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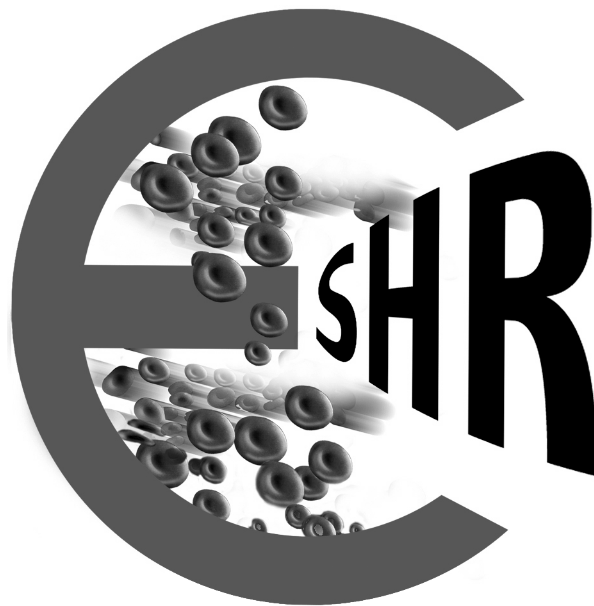

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Flow Cyometric Detection of Minimal Residual Disease (MRD) in Acute Lymphoblastic Leukemia: Correlation to Other Prognostic Parameters

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ABSTRACT

Background: Although clinical and biologic parameters can be used for treatment stratification of acute lymphoblastic leukemia (ALL), none of these prognostic factors is ideal. Measuring response to therapy as reflected by minimal residual disease (MRD) is now considered the most reliable prognostic parameter.

Objectives: To verify the value of MRD detection at different time points as a prognostic parameter in precursor-B ALL.

Patients and Methods: In this study flow cytometric detection of MRD was performed on 97 newly diagnosed precursor-B ALL cases (70 children and 27 adults) at day (D) 15, D28 and/or D42. The relationship between MRD and other clinical and biological prognostic factors was evaluated, as well as the clinical significance of MRD and its impact on the outcome of treatment regarding disease free survival (DFS) and overall survival (OS).

Results: In children, MRD positivity at D15 and D28, was significantly associated with cerebrospinal fluid (CSF) infiltration (p=0.03 and 0.01 respectively).

At D42 MRD positivity was significantly associated with CSF infiltration (p=0.01) and t(9;22) (p=0.045). Patients with MRD <0.01 at D28 and D42 had significantly better DFS (p=0.0002 and <0.0001 respectively) and OS (p=0.02 and 0.001 respectively).

In adults, a significant association was demonstrated between MRD D15 positivity and male gender (p=0.01) which was lost at D28 and D42. At D15, there was a trend for better OS in patients with MRD <0.1 (p=0.058) but no impact on DFS, however, it achieved significance for both at D28 (p=0.05). At D42, we demonstrated significant influence on OS (p=0.01) and DFS (p=0.02).

Conclusions: Using flow cytometry for MRD monitoring is a well-suited approach for the specific detection of minimal numbers of leukemic cells and, hence, could help obtain a more precise and early evaluation of response to therapy in patients with acute leukemia. A redefinition of complete remission according to MRD status is highly recommended.

Key Words: *ALL – MRD – Flow cytometry.*

INTRODUCTION

Despite recent advances in the treatment of acute leukemia, the disease remains a major cause of cancer-related mortality. In childhood acute lymphoblastic leukemia (ALL) there are still around 20% of patients who develop relapse, and those who survive suffer from major treatment related toxicities **[1,2]**, which reflects unadjusted treatment of the disease as a result of the lack of accurate prediction of response to therapy. Introducing methods for minimal residual disease (MRD) detection has revolutionized monitoring of treatment response in acute leukemia. The prognostic significance of MRD in childhood ALL was reported in many studies involving newly diagnosed patients, patients with first-relapse ALL, and those undergoing hematopoietic stem cell transplant **[3,4]**. It is considered the strongest prognostic predictor both in newly diagnosed or relapsed and in standard or intermediate risk ALL **[5,6]**. There is also strong evidence pointing to the clinical significance of MRD in adult ALL **[7-9]**. One of the distinctive markers of ALL cells is the clonal rearrangement of the genes encoding immunoglobulin and T-cell receptor proteins **[10]**. The test is accurate and sensitive (it allows the routine detection of one leukemic cell in 10,000 to 100,000 normal cells), however, the complexity of its set-up limits its routine appli-

cation. Leukemic lymphoblasts can also be recognized by the presence of chromosomal abnormalities and their resulting gene fusions and transcripts, such as *BCR/ABL*, *MLL/AF4*, *TCF3/PBX1*, and *ETV6/RUNX1* [10]. The most recurrent abnormalities are found in about one-third or less of patients and allow the detection of one leukemic cell in 1,000 to 100,000 normal bone marrow (BM) cells by reverse transcription-polymerase chain reaction (RT-PCR) [10]. Finally, ALL cells can be recognized by virtue of leukemia-associated cell markers combinations visualized with monoclonal antibodies and flow cytometry (FCM) at a sensitivity of detection of 1 leukemic cell in 10,000 normal cells [11].

Bone marrow samples collected after a temporary stop in chemotherapy, after the end of treatment, or after hematopoietic stem cell transplantation may contain a high proportion of recovering immature lymphoid cells whose morphology resembles that of ALL lymphoblasts "hematogones" [12-15]. Therefore, morphologic assessment of these samples is difficult and may result in erroneous conclusions; the application of MRD assays can clarify the identity of the morphologically ambiguous cells. Among MRD methods, flow cytometry is the one that is most affected by the state of bone marrow recovery [16]. In this regard, it is critical that flow cytometric analysis of MRD relies on markers that truly distinguish ALL cells from normal cells, including lymphoid progenitors; otherwise, the risk of false-positive MRD results is high [17,18].

In this study we aimed to determine the relationship between MRD and other clinical risk factors of precursor-B pediatric and adult ALL. We also aimed to find out the impact of the MRD status at different time points on the outcome of treatment regarding overall survival (OS) and disease free survival (DFS) in precursor-B ALL.

PATIENTS AND METHODS

The study was approved by the Institutional Review Board (IRB) of the National Cancer Institute (NCI), Cairo University and was conducted according to Helsinki declaration for studies involving human subjects. Informed consent was obtained from all patients, patients' parents/guardians.

Patients:

From March, 2009 to February, 2011, a total of 97 newly diagnosed precursor-B ALL patients were recruited from NCI. Among the 97 ALL cases, 70 were pediatric patients including 43 males and 27 females with an age range of 2 months – <18 years with a mean of 6±5.3 and a median of 7 years (Group I). The other 27 ALL cases were adults including 17 males and 10 females with an age range of 19-56, a mean of 30.6±10.78 and a median of 30 years (Group II).

Methods:

The diagnosis of ALL was based on standard morphologic, cytochemical, immunophenotypic and genetic studies. Flow cytometric immunophenotyping of BM aspirates at diagnosis was performed using a standard panel of antibodies and analyzed on Coulter EPICS XL-MCL flow cytometer. The monoclonal antibodies panel included CD45, CD1, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD22, cyto-μ, k, λ, CD13, CD14, CD33, MPO, TdT, anti class II MHC, CD56, CD38 and CD58 with relevant isotype controls. The antibodies were FITCI, PE, PerCP or Cy5 labelled, obtained from Coulter Hialeah, FL; Immunotech, Marseille, France; DACO, An Agilant Technologies company and/or/Becton Dickinson, Mountain View, California.

The presence of fusion genes in ALL including t(1;19) *TCF3 (E2A)/PBX1*, t(12;21) *ETV6 (TEL)/RUNX1 (AML1)*, t(9;22) *BCR/ABL* and t(4;11) *MLL/AF4* was examined following the standardized RT-PCR analysis of fusion gene transcripts for chromosomal aberrations in acute leukemia [19].

Patients were treated according to standard NCI Cairo University treatment protocols (www.nci.cu.edu.eg).

Disease free survival (DFS) and overall survival (OS) were estimated at 27 months in Group I and at 18 months in Group II.

Flow cytometric MRD analysis:

After initial immunophenotyping at diagnosis, monoclonal antibodies combinations were used to define leukemia- associated phenotypes (expressed on >50% of the blast cells). This step served to define a leukemia phenotypic fingerprint to be used in follow up samples. At

least 2 antibodies combinations were used to minimize pitfalls due to phenotypic switches. The panels included:

- CD34/CD19/CD10/CD58.
- CD34/CD19/CD10/CD38.
- CD34/CD19/CD10/CD45.
- Any aberrant myeloid (CD13 or CD33) or T markers (CD7, CD2).
- CD34/CD22/CD10/CD19: This one is helpful to recognize hematogones by the differentiation pattern.

Data acquisition was performed using the Coulter EPICS XL-MCL flow cytometer. At least 10,000 events were acquired and analyzed for identification of aberrant leukemic phenotypes at diagnosis, and at least 100,000 events were needed for MRD measurements. A detection limit of 0.01% (10/100,000 cells) could be achieved in most cases. MRD level was evaluated at day (D) 15, D28 and D42 and classified as negative (<0.01%) and positive (≥0.01-<0.1 and ≥0.1%). Examples of MRD detection by Flow Cytometry are presented in Fig. (1).

Statistical analysis:

SPSS version 17.0 was used for data management and data analysis. Mean±Standard deviation with median and range when appropriate described quantitative data. Parametric and non-parametric *t*-tests and ANOVA were used for comparing independent groups. Numbers with percentages described qualitative data. Chi-square test and Fisher exact tested proportion independence. Kaplan and Meier method estimated overall and disease free survival and log rank test compared survival curves. *p*-value, or calculated significance level, was considered significant at 0.05.

RESULTS

The study was performed on 97 newly diagnosed precursor-B ALL patients. Among the 97 ALL cases, 70 were pediatric patients including 43 males and 27 females with an age range of 2 months – <18 years with a mean of 6±5.3 and a median of 7 years (Group I). The other 27 ALL cases were adults including 17 males and 10 females with an age range of 19-56, a mean of 30.6±10.78 and a median of 30 years (Group II).

Group I: 70 Pediatric precursors B ALL:

According to risk stratification, patients were subdivided as 36 of favorable age group (≥1-<10 years) and 34 of unfavorable age group (<1-≥10 years).

Immunophenotyping revealed 39 common ALL (cALL), 27 Pre B and 4 Pro B cases. Aberrant CD33 expression was found in 7 cases while, CD2 and CD56 were found each in 2 cases.

DNA index of 1.06-1.16 was found in 23 patients and 47 had DNA index <1.06 or >1.16.

Cerebrospinal (CSF) fluid was free in 67 cases and 3 cases had infiltration.

Molecular studies by RT-PCR revealed t(12;21) in 8 and t(9;22) in 3 patients.

MRD level at different time points is presented in Table (1)

At D15 MRD was positive in 11/58 (19%) patients with no CSF infiltration and in all the 3 patients with CSF infiltration, (p=0.03).

There was no significant association between MRD and molecular studies. Neither was there an impact of MRD status on DFS or OS (Table 2).

At D28, MRD was positive in 10/50 (20.0%) patients with no CSF infiltration and in both patients with CSF infiltration, (p=0.01).

There was no significant association between MRD and molecular studies.

Patients with negative MRD had significantly better DFS (p=0.0002) and OS (p=0.02) than those with positive MRD (Table 2).

At D42, MRD was positive in 11/52 (21.2%) patients with no CSF infiltration and in both patients with CSF infiltration, (p=0.01).

MRD was positive in both cases with t(9;22) and in none of the 6 cases with t(12;21) (p=0.045).

Patients with negative MRD had significantly better DFS (p<0.0001) and OS (p=0.001) than those with positive MRD (Table 2).

There was no statistically significant association between MRD at D15, D28 or D42 and age, gender, total leucocytic count (TLC), lymphadenopathy, hepatosplenomegaly, DNA index or immunophenotyping.

Group II: 27 Adult precursors B ALL:

Immunophenotyping revealed 14 cALL and 13 Pre-B. Aberrant CD33 was found in two cases and CD2 in one case. Two patients had t(9;22).

At day 15, MRD was positive in 11/14 (78.6%) males and 2/9 (22.3%) females (p=0.01). The significance was lost at D28 and D42.

At D15, there was a trend for better OS in patients with MRD <0.1% (p=0.058) but no impact on DFS (Table 2).

At D28, at a cutoff of 0.1%; patients with lower MRD level had significantly better DFS and OS (p=0.05); the corresponding values at D42 were p=0.02 and p=0.01 (Table 2).

Lymphadenopathy, hepatosplenomegaly, TLC, immunophenotyping and molecular genetics showed no significant association with MRD.

Table (1): Minimal residual disease (MRD) level at different time points in precursor-B ALL.

Time point	Children			Adults		
	D15	D28	D42	D15	D28	D42
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
MRD level % \ No.	61	62	54	24	16	16
<0.01	31 (50.8)	49 (79.0)	44 (81.5)	9 (37.5)	8 (50.0)	7 (43.75)
>0.01<0.1	20 (32.8)	7 (11.3)	6 (11.1)	3 (12.5)	3 (18.75)	2 (12.5)
>0.1	10 (16.4)	6 (9.7)	4 (7.4)	12 (50)	5 (31.25)	7 (43.75)

Table (2): Disease free survival and overall survival in ALL patients in relation to minimal residual disease.

Survival Months	MRD level: Mean±SE			p
	<0.01	0.01-<0.1	>0.1	
Pediatrics:				
Day 15				
DFS	NC	22.8±1.3	20.3±2.97	0.299
OS	NC	25.4±1.04	22.8±2.3	0.22
Day 28				
DFS	25.7±0.8	–*	18.3±5.4	0.0002
OS	26.5±0.5	–*	20.8±1	0.02
Day 42				
DFS	25.5±0.7[a]	20.0±4.3[a]	10.2±3.4[b]	<0.0001
OS	NC	25.4±0.87	NC	0.001
Adults:				
Day 15				
DFS	NC	13.4±2.1	7.1±1.2	0.17
OS	NC[a]	13.3±1.7[a]	6.47±1.7[b]	0.058
Day 28				
DFS	14.5±2.2[a]	13.8±2.1[a]	6.1±1.1[b]	0.05
OS	14.7±2.1[a]	13.5±1.8[a]	6.3±1.2[b]	0.05
Day 42				
DFS	14.1±2.3[a]	14.4±1.5[a]	5.0±1.1[b]	0.02
OS	15.1±1.8[a]	14.8±1.97[a]	7.1±1.2[b]	0.01

NC: Survival estimates cannot be computed as all observations are censored.
*Only one case.

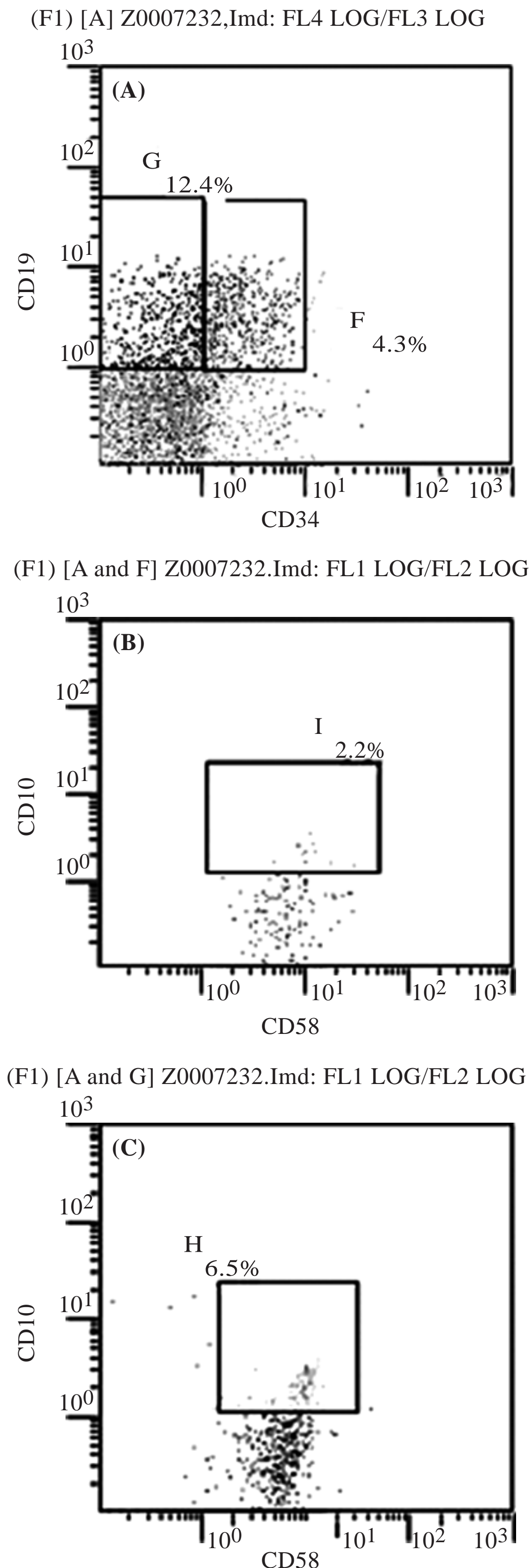

Fig. (1): MRD Detection in precursor B ALL case (CALL) at D15. A: Cells are gated: CD19+/CD34– (Gate G) and CD19+/CD34+ (Gate F). B: CD19+/CD34– in Gate F are analyzed for CD10 and CD58: Region I shows 2.2% coexpression of CD10 & CD58. C: CD19+/CD34+ cells in gate G are analyzed for CD10 & CD58 expression, region H shows 6.5. Both populations belong to the malignant clone as indicated by CD58 expression.

DISCUSSION

In this study flow cytometric detection of MRD was performed on 97 precursor-B ALL cases (including 70 pediatric patients and 27 Adults). The purpose of this study was to verify the value of MRD detection at different time points as a prognostic parameter in precursor-B ALL and to demonstrate the relationship between MRD status and other prognostic parameters. In children with ALL, measurements of MRD provide unique information on treatment response and have become a crucial component of contemporary treatment protocols. Flow cytometry-based assays are rapid and provide an accurate quantification of MRD while gaining information on the status of normal hematopoietic cells at the same time. Abnormal phenotype that can be used for MRD detection could be applied in 98% of cases of ALL with sensitivity up to 10^{-4}. The sensitivity of this approach depends on two main factors: The degree of dissimilarity between the immunophenotypes of leukemic cells and those of normal cells, and the number of cells available for study **[20]**. In the pediatric cohort, associations between MRD at D15, D28, and D42 post induction and other clinical and biological risk factors including age, gender, TLC, lymphadenopathy, hepatospleomegaly, molecular studies and DNA index were of no statistical significance. This is in agreement with other studies **[21-26]**, except for one that reported statistical association between gender and MRD **[27]**. This might be attributed to the different treatment protocols or to ethnic differences. In our study, no association was encountered between D15 MRD and OS or DFS at a cut off value of 0.01; however it was evident at D28 and D42. This suggests that D28 might be a good early indicator of early responders. Although this is consistent with another study **[22]**, it is not in line with others **[26,28-30]** The difference may be attributed to the short follow-up period as well as the sample size. Furthermore, these studies included both T and B ALL in their analysis and patients received different treatment protocols. In the current study, we detected statistically significant association between MRD positivity at D42 post induction and molecular findings (p=0.045). This is in agreement with some reports **[23,24]**. However, these issues were not addressed in other studies **[22,26]**. In the current study, we reported statistically significant

association between MRD positivity and CSF infiltration. These results are in concordance with previous reports [23,24]. In the current study, presence of MRD level of ≤0.01% at D42 post-induction had a significant impact on DFS and OS; this is consistent with one report [22]. but not in line with others [26,28-30]. The prognostic value of MRD detection in childhood ALL was most convincingly demonstrated by 3 large prospective studies [28-30]. They concluded that, flow cytometric MRD >0.1% on day 15 bone marrow was the most powerful early predictor of relapse. Multivariate analysis demonstrated that the MRD level was an independent prognostic factor with borderline significance [26].

Campana and his team recommended that patients with MRD at 0.1% level have to be reclassified as high-risk group who need therapy intensification and those with ≥1% are eligible for allogeneic BMT in the first remission [31].

In our cohort of adult ALL, a significant association was demonstrated between MRD on D15 post induction and gender (p=0.01). This was not reported in other studies [21,25]. The disagreement may be due to small sample size, as well as studying different ethnic groups.

At a cutoff of <0.1% there was a trend (p=0.058) for better OS at D15 but not DFS, border line for both at D28 (p=0.05) and significantly better at D42 (p=0.01 and 0.02 respectively). This is in line with other studies [32,33].

Associations between MRD at D42 post induction and other clinical and biological risk factors including gender, TLC, lymphadenopathy, hepatosplenomegly, and molecular findings were of no statistical significance. These results are in agreement with other studies [21,25].

Although the clinical significance of MRD has been studied less extensively in adult patients with ALL, there is considerable evidence supporting its potential usefulness [25,34]. In further work, it was claimed to be an independent prognostic parameter in both standard- and high-risk Philadelphia-negative ALL at a cutoff of 0.1% [33].

In conclusion, our study has validated the efficiency and practicability of Flow cytometry in evaluation of MRD status. It has further emphasized the prognostic value of MRD detection. The differences encountered between our study and others might be attributed to different treatment protocols or different response pattern in patients' cohorts from different countries. Accordingly, the time of testing and the cutoff of MRD that could best serve as a prognostic indicator has to be worked out in context of the specific patient cohort and the treatment protocols adopted. A longer follow-up period is needed to accurately determine the prognostic significance of MRD measurements at different time points and using different cutoff levels.

REFERENCES

1- Pui C, Robison L, Look A. Acute lymphoblastic leukaemia. Lancet. 2008; 371: 1030-1043.

2- Vrooman LM, Silverman LB. Childhood acute lymphoblastic leukemia: Update on prognostic factors. Curr Opin Pediatr. 2009; 21: 1-8.

3- Brisco MJ, Condon J, Hughes E, et al. Outcome prediction in childhood acute lymphoblastic leukaemia by molecular quantification of residual disease at the end of induction. Lancet. 1994; 343: 196-200.

4- Zhao XS, Liu YR, Zhu HH, et al. Monitoring MRD with flowcytometry: An effective method to predict relapse for ALL patients after allogeneic hematopoietic stem cell transplantation. Ann Hematol. 2012; 91: 183-92.

5- Thörn I, Forestier E, Botling J, et al. Minimal residual disease assessment in childhood acute lymphoblastic leukaemia: A Swedish multi-centre study comparing real-time polymerase chain reaction and multicolour flow cytometry. Br J Haematol. 2011; 152: 743-53.

6- Eckert C, Von Stackelberg A, Seeger K. Minimal residual disease after induction is the strongest predictor of prognosis in intermediate risk relapsed acute lymphoblastic leukaemia-long-term results of trial ALL-REZ BFM P95/96. Eur J Cancer. 2013; 49: 1346-55.

7- Brüggemann M, Raff T, Flohr T, et al. Clinical significance of minimal residual disease quantification in adult patients with standard-risk acute lymphoblastic leukemia. Blood. 2006; 107: 1116-23.

8- Gökbuget N, Kneba M, Raff T, et al. Adult patients with acute lymphoblastic leukemia and molecular failure display a poor prognosis and are candidates for stem cell transplantation and targeted therapies. Blood. 2012; 120: 1868-76.

9- Patel B, Rai L, Buck G, et al. Minimal residual disease is a significant predictor of treatment failure in non T-lineage adult acute lymphoblastic leukaemia: Final results of the international trial UKALL XII/ECOG 2993. Leukemia. 2011; 25: 254-8.

10- Brüggemann M, Schrauder A, Raff T, et al. Standardized MRD quantification in European ALL trials:

Proceedings of the Second International Symposium on MRD assessment in Kiel, Germany, 18-20 September 2008. Leukemia. 2010; 24: 521-35.

11- Coustan-Smith E, Campana D. Immunologic minimal residual disease detection in acute lymphoblastic leukemia: A comparative approach to molecular testing. Best Pract Res Clin Haematol. 2010; 23: 347-58.

12- Longacre TA, Foucar K, Crago S, et al. Hematogones: A multiparameter analysis of bone marrow precursor cells. Blood. 1989; 73: 543-52.

13- Rimsza LM, Larson RS, Winter SS, et al. Benign hematogone-rich lymphoid proliferations can be distinguished from B-lineage acute lymphoblastic leukemia by integration of morphology, immunophenotype, adhesion molecule expression, and architectural features. Am J Clin Pathol. 2000; 114: 66-75.

14- Van Wering ER, Van Der Linden-Schrever BE, Szczepanski T, et al. Regenerating normal B-cell precursors during and after treatment of acute lymphoblastic leukaemia: Implications for monitoring of minimal residual disease. Br J Haematol. 2000; 110: 139-46.

15- McKenna RW, Washington LT, Aquino DB, Picker LJ, Kroft SH. Immunophenotypic analysis of hematogones (B-lymphocyte precursors) in 662 consecutive bone marrow specimens by 4-color flow cytometry. Blood. 2001; 98: 2498-507.

16- Luria D, Rosenthal E, Steinberg D, et al. Prospective comparison of two flow cytometry methodologies for monitoring minimal residual disease in a multicenter treatment protocol of childhood acute lymphoblastic leukemia. Cytometry B Clin Cytom. 2010; 78: 365-71.

17- Patkar N, Abu Alex A, Bargavi B, et al. Standardizing Minimal Residual Disease by Flow Cytometry for Precursor B Lineage Acute Lymphoblastic Leukemia in a Developing Country. D Cytometry Part B (Clinical Cytometry). 2012; 82B: 252-258.

18- Solly F, Angelot F, Garand R, et al. CD304 is preferentially expressed on a subset of B-lineage acute lymphoblastic leukemia and represents a novel marker for minimal residual disease detection by flow cytometry. Cytometry A. 2012; 81: 17-24.

19- Van Dongen J, Macintyre EA, Gabert JA, et al. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease: Report of the BIOMED-1 concerted action: Investigation of minimal residual disease in acute leukemia. Leukemia. 1999; 13: 1901-1928.

20- Campana D. Status of minimal residual disease testing in childhood. Br J Haematol. 2008; 143: 481-489.

21- Bassan R, Spinelli O, Oldani E, et al. Improved risk classification for risk-specific therapy based on the molecular study of MRD in adult ALL. Blood. 2009; 113: 4153-62.

22- Borowitz MJ and Chan JK. Precursor lymphoid neoplasms. In: WHO classification of tumors of haematopoietic and lymphoid tissues. Swerdlow SH, Campo E, Harris NL, Jaff ES, Pileri SA, Stein H, Thiele J and Vardiman JW (eds.). International Agency for Research on Cancer (IARC). 2008; Chapter (9): 167-176.

23- Coustan-Smith E, Sancho J, Hancock ML, et al. Clinical importance of minimal residual disease in childhood acute lymphoblastic leukemia. Blood. 2000; 96: 2691-2696.

24- Coustan-Smith E, Ribeiro RC, Stow P, et al. A simplified flow cytometric assay identifies children with acute lymphoblastic leukemia who have a superior clinical outcome. Blood. 2006; 108: 97-102.

25- Mortuza FY, Papaioannou M, Moreira IM, et al. Minimal Residual Disease Tests Provide an Independent Predictor of Clinical Outcome in Adult Acute Lymphoblastic Leukemia. Clin Onc Jr. 2002; 20 (4): 1094-1104.

26- Koh N, Park M, Kim BE, et al. Prognostic significance of minimal residual disease detected by a simplified flow cytometric assay during remission induction chemotherapy in children with acute lymphoblastic leukemia. Korean J Pediatr. 2010; 53: 957-964.

27- Coustan-Smith E, Sancho J, Behm FG, et al. Prognostic importance of measuring early clearance of leukemic cells by flow cytometry in childhood acute lymphoblastic leukemia. Blood. 2002; 100: 52-58.

28- Cave H, Van Der Werfften Bosch J, Suciu S, et al. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia. European Organization for Research and Treatment O Cancer--Childhood Leukemia Cooperative Group. N Engl J Med. 1998; 339: 591-598.

29- Coustan-Smith E, Behm FG, Sanchez J, et al. Immunological detection of minimal residual disease in children with acute lymphoblastic leukaemia. Lancet. 1998; 351: 550-554.

30- Van Dongen JJ, Seriu T, Panzer-Grumayer ER, et al. Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. Lancet. 1998; 352: 1731-1738.

31- Campana D. Minimal residual disease in acute lymphoblastic leukemia. Hematology Am Soc Hematol Educ Program. 2010; 2010: 7-12.

32- Giebel S, Stella-Holowiecka B, Krawczyk-Kulis M. Status of minimal residual disease determines outcome of autologous hematopoietic SCT in adult ALL. BM Transpl. 2010; 45: 1095-1101.

33- Holowiecki J, Krawczyk-Kulis M, Giebel S, et al. Status of minimal residual disease after induction predicts outcome in both standard and high-risk Ph-negative adult acute lymphoblastic leukaemia. The Polish Adult Leukemia Group ALL 4-2002 MRD Study. Br J Haematol. 2008; 142: 227-237.

34- Sanchez J, Serrano J, Gomez P. Clinical value of immunological monitoring of minimal residual disease in acute lymphoblastic leukaemia after allogeneic transplantation. Br J Haematol. 2002; 116: 686-694.

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Role of Platelet Activation Markers CD62p and Annexine (V) in Hypertensive Patients With and Without Vascular Complications

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ABSTRACT

Background: Hypertension is a well known risk factor for cardiovascular and cerebrovascular events such as heart attack, renal failure and stroke. In addition, it is associated with earlier changes in target organ systems, such as LVH, proteinuria and carotid atherosclerosis, which are grouped under the term of "target organ damage" (TOD). There are many processes involved in the pathogenesis of TOD and some of these are endothelial dysfunction, platelet activation and increased thrombogenesis.

Aim of the Work: This study aimed to study the Level of expression of P-selectin (CD62p) and Annexin V as platelets activation markers in hypertensive patients as compared to control group, and comparison between the levels of these markers in hypertensive patients with and without vascular complications aiming at early detection of vascular complication in hypertensive patients.

Patients and Methods: *Our study included 50 patients divided into two groups:*

Group I: 20 patients suffering from hypertension without vascular complication. Group II: 30 patients suffering from hypertension with vascular complication which had renal, cardiac, cerebral and retinal affection. Also, 20 apparently healthy adults were chosen as control group. Patients were subjected to: Detailed history taking and clinical examination with emphasis on duration of disease and presence of complications. In the present study we investigated the levels of P-selectin (CD62p) and Annexin V expression on platelets by flow cytometry technique in patients with hypertension and control groups and compared the test characteristics of these markers. We also investigated the relation between P-selectin (CD62p) and Annexin V expression and various parameters;

Results: A highly statistically significant increase in CD62p mean florescent intensity was detected in all hypertensive patients (without and with complication) compared with control group ($p<0.001$ and $p<0.0001$). A significant increase in CD62p mean florescent intensity in hypertensive patients with vascular complications compared to patients without vascular complications ($p<0.05$). A highly statistically significant increase in Annexin V mean florescent intensity was encountered in all hypertensive patients (without and with complication) compared with control group ($p<0.01$ and $p<0.001$), and in hypertensive patients with compared with patients without vascular complications ($p<0.05$). The results also showed significant correlation between (CD62p) mean florescent intensity and Annexin V mean florescent intensity in hypertensive patients with vascular complications groups only. CD62 and Annexin V did not correlate with creatinine, triglycerides, total cholesterol, serum LDL cholesterol or HDL cholesterol in any of the studied groups.

Conclusion: The results of the present study support that activation of platelets in all hypertension patients is accompanied by high expression of P-selectin (CD62p) and Annexin V which is significantly associated with vascular complications. Hence, vascular complication may be predicted by an increase in the expression of these markers. The use of anti-platelet treatment in low-risk hypertensive patients and its effect on these markers needs to be investigated in long-term clinical outcome studies.

Key Words: *Hypertension – CD62p – Annexine (V) – Vascular complication.*

INTRODUCTION

Hypertension is a chronic medical condition, in which the blood pressure is chronically elevated. It is referred to as systemic arterial hypertension **[1]**. Hypertension is a well known risk factor for cardiovascular and cerebrovascular events such as heart attack, renal failure and stroke. In addition, it is associated with earlier changes in target organ systems, such as LVH, proteinuria and carotid atherosclerosis, which are grouped under the term of "target organ damage" (TOD). There are many processes involved in the pathogenesis of TOD and some of these are endothelial dysfunction, platelet activation and increased thrombogenesis **[2]**.

There is evidence that platelets and the endothelium, which both get activated in hypertension, have a crucial role in the increased thrombotic tendency seen in hypertension **[3]**. Abnormalities in platelet function may account for the pathogenesis and complications of thrombotic events associated with hypertension. Sev-

eral studies have reported that hypertensive patients show endothelial dysfunction and platelet hyperactivity and there is a positive linear relation between blood pressure and predisposition to platelet aggregation [4].

Platelets play a key role in arterial thrombosis and acute vascular events. Activated platelets translocate and secrete P-selectin from their alpha granules. Once exposed on activated platelets, P-selectin on platelets interacts with leukocytes and then induces inflammatory signals to potentiate vascular injury [5]. P-selectin is an adhesion molecule located in the platelet alpha granules and Weibel-Palade body of endothelial cells. P-selectin mediates the rolling of blood cells on the surface of the endothelium and initiates the attachment of leukocytes circulating in the blood to platelets, endothelial cells, and other leukocytes at sites of tissue injury and inflammation [6]. In addition, P-selectin induces the expression of tissue factor on monocytes, thus initiating the blood coagulation cascade. It also mediates fibrin deposition in the growing thrombus, and induces superoxide anion production by neutrophils and monocytes. It also regulates production of platelet activating factor and phagocytosis by monocytes [7]. Soluble P-selectin (sP-selectin) is a biomarker for platelet/endothelial activation and is considered a risk factor for vascular disease [8].

One of the most important signals accompanying platelet activation is the increase in intracellular calcium; annexins are highly conserved calcium-binding proteins, of which Annexin V is the major annexin in human platelets. The annexins are a family of proteins first described in 1990. All of the annexin proteins share the property of binding calcium and phospholipids. Annexin V has proven very useful as a marker for apoptosis and platelet activation [9].

Circulating Annexin V can be released from the cells of the vascular wall (endothelial cells, smooth muscle cells) or from secretor cells of the spleen and liver. Once it is in the plasma, it binds to blood cells (platelets and erythrocytes) or to endothelial cells [10].

Abnormalities in platelet function, endothelial function, and thrombotic markers have all been described in hypertension. Abnormal platelet aggregation, along with increased plasma markers of platelet activation, is also present in nonhypertension [11].

The aim of this work was to study the Level of expression of P-selectin (CD62p) and Annexin V as platelets activation markers in hypertensive patients as compared to control group, and comparison between the levels of these markers in hypertensive patients with and without vascular complications aiming to evaluate its potential value for early detection of vascular complications in hypertensive patients.

PATIENTS AND METHODS

Subjects:

This study was conducted on 50 patients with hypertension chosen from those attending Internal Medicine Department and Internal Medicine Clinic in AI-Zahraa University Hospital. They were 27 females (54%) and 23 males (46%) with an age ranging from 49 to 74 years old (Mean=60.24±7.25, Median=59). patient consider as hypertensive when either the systolic or the diastolic blood pressure value is >140/90 mmHg upon repeated sphygmomanometer measurements in the physician's office [12]. Twenty apparently healthy volunteers with matched age and sex were enrolled in the study as control group. They were 11 females (55%) and 9 males (45%). Cases with chronic inflammatory disease, infection, malignancy, Diabetes Mellitus, Smoking, and cardiac diseases due to causes other than hypertension were excluded.

The studied cases were divided into two groups:

Group I: Twenty patients suffering from hypertension without vascular complication. They were 13 females (65%) and 7 males (35%) with an age ranging from 49 to 60 years old (Mean=54±6, Median52).

These patients had no evidence of cardiac, retinal, cerebral, or renal affection.

Group II: Thirty patients suffering from hypertension with vascular complication who had renal, cardiac, cerebral and/or retinal affection. They were 14 females (46.7%) and 16 males (53.3%) with age ranged from 55-74 years old (Mean=67±7, Median62).

All patients and control were subjected to thorough history taking and complete clinical examination with emphasis on duration of disease, and presence of complications.

Sample collection:

Fasting sample: About 10ml of venous blood were withdrawn under sterile conditions in a

plastic syringe. The blood samples were divided into three parts:

1- Two ml added to (EDTA) tube for complete blood count (CBC) done using fully automated cell counter (Sysmix, Germany).

2- Five ml was left to clot and the serum was separated. Serum creatinine and lipid profile (triglyceride, HDL-Cholesterol, total cholesterol) were done using chemical auto analyzer (cobas 411) Germany and kits supplied by Roche Diagnostic Kits according to manufacture instructions.

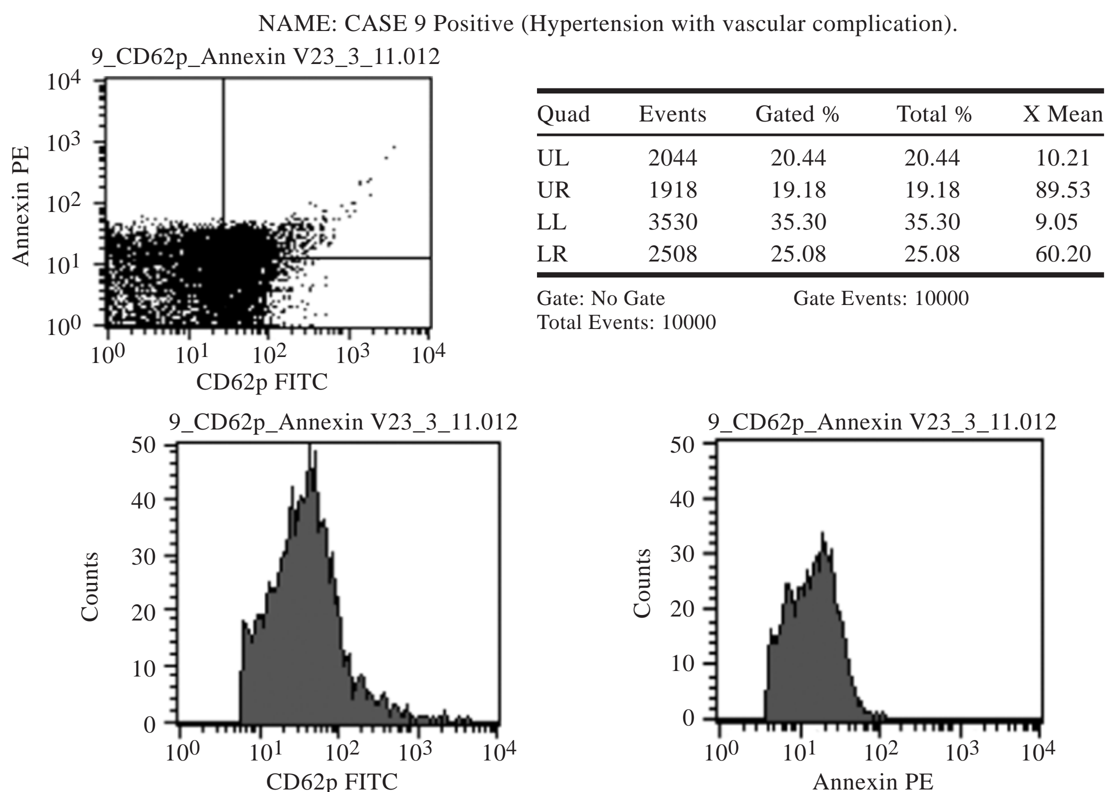
3- The third part of blood sample (2.7ml) were added to 300µl 3.2% trisodium citrate and centrifuged immediately at low speed (100g) for 10 minutes using a cooling centrifuge for separation of platelet rich plasma (PRP) for isolation of fresh platelets for immunophenotyping.

Flow Cytometric Measurement of CD62p and Annexin V:

Platelet rich plasma was subsequently centrifuged at high speed (1000Xg) for 10 minutes with Phosphate buffered saline (PBS) to obtain washed platelets. Platelet pellet was washed twice and then re-suspended in an equal volume of 1% paraformaldehyde. Fifty µl of platelet suspension were stained with a combination of 10µl of Fluorescein isothiocyanate (FITC) conjugated anti CD62p (P-selectin); Kit supplied by BD Biosciences Pharmingen™ and 5µl Phycoerythrin (PE) conjugated anti Annexin V produced by BD Biosciences Pharmingen™. Iso type negative control (PE and FITC) was obtained from BD Biosciences Pharmingen™. The mixture was incubated in dark room for 30 minutes; they were resuspended in 200µl phosphate buffer saline (PBS) and centrifuged at 1300g for 5 minutes for analysis to be done. Data acquisition and analysis were performed on cell quest program of the FACSCAN, BEKTON DICKENSON flow cytometry. Gating on platelets, 10000 events were acquired and statistical analysis was done by cell quest software. Results were expressed as mean florescence intensity (Figs. 1,2).

Statistical analysis:

Data was analyzed by Microsoft office 2003 (excel) and (SPSS) computer program (version 16). Parametric data was expressed as Mean± SD. Spearman correlations coefficient was used to test the relationship between various variables. Significant difference was considered when *p*-value <0.05, *p*-value<0.01 is considered highly significant [13].



Quad	Events	Gated %	Total %	X Mean
UL	2044	20.44	20.44	10.21
UR	1918	19.18	19.18	89.53
LL	3530	35.30	35.30	9.05
LR	2508	25.08	25.08	60.20

Fig. (1): CD62 and Annexin V expression on platelets from a case of hypertension with vascular complications.

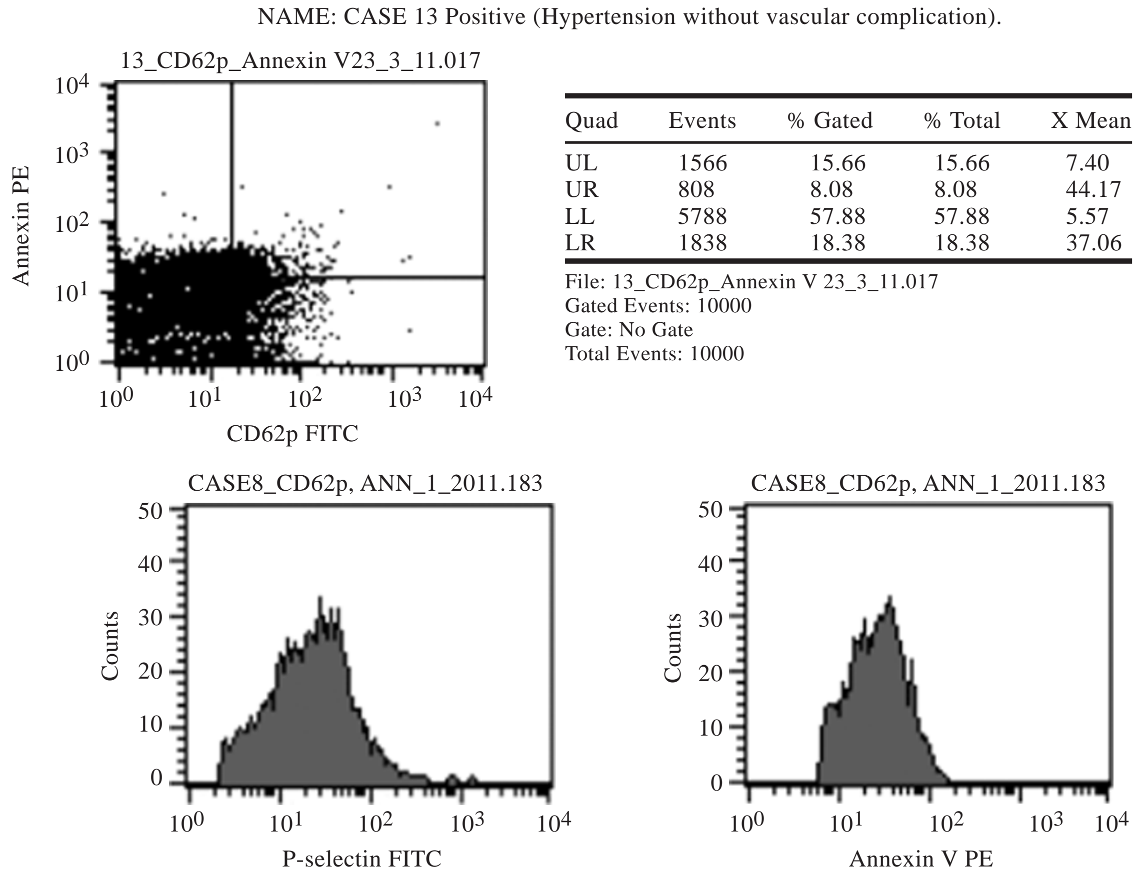


Fig. (2): CD62 and Annexin V expression on platelets from a case of hypertension without vascular complications.

RESULTS

The results of serum creatinine and lipid profile are presented in Table (1). The values of the different parameters in hypertensive cases without vascular complications were comparable to control group ($p>0.05$). HDL cholesterol values were comparable in all groups ($p>0.05$). Serum creatinine, triglycerides, total cholesterol and LDL cholesterol was significantly higher in hypertensive patients with vascular complications compared to control group $p<0.0001$ and compared to hypertensive cases without vascular complication $p<0.001$.

CD62 and Annexin V expression on the platelets of the different groups are presented in Table (2). The mean florescent intensity of both markers was found to be significantly higher in hypertensive cases as compared to control as well as in hypertensive patients with vascular complications as compared to those without vascular complication.

A significant increase in CD62p mean florescent intensity was detected in all hypertensive patients (without and with complication) compared with control group ($p<0.001$ and $p<0.0001$). A significant increase in CD62p mean florescent intensity in hypertensive patients with vascular complications compared to patients without vascular complications ($p<0.05$). A highly statistically significant increase in Annexin V mean florescent intensity was encountered in all hypertensive patients (without and with complication) compared with control group ($p<0.01$ and $p<0.001$), and in hypertensive patients with vascular complication compared with patients without vascular complications ($p<0.05$).

Figures (3,4) represents Study of Correlation coefficient "*r*" between CD62p and Annexin V mean florescent intensity, Patients without vascular complication showed no correlation "*r*" 0.185, $p>0.05$*, Correlation was encountered in hypertensive patients with vascular complication "*r*" 0.385, $p<0.05$*. No correlation was encountered between CD62 or Annexin V and the other parameters in the studied groups.

Table (1): Creatinine and lipid profile in hypertensive patients with and without vascular complications and control group.

Parameter	Hypertensive cases: Vascular complication		Control	p_1	p_2	p_3
	Absent	Present				
No.	20	30	20			
Creatinine (mg/dl)	0.87±0.28*	4.65±4.66	0.79±0.27	>0.05	<0.0001	<0.001
Triglycerides (mg/dl)	115.37±32.55	172.37±95.73	109.1±33.45	>0.05	<0.0001	<0.001
Total cholesterol (mg/dl)	107.63±30.04	199.73±44.70	90.70±27.11	>0.05	<0.0001	<0.001
LDL cholesterol (mg/dl)	52.66±21.12	143.30±54.55	47.40±20.53	>0.05	<0.0001	<0.001
HDL cholesterol (mg/dl)	31.42±8.38	28.09±5.81	31.30±9.76	>0.05	>0.05	>0.05

p_1: Hypertensive patients without vascular complications vs. control group.
p_2: Hypertensive patients with vascular complications vs. control group.
p_3: Hypertensive patients with vascular complications vs. hypertensive patients without vascular complications.

Table (2): P-selectin (CD62p) and Annexin V mean fluorescent intensity expression on platelets of hypertensive patients with and without vascular complications and control group.

Study group	No.	CD6p	*p*-value	Annexin V	*p*-value
Hypertensive patients without vascular complications.	20	17.98±0.91*	p_1<0.001	15.44±0.78*	p_1<0.01
Hypertensive patients with vascular complications.	30	24.06±0.93	p_2<0.0001	19.34±1.01	p_2<0.001
Control group.	20	6.29±1.01	$P3$<0.05	7.47±0.95	$P3$<0.05

*Mean florescent intensity±SD.
p_1: Hypertensive patients without vascular complications vs. control group.
p_2: Hypertensive patients with vascular complications vs. control group.
p_3: Hypertensive patients with vs. Hypertensive patients without vascular complications.

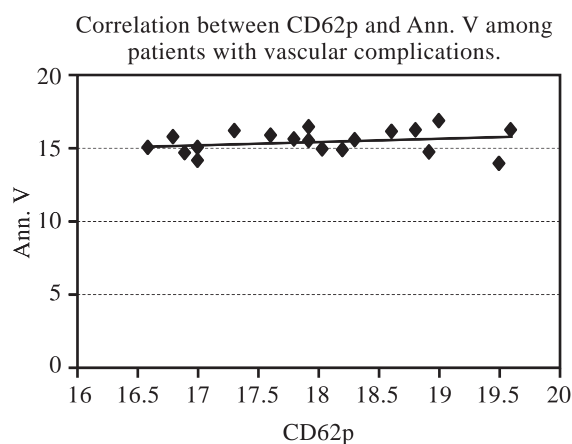


Fig. (3): Correlation coefficient "*r*" 0.185, *p*>0.05* between CD62p and Annexin V in hypertensive patients without vascular complications.

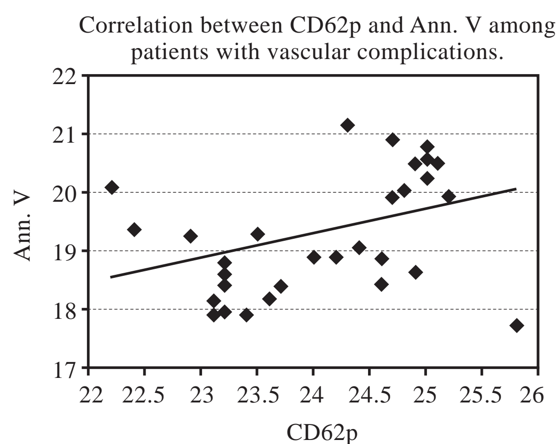


Fig. (4): Correlation coefficient "*r*" 0.385, *p*<0.05 between CD62p and Annexin V in hypertensive patients with vascular complications groups.

DISCUSSION

In hypertension, the delicate balance between the vasodilators and the vasoconstrictors is upset, leading to changes that then take place in the vascular beds, There is also increasing evidence that platelets and the endothelium, both get activated in hypertension, The main complications of hypertension (that is, myocardial infarction and stroke) are paradoxically thrombotic in nature rather than hemorrhagic- "The thrombotic paradox of hypertension". Certainly, increasing clinical and laboratory evidence suggests that hypertension per se may

confer a prothrombotic or hypercoagulable state, with abnormalities of coagulation, platelets, and the endothelium [14].

Platelets are activated by a large number of agonists that are released in the circulation during some pathologic conditions (e.g. hypertension and diabetes mellitus). Platelet activation and aggregation is involved in the development of hypertension in different ways. Indeed, upon activation, platelets change shape, release a number of autocrine factors that stimulate their adhesion to endothelial cells and the formation of blood clot. P-selectin and Annexin V have been proposed as useful biomarkers in various pathologic states in which platelets and/or endothelial cells are activated [15]. Hyperactive platelets may lead to capillary microembolisation secondary to the formation of microaggregates [16].

This study was conducted to compare the level of expression of P-selectin (CD62p) and Annexin V as platelets activation markers in hypertensive patients and control group, and to compare between the level of expression of these markers in hypertensive patients with and without vascular complication aiming to early detection of vascular complication in hypertensive patients.

In the current study, the mean value of CD62p expression were higher in hypertensive Patients without vascular complications compared with control group. There was highly significant increase in CD62p mean florescent intensity in hypertensive patients with vascular complications compared to control group. These findings were in agreement with several studies [17,18]. Stumpf et al., [19] found that Patients with mild arterial hypertension (AH) showed significantly enhanced expression of platelet P-selectin and concluded that platelets seem to play a significant role in mediating inflammation in AH, which could lead to target organ injury.

In the current study, the mean value of CD62p expression was higher in hypertensive patients with compared to patients without vascular complications. This finding is in agreement with that of Yang et al., [20] who reported that the expression of CD62p in essential hypertension group was higher than the control group, and the level of the expression of CD62p in essential hypertension Grade II & III groups was obviously higher than that in essential hypertension Grade I group. Preston et al., [21] also reported that platelet CD62 demonstrated a strong and graded association with both systolic and diastolic BP; that association persisted in the presence of multiple concomitant risk factors. They reported, as well, that platelet activation and platelet CD62 expression increase in a BP-dependent manner and this relationship persists at extreme levels of BP. They concluded that Platelet activation and platelet CD62 may participate in the accelerated target organ injury observed in high risk patients with severe hypertension.

In the current study, the mean values of Annexin V mean fluorescent intensity expression was higher in hypertensive patients with or without vascular complications compared with control group. These findings are in agreement with previous study [17]. Also Sinning et al., [22] reported that Annexin V is increased in patients with cardiovascular risk factors and impaired coronary endothelial function and that this elevation is associated with many cardiovascular risk factors, such as hypertension, obesity, hyperlipoproteinaemia, and diabetes.

In the current study, the mean value of Annexin V mean florescent intensity expression was higher in hypertensive patients with compared to patients without vascular complications. This finding is in agreement with that reported by Huang et al., [23] who reported that hypertensive patients with microalbuminuria or macroalbuminuria had significantly increased Annexin V which may contribute to atherosclerotic disease progression and enhanced cardiovascular risk in hypertensive patients with nephropathy.

Chen et al., [24] reported increased levels of Annexin V, endothelial MPs (EMP) and platelet-derived MPs (PMPs), in diabetics with or without hypertension. Ravassa et al., [25] reported that myocardial Annexin V upregulation is associated with hypertensive heart disease (HHD) and impairment of systolic function in hypertensive patients; this association being independent of apoptosis. They concluded that Plasma Annexin V can be a marker of myocardial Annexin V up regulation and systolic dysfunction in patients with HHD.

Our study showed that there was no significant difference in serum triglycerides, total

cholesterol, LDL cholesterol or HDL cholesterol in hypertensive patients without vascular complications compared to control group, but that there was significant increase in serum level of triglycerides, total cholesterol and LDL cholesterol in hypertensive cases with vascular complications compared to control group, These results are in agreement with Alexandru et al., **[26]** who reported that hypertension is associated with hypercholesterolemia (cholesterol and triglyceride) that induces major changes in morphology and signaling mechanisms operating in blood platelets that enhanced platelet activation and aggregation in cardiovascular disease.

Our work showed that there was no significant correlation between CD62p, Annexin V and any of the other parameters (triglycerides level, total cholesterol, HDL cholesterol or LDL cholesterol) in all studied groups This is against the results obtained by Pawelczyk et al., **[27]** who observed a significantly higher CD62p expression and percentage of CD62p-positive resting and thrombin-activated platelets in the hyperlipidemia as compared to the normolipidemia group. This contradictory result may be due to the small number of cases in our study.

In this study there was significant correlations between CD62p mean florescent intensity and Annexin V mean florescent intensity in hypertensive patients with vascular complication but no correlation found in hypertensive patients without vascular complication. The present findings suggested that enhanced Platelet activation and platelet CD62 and Annexin V may participate in the accelerated target organ injury observed in high risk patients with uncontrolled hypertension.

Most clinical events associated with hypertension have a thrombotic component, and there have been several reports on platelet activation in hypertensive patients **[28,29]**. Our results for CD62p, and Annexin V support the findings in these reports.

Conclusion:

The results of the present study support that activation of platelets in all hypertension patients is accompanied by high expression of P-selectin (CD62p) and Annexin V which may induce hypercoagulability in hypertensive patients and significantly associated with vascular complications and hence, vascular complication may be predicted by an increase in the expression of these markers. The use of anti-platelet treatment in low-risk hypertensive patients and its effect on these markers needs to be investigated in long-term clinical outcome studies.

REFERENCES

1- Carretero OA, Oparil S. “Essential hypertension” Part I: Definition and etiology. Circulation. 2000; 101 (3): 329-35.

2- Nadar SK, Tayebjee MH, Messerli F, Lip GY. Target organ damage in hypertension: Pathophysiology and implications for drug therapy. Curr Pharm Des. 2006; 12: 1581-1592.

3- Wong TY, McIntosh R. Hypertensive retinopathy signs as risk indicators of cardiovascular morbidity and mortality. Br Med Bull. 2005; 73-74:57-70.

4- El Haouari MA, Rosado JA. Platelet function in hypertension. Blood Cells, Mol Dis. 2009; 42: 38-43.

5- Cha JK, Jeong MH, Kim EK, Lim YJ, Ha BR, Kim SH. Surface expression of P-selectin on platelets is related with clinical worsening in acute ischemic stroke. J Korean Med Sci. 2002; 17 (6): 811-6.

6- Polek A, Sobiczewski W, Matowicka-Karna J. P-selectin and its role in some diseases. Postepy Hig Med Dosw (Online). 2009, 63: 465-70.

7- Gkaliagkousi E, Passacquale G, Douma S, Zamboulis C, Ferro A. Platelet activation in essential hypertension: implications for antiplatelet treatment. Am J Hypertens. 2010; 23 (3): 229-236.

8- Kisucka J, Chauhan AK, Zhao BQ. Elevated levels of soluble P-selectin in mice alter blood-brain barrier function, exacerbate stroke, and promote atherosclerosis. Blood. 2009; 113 (23): 6015-6022.

9- Tzima E, Walker JH. Platelet Annexin V: The ins and outs. Platelets. 2000, 11 (5): 245-51.

10- Cederholm A, Frostegård J. Annexin A5 as a novel player in prevention of atherothrombosis in SLE and in the general population. Ann NY Acad Sci. 2007; 1108: 96-103.

11- Minuz P, Patrignani P, Gaino S. Determinants of platelet activation in human essential hypertension. Hypertension. 2004; 43: 64-70.

12- Chobanian AV, Bakris GL, Black HR, et al. The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: The JNC 7 report JAMA. 2003; 2892560- 2572.

13- Sokal RR, James RF. Biometry; the principle and practice of statistics in Biological Research. 2nd ed. New York: WH. Freeman. 1995.

14- Lip GY. Hypertension, platelets, and the endothelium: The ‘‘thrombotic paradox’’ of hypertension (or ‘‘Birmingham paradox’’) revisited. Hypertension. 2003; 41: 199-200.

15- Nomura S, Kanazawa S, Fukuhara S. Effects of efonidipine on platelet and monocyte activation markers in hypertensive patients with and without type 2 diabetes mellitus. J Hum Hypertens. 2002; 16 (8): 539-47.

16- Packham MA, Mustard JF. The role of platelets in the development and complications of atherosclerosis. Semin Hematol. 1986; 23: 8-19.

17- Hoffbrand AV, Moss PAH, Pettit JE. Platelets, blood coagulation and haemostasis. In Hoffbrand AV, Moss PAH and Pettit JE (editors): Essential Haematology, 5th edition, Blackwell publishing, Oxford. 2006; 22: 264.

18- Nomura S, Shouzu A, Omoto S, Nishikawa M, Fukuhara S, Iwasaka T. Losartan and Simvastatin Inhibit Platelet Activation in Hypertensive Patients. J Thromb Thrombolysis. 2004; 18 (3): 177-85.

19- Stumpf C, John S, Jukic J. Enhanced levels of platelet P-selectin and circulating cytokines in young patients with mild arterial hypertension. J Hypertens. 2005; 995-1000.

20- Yang H, Lang S, Zhai Z, Li L, Kahr WH, Chen P, Brkić J, Spring CM. Fibrinogen is required for maintenance of platelet intracellular and cell-surface P-selectin expression. Blood. 2009; 114 (2): 425-36.

21- Preston RA, Coffey JO, Materson BJ, Ledford M, Alonso AB. Elevated platelet P-selectin expression and platelet activation in high risk patients with uncontrolled severe hypertension. Atherosclerosis. 2007; 192 (1): 148-54.

22- Sinning JM, Losch J, Walenta K, Böhm M, Nickenig G. Circulating CD31+/Annexin V+microparticles correlate with cardiovascular outcomes. Eur Heart J. 2010; 3 2 (16): 2034-2041.

23- Huang PH, Huang SS, Chen YH, Lin CP, Chiang KH. Increased circulating CD31+/Annexin V+apoptotic microparticles and decreased circulating endothelial progenitor cell levels in hypertensive patients with microalbuminuria. J Hypertens. 2010; 28 (8): 1655-1665.

24- Chen Y, Feng B, Li X, Ni Y, Luo Y. Plasma endothelial microparticles and their correlation with the presence of hypertension and arterial stiffness in patients with type2 diabetes. J Clin Hypertens. 2012; 14 (7): 455-60.

25- Ravassa S, González A, López B, Beaumont J, Querejeta R, Larman M, Díez J. Up regulation of myocardial Annexin A5 in hypertensive heart disease: Association with systolic dysfunction. Eur Heart J. 2007; 28 (22): 2785-91.

26- Alexandru N, Popov D, Dragan E, Andrei E, Georgescu A. Platelet activation in hypertension associated with hypercholesterolemia: Effects of irbesartan. J Thromb Haemost Jan. 2011; 9 (1): 173-84.

27- Pawelczyk M, Baj Z, Chmielewski H, Kaczorowska B, Klimek A. The influence of hyperlipidemia on platelet activity markers in patients after ischemic stroke. Cerebrovasc Dis. 2009; 27 (2): 131-7.

28- Levy PJ, Yunis C, Owen J, Brosnihan KB. Inhibition of platelet aggregability by losartan in essential hypertension. Am J Cardiol. 2000; 86: 1188-1192.

29- Tomoda F, Takata M, Kagitani S, Kinuno H, Yasumoto K, Tomita S, Inoue H. Different platelet aggregability during mental stress in two stages of essential hypertension. Am J Hypertens. 1999; 12: 1063-1070.

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Expression of Phosphorylated STAT5 in Chronic Myeloid Leukemia: Relation to Disease Stages

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ABSTRACT

Background: *BCR-ABL* expression is the whole mark of chronic myeloid leukemia (CML). It results in constitutive activation of signal transducer and activator of transcription (STAT) and essentially bypasses cytokine or growth factor-dependent activation of STAT5.

Methods: We investigated the state of STAT5 phosphorylation (pSTAT5) in relation to CML disease stages as a possible indicator of BCR/ABL tyrosine kinase activity. The study was conducted on 39 CML patients including 17 males and 22 females; 22 patients were in chronic phase (Group I) and 17 were in accelerated phase or blastic crisis (Group II). Patients were divided into 3 risk groups according to Hasford score: Low, Intermediate and High. pSTAT5 was measured using Flow Cytometry; its expression was evaluated in relation to various hematological and clinical parameters.

Results: pSTAT5 was expressed in all cases tested. The level was statistically significantly higher in advanced phases than in the chronic phase (p=0.006). CD34+ve cells % was 1.74±1.61% and 21.3±20.4% in Group I and II respectively (p=<0.001). All CD34 positive cells were pSTAT5 positive. CD34-ve cells were pSTAT5-ve (<10%) in 8/22 and 5/17 patients in Group I and II respectively. pSTAT5% expression was significantly higher in Group II as compared to Group I (56.4±27.6% vs. 33.9±21% respectively; p=.006). pSTAT5% expression showed significant +ve correlation with both peripheral blood and bone marrow blast percentage (r=0.39 and 0.37; p=0.017 and 0.02 respectively). No correlation was encountered between pSTAT5 expression on one side and age, Hasford score or duration of chronic phase on the other side.

Conclusions: The level of expression of pSTAT5 is higher in advanced phases of CML reflecting a higher tyrosine kinase activity of the BCR/ABL chimeric protein. This might help making therapeutic decisions.

Funding: The study was supported by Cairo University.

Key Words: *Chronic myeloid leukemia (CML) – Signal transducer and activator of transcription 5 (STAT5) – pSTAT5 – CD34.*

INTRODUCTION

Chronic myeloid leukemia (CML) was probably the first form of leukemia to be recognized as a distinct entity. The natural history of CML includes three distinct phases, the chronic phase, the accelerated phase and the blastic phase; CML typically presents in the chronic phase **[1]**.

It is generally believed that CML develops when a single, pluripotent, hematopoietic stem cell acquires a Ph chromosome carrying the *BCR-ABL* fusion gene, which confers on its progeny a proliferative advantage over normal hematopoietic elements and thus allows the Ph-positive clone gradually to displace residual normal hematopoiesis **[2,3]**.

It soon became clear that the BCR-ABL oncoprotein itself is the best molecular target presented by CML cells because it is not expressed by normal cells. Furthermore, the dissection of the signal transduction pathways affected by the deregulated kinase activity of BCR-ABL provided information on additional or alternative signaling steps that could be interrupted in an attempt to eliminate the oncogenic effect of BCR-ABL **[4]**. *BCR/ABL* is not only diagnostic but also pathogenic in CML **[5]**, although the translocation itself may not be sufficient to cause leukemia **[6,7]**.

The JAK/STAT pathway is the principal signaling mechanism for a wide array of cytokines and growth factors **[8]**. STAT5 plays an important role in hematopoiesis **[9]**. It has been implicated to play an important role in proliferation, differentiation and protection against

apoptosis during hematopoiesis. In addition, it is thought that STAT5 plays an important role in early myeloid differentiation [9,10] and to regulate survival in mature myeloid cells. STAT5 has also been implicated in myeloid differentiation induced by IL-3, G-CSF, and GM-CSF [11-13].

In this study, we investigated pSTAT5 expression in relation to CML disease stages as a possible indicator of BCR/ABL tyrosine kinase activity.

PATIENTS AND METHODS

Patients:

The study was conducted on 39 CML patients including 17 males and 22 females with an age range of 19-79, a mean of 39.88±15 and a median of 41.5 years. Twenty two patients were newly diagnosed cases in chronic phase (Group I) and 17 were in accelerated phase (4 cases) or blastic crisis (13 cases) (Group II). All patients presented to the Medical oncology Department of the NCI, Cairo University in the period 1999-2009. The study was conducted between February 2008 and December 2009 at the Bone Marrow Transplantation Lab Unit, Clinical Pathology Department, NCI, Cairo University.

The Study was conducted according to Helsinki declaration; it was approved by the NCI ethical committee and written informed consent was obtained from all patients.

Methods:

Patients were diagnosed as CML and stages defined according to the WHO classification of myeloid neoplasms [1]. Patient's files were revised to obtain data at diagnosis and newly diagnosed patients were followed-up for a period of 5-18 months. Patients were divided into 3 risk groups according to Hasford score: Low risk group: Score ≤780, Intermediate risk group: Score 781-1480 and High risk group: Score >1480. The score value for individual patients was calculated by accessing the website www.pharmacoepi.de [14]. Hasford score was available for 37 patients including 21 in Group I and 16 in Group II.

Cases were subjected to full history and clinical examination including organomegaly and lymphadenopathy. Routine investigations included abdominal ultrasound, complete blood picture, bone marrow (BM) aspiration, Leukocyte Alkaline Phosphatase (LAP) score, conventional karyotyping, and detection of *BCR/ABL* by RT-PCR. Immunophenotyping using monoclonal antibodies and analyzed on Coulter XL Flow Cytometer was performed for cases in blastic crisis.

Evaluation of STAT5 phosphorylation (pSTAT5):

Analysis of pSTAT5 was performed on EDTA BM samples using double labeling with CD34 PE for direct surface staining and purified antimouse pSTAT5 for indirect intracytoplasmic staining [15].

The intracellular pSTAT5 was performed after fixation and permeabilization using purified monoclonal antibodies followed by FITC-conjugated secondary antibody. The antibodies used are purified mouse anti-human phosphorylated STAT5 (clone 47) obtained from BD Biosciences (cat.No.611964) and PE-conjugated mouse anti-human CD34 monoclonal antibody (clone 563) obtained from BD Biosciences (cat.No.550619). FITC-conjugated polyclonal goat anti-mouse IgG obtained from BD Biosciences (cat.No.555988) served as a secondary antibody for mouse anti-human phosphorylated STAT5. Isotype control included mouse IgG2b/PE for CD34 staining and, as a negative control for indirect staining, FITC-conjugated secondary antibody was only added with no primary antibody.

Analysis of the samples was done using Coulter XL (Hialeah) flow Cytometer.

Results were expressed as percentage positivisty of pSTAT5 in the CD34+ve and CD34 -ve population as well as florescent ratio (FR) by dividing the channel number of the test by the channel number of the isotype (Figs. 1-3).

Statistical methods:

SPSS (Statistical package for social sciences version 17.0) was used for data analysis. Mean and standard deviation were estimates of quantitative data and median with range for non-normally distributed sets. Non-parametric *t*-test (Mann Whitney test) was used for comparison of means of two independent groups (Group I and II). Chi-square or Fischer exact tests checked the hypothesis of proportion independence. Non-parametric correlation analysis “Spearman rho” was used to test association. *p*-value is significant when ≤0.05.

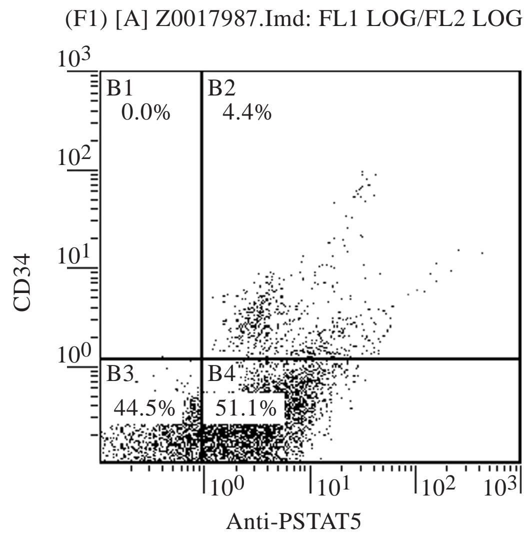

Fig. (1): Co-expression of CD34/STAT5p in a CML case in chronic phase.

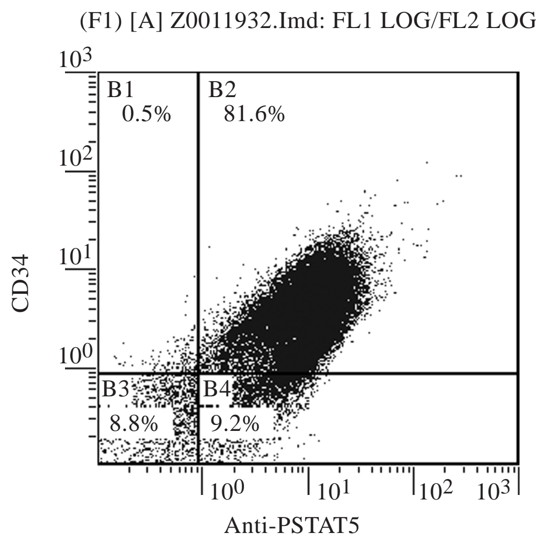

Fig. (2): Co-expression of CD34/STAT5p in a CML case in blastic crisis.

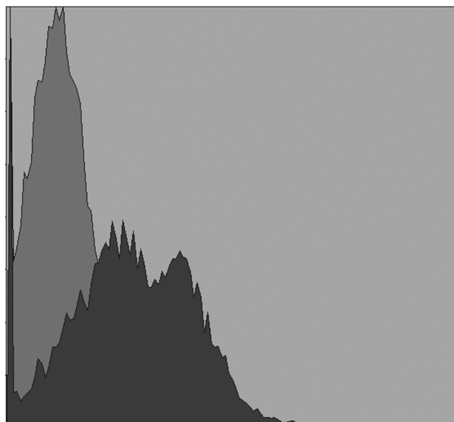

Fig. (3): Histogram showing the overlay of PSTAT5 on the isotypic control.

RESULTS

The patient cohort included 22 CML cases in chronic phase (Group I) and 17 in blastic crisis or accelerated phase (Group II). The latter group included 4 patients in blastic crisis at presentation. The duration of the chronic phase in the remaining 13 patients ranged between 3-96 with a mean of 46.5±26.8 and a median of 12 months. There was no statistically significant difference in the pSTAT5 findings between patients in accelerated phase (4 cases) and those in blastic crises (13 cases); hence they were dealt with as one group in further statistical analysis.

Comparison between Group I and Group II regarding hematological parameters is presented in Table (1). The blasts % was significantly higher in Group II both in PB and BM. Eosinophils % was significantly higher in Group II in the BM, while Hb % and PB granulocytes % were significantly higher in Group I.

Hasford risk stratification for the two patient's groups revealed 8/21 (38.1%) low, 8/21 (38.1%) intermediate and 5/21 (23.8%) high risk in Group I as compared to 4/16 (25%), 8/16 (50%) and 4/16 (25%) in Group II; the difference is statistically insignificant.

CD34 expression and the phosphorylation status of STAT5 are presented in Table (2). CD34 expression was higher in Group II than in Group I. All CD34+ve cells expressed pSTAT5. The pSTAT5% was significantly higher in patients in the accelerated phase or blastic crisis (Group II) than those in the chronic phase (Group I); however the florescent ratio was comparable in both groups. CD34-ve/pSTAT5 +ve% was higher in Group II than in Group I but the difference did not achieve statistical significance.

pSTAT5 % and pSTAT5 FR were comparable among patients in the three Hasford risk groups (Table 3).

Correlations between pSTAT5 expression and different variables:

A statistically significant positive correlation was encountered between pSTAT5 % and both BM and PB blasts % (r=0.37, p=0.02 & r=0.39, p=0.017 respectively) while a statistically significant negative correlation was encountered

between pSTAT5 % and granulocytes % in BM and PB (r=–0.438, p=0.005 and r=–0.435, p=0.007 respectively). A near significant positive correlation between pSTAT5% and Hasford score was encountered in the 37 patients (r=0.29, p=0.08). A fair positive correlation between pSTAT5% and Hasford score was encountered in Group II patients (r=0.28) although the p-value was statistically insignificant (0.28).

No correlation was encountered between CD34–ve/pSTAT+ve % on one side and any of the hematological or clinical parameters on the other side.

Table (1): Hematological parameters of 39 CML patients in relation to disease stage.

Parameter	Group I No=22	Group II No=17	*p* value
Peripheral blood:			
TLC x 10^9/L	157.5±81.9 (5.2-322)*	118.98±128.11 (2-550)	0.26
Hb g/dl	9.4±1.8 (6.3-12.5)	7.7±1.46 (4.1-10.4)	0.003
Platelets x 10^9/L	415.1±241.1 (125-988)	305.18±297.92 (12-949)	0.21
Blasts %	2.91±2.79 (0-10)	20.06±21.27 (1-95)	0.001
Basophils %	4.31±3.30 (0-14)	5.256±5.66 (3-23)	0.2
Eosinophils %	2.95±1.86 (0-6)	2.52±1.62 (0-6)	0.46
Granulocytes %	77.04±9.53 (60-91)	46.52±22.04 (3-87)	0.001
Bone marrow:			
Blasts %	2.5±2.29 (0-9)	27.78±20.66 (0-9)	0.001
Basophils %	2.52±2.29 (0-9)	7.72±7.77 (0-33)	0.06
Eosinophils %	3.78±3.84 (0-18)	4.33±3.82 (0-17)	0.001
Granulocytes %	76.83±12.59 (46-90)	38.77±23.36 (2-78)	0.65

Mean±SD, (range).

Table (2): CD34 and pSTAT5 expression in 39 CML patients.

Parameter	All patients No. 39	Group I† No. 22	Group II‡ No. 17	*p* value
CD34%	10.27±16.55* (0-86)	1.74±1.61 0-7	21.32±20.42 0-86	<0.001
pSTAT5%	43.66±26.32 (2-97)	33.85±21.05 2-75	56.35±27.58 14-97	0.006
pSTAT5 FR§	(1.48-46.03) 13.82±10.64	13.32±10.89 1.48-46.03	14.47±10.61 2.67-39.74	0.74
CD34-ve/pSTAT5+ve %	28.64±26.87 (0-97)	23.35±22.46 0-75	35.18±30.94 2-97	0.19

*Mean±SD, (range).
§FR: Fluorescent ratio.
†Group I : Chronic phase.
‡Group II: Accelerated phase and blastic crisis.

Table (3): pSTAT5 expression in 37 CML patients in relation to risk groups according to Hasford score.

Risk group	No.	pSTAT5 %	pSTAT5 FR†
Low	12	39.6±31.7 (2-90)*	15.7±12.5 (1.5-46)
Intermediate	17	45.1±23.2 (6.8-88)	12±8.5 (2.5-27.8)
High	8	45.2±30.1 (20-97)	13±8.6 (4.7-27.2)
Total	37	43.4±27 (2-97)	13.4±9.8 (1.5-46)
p-value		0.79	0.76

*Mean±SD, (range).
†FR: Fluorescent ratio.

DISCUSSION

Leukemogenesis in CML is a complex and incompletely understood process wherein BCR-ABL plays a central role as it influences a large number of signal transduction routes in parallel. Most signal transduction pathways that are involved in the pathogenesis of CML converge at the level of transcription factors, like STAT proteins and C-MYC, and BCL-2 family. These proteins all act synergistically to induce proliferation, while promoting cell survival **[16]**.

STAT proteins are known to be regulated by cytokine receptors and are critical for driving transcription necessary for growth, survival, and differentiation of hematopoietic cells. Experimental evidence indicates that BCR-ABL activates predominately STAT5 and to a lesser extent STAT3 and STAT1. BCR-ABL may activate STAT5 by direct phosphorylation or via phosphorylation by JAK2 **[18]**.

We have investigated the pSTAT5 in 39 patients. pSTAT5 was detected by flow cytometry in all BM samples from CML patients either in chronic or advanced cases in all CD34+ve cells indicating constitutive activation of STAT5 in CML progenitor cells. Gutierrez-Castellanos et al., **[19]** determined pSTAT5 in 27 CML patients including 11 in chronic, 6 in accelerated and 10 in blastic crisis phase. In their study 19 patients (70.3%) were positive for pSTAT5. Frequency of pSTAT5 was higher in patients in blastic crisis (100%) than in patients in accelerated phase and chronic phase (66.6 and 45.3%, respectively, p=0.022). However they tested pSTAT5 by immuneprecipitation and western blotting; the CD34+ve cells in the chronic phase which are pSTAT5+ve are too few to reach the detection limit of this technique. In their work they tested for the pSTAT5 in CD34+ve cells by confocal microscopy but only in the cases that were positive by western blot. In spite of the difference in the technique, our findings are matching theirs with regards to the significantly higher expression of pSTAT5 in advanced phases (blastic crisis and accelerated phase) than in chronic phase (p=0.006). In agreement with our results; Horita et al., **[20]** showed that all CML patients included in their study had pSTAT5 independent of disease stage.

An increase in BCR-ABL level is seen in advanced-phases of CML; blast phase is accompanied by an increase in both BCR-ABL mRNA and protein level and this increase is accompanied by increased tyrosine kinase activity of the BCR-ABL protein **[16]**. In view of our findings related to lower levels of pSTAT5 in chronic phase than in blast crisis, it is suggested that pSTAT5 may be taken as an indicator of BCR-ABL kinase activity.

In our study, pSTAT5 was expressed in 100% of CD34+ cells. In agreement with these results, Gutierrez-Castellanos et al. **[19]** demonstrated pSTAT5 in CD34+ cells in CML patients at different disease stages; however they tested only those cases that were positive by western blot.

Also other studies showed evidence of constitutive STAT5 and/or STAT1 activity in *BCR/ABL*- positive cell lines, peripheral-blood samples from CML patients, and hematopoietic cell lines transfected in vitro with *BCR/ABL*, leading to malignant transformation **[20,21]**.

Although the important role of STAT5 in the pathogenesis of CML was demonstrated in many studies; its role in transformation of the disease from chronic phase into advanced phases was not that much addressed. Up to our best knowledge, only the study conducted by Gutierrez-Castellanos et al., **[19]** investigated the role of STAT5 in the transformation of CML. In a recent study Warsch et al., **[22]** have shown that STAT5 over-expression leads clearly to a TKI-resistant phenotype whereas STAT3 and STAT1 have no effect. High levels of STAT5 protected leukemic cells from TKI toxicity in the absence of JAK2 expression, suggesting that BCR-ABL (or v-ABL) induced JAK2 phosphorylation is not required for STAT5 activation in leukemic cells. In BM samples from patients in advanced CML, high levels of STAT5 mRNA levels have also been shown to correlate with TKI resistance and accordingly, STAT5A and STATB expression (2 highly homologous STAT5 gene products) were found to be increased in CML patients with advanced stage with or without ABL-kinase mutations. These findings together with the report of Nelson et al., **[23]** suggest that STAT5 phosphorylation is a marker of CML progression and it could be an attractive target to circumvent TKI resistance in CML.

In our study, there was a statistically significant positive correlation between pSTAT5%

and blasts % in both BM and PB (r=0.37, p=0.02 and r=0.39, p=0.017 respectively) indicating the relation between the transformation and the STAT5 phosphorylation. This result is in agreement with Gutierrez-Castellanos et al. [19] who stated that the percentage of immature cells in chronic phase and advanced phases can influence phosphorylation of STAT5. Our reciprocal finding of a statistically significant negative correlation between the pSTAT5% and the granulocytes % both in the BM and PB (r=-0.438 and p=0.005, –0.435 and p=0.007) indicates again that the phosphorylation of STAT5 is dependent on the immature cells.

A near significant positive correlation between pSTAT5% and Hasford score was found in our study (r=0.29, p=0.08). Also pSTAT5% was higher in high risk group than other groups though it was not statistically significant. These findings, though lacking statistical significance, are online with the assumption that pSTAT5 reflects the level of tyrosine kinase activity and the state of disease progression.

Because neoplastic cells are dependent on constitutive STAT activation; targeting STATs causes preferential cancer cell killing with minimal effects on normal cells [24]. Although imatinib is very active and well-tolerated in the majority of patients with CML, cure cannot be attained with this or second generation TKI's, necessitating lifelong treatment. Moreover, a substantial number of patients demonstrate insufficient response to these drugs. Quiescence and multiple other mechanisms render CML stem cells drug resistant [16]. It is likely that optimal strategy for eliminating leukemic stem cells will involve targeting multiple pathways asking for combination of several agents. Targeting STAT5 could be an appealing approach being downstream of multiple signaling pathways. Being expressed in all CD34+ve cells, the fraction including the leukemia stem cells would further support this approach. Although not tried in CML yet; those new approaches may be of benefit to shut down the signal transduction pathways involved in the progression into advanced phases.

In conclusion, we have reported activation of STAT5 as evidenced by expression of pSTAT5 in all CD34+ve cells in CML patients regardless the disease stage. Its expression is higher in advanced stages known to be associated with higher levels of *BCR/ABL* expression. pSTAT5 may serve as an indicator of the level of *BCR/ABL* expression and STAT5 may serve as a potential therapeutic target in CML.

REFERENCES

1- Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood. 2002; 100: 2292-2302.

2- Eaves CJ, Eaves AC. Stem cell kinetics. Baillieres Clin Haematol. 1997; 10: 233-57.

3- Goldman JM, Melo JV. Chronic Myeloid Leukemia-Advances in Biology and New Approaches to Treatment. N Engl J Med. 2003; 349: 1451-64.

4- Melo JV, Hughes TP, Apperley JF. Chronic myeloid Leukemia. Am Soc Hematol. 2003; 132-152.

5- Sawyers CL: Chronic myeloid leukemia. N Engl J Med. 1999; 340: 1330-40.

6- Biernaux C, Loos M, Sels A, Huez G, Stryckmans P. Detection of major bcr-abl gene expression at a very low level in blood cells of some healthy individuals. Blood. 1995; 86: 3118-22.

7- Bose S, Deininger M, Gora-Tybor J, Goldman JM, Melo JV. The presence of typical and atypical BCR-ABL fusion genes in leukocytes of normal individuals: biologic significance and implications for the assessment of minimal residual disease. Blood. 1998; 92: 3362-7.

8- Rawlings JS, Rosler KM, Harrison DA. The JAK/STAT signaling pathway. J Cell Sci. 2004; 117: 1281-83.

9- Bunting KD, Bradley HL, Hawley TS, et al. Reduced lymphomyeloid repopulating activity from adult bone marrow and fetal liver mice lacking expression of STAT5. Blood. 2002; 99: 479-487.

10- Buitenhuis M, Baltus B, Lammers J-WJ, et al. Signal transducer and activator of transcription 5A (STAT5A) is required for eosinophil differentiation of human cord blood-derived CD34+ cells. Blood. 2003; 101: 134-142.

11- Ilaria RL Jr, Hawley RG, Van Etten RA. Dominant negative mutants implicate STAT5 in myeloid cell proliferation and neutrophil differentiation. Blood. 1999; 93: 4154-4166.

12- Nosaka T, Kawashima T, Misawa K, et al. STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells. EMBO J. 1999; 18: 4754-4765.

13- Kieslinger M, Woldman I, Moriggl R, et al. Antiapoptotic activity of STAT5 required during terminal stages of myeloid differentiation. Genes Dev. 2000; 14: 232-244.

14- Hochhaus A. Prognostic factors in chronic myeloid leukemia. Onkologie. 2008; 31: 576-578.

15- Jacobberger JW,1, Sramkoski RM,1 Frisa PS, et al. Immunoreactivity of STAT5 Phosphorylated on Tyrosine as a Cell-Based Measure of Bcr/Abl Kinase Activity. Cytometry Part A. 2003; 54A: 75-88.

16- Janssen JJ, Schuurhuis GJ, Terwijn M, et al. Towards cure of CML: Why we need to know more about CML stem cells? Current stem cell research and Therapy. 2009; 4: 224-236.

17- Ilaria RL Jr, Van Etten RA. P210 and P190 (BCR/ABL) induce the tyrosine phosphorylation and DNA binding activity of multiple specific STAT family members. J Biol Chem. 1996; 271 (49): 31704-31710.

18- Samanta AK, H Lin, T Sun, H Kantarjian, RB. Arlinghaus. 2006. Janus kinase 2: A critical target in chronic myelogenous leukemia. Cancer Res. 66: 6468-6472.

19- Gutiérrez-Castellanos S, Cruz M, Rabelo L, et al. Differences in BCL-XL expression and STAT5 phosphorylation in chronic myeloid leukemia patients. Eur J Haematol. 2004; 72: 231-238.

20- Horita M, Andreu EJ, Benito A, et al. Blockade of the Bcr-Abl kinase activity induces apoptosis of chronic myelogenous leukemia cells by suppressing signal transducer and activator of transcription 5-dependent expression of Bcl-XL. J Exp Med. 2000; 191: 977-984.

21- De Groot RP, Raaijmakers JA, Lammers JW, et al. STAT5-dependent Cyclin D1 and Bcl-XL expression in Bcr-Abl-transformed cells. Mol Cell Biol Res Commun. 2000; 3: 299-305.

22- Warsch W, Kollmann K, Eckelhart E, et al. High STAT5 levels mediate imatinib resistance and indicate disease progression in chronic myeloid leukemia. Blood. 2011; 117: 3409-3420.

23- Nelson EA, Walker SR, Weisberg E, et al. The STAT5 inhibitor pimozide decreases survival of chronic myelogenous leukemia cells resistant to kinase inhibitors. Blood. 2011; 117: 3421-3429.

24- Benekli M, Baumann H, Wetzler M. Targeting signal transducer and activator of transcription signaling pathway in leukemias. J Clin Oncol. 2009; 27: 4422-4432.

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Parathormone Hormone Levels, Myocardial Iron Overload and Cardiac Functions in Patients with ß-Thalassaemia Major

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ABSTRACT

Background: Iron overload cardiomyopathy (IOC) represents the leading cause of death in patients with ß-thalassemia major (TM). There is increasing evidence that high parathormone hormone levels are associated with increased cardiac iron content.

Aim of the Work: In this study we aimed to assess the level of serum parathormone and its relation to cardiac functions, cardiac magnetic resonance T2* and liver iron concentration in children and adolescents with ß-thalassemia major.

Patients and Methods: In seventy TM patients and 30 age and sex matched healthy subjects, levels of intact parathormone (iPTH) levels, calcium, phosphorus, and alkaline phosphatase were measured in serum and cardiac functions were assessed by complete M-mode and pulsed Doppler echocardiography and myocardial performance index (MPI) was calculated for both the left and right ventricles. Myocardial iron content and liver iron content were assessed by Magnetic resonance imaging T2* and results were derived from patients' medical records. Average values of serum ferritin were calculated for 12 months prior to the study.

Results: The results of iPTH revealed that 72.5% had normal values, 18.5% had below normal values and 9% had elevated levels. We observed weak positive correlation between iPTH and LVEF and LIC but no correlation was found between iPTH and serum ferritin or cardiac T2*. Cases with increased cardiac iron (T2*<20ms) had insignificantly lower iPTH, higher mean age, more frequent transfusions, higher LIC, higher ferritin and lower calcium. Mean calcium values were significantly lower among cases while serum phosphorus and alkaline phosphatase were significantly higher among the cases compared to the control. There was no statistically significant difference in fractional shortening (FS %), left ventricular ejection fraction (LVEF %), or mean MPI of the left ventricle between the cases and control group. However, the left ventricular diastolic E and E/A indices were significantly higher amongst the cases, indicating restrictive pattern of iron overload cardiomyopathy.

Conclusions: In ß-thalassemia, cardiac iron overload can occur in spite of the low levels of PTH. Subclinical cardiac dysfunction is common in TM patients but not dependant on PTH level. Early and adequate vitamin D and calcium supplementation is mandatory to prevent secondary hyperparathyroidism and to delay the onset of cardiac dysfunctions in pediatric patients with ß-thalassemia major.

Key Words: *Thalassemia major – Magnetic resonance imaging – Cardiac functions – Iron overload – Parathormone.*

INTRODUCTION

ß-Thalassemia is a hereditary anemia resulting from defects in hemoglobin production. ß-thalassemia, which is caused by a decrease in the production of ß-globin chains, affects multiple organs and is associated with considerable morbidity and mortality [1]. Heart complications represent the leading cause of mortality in thalassemia major, even though, following the introduction of chelating therapies, an important and progressive increase of life expectancy mainly due to a reduction in mortality due to cardiac dysfunction has been demonstrated [2].

Vitamin D deficiency is quite common in thalassemia major patients' due to increased metabolic demands, chronic medical care, and iron overload [3,4]. It was reported that low D25-OH levels produce reciprocal increase in serum parathyroid levels, leading to higher heart rates, higher cardiac intracellular calcium levels, and hypertrophy. However, this data is the first to suggest an association between cardiac iron uptake and vitamin D25-OH deficiency [5].

Both parathyroid hormone and vitamin D1-25OH appear to stimulate transmembrane cal-

cium movement via L-type calcium-dependent channels (LTCC); although the details of this interaction remain poorly characterized [5]. Murine data indicate that LTCC are important in transporting non-transferrin bound iron (NT-BI) into the myocardium. Thus, LTCC modulation represents the logical link between vitamin D deficiency, cardiac iron, and cardiac function [6]. Recent studies have correlated increased myocardial iron content to decreased levels of vitamin D in thalassemic patients and reported increased PTH levels as the major predictor of increased myocardial iron [7].

When putting the aforementioned facts together, it is reasonable to hypothesize that secondary hyperparathyroidism may be common in pediatric patients with TM and could be associated with increased myocardial iron content and a higher incidence of myocardial dysfunction in these patients.

In this study we aimed to assess the level of serum parathormone and its relation to cardiac systolic and diastolic dysfunction, cardiac magnetic resonance T2* and liver iron concentration in children and adolescents with *ß*-thalassemia major.

PATIENTS AND METHODS

This was a case-control study conducted on 70 children with established diagnosis of *ß*-thalassemia major and 30 age and sex matched healthy subjects taken as control group, after obtaining consents from their legal guardians. All recruited patients were attending routine follow-up visits at the outpatient clinic, New Children's Hospital, Cairo University, Cairo, Egypt, during the study period (1st August 2011 to 1st February 2012). We excluded patients aged less than 6 years or older than 18 years and those with significant valvular or congenital heart disease. The study protocol was approved by the Institutional Ethical Committee and was conducted in accordance with the Institutional Committee for the Protection of Human Subjects and adopted by the 18th World Medical Assembly, Helsinki, Finland.

Detailed history-taking with emphasis on age, age at diagnosis, splenic status, onset of blood transfusion and transfusion frequency, calcium and vitamin D supplementation and chelation therapy was carried out. Serum ferritin concentrations were derived from patients' records, and mean values were calculated for 1 year prior to the study. Complete blood picture with blood indices was assessed by Coulter Counter (Cell-Dyn® 1700CS; Abbott). Parameters of calcium homeostasis including serum calcium, serum phosphate and alkaline phosphatase (ALP) were measured by conventional laboratory methods. Two ml of whole blood were collected into EDTA-containing tubes and were kept cold throughout the collection and separation process for measurement of intact Parathormone levels (iPTH) by chemiluminescent assay [8]. Data of myocardial and Liver iron concentration (LIC) were assessed with magnetic resonance imaging by means of T2* based on a method that has been previously described and validated [9], and data were extracted from patients' medical records. Increased cardiac iron is defined as (T2*<20ms).

Echocardiography examination: Was performed at Echocardiography Laboratory of Abu El-Reesh Hospital, Faculty of Medicine, Cairo University. Transthoracic two dimentional (2D) guided (M Mode) and Doppler echocardiogram was performed with a Hewlett-Packard 5500 SONOS ultrasonic machine phased array sector scanner with the 4 and 8 MHZ probes according to age. Patient's recordings were taken while patients were in supine position without breath holding. M-mode, 2D and Doppler echocardiographic parameters were averaged over 3 cardiac cycles and all echocardiographic measurements were performed according to the guidelines for performance of a pediatric echocardiogram by American Society of Echocardiography. The MPI is a pure number and is calculated from the ratio of time intervals (a-b/b) derived with the aid of pulsed Doppler echocardiography [10].

Statistical analysis: Patients' data were analyzed using SPSS 17.0 for windows 7. Quantitative variables were expressed by mean and SD (Standard deviation), median and range. Comparison of Quantitative variables between groups was done using *t* student test for parametric data or Mann Whitney test for nonparametric data. Correlations were done to test for linear relations between variables. Qualitative variables were expressed as numbers (frequency) and percent and compared between groups using Chi-square test or Fisher exact test for

small values. Receiver's operating characteristics (ROC) curve was made and area under the curve (AUC) was calculated for the ability of elevated PTH to predict cardiac iron overload. All *p*-values are two tailed and considered statistically significant If ≤0.05.

RESULTS

The study group consisted of 41 (58.6%) males and 29 (41.4%) females with male sex predominance (M/F ratio 1.4). Mean patients' age was 14.8±2.9 years (range: 6-18 years). The mean age at diagnosis was 12.5 months and the mean age of first transfusion was 12.6 months. At the time of enrollment; 58 (83%) patients were splenectomized at a median age of 6 years (range 3-17 yrs) and 12 (17%) patients had splenomegaly. All patients received blood transfusions at 4-8 week intervals to maintain a mean hemoglobin level of 7.6±1.6g/dl. Fifty-five (78.6%) patients had begun chelation with deferoxamine in 30 (42.9%) patients, deferiprone in 16 (22.9%) while 9 (12.9%) were currently on deferasirox. Folate, calcium and vitamin D supplementation were prescribed to all patients; no patients were taking cardiac medications.

The study populations were mild to severely iron overloaded with serum ferritin ranging from 263 to 12128ng/ml with a median of 2879.0ng/ml, average liver iron was 25.5±12.8 mg/g dry weight (rang: 2.3-43.0) and mean cardiac T2* was 27.3±14.0 (4.6-60.0) (Table 1).

Table (2) summarizes the comparison of the hemoglobin level, biochemical parameters and cardiac functions of cases and controls. Mean parathormone of cases was 38.0pg/ml (range 2.3-195pg/ml) versus 34.8±25.5pg/dl (range 3-95.9pg/dl) among the controls and this difference was not statistically significant (*p*=0.6). The results of parathormone revealed that 51 (72.8%) of the studied patients had normal values, 13 (18.6%) had below normal values and 6 (8.6%) patients had high levels exceeding the upper limit of the reference range. The echocardiographic parameters showed no statistically significant differences between cases and control as regards: Fraction shortening (*p*=0.8), ejection fraction (*p*=0.3), MPI (lt) (*p*=0.2) and MPI (rt) (*p*=0.09); while right E velocity, right A velocity, left E velocity and the left A velocity values were significantly higher among cases (*p*=0.01, 0.002, 0.001 and <0.001 respectively).

Table (1): Parameters of iron overload among thalassemia major cases.

Parameter	The studied cases (n=70)
Serum ferritin (ng/ml):	
Mean±SD (Range)	3637.6±2540.9 (263-12128)
Median (IQR[a])	2879.0 (1854.8-4775.5)
LIC[b] mg/dl (n=43):	
Mean±SD (Range)	25.5 ±12.8 (2.3->43.0)
Median (IQR[a])	23.8 (15.5-36.9)
LIC[b] mmol/kg (n=43):	
Mean±SD (Range)	453.4±228.8 (18.8->769.0)
Median (IQR[a])	426 (276.8-661.0)
Cardiac T2 ms (n=43):*	
Mean±SD (Range)	27.4±14.1 (4.6-60.0)
Median (IQR[a])	26.5 (15.2-34.7)

[a]IQR : Inter-quartile range.
[b]LIC : Liver iron concentration.

There was no correlation between PTH and age, frequency of blood transfusion, serum ferritin, cardiac T2*, Calcium, Phosphorus or Alkaline Phosphatase. No correlation was proved between PTH and fractional shortening (FS) (*p*=0.148), ejection fraction (EF) (*p*=0.313), left ventricular MPI (*p*=0.070), right ventricular MPI (*p*=0.187), T E/A velocity (*p*=0.339), M E/A velocity (*p*=0.586). There was a weak correlation between PTH and LIC, LVEF (*r*=0.3, *p*=0.03; *r*=0.3, *p*=0.02 respectively) (Fig. 1).

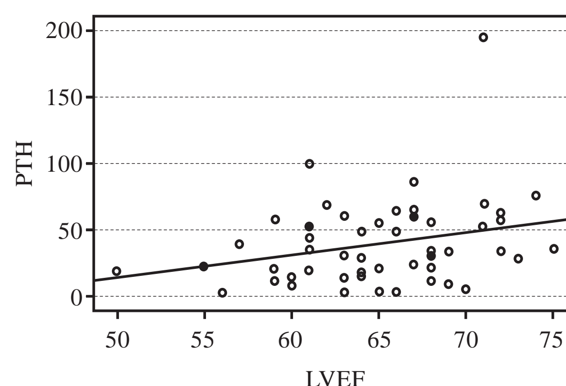

Fig. (1): Correlation between Parathormone and left ventricular ejection fraction in 70 thalassemia major patients (*r*=0.27, *p*=0.047).

Table (2): Comparison of laboratory data and cardiac functions of thalassemia major cases and control group.

Parameter	Cases (n=70)		Control (n=30)		*p* value
	Mean±SD	Range	Mean±SD	Range	
Hemoglobin (g/dl)	7.6±1.6	3.4-12.0	12.4±1.7	8.9-17	<0.001*
iPTH[a] (pg/mL)	38.0±3.5	2.3-195	34.8±25.5	3.0-95.9	0.550
Calcium (mg/dl)	8.6±0.9	6.4-10.1	9±1.1.0	5.3-10.5	0.022*
Phosphorus (mg/dl)	5.6±0.2	3.3-10.5	4.8±0.6	3.4-6.1	<0.001*
Alkaline phosphatase (U/L)	359.4±170.6	79-796	237±90	118-502	<0.001*
Fraction shortening (%)	35.8±5.7	29-69	36.5±5.2	29-48	0.757
Ejection fraction (%)	64±6.4	38-79	65.7±6.3	57-77	0.333
MPI (lt)	0.33±0.04	0.22-0.39	0.32±0.04	0.23-0.4	0.183
MPI (rt)	0.29±0.05	0.19-0.48	0.27±0.06	0.16-0.38	0.090
Right E velocity (m)(m/s)	0.75±0.1	0.51-0.94	0.7±0.1	0.46-0.9	0.012*
Right A velocity (m)(m/s)	0.49±0.06	0.34-0.61	0.44±0.07	0.32-0.55	0.002*
Left E velocity (m)(m/s)	1.03±0.1	0.78-1.29	0.93±0.14	0.63-1.29	>0.001*
Left A velocity (m)(m/s)	0.6±0.06	0.44-0.76	0.55±0.08	0.39-0.77	>0.001*

[a]PTH: Parathormone.
MPI (lt) myocardial performance index of the left ventricle.
MPI (rt) myocardial performance index of the right ventricle.
**p*-value is significant If <0.05.
Right E vel : E velocity across the Tricuspid valve.
Right A vel : A velocity across the Tricuspid valve.
Left E vel : E velocity across the mitral valve.
Left A vel : A velocity across the mitral valve.

On revising the results of cardiac T2* of 47 cases, we found 15 (32%) cases with T2*<20 while 32 (68%) had T2*<20 (Fig. 2).

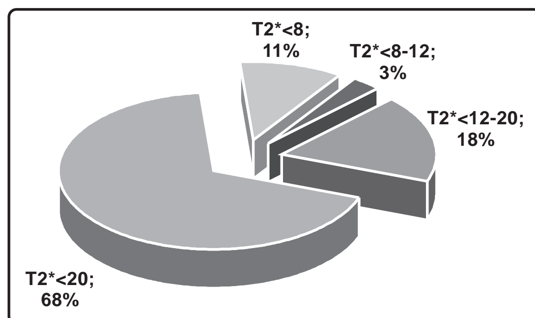

Fig. (2): Distribution of 47 thalassemia major cases according to Cardiac T2* Level.

Table (3) shows a comparison between cases with T2*<20 and cases with T2*<20. Cases with increased cardiac iron (T2*<20) were older, had more frequent transfusions, higher LIC, higher serum ferritin, lower PTH level and lower calcium but all did not reach level of significance (*p*>0.05).

ROC curve for parathormone in T2 groups:*

Receiver's operating characteristics (ROC) curve was performed to test for the ability of elevated PTH to predict cardiac iron overload among the studied patients with thalassemia major. The area under the ROC curve was 0.6 indicating that overall predictability of PTH is statistically insignificant (*p*>0.05).

Table (3): Comparison of thalassemia major cases with T2*<20ms and T2*>20ms.

Parameter	T2<20 (n=15)		T2>20 (n=32)		*p* value
	Mean	Std. Deviation	Mean	Std. Deviation	
Age	16.17	2.662	14.79	2.506	0.075
Bl Tr/year	15.67	5.499	12.68	4.839	0.058
Ferritin (ng/ml)	4553.78	3133.694	3600.08	2211.423	0.201
Ca (mg/dl)	8.21	1.018	8.69	0.780	0.054
Ph (mg/dl)	5.976	1.6627	5.549	0.9074	0.22
ALP (units/L)	421.61	187.804	356.16	164.539	0.19
iPTH (pg/ml)	31.35	26.131	43.83	33.062	0.166
LIC (mg/gm)	24.394	14.3699	25.067	10.8402	0.863
LVEF (%)	63.94	5.620	65.46	4.828	0.306

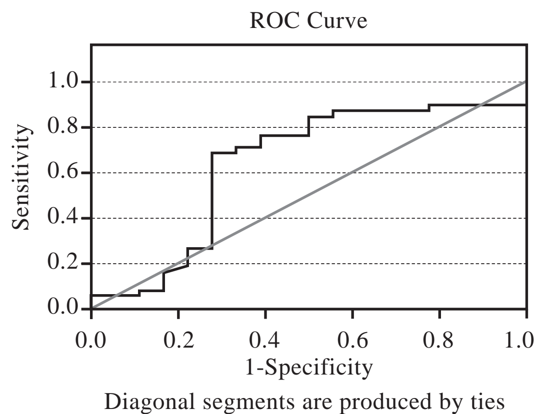

Fig. (3): ROC curve for the ability of elevated PTH to predict cardiac iron overload in patients with thalassemia major (n=47). The area under the ROC curve was 0.6 (*p*-value >0.05).

DISCUSSION

Contrary to expectations, we found low level of PTH in patients having increased myocardial iron deposition. In addition, there was a weak correlation between PTH level and LVEF and no correlations were found between PTH and other echocardiographic parameters and the ROC curve failed to prove the ability of elevated PTH to predict cardiac iron overload among our cases. This oppose the data reported in two recent studies carried out by Wood et al., [11] and Dimitriou et al., [7] that linked vitamin D deficiency and secondary hyperparathyroidism with cardiac iron uptake.

The pathogenetic mechanism that links PTH and cardiac iron uptake involves the transport of non-transferrin-bound iron (NTBI) in cardiomyocytes through the L-type Calcium channels (LTCCs), as this was initially described by Tsushima et al. [12], as elevated PTH levels have been shown to increase LTCCs activity. Further evidence was obtained from studies showing reduced intracellular myocardial iron accumulation using LTCCs blockers, like amlodipine and verapamil, in mouse models [6].

However, in the literature, not only the elevated PTH levels; but also the level and duration of NTBI exposure are important components for cardiac iron uptake [13]. Thus, secondary increases in PTH would not be expected to produce cardiac iron loading in the absence of elevated circulating NTBI, but might independently impair myocardial calcium cycling and cardiac function.

In addition, previous studies reported that the age of onset of parathyroid dysfunction in transfusion-dependant TM patients is usually around the age of 10 years [14-16] and abnormal cardiac T2* is rarely found before the age of 10 years, even in patients with high liver iron concentrations [17]. This way, the presence of parathyroid hypofunction with subsequent decreased functional reserve might lead to failure to compensate vitamin D deficiency which may induce cardiac iron uptake by mechanisms other than increased PTH. This may explain our findings of cases with increased myocardial iron that were older and had lower PTH and highlighted the fact that cardiac iron deposition is multifactorial and could occur in spite of low PTH.

Our data showed that about one fourth of children and adolescents with TM had abnormal parathormone (PTH) levels with the frequency of low PTH reaching twice that of high PTH. However, mean parathormone of cases was comparable to that of the controls. PTH did not correlate with age, transfusion frequency, serum ferritin, calcium, phosphorus or alkaline phosphatase. We observed weak positive correlation between PTH level and LIC. Our data support the prior evidence that parathyroid dysfunction is one of the common endocrinopathies of *ß*-thalasemia major due to chronic anemia, hypoxia and iron overload [18,19].

Among our cases, there were no statistically significant differences in fractional shortening (FS %), left ventricular ejection fraction (LVEF %) or MPI between the cases and control group. This is in agreement with the results reported in previous studies [20,21]. However, several studies reported a significantly lower LVEF % in thalassemic patients in comparison with healthy age and sex matched individuals [22-25] and that MPI of TM cases was significantly increased in comparison to the control group [24]. This may be explained by the younger age group included in our study. In addition, supplementary vitamin D at our center is usually prescribed very early and it was reported that vitamin D may improve left ventricular systolic functions [26].

However, the left ventricular diastolic E and E/A indices were significantly higher amongst the cases, indicating restrictive pattern of iron

overload cardiomyopathy and reflecting left ventricular chamber stiffness; this is in agreement with that reported by other studies [25]. It is to be mentioned that none of the patients had history of clinically manifesting cardiomyopathy or was receiving any kind of medicine that would affect cardiac functions.

Despite the early introduction of blood transfusion and chelation therapy, the majority of our study populations had post-transfusion hemoglobin below 9g/dl and were moderate to severely iron overloaded as evidenced by serum ferritin, LIC and cardiac T2*. This may be explained by the restrictive transfusion regimen adopted at our center as well as the bad compliance of our cases to chelators. In fact, the financial aspects also share in the problem; due to frequent interrupted availability of these chelators.

In spite of the routine supplementation with calcium and vitamin D, our cases showed biochemical parameters suggestive of hypocalcemia and vitamin D deficiency which may induce parathyroid hyper function [5]. This confirms the data reported in the majority of published literature on the high frequency of vitamin D deficiency in TM cases [3,4,7,11].

In fact, our study has some limitations including lack of data about the level of NTBI and vitamin D metabolites among our cases which could impair the strength of our conclusions. But one of the points of strength in this study is the number of cases which was relatively larger than previous reports.

Conclusion: In *ß*-thalassemia, cardiac iron overload can occur in spite of the low levels of PTH. Subclinical cardiac dysfunction is common in TM patients but not dependant on PTH level. Early and adequate vitamin D and calcium supplementation is mandatory to prevent secondary hyperparathyroidism and to delay the onset of cardiac dysfunctions in pediatric patients with *ß*-thalassemia major.

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REFERENCES

1- Rund, Rachmilewitz E. Beta thalassemia. N Engl J Med. 2005; 353: 1135-1146.

2- Borgna-Pignatti C, Cappellini MD, De Stefano P, Del Vecchio GC, Forni GL, Gamberini MR, et al. Survival and complications in thalassemia. Ann N Y Acad Sci. 2005; 1054: 40-7.

3- Moulas A, Challa A, Chaliasos N, Lapastsanis PD. Vitamin D metabolites (25-hydroxy vitamin D, 24-25 dihydroxyvitamin D and 1-25 dihydoxyvitamin D) and osteocalcin in beta thalassemia. Acta Paediatrica. 1997; 86: 594-599.

4- Napoli N, Carmina E, Bucchieri S, Sferrazza C, Rini GB, Di Fede G. Low serum levels of 25-hydroxy vitamin D in adults affected by thalassemia major or Intermedia. Bone. 2006; 38: 888-892.

5- Smogorzewski M, Massry SG. Uremic cardiomyopathy: Role of parathyroid hormone. Kidney Int. 1997: 62: S12-14.

6- Oudit GY, Sun H, Trivieri MG, Koch SE, Dawood F, Ackerley C, et al. L-type Ca (2+) channels provide a major pathway for iron entry into cardiomyocytes in iron-overload cardiomyopathy. Nat Med. 2003; 9: 1187-1194.

7- Dimtriadou M, Christfordis A, Econumou M, Tsatra I, Vlachaki E, Fidani L, Katzos G, et al. Elevated serum parathormone levels are associated with myocardial iron overload in patients with beta thalassemia major. Eur J Haematol. 2010; 84 (1): 64-71.

8- Nussbaum SR. Immunometric assay of parathyrin in the diagnosis of hypercalcemic individuals. Clinical Chem. Clin Chem. 1991; 37 (2): 142-3.

9- Anderson LJ, Holden S, Davis B. Cardiovascular T2-star (T2*) magnetic resonance for the early diagnosis of myocardial iron overload. Eur Heart J. 2001; 22: 2171-9.

10- Lakoumentas JA, Panou FK, Kotseroglou VK, Aggeli KI, Harbis PK. The Tei index of myocardial performance: Applications in cardiology. Hellenic J Cardiol. 2005; 46 (1): 52-8.

11- Wood JC, Claster S, Carson S, Menteer JD, Hofstra T, Khanna R, et al. Vitamin D deficiency, cardiac iron and cardiac function in thalassaemia major. Br J Haematol. 2008; 141: 891-894.

12- Tsushima RG, Wickenden AD, Bouchard RA, Oudit GY, Liu PP, Backx PH. Modulation of iron uptake in heart by L-type Ca2+ channel modifiers: Possible implications in iron overload. Circ Res. 1999; 84: 1302-1309.

13- Wood JC, Enriquez C, Ghugre N, Otto-Duessel M, Aguilar M, Nelson MD, et al. Physiology and pathophysiology of iron cardiomyopathy in thalassemia. Ann N Y Acad Sci. 2005; 1054: 386-95.

14- Rombos Y, Tzanetea R, Konstantopoulous K. Chelation therapy in patients with thalassemia using the orally active iron chelators deferiprone. Hematol. 2000; 85: 115-7.

15- Toumba M, Sergis A, Kanaris C, Skordis N. Endocrine complications in patients with Thalassaemia Major. Pediatr Endocrinol Rev. 2007; 5: 642-648.

16- Habeb AM, Al-Hawsawi ZM., Morsy MM, Al-Harbi AM, Osilan AS, Al-Magamsi MS, et al. Endocrinopathies in beta-thalassemia major. Prevalence, risk factors, and age at diagnosis in Northwest Saudi Arabia. Saudi Med J. 2013; 34 (1): 67-73.

17- Wood JC, Tyszka JM, Carson S, Nelson MD, Coates TD. Myocardial iron loading in transfusion-dependent thalassemia and sickle-cell disease. Blood. 2004; 103: 1934-1936.

18- Oerter KE, Kamp GA, Munson PJ, Nienhis AW, Cassorla FG, Manasco PK. Multiple hormone deficiencies in children with haemochromatisis. J Clin Endoc Metab. 1993; 76: 357-361.

19- Rund D, Rachmilewitz E. Thalassemia major, older patients, new therapy. Blood Rev. 1995; 9: 25-32.

20- Garadah T, Kassab S, Mahdi N, Abu-Taleb A, Jamsheer A. Pulsed and Tissue Doppler Echocardiographic Changes in Patients with thalassemia major Clinical Medicine Insights: Cardiology. 2010; 2: 1-8.

21- Spirito P, Gabriele Lupi, Caterina Melvendi, Cario Vecchio. Restrictive Diastolic Abnormalities Identified by Dopler Echocardiography in Patients with Thalassemia Major. Circulation. 2010; 82 (1): 89-93.

22- Vaccari M, Crepaz R, Fortini M, Gamberini MR, Scaria S, Pitscheider W. Left ventricular remodeling, systolic function and diastolic function in young adults with beta thalassemia intermedia: A Doppler echocardiography study. Chest. 2002; 121: 506-512.

23- Bosi G, Crepaz R, Gamberini MR. Left ventricle remodeling and systolic and diastolic function in young adult with beta thalassemia major: A Doppler echocardiographic assessment and correlation with hematological data. Heart. 2003; 89 (7): 762-6.

24- Noori NM, Mehralizadeh S. Echocardiographic evaluation of systolic and diastolic heart function in patients suffering from beta-thalassemia major aged 5-10 years at the Zahedan Research Center for Children and Adolescent Health. Anadolu Kardiyol Derg. 2010; 10 (2): 150-3.

25- Aessopos A. Cardiac status in well-treated patients with thalassemia major. Eur J Haematol. 2004; 73: 359-66.

26- Nappi S, Saha H, Virtanen V, Limnell V, Sand J, Salmi J, et al. Left ventricular structure and function in primary hyperparathyroidism before and after parathyroidectomy. Cardiology. 2000; 93: 229-33.

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Bone Marrow Biopsy Angiogenesis in Multiple Myeloma: Computerized Image Analysis and Correlation with Clinico-Pathologic and Laboratory Prognostic Factors

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ABSTRACT

Background: Increased angiogenesis has been found to be an adverse prognostic factor in solid tumors. Evidence shows that angiogenesis plays an important role in hematological malignancies including multiple myeloma (MM) as well.

Aim of the Study: We aimed in the present study to investigate the various angiogenesis parameters; namely microvessel density (MVD) and total vascular area (TVA), using a standardized computer assay instead of the routinely used hot-spot technique, on bone marrow biopsy (BMB) of de novo MM patients. We also correlated BMB angiogenesis with clinico-pathologic and laboratory parameters established to have prognostic impact on MM.

Patients and Methods: BMB from thirty eight newly diagnosed cases of MM and twenty morphologically and immunohistochemically negative Hodgkin's lymphoma de novo patients as a control group, were examined using a computerized image analyzer. Bone marrow biopsies of test cases (n=38) were immunohistochemically stained with CD34 for visualization of microvessels, and MVD and TVA were measured.

Results: MVD and TVA were significantly increased in MM patients Vs controls (p<.0.001 for both). Angiogenesis was correlated with higher tumor burden, higher B_2 microglobulin level, higher M protein level and diffuse pattern of marrow infiltration.

Conclusion: Angiogenesis in BMB of MM cases is correlated to all other prognostic parameters which qualify it as a potential prognostic parameter. Standardization of the method is mandatory before testing it as an indicator of treatment outcome.

Key Words: *Angiogenesis – Multiple myeloma – Bone marrow biopsy – Microvessel density – Total vascular area – Computerized image analysis.*

INTRODUCTION

Multiple myeloma (MM) accounts for 1% of all malignancies and >10% of malignant hematological neoplasms **[1]**. It is characterized by the presence of a monoclonal (M) protein, lytic bone lesions, and increased plasma cells in the bone marrow and may be associated with anemia, renal failure, and hypercalemia **[2]**. Angiogenesis, the formation of new blood vessels, occurs physiologically during embryonal growth, wound healing, and in the female genital system during the menstrual cycle. It is important for the proliferation and metastasis of most malignant neoplasms. In the absence of angiogenesis, tumors cannot grow beyond 1-2mm in size **[3]**. Increased angiogenesis has been found to be an adverse prognostic factor in solid tumors but evidences show that angiogenesis also plays an important role in hematological malignancies including multiple myeloma **[4]**.

The limited success achieved by targeting only myeloma cells with the existing conventional and/or high-dose chemotherapy highlights the importance of understanding the role of the bone marrow microenvironment and its contribution to myeloma genesis. The microenvironment in multiple myeloma is composed of clonal plasma cells, bone marrow stromal cells, extracellular matrix proteins, inflammatory cells, and microvessels **[5]**. There is substantial evidence that interactions between these components have a crucial role in the proliferation and survival of myeloma cells and their acquisition of drug resistance and disease progression **[6]**.

There is growing evidence that not only increased bone marrow angiogenesis occurs in multiple myeloma, but also it is related to disease activity. The mechanism behind the increased angiogenesis in myeloma is not fully understood. There are data that myeloma cells express the potent angiogenic cytokines, VEGF and basic fibroblast growth factor [7]. Preliminary data using reverse transcription-PCR techniques indicate that VEGF isoforms, VEGF121 and VEGF165, are expressed by myeloma cells both in studies of bone marrow samples from patients with myeloma and on various myeloma cell lines [4,8]. Targeting angiogenesis with antiangiogenic agents is a promising and exciting therapeutic approach and is the subject of intense investigation [9].

The aim of our study is to investigate the extent of angiogenesis in bone marrow biopsy samples of newly diagnosed multiple myeloma patients compared to controls, using a computer based image analyzer soft ware, and to find out its correlation with other established clinicopathologic and laboratory prognostic parameters.

PATIENTS AND METHODS

Thirty eight newly diagnosed multiple myeloma patients were enrolled in our study. Patients were diagnosed to have multiple myeloma according to the WHO criteria such as bone marrow aspirate plasma cell percentage, lytic bone lesions on skeletal survey, anemia, hypercalcemia, renal insufficiency, Immunophenotyping, monoclonal gammopathy and Bence Jones proteinuria [10]. Pretreatment bone marrow trephine biopsy samples were available from all the patients.

Information on prognostic factors included clinical staging according to the Durie and Salmon staging system [11], serum M-protein levels, percentage of bone marrow aspirate plasma cells, B_2 microglobulin levels (B_2M), pattern of bone marrow biopsy plasma cell infiltration, and extent of marrow fibrosis; they were correlated with the degree of bone marrow angiogenesis. Twenty age- and sex-matched bone marrow biopsies performed for staging of Hodgkin's lymphoma for patients who proved to have no evidence of infiltration, based on morphology and immunohistochemistry for CD30, and who received no previous chemotherapy were included in the study as a control group.

Bone marrow biopsy preparation, immunohistochemical staining and assessment:

Biopsies were fixed in 10% formalin, decalcified in 10% EDTA for 48 hours, and embedded in Paramat extra (BDH, Poole, Dorset, UK). Initially, haematoxylin and eosin stained, 3µm thick sections were examined by light microscopy. All slides (H & E) were evaluated for confirmation of the original diagnosis. The pattern of infiltration of the bone marrow by MM was highlighted by immunostaining the neoplastic plasma cells with a monoclonal antibody to CD138 (clone MI15, m7228, DAKO, Glostrup, Denmark). Microwave antigen retrieval was done in the presence of 1mmol/L EDTA (pH 8.0) buffer. Slides were then incubated with anti-CD138 monoclonal antibody at 1:25 dilutions for 30 minutes at room temperature. Immunoglobulin k (No. 40191, DAKO) and immunoglobulin λ (No. 40193, DAKO) antibodies to detect the restriction of immunoglobulin light chains and to confirm monoclonality were done.

Monoclonal antibody anti-CD34 class II (m7165 clone QBEnd10, DAKO) was used to highlight endothelial cells. Epitope retrieval was achieved by immersing slides in Tris-EDTA (Merck, Damstadt, Germany) buffer (pH 9.0) and boiling for 15 minutes in a water bath at 97°C. Slides were then incubated with CD34 monoclonal antibody at 1:100 dilutions for 30 minutes at room temperature.

Computerized image analysis (CIA) of angiogenesis:

All slides stained with anti-CD34 were scanned and analyzed with Alphelys Spot Browser 2 integrated system (Alphelys, France), using a software-controlled (Alphelys Spot Browser 2.4.4, Alphelys), stage-positioning Nikon Eclipse 50i microscope (Nikon, Japan) mounted on a 1360x1024 resolution Microvision CFW-1310C digital camera (Microvision Instruments, France). Slides were scanned at x 20 magnifications to identify the section area of slide and then scanned at x 200 magnifications to create images for quantification.

Computer-assisted image analysis was used to determine the total count of microvessels per square millimeter and total area occupied by microvessels (as a percentage of the total section

area). During digital image analysis, the software detected objects of interest based on pixel color properties (wavelength, intensity, and saturation) and morphometry (size and shape). The analysis software used created color segmentation algorithms on all slides which were designed for the detection and elimination of empty space, determination and count of positive areas, and measurement of mean vessel diameter. These measurements were used to calculate average MVD (quantity of microvessels per square millimeter) and TVA (percentage of microvessel area in total section area).

Analysis of data:

Statistical analysis was performed using SPSS version 18 software. The measures of central tendency for continuous data such as plasma cell infiltrates, MVD and TVA were compared by using the Student-*t*-test or Mann-Whitney test, depending on data distribution. The Fisher exact test and the Chi-square test were used to compare differences in nominal variables. Correlation between microvessel density, total vascular areas and other laboratory and clinical data was done by w2 test (univariate analysis).

RESULTS

A total number of 38 newly diagnosed multiple myeloma patients were enrolled in the present study. They were 29 males and 9 females with a male to female ratio of 3.2:1. 52.6% of the patients had monoclonal IgG, while 26.3% of patients had monoclonal IgA. The age of the patients ranged from 47 to 63 with a mean of 54.8±6.3 years. B_2 microglobulin level ranged from 2400 to 13,500 with a mean of 5800±2900 ng/dl. Bone marrow aspirate plasma cell percentage ranged from 13% to 76% with a mean of 45±12%. Table (1) shows the different clinical and laboratory parameters of the patient's group and Figs. (1-4) demonstrate morphologic patterns of marrow infiltration, immunohistochemistry and angiogenesis.

Table (1): Clinico-pathologic and laboratory parameters of the multiple myeloma patient group.

	Number (38)	Percent (100%)
Histological pattern:		
Interstitial	13	34.2
Sheets	9	23.7
Nodular	2	5.3
Diffuse	14	36.8
Degree of marrow fibrosis:		
No fibrosis	17	44.7
Grade I	10	26.3
Grade II	11	29
M protein level:		
<2gm%*	6	15.8
2-4gm%	20	52.6
>4gm%	12	31.6
Durie and salmon staging:		
Stage I	8	21.1
Stage II	8	21.1
Stage III	22	57.8
Plasma cell % in bone marrow aspirate:		
<20%	9	23.7
20%-50%	18	47.4
>50%	11	28.9
B_2 microglobulin level:		
<3400ng/dl	16	42.1
3400-10000ng/dl	19	50
>10000ng/dl	3	7.9

*The level of 2gm% was considered due to the percentage of IgA.

MVD (the quantity of microvessels/square mm) and TVA (percentage of microvessel area in the total section area) were measured for all patients and controls. MVD ranged from 12.3-430.5 with a median of 163.6 for the control group and ranged from 13.7-1079.1 with a median of 174.4 for the multiple myeloma group. TVA ranged from 0.1-16.4 with a mean of 1.9±1.3% for the control group and ranged from 18.2-36.1 with a mean of 6.4±6.5% for the multiple myeloma group. There was a significant difference between MVD and TVA in the myeloma group versus the control group ($p<0.001$ for each). Patients with higher angiogenesis had significantly higher B_2 microglobulin levels ($p=0.012$ for MVD and $p=0.029$ for TVA) and higher levels of M protein ($p=0.039$ for MVD). MVD and TVA were significantly higher in diffuse pattern of marrow infiltration compared to other patterns of infiltration ($p=0.008$ and $p=0.023$ respectively).

Angiogenesis was significantly correlated with the percentage of plasma cells, for MVD $r=0.48$ and $p=0.001$ and for TVA $r=0.45$ and $p=0.003$. It was also significantly correlated with the level of M protein ($r=0.44$ and $p=0.004$ for MVD), with no significant correlation with TVA ($p=0.066$). No significant correlation could be detected between angiogenesis and either the clinical staging of the disease ($p=0.089$ for MVD and $p=0.113$ for TVA) or the degree of marrow fibrosis ($p=0.069$ for MVD and $p=0.093$ for TVA).

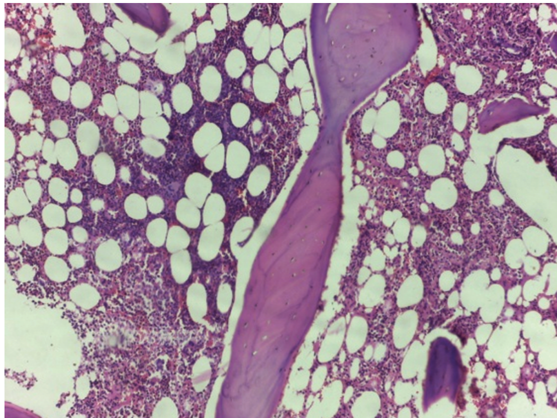

Fig. (1): Sheet of mature plasma cells (x20).

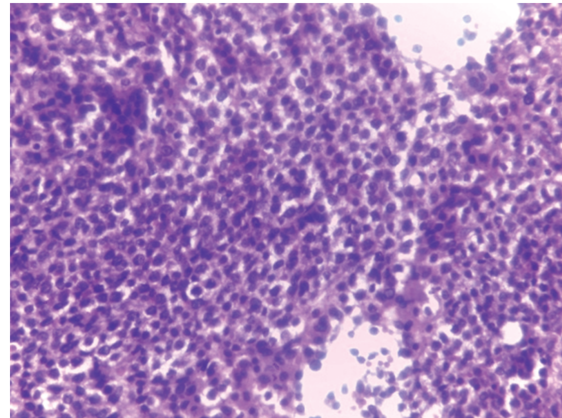

Fig (2): Diffuse infiltration with plasma cells (x40).

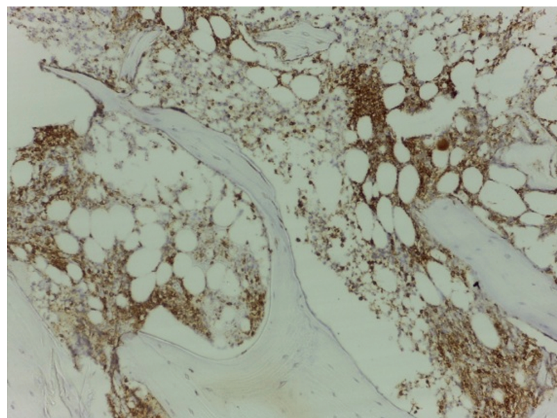

Fig. (3): CD138 positive plasma cells (x20).

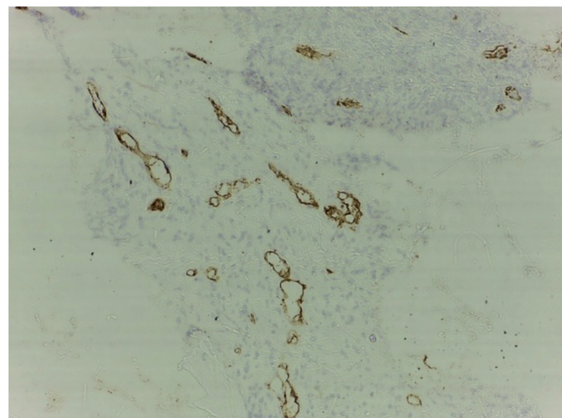

Fig. (4): Abundant CD34 lined newly formed vessels (x40).

DISCUSSION

In a malignancy such as MM, in which multiple therapeutic approaches with various mechanisms of action are available, accurate and standardized techniques for monitoring patients is critical to confirm disease responsiveness, to enable prompt detection of ineffective therapy, and to detect relapse before the occurrence of organ damage **[12]**. Not much attention is given to the morphologic characteristics of the bone

marrow, although it has been shown in several studies that the use of BMB is a more accurate method for the evaluation of plasma cell infiltration [13]. Therefore, the aim of the present study was to analyze accurately one of the most important components of the bone marrow microenvironment in BMB samples of patients with MM; namely angiogenesis. Most of the previous studies included only simple quantitative evaluation of MVD and used methods developed primarily for the characterization of angiogenesis in solid tumors. Such studies measured MVD in the hot spots, which are areas of the bone marrow biopsy carrying the highest number of microvessels, based on conventional light microscopy. Our study measured angiogenesis: MVD, and TVA on the whole area of the slides. Therefore, the usually applied, so-called hot-spot technique should be amended by an appropriate and more elaborate computer-assisted morphometric analysis of the microvessel structures. Such a computer standardized spectrum of information regarding quantity and quality of angiogenesis enables further understanding of the morphologic changes in the course of the disease and accordingly on the effect of various therapies on bone marrow vascularization.

The results of our study linked plasma cell infiltration with angiogenic activity in MM because both angiogenic parameters, MVD and TVA, correlated with plasma cell percentage "Tumor burden" and the diffuse pattern of marrow infiltration. This association is in accordance with other studies [14-17]. We also correlated MVD and TVA with increased levels of B_2 microglobulin, above 3400ng/dl as reported by Bhati et al., [15] and with increased level of M protein as reported by Babarović et al., [17]. A limitation of the present study is the small number of patients, which might have impacted our failure to detect an association between angiogenesis parameters and either of the extent of marrow fibrosis and the clinical staging of the disease.

Our study underlines the role of BMB not only in establishing the diagnosis of MM but also in patient monitoring and providing important prognostic information, as well as highlighting the importance of angiogenesis being correlated to other prognostic parameters, which qualify it as a potential prognostic one.

REFERENCES

1- Landis SH, Murray T, Bolden S, Wingo PA. Cancer statistics, CA Cancer J. Clin. 1999; 49: 8-31, 1999.

2- Bataille R, Harousseau JLS. Multiple myeloma. N Engl J Med. 1997; 336: 1657-1664.

3- Folkman J. Seminars in medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. N Engl J Med. 1995; 333: 1557-1563.

4- Rajkumar SV, Leong T, Roche PC, et al. Prognostic value of bone marrow angiogenesis in multiple myeloma. Clin Cancer Res. 2006; 3111.

5- Ribatti D, Vacca A. The role of microenvironment in tumor angiogenesis. Genes Nutr. 2008; 3: 29-34.

6- Fowler JA, Edwards CM. Croucher PI. Tumor-host cell interactions in the bone disease of myeloma. Bone. 2011; 48: 121-128.

7- Ito A, Hirota S, Mizuno H, Kawasaki Y, Takemura T, Nishiura T, Kanakura Y, Katayama Y, Nomura S, Kitamura Y. Expression of vascular permeability factor (VPF/VEGF) messenger RNA by plasma cells: Possible involvement in the development of edema in chronic inflammation. Pathol. Int. 1995; 45: 715-720.

8- Bellamy WT, Richter L, Frutiger Y, Grogan TM. Expression of vascular endothelial growth factor and its receptors in hematopoietic malignancies. Cancer Res. 1999; 59: 728-733.

9- Vacca A, Ribatti D, Roncali L, Dammacco F. Angiogenesis in B cell lymphoproliferative diseases. Biological and clinical studies. Leuk. Lymphoma. 1995; 20: 27-38.

10- Mckenna RW, Kyle RA, Kuehi WM, et al. Plasma cell neoplasms. In: Swirlow S, Campo E, Harris N, et al. eds. WHO classification of tumors of hematopoietic and lymphoid tissues. International Agency for research on cancer, Lyon. 2008; pp. 200-213.

11- Durie BG, Salmon SE. A clinical staging system for multiple myeloma: Correlation of measured myeloma cell mass with presenting clinical features, response to treatment and survival. Cancer. 1975; 36: 842.

12- Chee CE, Kumar S, Larson DR, et al. The importance of bone marrow examination in determining complete response to therapy in patients with multiple myeloma. Blood. 2009; 114: 2617-2618.

13- Štifter S, Babarović E, Valković T, et al. Combined evaluation of bone marrow aspirate and biopsy is superior in the prognosis of multiple myeloma. Diagn Pathol. 2010; 5: 30. Doi: 10.1186/1746-1596-5-30.

14- Rajkumar SV, Mesa RA, Fonseca R, et al. Bone marrow angiogenesis in 400 patients with monoclonal gammopathy of undetermined significance, multiple myeloma, and primary amyloidosis. Clin Cancer Res. 2002; 8: 2210-2216.

15- Bhatti SS, Kumar L, Dinda AK, et al. Prognostic value of bone marrow angiogenesis in multiple myeloma: Use of light microscopy as well as computerized image analyzer in the assessment of microvessel

density and total vascular area in multiple myeloma and its correlation with various clinical, histological, and laboratory parameters. Am J Hematol. 2006; 81: 649-656.

16- Rana C, Sharma S, Agrawal V, et al. Bone marrow angiogenesis in multiple myeloma and its correlation with clinicopathological factors. Ann Hematol. 2010; 89: 789-794.

17- Babarović, Valković T, Štifter S, et al. Assessment of bone marrow fibrosis and angiogenesis in monitoring patients with multiple myeloma. Am J Clin Pathol. 2012; 137: 870-878.