risk factors showed the highest statistically significant recurrence rate (p= 0.000). Ten of our patients developed recurrent thrombosis in the unaffected leg and three developed isolated PE. These results are in accordance with Paolo et al. **[23]** who confirmed that both idiopathic cases and those with a definite risk factor are associated with increased risk for recurrent VTE. Also, one third of their cases developed recurrent thrombosis in the initially unaffected leg and another third developed isolated PE.

The presence of an identifiable cause among our patients was associated with an increased risk for incomplete patency and development of ilio-femoral rather than peripheral DVT when compared to the idiopathic group. There was a strong correlation between recurrence and incomplete patency, the latter showed statistical significance in group 3 patients with both endogenous and exogenous risk factors.

Ginsberg and others suggested that the risk for recurrence is considerably higher in patients with residual venous thrombosis on repeated ultrasonography than in patients with early vein recanalization **[24,25]**. Residual thrombosis could impair venous outflow, resulting in blood stasis with consequent clot formation. Piovella and colleagues suggest that the thrombus in proximal venous segments (ilio-femoral) increase the incidence of recurrence risk **[26]**.

Prins and Marchios suggested that patients with proximal venous thrombosis whose veins do not recanalize are likely to develop recurrent thrombotic events after withdrawal of oral anticoagulant therapy **[6]**. Recurrence of DVT, in our study, occurred in 3 out of 8 patients (37.5%) who did not receive adequate warfarin treatment. This was statistically significant in comparison to those who received adequate treatment; 5 out of 117 patients (4.2%).

In patients with thrombosis associated with time-limited, reversible risk factors, oral anticoagulant therapy can be limited to 3 months after the elimination of the risk factor. More prolonged courses of anticoagulant therapy are recommended for patients in whom thrombosis is associated with persistent risk factors or idiopathic thrombosis **[2]**. However, in all patients, the risk for recurrence after a short, fixed period of anticoagulation varies greatly. Approximately 70% of patients with unexplained thrombosis do not develop a recurrence, and 10% of patients with transient risk factors do **[27]**.

In their study, Angelli et al., reported that prolonging anticoagulant therapy beyond 3 months in patients with idiopathic DVT simply delays recurrence until anticoagulant therapy is stopped, rather than reducing the risk of recurrence **[2]**. Pinede et al., showed equivalence between two treatment regimens for recurrence, namely, 6 or 12 weeks of anticoagulant therapy for isolated calf DVT and 3 or 6 months for proximal DVT and/or PE without a significant increase in bleeding complications **[28]**.

Christiansen et al., stated that prothrombotic abnormalities do not appear to play an important role in the risk of a recurrent thrombotic event. Testing for prothrombotic defects has little consequence with respect to prophylactic strategies. Clinical factors are probably more important than laboratory abnormalities in determining the duration of anticoagulation therapy **[29]**.

There is no doubt that discrepancy of results among the many studies now available on the role of thrombophilia in predisposing to VTE recurrences are largely due to the complexity of interactions of component causes. Unfortunately, discrepancies of results often generate discordance in the management of thrombotic patients. Future studies with the potential to provide physicians with consistent and reassuring answers are warranted.

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Factor FVII Arg^{353}Gln Polymorphism and its Relevance to Ischemic Complications Following Coronary Catheter Interventions

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ABSTRACT

Factor VII polymorphisms have been suggested in some studies to show an association with coronary artery disease (CAD) especially its fatal outcome myocardial infarction, and there is a known association between FVII levels and polymorphic variants in the gene. The aim of this study was to study the influence of FVII Arg^{353}Gln polymorphism on the plasma levels of both FVIIc and FVIIa and to assess its role as a risk predictor of complications following coronary catheter interventions. A total of 24 patients with CAD who had undergone percutaneous catheter intervention (PCI) were followed up for 30 days and assessed for occurrence of ischemic complications which included myocardial infarction (MI), death or need for target vessel revascularization. A control group of 20 age and sex matched subjects was also included. The FVII Arg^{353}Gln polymorphism was determined by PCR/REFLP assay. The frequencies of FVII genotypes did not show significant differences between the CAD group and the controls or between the males and the females. Carriers of the Gln^{353} allele had a significantly lower levels of total FVII activity (FVIIc, -37%, p<0.01) and of activated circulating FVII (FVIIa, -50%, p<0.01). In this study 5 (21%) patients suffered from major complications during the 30 day follow up after PCI. No patients with Gln^{353} allele had complications following PCI, while 26% of patients with Arg^{353}/Arg^{353} genotype had complications. In this study, we confirmed that Factor VIIa levels and VIIc levels, are influenced by factor VII gene codon 353 polymorphism and our preliminary results indicate that the Gln^{353} allele might be protective against the thrombotic complications following PCI, yet it did not reach statistical significance mostly due to the small number of cases. Further prospective studies are needed to assess this protective role of the Gln^{353} against thrombogenesis.

Key Words: *Factor VII polymorphism catheter intervention.*

INTRODUCTION

Despite recent advances in angiography technology, acute complications including death, delayed abrupt closure and periprocedural myocardial infarction (MI), continue to occur in 10-15% of patients undergoing percutaneous coronary intervention (PCI) **[1]**. Because numerous observational studies have now confirmed a close association between ischemic complications of PCI and late mortality, prevention of such complications remains a central goal of the practicing intervention cardiologist **[2]**.

In recent years, it has become increasingly clear that the coagulation cascade plays a critical role in the pathogenesis of such complications. All forms of catheter intervention based revascularization produce local endothelial injury, thus exposing underlying tissue factor, which binds to and activates circulating factor VII (FVII). The activated form of factor VII is then free to activate other clotting factors (including factors IX and X), thus leading to the generation of thrombin, platelet activation and ultimately to the formation of thrombus at the site of the catheter induced arterial injury **[3]**. It might be that the risk of subacute ischemic complications after catheter-induced endothelial injury should be influenced by circulating levels of FVII. In theory, higher levels of circulating FVII should increase the risk of an ischemic complication, while lower levels of circulating FVII would be protective

Elevated levels of plasma FVII may lead to a prothrombotic state and increase vascular events. The Northwick Park Heart Study showed that elevated FVII coagulant activity (FVIIc) levels in western countries are risk factors for

ischemic heart disease (IHD), particularly its fatal outcome **[4]**. Other studies **[5,6]** also indicated that plasma FVII levels were related to coronary heart disease.

Plasma levels of FVII are influenced by environmental and genetic factors. Several polymorphisms influencing FVII activity have been recently identified. The substitution of glutamine for arginine at position 353 in the catalytic domain (R353Q) and a 10-bp insertion in the promoter region (5'F7) may be responsible for one third of the variations in plasma FVII levels **[7]**. The -401 G/T polymorphism may account for 18% of the variations in FVII Ag and FVIIa levels **[8]**. The FVII gene is also characterized by a polymorphism involving a variable number of 37-bp repeats in intron 7(IVS7) **[9]**. The rare alleles of the above polymorphisms are generally associated with the decreased levels of FVII. In contrast, the rare -402A allele of the -402G/A polymorphism, which may account for 28% of the variation in plasma FVII levels, is associated with the increased plasma FVII levels **[8]**. Allelic frequencies among populations were different **[10]**.

It has been suggested that the Gln^{353} allele protects against myocardial infarction **[11]**. Thus, presence of the Gln^{353} allele may consequently also be protective in other situations in which thrombus formation is a fundamental pathophysiological mechanism, as it is for adverse events complicating coronary catheter interventions.

This study aimed at investigating the relationship between FVII Arg^{353}Gln polymorphism and the plasma level of FVII coagulant activity and activated FVIIa in patients with coronary artery disease (CAD). Also, the possible role of this polymorphism as a risk predictor of complications following coronary catheter interventions has been evaluated.

PATIENTS AND METHODS

This study was carried out on 24 patients suffering of coronary artery disease and were receiving coronary catheter intervention at the National Heart Institute. Coronary artery disease was defined on the basis of angiographic criteria as stenosis ≥50% in a major coronary artery or its major branches. Our study included 8 (33.3%) patients with stable angina, 10 (41.7%) patients with unstable angina and 6 (25%) patients with myocardial infarction. They were17 males and 7 females with a mean age of 50±8.5 years (range 38-68 years). Patients were followed up for one month after coronary catheter interventions and assessed for occurrence of complications as the need for target-vessel revascularization, periprocedural MI or death. We routinely performed post-procedure creatine kinase, MB fraction levels for all patients. The CK levels greater than twice normal were established as criteria for suspecting MI. In addition, a post-procedural electrocardiogram was carried out. In our study, 5 patients presented with complications following the intervention; 2 patients needed target vessel revascularization while 3 patients had periprocedural MI. A control group of 20 age and sex matched healthy subjects were also included in the study. All patients were subjected to the following:

1- Clinical examination and history taking including smoking, diabetes mellitus, hypertension, use of contraceptive pills for females and family history of coronary artery disease.

2- ECG to determine the type of ischemic heart disease.

3- Coronary angiography was performed according to standard techniques and type of lesion was assessed as follows:

Type A lesion: High success rate-low risk-discrete <10mm-concentric-smooth contour-little or no calcification-less than totally occlusive-absence of thrombus.

Type B lesion: Moderate success rate-60-85%-moderate risk-tubular 10-20mm-eccentric-irregular contour-moderate to heavy calcification-total occlusion <3 month-some thrombus are present.

Type C lesion: Low success rate <60%-high risk-diffuse >2cm-total occlusion >3 month-some thrombus are present.

4- Routine laboratory investigations: Included complete blood picture, erythrocyte sedimentation rate, liver function tests, kidney function tests, blood glucose level, cardiac enzymes and lipid profile.

5- Coagulation studies: Estimation of prothrombin time & concentration, INR, activated partial thromboplastin time and fibrinogen level.

6- Assay of factor VII level:

a- Total activity of Factor VII (FVIIc) was determined using a clotting assay with STA-Deficient VII supplied by Diagnostica-Stago, France (Cat.No.00743) **[12]**.

b- Activated Factor VII (FVIIa) was measured using Staclot VIIa-rTF, Diagnostica-Stago, France (Cat.No.00281) **[13]**.

7- Genetic analysis of factor VII $Arg^{353}Gln$ polymorphism by PCR/REFLP:

Genomic DNA was extracted from peripheral-blood lymphocytes by phenol chloroform method. Amplification conditions were an initial cycle at 93°C for 3min, then subsequent 35 cycles for 60 seconds at 93°C, 60 seconds at 55°C and 2min at 72°C, then a final elongation step at 72°C for 5min. (Perkin Elmer 9600, USA). Primers for the $Arg^{353}Gln$ polymorphisms were 5'-GGG AGA CTC CCC AAA TAT CAC-3' and 5'-ACG CAG CCT TGG CTT TCT CTC-3' **[14]**. Twenty microliters of the PCR amplification product (312bp) were digested with 10 units Msp1 restriction enzyme under the conditions described by the manufacturer (Promega, USA). Fragments of 206bp, 67bp and 39bp were detected in the presence of the Arg^{353} allele, and 273bp and 39bp bands indicated the Gln^{353} allele.

Statistical analysis:

Data management and statistical analysis of this work was performed using SPSS 11 computer system. Analysis included descriptive statistics with calculation of mean and SD and frequency distribution. Mean values were compared using student *t* test and *p*-value was calculated. Identifying relationships between different variables was performed using chi-square test.

RESULTS

General characteristics of patients in the study:

The prevalence of the potential risk factors in our studied patients did not differ between the two genotypes (Table 1) except for the type of lesion in coronary angiography. Patients having the Arg^{353}/Gln^{353} genotype were significantly associated with type A lesion ($p<0.01$), while Arg^{353}/Arg^{353} genotypes were significantly associated with types B and C lesions.

Prevalence of FVII polymorphism:

The Arg^{353}/Gln^{353} genotype was found in 5 (20.8%) patients and 4 (20%) normal controls, while the Arg^{353}/Arg^{353} genotype was found in 19 (79.2%) patients and in 16 (80%) control subjects (Table 2). The distribution of these two genotypes was not significantly different between patients and controls ($p>0.05$) nor between males and females. The allelelic frequencies of Arg^{353} and Gln^{353} were 89.6%, 10.4% and 90%, 10% in the CAD group and controls, respectively. No homozygous (Gln^{353}/Gln^{353}) cases were found in this study.

FVII polymorphism and FVII level:

Genotype-phenotype relationship analysis was performed with data from the entire study population. On comparing the plasma level of FVIIc and FVIIa between the two genotypes, it was evident that the plasma levels of factor VIIa and FVIIc were significantly influenced by FVII polymorphism (Table 3). The mean level of factor VIIa was 50 percent lower in patients with the Arg^{353}/Gln^{353} genotype than in those with the Arg^{353}/Arg^{353} genotype and FVIIc was 37 percent lower in patients with the Arg^{353}/Gln^{353} genotype than in those with the Arg^{353}/Arg^{353} genotype. The plasma FVIIa mean values were significantly higher in patients who showed complications after PCI versus the non-complicated group (Table 5).

FVII polymorphism and ischemic complications following PCI:

No patients with Arg^{353}/Gln^{353} genotype had complications following PCI, while 26% of patients with Arg^{353}/Arg^{353} genotype allele had complications. In this study the Gln^{353} allele appears to be protective against complications following PCI, yet it did not reach statistical significance mostly due to the small number of cases (Table 4).

Table (1): Comparison of baseline potential risk factors stratified by genotype.

	Arg^{353}/Arg^{353}	Arg^{353}/Gln^{353}	*p* value
N	19	5	
Age (Yrs)	49.6±8.4	52.2±9.6	NS
Male/Female (%)	14/5	3/2	NS
Smoking (%)	52.6	20.0	NS
Diabetes (%)	21.1	40	NS
Hypertension (%)	36.8	40	NS
Hypercholesterolemia (%)	78.9	100	NS
Acute MI (%)	26.3	20	NS
Lesion type A (%)	5.3	100	<0.01
Lesion type B (%)	68.4	0	<0.01
Lesion type C (%)	26.3	0	<0.01

Table (2): Distribution frequencies of FVII genotypes between patients and controls.

Genotypes	Patients (n=24)	Controls (n=20)	*p* value
Arg^{353}/Arg^{353}	19 (79.2%)	16 (80%)	NS
Arg^{353}/Gln^{353}	5 (20.8%)	4 (20%)	

Table (3): Comparison of FVIIc and FVIIa levels between the different genotypes.

	Genotype	Mean	S.D	*p* value
FVIIc (%)	Arg^{353}/Arg^{353} (n=35)	84.9	19.2	<0.01*
	Arg^{353}/Gln^{353} (n=9)	53.2	17.6	
FVIIa (mU/dl)	Arg^{353}/Arg^{353} (n=35)	43.29	16.39	<0.01*
	Arg^{353}/Gln^{353} (n=9)	21.44	8.63	

Table (4): Distribution frequencies of FVII genotypes in CAD patients with and without complicated PCI.

Genotypes	Complicated PCI (n=5)	Non-complicated PCI (n=19)	*p* value
Arg^{353}/Arg^{353}	5 (100%)	14 (73.7%)	NS
Arg^{353}/Gln^{353}	0 (0%)	5 (26.3%)	

Table (5): Comparison of FVIIc and FVIIa levels between patients with and without complicated PCI.

		Mean	S.D	*p* value
FVIIc (%)	Non-complicated PCI (n=19)	70.89	22.70	NS
	Complicated PCI (n=5)	81.40	15.78	
FVIIa (mU/dl)	Non-complicated PCI (n=19)	31.69	15.48	<0.01*
	Complicated PCI (n=5)	53.75	16.52	

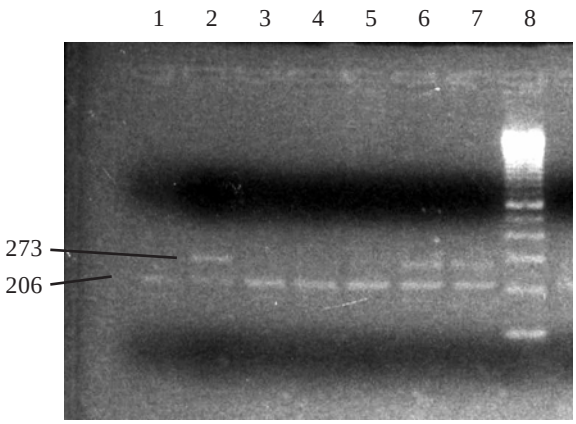

Fig. (1): Gel electrophoresis showing FVII Arg^{353}Gln polymorphism, lanes 2,6,7: heterozygous FVII Arg^{353}/Gln ^{353} cases, lanes 1,3,4,5: Homozygous FVII Arg^{353}/Arg^{353} cases, lane 8: Molecular weight marker.

DISCUSSION

During catheter interventions the protective endothelial lining of the arterial wall is disrupted, and such catheter-induced endothelial injury may trigger thrombogenesis. Increased FVII activity represents a risk factor for thrombotic events complicating coronary catheter interventions. Previous studies had shown an association between FVII levels and polymorphic variants in the gene. Several investigators found that Arg^{353}Gln polymorphism was protective against the development of myocardial infarction in patients with CAD **[15,16]**. The aim of this study was to investigate the relationship between FVII Arg^{353}Gln polymorphism and the plasma levels of both FVIIc and FVIIa, as well as the possible role of this polymorphism in the protection against complications following coronary catheter interventions.

In this study, the allelelic frequencies of Arg^{353} and Gln^{353} were 89.6%, 10.4% and 90%, 10% in the CAD group and controls, respectively. No homozygous (Gln^{353}/Gln^{353}) cases were found in this study. The frequency of the Arg^{353}/Arg^{353} and the Arg^{353}/Gln^{353} genotypes were 79.2% and 20.8% respectively in our CAD patients with no homozygous cases. These frequencies were similar to that reported by other investigators as Girelli et al. **[15]** who reported frequencies of 69.1%, 28.6% and 2.3% in the Arg^{353}/Arg^{353}, Arg^{353}/Gln^{353} and Gln^{353}/Gln^{353} respectively. The frequency of Gln^{353} allele in our controls was similar to those

reported in controls in other studies from northern Italy (for example, 10 percent of our controls carried the Gln^{353} allele, as compared with 16.5 percent of the healthy controls in the study by Ardissino et al. **[17]** and 16.2 percent in the study by Girelli et al. **[15]**.

It has been evidenced by our findings (Table 3) that the Gln^{353} mutation possesses functional importance, that consists in a FVIIc reduction of 37% in Gln^{353} carriers. An analogous phenomenon could be observed in FVIIa levels, with a more reduction of 50% in the Gln^{353} heterozygotes. These phenotypical findings accord well with the literature **[11,14,18,19]** and may explain the expected protective effect of the Gln^{353} allele. The $Arg^{353}Gln$ site was noted initially to associate with a 20% to 30% variance in factor VII levels in males and females and in different ethnic groups **[10]**. Many subsequent studies have confirmed that carriers of the allele coding for Gln^{353} have lower factor VII levels.

An interesting finding in this study is the significant association of FVII Arg^{353}/Gln^{353} genotypes with the atheromatous lesion type A, (Table 1) which is characterized by having no thrombus while FVII Arg^{353}/Arg^{353} genotypes were either type B or C which may have a thrombus.

In this study five (21%) patients suffered from major complications during the first 30 days after PCI, in form of myocardial infarction and need for target vessel revascularizton. When evaluating the role of FVII $Arg^{353}Gln$ genotype as a risk predictor of these ischemic complications, we found that the incidence of complications was 0% in Arg^{353}/Gln^{353} genotype and 26% in Arg^{353}/Arg^{353} genotype. Although all complicated cases were of the Arg^{353}/Arg^{353} genotype and none of them was of the Arg^{353}/Gln^{353} genotype, the difference was statistically insignificant. This might be due to the relatively small number of subjects studied in our work, yet there seems to be a trend towards a protective role for the Gln^{353} allele in prevention of complications following PCI. This relationship has been evaluated by Mrozikiewicz et al. **[14]** and they provided a clear evidence that the Gln^{353} allele of coagulation FVII is associated with a substantial risk reduction by two thirds following coronary catheter interventions.

In the present study, the mean plasma FVIIa level was significantly higher in the complicated group ($p<0.01$), however, FVIIc levels were higher in the complicated group when compared to the non complicated, yet it did not reach statistical significance. It is well accepted that precise genetic markers may provide a better measure of individual lifelong exposure to a putative risk factor than related plasma measurements, which may vary over time **[20]**. This may be particularly true of factor VIIc levels. Whereas genetic markers are probably the strongest determinants of these levels, **[21]** a number of well-known, transient, environmental influences, **[22,23]** may obscure the relation with thrombotic complications when a single measurement is made. By contrast to functional variations of FVIIc or FVIIa levels, the FVII genotype is a constant predictor of the lifelong tendency of individual FVII activity. Similar explanation was reported by Girelli et al. **[15]** who concluded that Gln^{353} allele was protective in their CAD patients against MI yet they found that the mean levels of factor VIIa did not differ significantly between those with a history of myocardial infarction and those without it.

We concluded that the polymorphism strongly influenced the plasma levels of both FVIIc and FVIIa. Also, our preliminary results indicate that absence of the Gln^{353} allele in patients who suffered ischemic complications following PCI might shed light on its protective role against the risk of such complications. Because the number of subjects included in this study was small, these results require further confirmation in larger scale prospective studies.

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Hyperhomocysteinemia in Recurrent Miscarriage

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ABSTRACT

Objective: An elevated total plasma homocysteine level has been suggested as a possible risk factor in women suffering from recurrent miscarriage. The current study was undertaken to assess the association between homocysteine, folate, cobalamin (vitamin B12) and the risk of recurrent miscarriage.

Setting: Recurrent Miscarriage Clinic, National Research Centre, in collaboration with the Radioisotope Department, Nuclear Research Centre and the Obstetrics and Gynecology Department, Kasr Al Aini University Hospital.

Design: Case-control study.

Materials and Methods: The study included 57 non-pregnant Egyptian women. They were classified according to their obstetric history into 2 groups: 32 cases with at least two consecutive miscarriages (Study group), and 25 cases with normal obstetric history (Control group). All cases were tested for plasma total homocysteine, serum folate and cobalamin (vitamin B12).

Results: The fasting total homocysteine was significantly higher in the study group as compared to the control group. While the median concentrations for the vitamins studied were significantly lower in women of the study group as compared to the controls. Elevated homocysteine and reduced vitamin B12 can be considered risk factors for recurrent miscarriage with odds ratio (OR) and 95% confidence intervals (95% CI) of 1.839 (1.286, 2.63) and 1.993 (1.346, 2.951) respectively in the group of recurrent miscarriages. The OR (95% CI) in the study population for low serum folate concentrations was 1.23 (0.776, 2.256).

Conclusion: Elevated homocysteine and reduced serum vitamin B12 are risk factors for recurrent miscarriage. Low serum folate did not seem to be a risk factor for recurrent miscarriage. Testing for homocysteine levels in women suffering from unexplained recurrent miscarriage and pre-conceptional supplementation with vitamin B12 might be beneficial to improve pregnancy outcome.

Key Words: *Homocysteine - Miscarriage.*

INTRODUCTION

Miscarriage is the most common adverse pregnancy outcome, affecting between 10 and 15% of clinically recognized pregnancies **[1,2]**. As many as 5% of all couples attempting to conceive have two successive pregnancy losses, and 1% have three or more consecutive losses **[3]**. Recurrent miscarriage is usually defined as the loss of three or more consecutive pregnancies before viability. In many clinical situations, the definition is altered to two or more consecutive spontaneous miscarriages **[4]**.

An increased miscarriage rate has been observed in pregnancies preceding that of fetuses or newborn infants with neural tube defects (NTDs). Carmi et al. **[5]** found a significantly higher miscarriage rate (48%) in pregnancies preceding those of fetuses with NTDs, compared to those with other birth defects (20%).

It has been hypothesized that both forms of reproductive failure could have one factor in common: Hyperhomocysteinemia (HHcy), which interferes with embryonic development, as well as with vascular function **[6]**. The hypothesis that homocysteine (Hcy) might induce vascular disease was originally advanced by Mc Cully **[7]**, based on the observation that thromboembolism and atherosclerosis were features in children with inherited disorders of Hcy metabolism.

The introduction of the antiphospholipid syndrome (APLS), in the early 1980s, as an etiological cause for recurrent pregnancy loss, has substantiated the "thrombosis theory" of repeated fetal loss **[8]**. Hyperhomocysteinemia

belongs among the familial thrombophilias and is a long known vascular disease risk factor. In vitro, Hcy has been shown to directly damage endothelial cells that predispose thrombogenesis and arteriosclerosis **[9]**. In addition, Hcy induces tissue factor (TF) expression in vitro, which is the initiator of blood coagulation in vivo **[10]**.

Homocysteine is a non-protein forming sulfur amino acid, whose metabolism is at the intersection of two metabolic pathways remethylation and transsulfuration. Remethylation requires the cofactors, folate and cobalamin (vitamin B12) **[11]**.

Hyperhomocysteinemia may be caused by genetic defects of the enzymes involved in its metabolism, nutritional deficiencies or absorption deficiencies of the vitamin cofactors of these enzymes, chronic diseases or administration of some drugs **[12]**.

The current study was undertaken to evaluate the prevalence of hyperhomocysteinemia, folate or cobalamin deficiency in non-pregnant women with history of two or more consecutive miscarriages and no known risk factors for such events, in comparison to non pregnant women with normal obstetric outcome and no history of miscarriage.

MATERIAL AND METHODS

The study was conducted in the Recurrent Miscarriage Clinic of the Prenatal Diagnosis and Fetal Medicine Department, National Research Centre, in collaboration with the Radioisotope Department, Nuclear Research Centre, and the Obstetrics and Gynecology Department, Kasr Al Aini University Hospital.

The study included 57 non-pregnant Egyptian women of the same age range. The cases were classified according to their obstetric history into 2 groups. Control group consisted of 25 currently non-pregnant women, with no history of miscarriage, stillborn or intrauterine growth retardation, and having at least one normal living baby. The study group consisted of 32 non pregnant women with a history of at least 2 consecutive miscarriages before the 20th week of gestation.

The exclusion criteria included: Ectopic pregnancy, elective termination of pregnancy, miscarriage associated with anembryonic pregnancy or fetal malformation detected by ultrasound or pathological examination, immunological disorders confirmed by tests for anticardiolipin antibodies and lupus anticoagulant. Severe uterine (anatomical) abnormalities were ruled out by hysterosalpingography or high resolution ultrasonography. The patients were tested at least 10 weeks post-termination.

The patients and the control subjects did not receive oral contraception, vitamin supplementation, or any medication known to influence homocysteine metabolism within at least a period of 6 months. None of the subjects of both groups had a known endocrine dysfunction, or suffered from gastrointestinal, hepatobiliary, renal or vascular diseases.

Blood samples were collected in the morning after an overnight fast. Blood samples for measurement of total Hcy were collected into tubes containing EDTA. Plasma was then separated immediately by centrifugation at 3000xg for 20 minutes and aliquots were stored at -70ºC until analysis. Plasma was separated immediately to avoid artifactual increase due to the synthesis by blood cells in vitro. Blood samples, for measurement of serum folate and vitamin B12, were collected into empty glass tubes and aliquots were stored at -70ºC until analysis.

Total Hcy (free plus protein bound) levels were measured using a commercially available immunoassay kit Axis (Axis-Shield AS, Norway). It is a solid phase enzyme immunoassay based on competition between S-adenosyl-L-Homocysteine (SAH) in the sample and immobilized SAH bound to the wall of the microtitre plate for binding sites on a monoclonal anti-SAH antibody. Homocysteine is reduced by the use of dithiothreitol (DTT) and then enzymatically converted to SAH by the use of SAH hydrolase in a separate procedure prior to the immunoassay.

Serum folate and vitamin B12 were measured simultaneously by radioimmunoassay technique using commercially available kit produced by DPC (Diagnostic Product Corporation). The test is a dual count solid phase, no boil immunoassay for both folic acid and vitamin B12, based on the principle of: Alkaline denaturation of endogenous proteins, competition for purified binder at pH 9.3, then solid

phase separation. The procedure was done following the instruction manual. Calibration range for vitamin B12: 50-2400pg/ml and 0.5-24ng/ml for folic acid. Analytical sensitivity for vitamin B12: 34pg/ml and for folic acid 0.3ng/ml.

Statistical analysis: Data were analyzed using SPSS statistical package version 10. Numerical data were expressed as mean ± SD, or median and range as appropriate. Comparison between cases and controls were done with Mann-Whitney U tests. Probability *p*-value less than 0.05 was considered significant and less than 0.001 highly significant. Odds ratio (OR) and its 95% confidence interval was the test used to estimate risk of cases in relation to controls.

RESULTS

The study group comprised 32 women with history of recurrent miscarriage (median 5; range 2-9). The median age was 28.0 years (range 20-40 years). The control group, consisting of 25 parous women with no history of miscarriage, had a median parity of 2 (range 1-3 babies), and the median age was 26.0 years (range 20-40 years).

Women with recurrent miscarriage had significantly lower median serum folate and vitamin B12 concentrations, and significantly higher median homocysteine concentrations compared with control. The results are presented in Table (1).

Hyperhomocysteinemia (fasting Hcy greater than 15µmol/L) was detected in 28.1% (9/32) of cases, compared with 4% (1/25) in the control group, giving an OR (95% CI) of 1.839 (1.286, 2.63). Although there appears to be a marked difference in the median folate concentrations regarding the study group (4.4ng/ml) and the control group (8.5ng/ml), a serum folate deficiency (<3.0ng/ml) was found in 15.6% (5/32) of the study group and 8% (2/25) of the control group, giving an OR (95%CI) of 1.23 (0.776, 2.256) (Table 2).

Low serum vitamin B12 (<200pg/ml) was diagnosed in 43.8% (14/32) of the study group compared with 8.0% (2/25) of the controls, giving an OR (95% CI) of 1.993 (1.346, 2.951) (Table 2).

Table (1): Median levels of homocysteine, serum folate and vitamin B12 in the studied groups.

	Study Group (n=32)	Control Group (n=25)	*p* value
Homocysteine µmol/L	10.0 (3.4-40.0)	6.8 (3.4-19.5)	0.011
Serum folate ng/ml	4.4 (1.9-17.0)	8.5 (2.1-16.0)	0.001
Serum vitamin B12 pg/ml	260 (60-800)	350 (100-700)	0.013

The values are expressed as median (range).

Table (2): Estimated risk of recurrent miscarriage for the studied biochemical markers.

	RM (n)	Control (n)	OR (95% CI)
Homocysteine >15µmol/L	9	1	1.839 (1.286,2.63)
Serum folate <3.0ng/ml	5	2	1.23 (0.776,2.256)
Serum vitamin B12 <200pg/ml	14	2	1.993 (1.346,2.951)

OR: Odds Ratio. CI: Confidence Interval.
RM: Recurrent Miscarriage. n : number of cases.

DISCUSSION

The etiology of recurrent miscarriage is considered to be multifactorial **[13]**. Homocysteine has received increasing attention during the past decade and elevated plasma homocysteine levels have been implicated in a variety of clinical conditions **[14]**. Normal levels of fasting plasma homocysteine are considered to be between 5 and15µmol/L **[15]**. Hyperhomocyteinemia can cause obstetrical diseases that are connected with vascular disorders of pregnancy or the uteroplacental unit **[16,17]**. We found that the incidence of hyperhomocystenemia in the study group was about seven times higher (28.1%) than the control (4.0%). The study by Coumans et al. **[18]** also showed this discrepancy (17.1% vs. 4.5%). Gris et al. **[19]** reported an association between increased levels of Hcy and a first early pregnancy loss. Del Bianco et al. **[20]** found 25% of women with recurrent pregnancy loss to have hyperhomocysteinemia or at least a pathological methionine loading test. Kumar et al. **[21]** reported no sig-

nificant difference in the median fasting total plasma Hcy concentrations between women with recurrent miscarriage and the controls. However, elevated Hcy levels >18μmol/L was considered by the authors to be a risk factor for recurrent pregnancy loss **[21]**. Nelen et al. **[22]** studied women with recurrent miscarriage and found a direct relationship between high levels of Hcy and defective chorionic vascularization.

Thrombophilias are suggested to play a role in recurrent miscarriage **[23]**. Krabbendam et al. **[24]** evaluated the literature of the past ten years regarding the association between thrombophilias and recurrent miscarriage. No relation was found between recurrent miscarriage and the methylenetetrahydrofolate reductase C 667T mutation, the levels of antithrombin, protein C and protein S. They concluded that there is only justification for testing for homocysteine levels, antiphospholipid antibodies and factor V Leiden in women with recurrent miscarriage.

Selhub and coworkers **[25]** suggested that most individuals with increased plasma Hcy concentrations have inadequate concentrations of one or more of the vitamins required for Hcy metabolism. In the study we evaluated the folate and cobalamin serum levels in women with recurrent miscarriage. The levels showed significant differences between the cases and the controls. This result is in contrast with the study conducted by Sutterlin et al. **[26]** where no significant differences in folate and cobalamin serum levels were found between the cases and the controls.

Coumans et al. **[18]** observed a weak significant correlation between the number of previous miscarriages and folate values. In this study, the number of previous miscarriages did not have any significant influence on folate values. This study demonstrated that low serum folate did not seem a risk factor for recurrent miscarriage.

Reznikoff-Etievant et al. **[27]** was in agreement with our data that vitamin B12 is significantly low in women with recurrent miscarriage. They recommended vitamin B12 assay in women with recurrent miscarriage whether or not hematological abnormalities are present. Candito et al. **[28]** found that vitamin B12 is one of the causes of recurrent pregnancy loss associated with HHcy and that parental B12 therapy led to normal Hcy level within 2 months and to a successful pregnancy.

Although folate deficiency is one of the factors that may lead to alterations in DNA synthesis and chromosome structure in rapidly dividing cells and the serum concentration is a sensitive indicator of the folate available for replicating cells **[29]**, Abir et al. **[30]** found the mean serum concentration of folic acid to be similar in the so called "high risk sera" from women with at least two abortions and in the control sera.

Ronnenberg et al. **[31]** found that the risk of spontaneous miscarriage was four fold high among women with suboptimal plasma concentrations of folate, while Hcy and vitamin B12 status were not associated with spontaneous miscarriage risk. In the current study, we found that the risk of spontaneous miscarriage was two fold high among women with suboptimal plasma concentration of vitamin B12. The apparent inconsistencies can be explained on the basis of the study population, both with respect to genetic background and dietary habits. It should be remembered that certain population, especially Mediterranean population already have adequate intake of folate **[32]**.

In conclusion, elevated homocysteine and reduced serum vitamin B12 are risk factors for recurrent miscarriage. Low serum folate did not seem to be a risk factor for recurrent miscarriage. Testing for homocysteine levels in women suffering from unexplained recurrent miscarriage and pre-conceptional supplementation with vitamin B12 might be beneficial to improve pregnancy outcome.

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Effect of Renal Failure and Hemodialysis on Some Procoagulant Aspects of Hemostasis

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ABSTRACT

Background: At present, the incidence of bleeding is CRF patients is apparently declining, whereas thrombotic complications have become the predominant causes of mortality. The objective of this study was to explore the effect of renal insufficiency and maintenance hemodialysis on some hemostatic parameters with possible role in increased risk of thrombosis.

Methods: This case control study was conducted on 30 patients with chronic renal failure under maintenance hemodialysis and 30 control subjects. An extended hemostatic assessment was performed including study of factor VIIa-rTF coagulant activity by clotting assay, factor VII Ag using ELISA technique, and assessment of monocyte procoagulant activity by measuring its tissue factor expression by flow cytometry before and immediately after dialysis.

Results: Thrombotic events were reported in 40% of patients. CRF patients showed increased monocyte tissue factor expression and increased activation of tissue factor pathway and factor VII Ag. Moreover the maintenance HD process significantly affected the levels of activated factor VII, monocyte tissue factor expression, and factor VII antigen with possible role in thrombotic events.

Conclusion: CRF patients under maintenance HD are at increased risk of thrombosis especially those with recurrent vascular access obstruction. An extended coagulation profile study including monocyte tissue factor expression, assay of (VIIa-rTF), factor VII antigen and protein C should be included in workup of these patients with possible role in considering prophylactic anticoagulant therapy.

Key Words: *Renal failure - Hemodialysis - Coagulation.*

INTRODUCTION

Although renal failure has classically been associated with a bleeding tendency, thrombotic events are common among patients with end stage renal disease (ESRD) under maintenance haemodialysis. Dialysis patients experience an exceedingly high incidence of thrombotic complications including cardiovascular disease, DVT, thrombotic cerebral accidents and vascular access-related complications **[1]**.

Studying of Hypercoagulability in ESRD patients and renal transplant recipients to identify patients at risk of thrombosis and evaluating strategies for prevention by Irish, (2004), recommended further studies to determine whether routine clinical screening for thrombophilic factors is justified **[2]**.

The objective of this study was to explore the effect of renal insufficiency and maintenance hemodialysis on some hemostatic parameters with possible role in increased risk of thrombosis.

SUBJECTS AND METHODS

This case control study was conducted on 30 patients with chronic renal failure under maintenance hemodialysis and 30 control subjects. The group under study was selected from patients on maintenance hemodialysis in Nephrology and Urology Unit (King Fahd Unit), Kasr Eleini Hospitals. Selection of the cases was randomly made with no segregation due to sex or age. All study participants were subjected to the following investigations:

1- Clinical Assessment included recording of age, sex, age of onset, clinical history with special emphasis on personal and family history of thrombosis, shunt operation and shunt failure, cardiac or cerebral accidents; intake of erythropoitien and anticoagulant drugs.

2- Routine laboratory tests included blood sugar, serum lipid profile, hemoglobin level, platelet count, kidney function and liver function tests.

3- Hemostatic screening: Hemostatic screening started with prothrombin time (PT), activated partial thromboplastin time (aPTT). Further coagulation assessment included assay of fibrinogen level, protein C (Quantitative determination of protein C by the synthetic chromogenic substrate method) **[3]**. In addition, protein S was measured (Quantitative determination of protein S by microlatex-mediated Immunoassay), using commercial kit from Diagnostica Stago (Cat. No. 00570), France **[4]**. Anticardiolipin IgG and IgM measurement by ELISA technique using commercial kit from Immco-Diagnostics, Inc. USA (Cat. No. 1118G for IgG and 1118M for IgM) **[5]**.

4- Specific coagulation parameter: Clotting assay of activated factor VII (Factor VIIarTF), using commercial kit from Diagnostica Stago (Cat. No. 00281), France **[6,7]**. Factor VII antigen (VII:Ag) Assay of factor VII antigen by enzyme immunoassay using commercial kit from Diagnostica Stago (Cat. No. 00241), France **[8]**. Monocyte tissue factor expression using Becton Dekenson "FACS Calibur" Flow cytometric analyse. Monocyte tissue factor expression assay included direct labelling of cells with FITC conjugated mouse antihuman CD14 antibodies and PE conjugated goat antihuman CD142 antibodies. Flow cytometric acquisition followed starting with isotypic control samples, followed by test samples. Two dot blots were created for each sample one representing FSC againest SSC and the other represents FL1 againest FL2. Analysis followed where Monocyte population was gated on forward scatter and side scatter blot. nonspecific fluorescence was excluded. Percent monocytes expressing TF were obtained as fluorescence in the upper right side of the dot blot FL1 againest FL2 **[9,10]**.

Statistical analysis:

The data were coded and entered using the statistical package SPSS version 11.01. The data were summarized using the mean and standard deviation (S.D.) for quantitative data and the frequency distribution for qualitative data.

The student's *t*-test was used to assess statistical differences between two groups of quantitative data, paired *t*-test was used to compare data before and after dialysis. ANOVA test was used to assess differences between multiple groups. As for the qualitative data, statistical associations were assessed using Chi-Square test.

RESULTS

Patient's characteristics are shown in Table (1). Frequency of shunt manipulation is illustrated in Table (2). The frequency distribution of other thrombotic events history (Deep venous thrombosis (DVT), ischemic heart disease is also summarized in Table (3). Comparison of routine coagulation parameters in CRF patients versus controls is summarized in (Table 4). Table (5) and Fig. (1-3) summarize the difference between procoagulant parameters in CRF patients compared to control subjects while Table (6), Figs. (4-6) show the effect of hemodialysis on the same parameters by comparing procoagulant parameters before versus immediately after hemodialysis session.

Comparison of procoagulant parameters in patients with history of thrombosis versus patients with no history of thrombosis is shown in (Table 7), calculated procoagulant level differences (before-After dialysis) were also compared in groups bases on history of thrombosis in Table 8 (Fig. 7).

Table (1): CRF patient characteristics and summary of history records.

Mean Age (years)	39.4±12.93
Mean age of onset (years)	7.6±4.69
Mean duration of dialysis (years)	6.4±3.9
Male / Female ratio	13/17
Family history of thrombosis	1/30 (3.1%)
Smoking	3/30 (10%)
Conraceptive pills	0/30 (0%)
Hypercholesterlemia	3/30 (10%)
DM	6/30 (20%)
Hypertension	8/30 (26.6%)
EPO Therapy	9/30 (30%)

Table (2): The Frequencies of shunt manipulation of the CRF patients under maintenance hemodialysis.

Number of shunt manipulation	Frequency	Percent
1	18	60.0
2	7	23.3
3	4	13.3
5	1	3.3

Table (3): Frequency distribution of thrombotic events among the CRF patients under maintenance hemodialysis.

Thrombotic events	Number	Percent
Deep venous thrombosis	3	10
Ischemic heart disease	2	6.6
Failure of shunt operation	12	40

Table (4): Comparison of coagulation parameters of the CRF patient group versus the control group under study.

Type of analysis	Patients Mean ± SD	Control Mean ± SD	*p* value
Prothrombin conc. (%)	92.8±9.38	97.9±3.67	0.173
Fibrinogen (mg/dl)	317.7±52.48	299.4±26.35	0.350
Protein C (%)	66.3±20.54	102.0±21.33	0.001/HS
Protein S (%)	112.1±19.51	100.8±13.91	0.304
Platelet count (x 1000/mm^3)	187.1±55.19	210.4±38.95	0.273

p value >0.05 is non significant (NS).
p value <0.05 is significant (S).
p value <0.01 is highly significant (HS).

Table (5): Effect of renal impairment on activated factor VII (VIIa-rTF complex), Monocyte TF Expression and Factor VII Antigen.

Sample status	VIIa-rTF level in mU/ml Mean ± S.D.	*p* value
Patients before dialysis Control	200.9±49.39 75.3±5.44	< 0.001/HS
	Monocyte TF Expression % Mean ± S.D.	
Patients before dialysis Control	5.2±4.14 1.5±1.08	< 0.001/HS
	Factor VII:Ag % Mean ± S.D.	
Patients before dialysis Control	108.7±10.96 101.3±13.80	NS

Table (6): Effect of hemodialysis on activated factor VII (VIIa-rTF complex), Monocyte TF Expression and Factor VII Antigen.

Sample status	VIIa-rTF level in mU/ml Mean ± S.D.	*p* value
Patients before dialysis Patients after dialysis	200.9±49.39 273.1±52.55	<0.001/HS
	Monocyte TF Expression % Mean ± S.D.	
Patients before dialysis Patients after dialysis	5.2±4.14 13.6±11.09	<0.001/HS
	Factor VII:Ag % Mean ± S.D.	
Patients before dialysis Patients after dialysis	108.7±10.96 116.2±11.18	<0.01/HS

Table (7): Specific procoagulant parameters measured after dialysis in patients with history of thrombotic events versus patients with no thrombotic events.

Procoagulant parameter	Patients with thrombotic events Mean ± SD	Patients with no thrombotic events Mean ± SD	*p* value
VIIa-rTF level (mU/ml)	289.4±51.05	260.7±51.66	NS
Monocyte TF Expression (percent)	14.4±12.38	13.0±10.34	NS
VII:Ag (%)	118.5±9.28	114.5±12.42	NS

Table (8): Difference between specific procoagulant parameters before and after dialysis (Δ change) in patients with thrombotic events versus patients with no thrombotic events.

Procoagulant parameter	Patients with thrombotic events Mean ± SD	Patients with no thrombotic events Mean ± SD	*p* value
VIIa-rTF level (mU/ml)	93.0±56.95	56.4±41.55	<0.05/S
Δ Monocyte TF Expression (percent)	9.5±11.43	8.3±9.19	NS
Δ VII:Ag (%)	9.2±10.06	6.3±12.26	NS

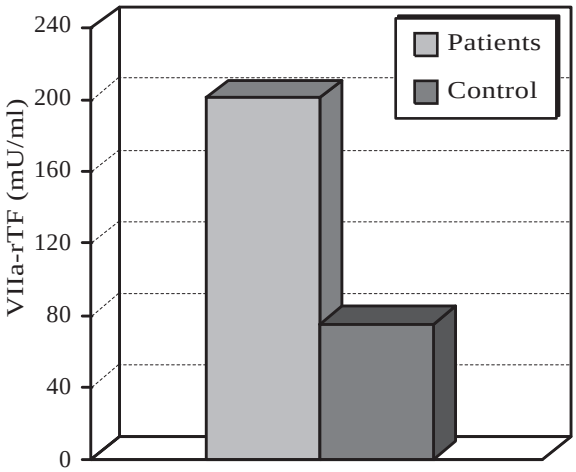

Fig. (1): Activated factor VII mean value before dialysis among CRF patients under HD versus control group.

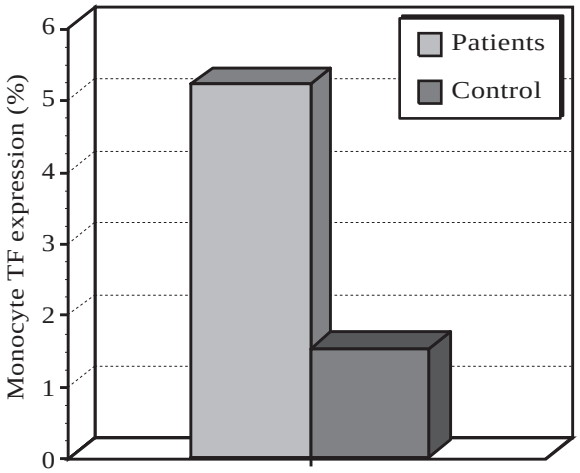

Fig. (2): Monocyte tissue factor expression mean value before dialysis among CRF patients under HD versus control group.

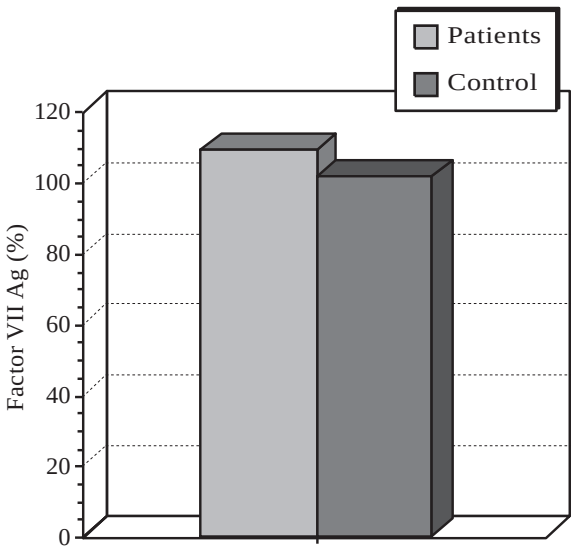

Fig. (3): Factor VII antigen mean value before dialysis among CRF patients under HD versus control group.

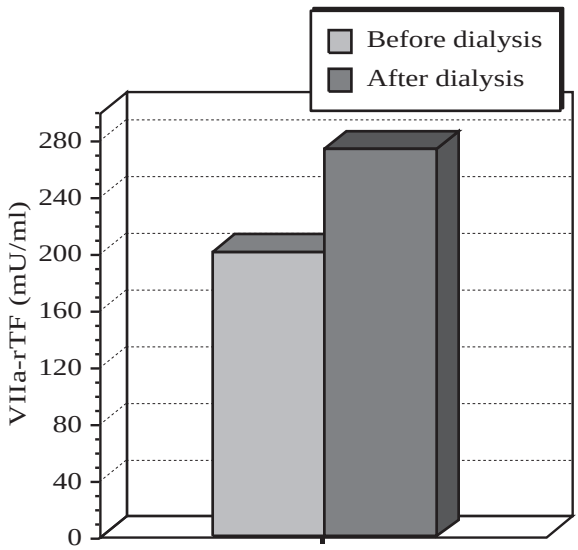

Fig. (4): Activated factor VII mean value before and after dialysis among CRF patients.

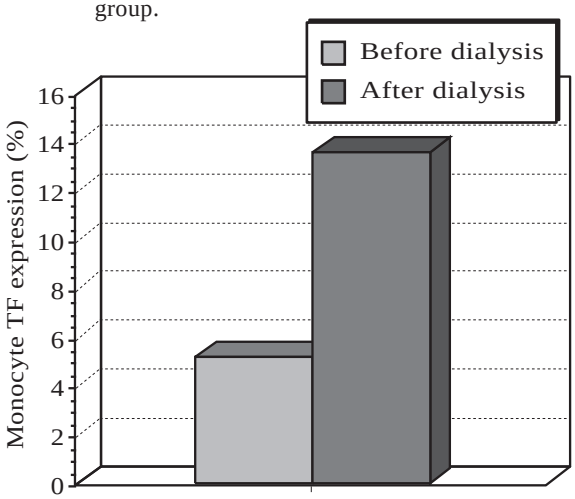

Fig. (5): Monocyte tissue factor expression mean value before and after dialysis among CRF patients.

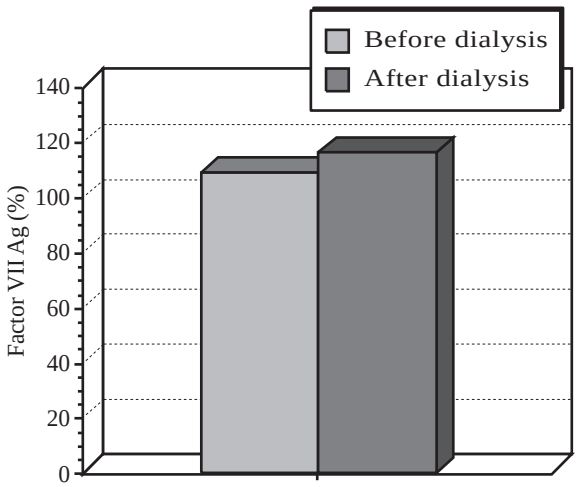

Fig. (6): Factor VII antigen mean value before and after dialysis among CRF patients.

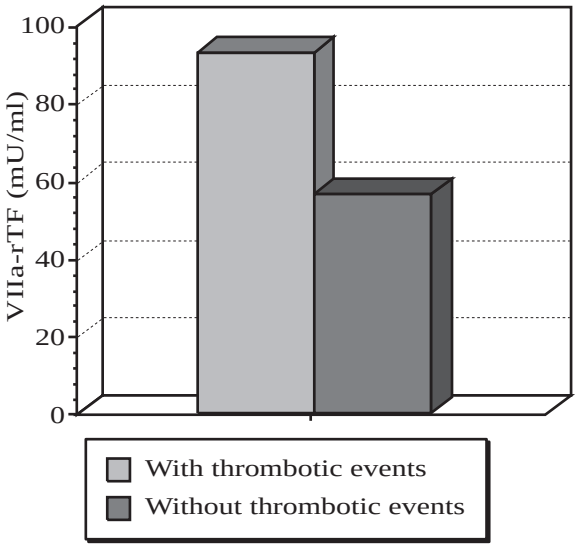

Fig. (7): Difference between activated factor VII mean value before and after dialysis in patients with thrombotic events versus patients with no thrombotic events.

DISCUSSION

At present, the incidence of bleeding is CRF patients is apparently declining, whereas thrombotic complications have become the predominant causes of mortality. The most important suggested determinants of the pathogenesis of the prothrombotic state in uremia are increased levels of clotting factors and decreased levels of clotting inhibitors, diminished fibrinolytic activity, hyperfibrinogenemia, and platelet hyper-aggregability **[11]**. Endothelial activation and endothelial injury in uremia can also induce TF exposure on its surface by a wide range of events, from inflammatory cytokines to mechanical arterial injury.

The objective of this study was to explore the effect of renal insufficiency and maintenance hemodialysis on hemostatic parameters with possible role in increased risk of thrombosis.

In the current study twelve CRF patients needed more than one shunt manipulation (40%), 3 (10%) of them had past history of DVT and 2 (6.6%) had past history of ischemic heart disease; where 18 CRF patients needed only one shunt operation and no past history of thrombosis (Tables 2,3). This finding suggest CRF and maintenance hemodialysis as a potential risk factor for thrombosis (Odds 19.33 CI 2.24-432.34 $p<0.01$).

Comparison of the coagulation parameters in ESRD patients versus controls showed no statistically significant difference in prothrombin concentration, fibrinogen level, Protein S or platelet count (Table 4). However, Protein C activity was significantly lower in ESRD patients compared to controls. Fifty eight percent of the patient group showed deficiency in protein C activity. These results agreed with the finding of Nampoory et al. (2003) who reported the presence of deficiency in protein C in end stage renal disease ESRD patients. When parameters were compared between patients with and without vascular access thrombosis (VAT) episodes. PC levels were significantly lower in those who experienced VAT. It was also reported that these deficiencies were completely corrected after renal transplantation **[12]**.

Values of VIIa-rTF in CRF patients under maintenance hemodialysis was significantly elevated compared to control group value. The same was observed comparing monocyte tissue factor expression percentage in patients versus controls where the mean value of the patient group showed significantly higher value compared to control value (Table 5, Figs. 1,2). Yu et al. (2003) confirmed the obtained results in this study when reported that elevated baseline levels of tissue factor in hemodialysis patients, compared to normal reference ranges **[13]**. In addition, Mercier et al. (2001) reported similar results of the current study showing increased values of FVIIa, VIIa/FVIIAg ratio, soluble tissue factor, and tissue factor monocyte procoagulant activity, along all the healthy control group, nondialyzed CRF group, and CRF on hemodialysis group in ascending manner (1). Level of factor VII antigen in plasma showed no significant difference in the patient group compared to control group (Table 5 and Fig. 3). This result agreed with the finding of Mercier et al. (2001) who did not detect any significant differences between factor VII antigen in CRF patients before dialysis and control group (1). The difference elicited comparing studied hemostatic parameters in ESRD patients versus controls suggest CRF as a hypercoagulable state.

To evaluate direct effect of hemodialysis on the same hemostatic parameters, comparative study of these parameters before and immediately after dialysis was performed. Activated

factor VII was significantly increased after dialysis compared to before dialysis values. (Table 6 and Fig. 4). The level of factor VII antigen in plasma was significantly elevated in patients immediately after dialysis compared to levels before dialysis. Procoagulant activity of monocytes, measured as increased expression of tissue factor (CD 142) on the surface of monocytes, showed a highly significant increase in TF expression immediately after dialysis compared to before dialysis values (Table 6 and Figs. 5,6). Theses results are in accordance with the data reported by Camici et al. (1997) who evaluated the behavior of factor VIIa before and after dialytic treatment in patients on maintenance hemodialysis and observed significant increase of factor VIIa after hemodialysis compared with before dialysis **[14]**.

Also Mercier et al. (2001) confirmed the result obtained from this study when they investigated FVIIa, soluble tissue factor, and monocyte procoagulant activity (represented by tissue factor expression of monocytes) before and immediately after dialysis and found that dialysis induced a significant increase of FVIIa, soluble tissue factor, and monocyte procoagulant activity. Fang et al. (2004) also showed enhanced levels of coagulation factor VII in chronic renal failure which might be aggravated by hemodialysis **[15]**.

The specific coagulation parameters performed in this study reflected abnormal coagulation parameters of CRF group on maintenance hemodialysis. Such abnormalities were more evident immediately after hemodialysis.

To evaluate potential role of studied parameters in thrombophilic events, patients were divided into 2 groups, group 1 with history of thrombosis and group 2 with no history of thrombosis. Procoagulant parameters measured before and after dialysis of these two groups of patients were statistically analyzed and no significant differences were observed (Table 7). The difference of the procoagulant parameters between before and after dialysis was then calculated and the new calculated data were statistically analyzed to detect the differences between the two above-mentioned groups. Factor VIIa change was the only parameter that showed significant difference between the two groups with or without history of thrombosis. VIIa-rTF change (Δ VIIa-rTF) was significantly higher in patients with history of thrombosis compared to patients with no history of thrombosis (Table 8, Fig. 7), a finding that can point to possible role of factor VII in dialysis induced enhanced coagulation.

LeSar et al. (1999) reported that warfarin therapy should be instituted when hypercoagulable states are found in CRF patients under maintenance hemodialysis, unless otherwise contraindicated, and prothrombin INR maintained at 2.7-3.0 to decrease morbidity and frequency of graft thrombosis **[16]**.

The study concluded that, chronic renal failure patients are more liable to thrombotic complications reflected by decreased protein C activity and increased procoagulant activity of monocytes and increased activation of tissue factor pathway and factor VII activation. Moreover the maintenance hemodialysis process increases the liability to thrombosis. Hypercoagulability has been a major etiologic factor in vascular access thrombosis which is a frequent cause of morbidity in patients on hemodialysis. Evaluation of CRF patients under maintenance hemodialysis should include an extended thrombophilia profile specially in patients with recurrent vascular access thrombosis (VAT). Thrombotic risk factors whether inherited or acquired should be investigated in all CRF patients as a step to identify high-risk patients and to consider anticoagulant therapy.

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