The Journal of the Egyptian Society of Haematology & Research

The Official Journal of the Egyptian Society of Haematology & Research

Volume 7 * Number 1 * March 2011

CONTENTS

Journal of the Egyptian Society of Haematology & Research, Vol. 7, No. 1, March: 1-9, 2011

In Vitro Assessment of Different Prepared Platelet Concentrates During 8 Days Storage

AZZA MOUSTAFA, M.D.[1]; HAMDA EL-SAYED, M.D.[2]; RANIA FAWZY, M.D.[1]; AZZA HASSANEIN, M.D.[2]; NADIA SWELAM, M.D.[1]; HEBA ABOU-ELEW, M.D.[1] and EMAD YACOUB, M.Sc.[2]

The Departments of Clinical Pathology, Faculty of Medicine, Cairo University[1] and Hematology, Theodor Bilharz Research Institute[2]

ABSTRACT

Background: Adequate prolongation of platelet (PLT) shelf life can achieve improved availability, logistical management and decreased wastage. The coupling of reliable methods of bacterial detection and optimum methods of platelet preparation can preserve the quality of platelets with extended storage.

Objectives: This study aimed at evaluating the applicability of extending platelet shelf life up to 8 days, using different methods of platelet preparation.

Subjects and Methods: Thirty six platelet concentrates (PCs) were collected and divided into 3 equal groups, according to preparation procedure: Group (1): Non-leucofiltered Random-donor PLTs (RDPs); Group (2): Leucofiltered RDPs; and Group (3): Single-donor aphaeresis PCs. All units were stored at 22-24ºC on a flatbed agitator for 8 days. PLT characteristics and metabolic variables were assessed on days 1,5 and 8 of storage. Besides, automated bacterial screening was performed on days 1 and 8.

Results: Until the end of shelf life, the mean PLT recovery, mean PLT volume (MPV), PLT distribution width (PDW), swirling scores, glucose and lactate dehydrogenase (LDH) levels showed best suitable values among the aphaeresis units, compared to the other 2 groups. pH was maintained >6.8 in all groups. Also, the lowest expression of CD62p and CD63 was found among group 3, on day 8, compared to the other groups. However, RANTES (regulated on activation normal T expressed and secreted cytokines) results showed highly significant lower levels in groups 2 and 3 compared to group 1 on all days. No bacteriological growth was detected in all PC units, till day 8 of storage.

Conclusion: Aphaeresis units could provide the highest quality possible with 8 days storage, particularly when assisted by a good and rapid bacterial detection system. Thus, the choice between different methods of preparing PCs with extended shelf life should depend on a critical balance between safety, quality and cost.

Key Words: *Platelet storage – Non-filtered random PCs – Leucofiltered random PCs – Aphaeresis PCs – BACTEC.*

INTRODUCTION

Platelet transfusion is often considered a life-saving measure, being essential for the prevention and treatment of bleeding in patients who have quantitative and/or functional platelet disorders **[1]**. Nowadays, in many western countries, the demand for platelet concentrates (PCs) is obviously growing, almost up to 80% increase, compared to a decline in the use of packed red blood cells **[2]**.

The development of potentially curable chemo/radiotherapeutic regimens, which lead to prolonged periods of severe myelosuppression and which in turn place a considerable pressure on the logistics of platelets supply, necessitate intensive research into the biology of platelets, methods and devices needed for their collection and storage, and platelet transfusion practices **[3,4]**.

Studies conducted with PCs revealed that these cells lose their viability very quickly during the storage period, implying the need for continuous renewal of stock **[5,6]**. So, in order to reduce the bulk of outdated and wasted products, many studies have been directed towards extending the platelet storage time for more than 5 days and assessing their acceptability in vivo performance. If this can be successfully achieved, it will confer additional advantage and flexibility to blood banks and transfusion services **[7]**.

In fact, PLTs undergo a number of operations during collection, processing and storage that adversely affect their structure, resulting in reduced post-transfusion recovery and functionality. The aim of this study is to evaluate the applicability of extending platelet shelf life up to 8 days using different standard methods of platelet preparation namely, whole blood derived-PCs (WBD-PCs) either filtered or non-filtered and aphaeresis PCs and comparing their in vitro viability during the extended storage period. This will be an important step in transfusion services if platelets can be rendered available in a timely manner while diminishing the wastage of time-expired platelets.

MATERIALS AND METHODS

Platelet collection and storage:

Thirty-six PC units freshly collected, from healthy blood donors who visited the blood bank of Theodor Bilharz Research Institute, were enrolled in this study. They were classified equally into 3 groups according to the preparation method applied:

A- Group 1, included 12 non-filtered random donor PCs (RDPs) derived from whole blood by platelet-rich plasma (PRP) method.

B- Group 2, as well, included 12 random donor PCs, yet leucofiltered using pre-storage platelet leucoreduction filter (Fresenius Kabi BIO P Plus Filter).

C- Group 3 in which single donor PCs were prepared from the remaining 12 donors using aphaeresis device (COBE Spectra ™v. 7.0 LRS Turbo).

The study was approved by the local ethics board (Institutional Board Review) and an informed consent was obtained from all subjects.

Following preparation, PCs were left for 1 hour without agitation at room temperature. Subsequently, all the 36 units were kept on a flatbed platelet agitator (Helmer, Inc, USA) then stored with continuous gentle agitation to prevent clumping at 22-24ºC for a total of 8 days.

Analysis of PLT characteristics and metabolic variables during storage:

Samples were drawn aseptically under a laminar flow hood from all units at days 1,5 and 8 of storage. For each sample, PLT count, and indices (MPV, PDW), together with the white blood cell (WBC) count of the non-filtered units were estimated using the automated cell counter (Beckman Coulter Act Diff III). However, residual WBCs count in leucoreduced PCs either the leucofiltered RD-PLTs or aphaeresis units, was assessed by flowcytometric enumeration using (BD LeucocountTM kit, Flowcytometer Epics ® Elite "Coulter" system) **[8]**.

Swirling phenomenon was evaluated by examining the gently rotated PC units against the light. The normal discoid platelets refract light and produce swirling pattern, which can be identified and scored (0-3) by visual inspection of trained personnel in blood bank **[9]**.

pH of all samples was assessed immediately after sampling at a temperature of 22ºC using hand-held pH meter (HANNA Instruments HI 98103 Checker pH Tester, Italy). Glucose and lactate dehydrogenase (LDH) enzyme as indicators of platelet metabolism were determined according to the standard methods by using the semi-automated, single-beam filter photometer (RIELE 5010).

To detect platelet activation markers, PRP was separated and freshly tested for CD62p and CD63 expression using fluorescein isothiocyanate conjugated (FITC) monoclonal antibodies (moAbs), Mouse Anti-Human CD62p and CD63 antibodies (BD Biosciences.Com, PharmingenTM). A non-specific Isotype Control was used with each sample. All antibodies were of the IgG1k Isotype and Flowcytometer Epics ® Elite "Coulter" system was used for the analysis **[10]**. Results were expressed as specific CD62p and CD63 percentage of positive platelets, calculated by subtracting the nonspecific fluorescence of the isotype control from the specific fluorescence of the moAbs. For subtraction, the manufacturer's software was used.

Platelet poor plasma (PPP) samples were also separated and stored frozen at-70ºC for testing of the platelet derived cytokine, regulated on activation normal T expressed and secreted (RANTES; CC chemokine ligand 5) using enzyme-linked immunosorbent assay (ELIZA) (Quantikine) **[11]**.

Bacteriological screening of PC units:

The study had a standardized testing protocol that used aerobic culture bottles (BACTEC Plus Aerobic/F bottles) inoculated with 6ml of PLT

samples, and BACTEC 9050 System (BD Microbiology, Cockeysville, MD). These cultures were carried out for each PC unit on day 1 (24 hours post collection) and day 8. Continuous monitoring blood culture system in the incubator (37ºC) for 8 days after inoculation was performed for the detection of bacterial contaminants in PLT preparations. Even though, an automated system was used, the cultures were also controlled visually for signs of growth, cloudiness or a color change in the broth and gas bubbles or clumps of bacteria.

Statistical methods:

Results were expressed as mean±standard deviation (SD) or number (%). Comparison between the mean values at different dates within the same group was performed using paired *t*-test. Comparison between the mean values of different parameters between the different groups were performed using one way analysis of variance (ANOVA) of the mean percent change of each parameter with post hoc using the least significant difference. Correlation between parameters was performed using Spearman's rank correlation coefficient. SPSS computer program (version 18 windows) was used for data analysis. *p*-value ≤0.05 was considered significant and *p*-value <0.01 was considered highly significant. The percent change of each parameter was calculated by subtracting the baseline (day 1) result from the final result (day 8), then dividing the result of this subtraction by the baseline result, and finally multiplying by 100.

RESULTS

The mean volume of aphaeresis units on day 1 was about 228.33±20.74ml, which was obviously much higher than the other 2 groups (non filtered and filtered PRP-PC, 59.58±8.39 and 61.00±8.19, respectively). However, the volume of all units was decreased gradually during storage due to the sampling (10ml) each time, on days 1,5 and 8, in order to monitor the studied parameters.

Table (1) sums up all the studied parameters on different storage intervals. Intergroup comparison revealed highly significant difference regarding mean PLT count/unit among the aphaeresis group compared to the other 2 groups ($p<0.01$) during all studied storage times. Meanwhile, group 2 showed significant lower PLT count than group 1 only on days 1 and 5 ($p<0.01$ and $p<0.05$, respectively). As regards the PLT indices, PDW was significantly lower among group 3 compared to the first 2 groups on all storage days ($p<0.01$) and among group 2 compared to group 1 on day 1 ($p<0.01$). On the contrary, the MPV was significantly high among group 3 in comparison to group 1 on day 1 ($p<0.01$) and in comparison to group 2 on day 5 ($p<0.05$).

Mean WBC counts were significantly low among both leucoreduced groups (group 2 and 3), in relation to group 1 on all studied storage times ($p<0.01$) and also among group 3 in relation to group 2 on days 5 and 8 ($p<0.05$ and $p<0.01$, respectively).

The metabolic characteristics of the studied PCs during storage revealed that, the pH level was maintained above >6.8, with no significant difference between all groups all over the storage period. However, glucose and LDH results showed significantly lower levels among the aphaeresis group compared to the other groups ($p<0.01$) on all storage days. Also, significant high LDH levels were found among group 2 compared to group 1 on days 1,5 ($p<0.01$) and 8 ($p<0.05$).

Swirling scoring showed a non-significant difference between all groups on day 1, however, a highly significant lower score was reported in group 2 compared to the others groups on day 5 ($p<0.01$). On day 8, there was a significant high score in aphaeresis group compared to the first group and there was a significant lower score in group 2 compared to groups 1 and 3 ($p<0.01$).

Regarding PLT activation markers, intergroup comparison revealed significantly lower CD62p expression among the aphaeresis group compared to group 2 on day 1,5 and 8 ($p<0.05$, $p<0.01$ and $p<0.01$, respectively) and compared to group 1, only on day 8 ($p<0.01$). However, CD63 showed only significantly lower expression among the aphaeresis group compared to group 1 on day 8 ($p<0.01$). In addition, analysis of the results revealed significantly lower RANTES levels among the aphaeresis group compared to group 1 on all days and compared to group 2 on days 1 and 8 only ($p<0.01$).

Mean percent change of evaluated parameters among the 3 studied groups are were shown in Table (2). Correlation studies between WBC count versus PLT count, swirling, MPV, RANTES, CD62 and CD63 in different platelet products on day 8 of storage are were illustrated in Table (3), while between pH versus PLT count, swirling, MPV, LDH, Glucose, CD62, CD63 in different platelet products on day 8 of storage are were shown in Table (4).

No bacteriological growth was observed in all units within the studied groups neither in cultures performed on day 1 nor on day 8 which is actually considered as confirmatory culture to day 1.

Table (1): Storage changes as regards; PLT count, indices, residual WBC counts, metabolic parameters and mean CD62p and CD63 expression and RANTES levels.

	Donor	Day 1	Day 5	Day 8
PLT Count (x10^{10}/unit)				
Non-filtered PRP		6.30±1.43	4.98±0.96^{aa}	4.03±0.72^{aabb}
Filtered PRP		5.05±0.88	4.38±0.59^{aa}	4.23±0.73^{aa}
Apharesis PC		50.83±6.55	48.33±6.51^{aa}	47.42±7.69^{a}
MPV (fl)				
Non-filtered PRP	4.84±1.01	5.50±0.86**	6.85±0.68** ^{aa}	7.65±0.97**^{aabb}
Filtered PRP	5.19±0.59	6.18±0.65**	6.81±0.64** ^{aa}	7.57±0.86**^{aabb}
Apharesis PC	5.57±0.84	6.81±0.84**	7.56±0.87** ^{aa}	7.94±0.79**^{aabb}
PDW (fl)				
Non-filtered PRP	18.09±0.90	19.50±0.85**	20.35±0.97** aa	20.97±0.63**^{aab}
Filtered PRP	17.82±0.94	19.23±0.90**	20.35±0.97** aa	20.69±0.64**^{aa}
Apharesis PC	17.39±0.87	18.77±0.79**	19.66±0.68** aa	20.04±0.50**^{aab}
WBCs (x10^{6}/Unit)				
Non-filtered PRP		185.50±67.44	144.43±49.51^{aa}	113.87±39.66^{aabb}
Filtered PRP		0.67±0.41	0.57±0.37^{aa}	0.43±0.31^{aabb}
Apharesis PC		0.44±0.19	0.28±0.13^{aa}	0.17±0.07^{aabb}
Swirling score				
Non-filtered PRP		3.00±0.00	2.83±0.39	2.42±0.67^{ab}
Filtered PRP		3.00±0.00	2.08±0.29^{aa}	1.50±0.52^{aabb}
Apharesis PC		3.00±0.00	3.00±0.00	2.92±0.29
pH				
Non-filtered PRP		7.39±0.15	7.38±0.11	7.17±0.20^{aabb}
Filtered PRP		7.27±0.33	7.18±0.29	6.95±0.29^{aabb}
Apharesis PC		7.33±0.14	7.26±0.17	7.13±0.17^{aa}
Glucose (mg/dl)				
Non-filtered PRP		405.17±3.69	392.33±7.69^{aa}	361.25±26.79^{aabb}
Filtered PRP		409.42±32.40	399.67±44.68	379.92±54.62
Apharesis PC		344.53±29.93	299.67±39.07^{aa}	248.08±45.02^{aabb}
LDH (U/L)				
Non-filtered PRP		287.47±35.34	316.18±61.34	346.69±78.82^{ab}
Filtered PRP		387.23±85.47	419.90±95.13	442.15±108.45^{a}
Apharesis PC		171.69±61.42	179.31±58.58	188.35±68.58^{a}
CD62p (%)				
Non-filtered PRP	3.36±2.86	24.55±9.65**	37.78±12.45**^{aa}	51.38±12.47**^{aabb}
Filtered PRP	3.73±1.76	33.96±12.49**	43.78±9.50**^{aa}	46.73±13.16**^{aa}
Apharesis PC	2.54±1.08	24.75±6.09**	27.57±8.63**^{a}	29.58±7.83**^{aa}
CD63 (%)				
Non-filtered PRP	6.08±3.53	9.82±4.83**	15.01±6.97**^{aa}	24.64±8.69**^{aa}
Filtered PRP	4.97±2.59	15.38±12.33**	21.10±15.01**^{a}	25.10±15.63**^{aa}
Apharesis PC	6.81±2.61	13.47±3.87**	13.73±3.81**	15.30±4.69**^{ab}
RANTES (ng/ml)				
Non-filtered PRP	1.60±20.50	196.8±48.88**	241.4±53.32**^{aa}	314.73±100.86**^{aabb}
Filtered PRP	1.20±0.45	96.59±42.23**	117.9±49.73**^{aa}	168.54±58.85**^{aabb}
Apharesis PC	1.73±0.41	11.88±1.98**	130.2±60.43**^{aa}	205.92±44.02**^{aabb}

*$p \leq 0.05$; **$p < 0.01$ (relative to donor). $^{a}p \leq 0.05$; $^{aa}p < 0.01$ (relative to day 1). $^{b}p \leq 0.05$; $^{bb}p < 0.01$ (relative to day 5)

Table (2): Mean percent change of evaluated parameters between different studied groups.

	Non-filtered PRP-PCs (n = 12)	Filtered PRP-PCs (n =12)	Aphaeresis PCs (n =12)	F- value	*p*- value
Platelet count	-35.980	-16.34^{aa}	-11.150^{aa}	12.590	0.000
Swirling	-19.440	-50.000^{aa}	-2.780^{abb}	23.140	0.000
MPV	39.090	22.370^{aa}	16.590^{aa}	9.532	0.001
PDW	7.520	7.630	6.790	0.199	0.820
pH	-3.020	-4.470	-4.120	1.276	0.292
LDH	20.600	20.720	17.710	0.173	0.842
Glucose	-10.840	-7.210	-27.990^{aabb}	12.621	0.000
Residual WBCs	-38.620	-35.720	-62.050^{aab}	5.259	0.010
RANTES	59.860	74.490	1995.200^{aabb}	122.821	0.000
CD62	109.300	37.600^{aa}	48.120^{aa}	7.774	0.002
CD63	151.020	63.230^{a}	27.830^{aa}	8.585	0.001

Data are expressed as mean percent change. $^{a}p<0.05$; $^{aa}p<0.01$ relative to non-filtered PRP. $^{b}p<0.05$; $^{bb}p<0.01$ relative to filtered PRP. ^{a,b} = Significant difference. $^{aa.bb}$ = Highly significant difference.

Table (3): Correlation between WBCs counts/Unit versus PLT count, swirling, MPV, RANTES, CD62 and CD63 in different platelet products on day 8 storage.

	Non-filtered PC		Filtered PC		Aphaeresis PC	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value
Plt conc.	-0.634	0.027*	-0.601	0.039*	0.375	0.230^{NS}
Swirling	0.198	0.536^{NS}	-0.411	0.185^{NS}	0.322	0.307^{NS}
MPV	0.611	0.035*	0.442	0.150^{NS}	0.705	0.010*
RANTES	0.258	0.418^{NS}	0.196	0.542^{NS}	0.050	0.878^{NS}
CD62	0.111	0.731^{NS}	0.239	0.454^{NS}	0.683	0.014*
CD63	0.237	0.458^{NS}	0.702	0.011*	0.095	0.769^{NS}

r = correlation coefficient. *p*= p value. ^{NS} = Not significant (*p*> 0.05). # = Correlation was invalid.
* = Correlation is significant at the 0.05 level (2-tailed) (Significant correlation).
** = Correlation is significant at the 0.01 level (2-tailed) (Highly significant correlation).

Table (4): Correlation between pH versus Platelet count, swirling, MPV, LDH, Glucose, CD62, CD63 in different platelet products on day 8 storage.

	Non-filtered PC		Filtered PC		Aphaeresis PC	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value
Plt conc	0.254	0.426^{NS}	-0.201	0.530^{NS}	0.123	0.704^{NS}
Swirling	-0.407	0.189^{NS}	#	#	0.251	0.430^{NS}
MPV	-0.582	0.047*	0.103	0.750^{NS}	-0.511	0.090^{NS}
LDH	-0.355	0.258^{NS}	-0.182	0.570^{NS}	-0.400	0.198^{NS}
Glucose	-0.253	0.427^{NS}	-0.106	0.743^{NS}	-0.414	0.181^{NS}
CD62	-0.082	0.801^{NS}	-0.145	0.654^{NS}	-0.670	0.017*
CD63	-0.195	0.544^{NS}	-0.480	0.114^{NS}	-0.285	0.368^{NS}

r = correlation coefficient. *p* = *p*-value. ^{NS} = Not significant (p> 0.05). # = Correlation was invalid.
* = Correlation is significant at the 0.05 level (2-tailed) (Significant correlation).
** = Correlation is significant at the 0.01 level (2-tailed) (Highly significant correlation).

DISCUSSION

It has been reported that three fundamental quality standard parameters, namely PLT counts, PLT activation and metabolic alterations, must be considered for a proper evaluation of the effect of prolonging PLT shelf-life **[12]**.

The significant reduction in PLT counts at the studied time-points of storage, in the 3 groups, indicates an increase in platelet elimination with storage, which could be attributed to platelet senescence, as the platelets' life span is 7-10 days **[12]**. Fortunately, despite this decline in PLT counts in all groups, only the studied

aphaeresis units till day 8, were fulfilling the quality criteria of aphaeresis units (>24.0 x 10^{10}/unit) as mentioned by Vasconcelos et al. **[14]**.

Concurrently, on performing the intergroup comparison, we have estimated the mean percent change of studied parameters, assuming that it would eliminate the changeable irrelevant factors such as, variability of donor's criteria, baseline levels and discrepancy in units' volume among the studied groups. The observed higher mean percent reduction of PLT count in the non-filtered compared to other groups could be referred to their much higher leucocytic counts which in turn showed highly significant reduction particularly on day 8 compared to day 1. It seems likely, that lyses of WBCs probably resulted in release of their cytokines and proteolytic enzymes that affect platelet viability.

This is in accordance with the findings of Kaufman and his colleagues **[12]** who noticed that the quality of stored platelets could be improved by leucoreduction. It seems likely that stored platelets are exposed to proteolysis by enzymes released from leucocytes and from activated platelet themselves, such as metalloproteases **[16]**.

Nevertheless, on comparing both leucoreduced groups (filtered WBD-PCs versus aphaeresis PCs), it has been noticed that filtration had a negative effect on platelet yield. Herein, the mean platelet count had dropped from 7.55±0.65 x10^{10}/unit before filtration to 5.05±0.88 x10^{10}/unit after filtration (i.e. presenting 66.2±9.8% of the pre-filtration value). On the other hand, in the aphaeresis PCs, the leucoreduction was performed automatically during the collection and had no effect on platelet yield and was completely dependent on the previously programmed centrifugal separation of the aphaeresis device protocol.

PLT indices namely MPV and PDW, evaluated in conjunction with PLT counts, constitute further indicative parameters in assessment of the PC quality **[12]**. In our study, as part of PLT count analysis, MPV and PDW were recorded and showed significant increase on comparing days 5 and 8 versus day 1. These findings were the same in all groups, denoting that the effect of storage under the blood bank conditions was constant for all studied units. These changes in MPV and PDW values during storage were accounted for by the gradual change in platelet shape from discoid to a spherical shape **[14]**. Similarly, platelets derived from whole blood and aphaeresis procedures come in contact with various artificial surfaces that may promote changes in membrane lipids aggregation, microvesiculation and contact activation during collection, processing and storage **[17]**.

Derived data demonstrated that the aphaeresis group showed the least mean percent increase in MPV followed by the filtered group. It has to be mentioned that a mixture of small and large platelets may give a normal MPV but a high PDW, this being indicative of active platelet release and consequent unsuitability of the product. Taken together MPV and PDW can thus provide a more reliable description of the platelet volume distribution than if MPV is considered alone.

The pH measurement is considered a global indicator of the platelet environment, demonstrating the balance between platelet metabolism, bacterial contamination if present and the buffer capacity of the medium, with an acceptable range of 6.4-7.4 at 22°C in Europe and >6.2 in USA in order to retain platelet function **[18]**.

The current study recorded a significant decrease in pH level in both non-filtered and filtered-PRP-PCs, on day 8 versus days 1 and 5, while for the aphaeresis group, on day 8 versus day 1 only. This decrease in pH, which was within the acceptable range, could be attributed to the production of lactic acid and carbon dioxide by platelet metabolism during storage. However, the difference in pH levels between all 3 groups was insignificant even at all studied days of storage and the mean percent decrease in pH as well, was insignificantly different between them. This limitation in pH decline could be explained by the absence of bacterial contamination as demonstrated by negative culture in all studied PCs and by the fact that the quality of the storage containers allowed proper exchange of oxygen and CO2 between the outside air and the suspended platelets **[19]**.

In accordance to the prior study done by Singh and his colleagues in 2009 **[20]**, the higher leucocyte contamination in the non-filtered PCs

group included in our work, resulted in significant glucose consumption over time and consequently its concentration showed significant decrease on day 8 versus days 1 and 5. However, on comparing the 3 PCs groups, the aphaeresis PCs showed the lowest mean glucose concentration and within this same group, its mean concentration showed significant decrease on days 5 and 8 versus day 1 and in day 8 versus day 1. This gradual drop in glucose concentration could be attributed to its high cellular platelet compartment, which entails relatively higher glucose consumption during metabolism over the storage time. On the other hand and in concomitance with a prior report **[21]**, the glucose concentration showed insignificant drop among the filtered PCs group all over the eight days. This is possibly due to the lower platelet count in comparison with the aphaeresis group, and lower white blood cell count in comparison with the non-filtered PCs group.

Intergroup comparison of the mean LDH level revealed highly significant lower results among the aphaeresis PCs compared to the other 2 groups, meanwhile significant higher levels among the filtered-PRP-PCs compared to the non-filtered. These findings may be attributed to the process of filtration resulting in subsequent platelet damage and evidenced by lower post-filtration platelet yield. Measurement of LDH helps in evaluating the extent of cell damage for both platelets and leucocytes. Nevertheless, it has to be mentioned that the LDH cannot be considered an indicative marker of platelet status in the case of non-filtered-PRP-PCs, as the contaminant leucocytes have an effect, which cannot be neglected, and definitely contribute to remarkable LDH increase **[22]**.

Aphaeresis collected PCs included in this study, showed the best swirling score and the lowest mean percent change decrease during the storage period, in contrast with the single filtered-PRP-PCs, which showed the highest mean percent decline. However, it has to be mentioned that swirling phenomena in all groups were within the acceptable range till day 8 of storage. From these findings, we deduce that aphaeresis process had minimal effect on platelet viability, whereas, the filtration process had a negative impact on platelet viability; but it is yet to be confirmed whether it is reversible or irreversible in vivo.

It has to be mentioned that, loss of swirling is associated with major pH derangement, poor morphology and loss of platelet viability; it may also be considered a gross measure of apoptosis in the vast majority of platelets in PCs. Accordingly, it is a reliable index of subsequent poor platelet survival and function. However, it may be very sensitive when the irreversible damage affects fewer numbers of platelets in PCs **[9]**.

It has been demonstrated that, the extent of platelet activation depends mainly on methods of collection, processing and to a lesser extent on the duration of storage and the storage medium of PCs **[23]**. Upon activation, granule membrane proteins such as CD62p and CD63 are expressed on the external membrane of the platelet **[24]**. Our findings revealed a significantly lower CD62p expression among the aphaeresis PCs, most probably, because those aphaeresis units were collected, separated and leucoreduced with less handling procedures than the whole blood derived PCs. Also, on day 8 there was a highly significantly lower CD63 expression among the same aphaeresis PCs, which was consistent with the findings reported by Vassallo and Murphy **[25]**.

The current findings appear to be compatible with previous in vivo studies which have observed superior radiolabel recovery and post-transfusion increments for platelets derived from aphaeresis compared with platelet-rich plasma whole blood-derived platelets **[25,26]**. Moreover, CD62 expression has shown to be inversely correlated with the platelet count increment and recovery of platelets, so it may serve as a useful quality control measurement **[27]**.

As regards the platelet derived cytokine (RANTES) and in accordance with prior studies **[28,29]**, analysis of the results revealed highly significant low levels among the leucoreduced PCs compared to the non-filtered-PRP PCs at all studied storage days, together with a gradual significant increase in its levels over time among all collected PC units. It has been suggested in previous studies that under normal storage conditions mononuclear cells in PCs in particular monocytes, which are a major constituent of the leucocyte population, have the ability to synthesize and secrete cytokines including

RANTES for at least 5 days **[30]**. More and above, it has been noticed that the substantial accumulation of storage time dependent platelet-derived bioactive substances takes place in all PCs, presumably as a consequence of platelet activation or disintegration **[31]**.

Data derived from this study revealed that the 36 enrolled PCs, showed negative results for bacterial culture on the 1st and 8th day of storage. In fact, culture testing on day 8 can be considered as confirmatory to that of day 1, added to the acceptable limited metabolic biomarker changes, namely pH and glucose, and the maintained swirling pattern in all units. These findings support the concluded facts of 2 previous studies concerning sensitivity and rapidity of BACTEC system and which supported the feasibility of its performance for bacterial testing in PCs **[32,33]**.

To sum up, during the 8 days storage period, aphaeresis collected PC units were superior to the whole blood derived platelets whether filtered or non-filtered as evidenced by: Highest platelet count per unit, better viability of platelets with highest swirling score, least metabolic changes of the plasma media, and least expression of platelet activation markers namely CD62p and CD63. Taken in consideration, that PLT counts in both WBD-PCs groups were not fulfilling the standard quality criteria at the end of 8 days storage. However, despite the definite superiority in quality, aphaeresis units were costly. The procedure of donation using the aphaeresis device is safe, yet we found difficulties in recruitment of voluntary thrombocytapheresis donor as the time needed to complete the donation was long, and the dual needle technique adds to the donor fears of the process of donation.

Our choice between different methods of preparing PCs should actually depend on a critical balance between safety, quality and cost. To minimize outdating of PCs that are licensed now for a maximum of 5 days, we can conclude that PCs obtained by aphaeresis could provide the highest quality possible, when coupled with a good and rapid bacterial detection system to assure the sterility of PCs, kept at 22-24ºC for 8 days. Yet, studies in therapeutic efficacy in PLT products should be made to promote appropriate transfusion practice.

REFERENCES

1- Fabris F, Soini B, Sartori R, Randi ML, Luzzatto G, Girolami A. Clinical and laboratory factors that affect the post-transfusion platelet increment. Transfus Sci. 2000; 23: 63-68.

2- Verma A, Agarwal P. Platelet utilization in the developing world: Strategies to optimize platelet transfusion practices. Transus Apher Sci. 2009; Oct, 41 (2): 145-149.

3- Bodensteiner DC, Doolittle GC. Adverse haematological complications of anticancer drugs. Clinical presentation, management and avoidance. Drug Saf. 1993; Mar, 8 (3): 213-224.

4- Slichter SJ. Platelet transfusion therapy. Hematol Oncol Clin North Am. 2007; Aug, 21 (4): 697-729.

5- Gulliksson H. Platelet storage media. Transfus Apher Sc. 2001; 24: 241-244.

6- Wildt-Eggen J, Schrijiver JG, Bins M, Gulliksson H. Storage of platelets in additive solution: effect of magnesium and/or potassium. Transfusion. 2002; 42: 76-80.

7- Ezuki S, Kanno T, Ohto H, Herschel L, Ito T, Kawabata K, Seino O, Ikeda K, Nollet KE. Survival and recovery of aphersis platelets stored in a polyolefin container with high oxygen permeability. Vox Sang. 2008; May, 94 (4): 292-298.

8- Venglen-Tyler V, ed. Leukoreduction of RBC and platelet units. American Association of Blood Banks. 1996; 722-725.

9- Arnold DM, Heddle NM, Kulczycky M, Carruthers J, Sigouin C, Blajchman MA. In vivo recovery and survival of aphersis and whole blood-derived platelets: a paired comparison in healthy volunteers. Transfusion. 2006; Feb, 46 (2): 257-264.

10- Kishimoto T, Goyert S, Kikutani H, Mason D, Miyasaka M, Moretta L, Ohno T, Okumura K, Shaw S, Springer TA, Sugamura K, Sugawara H, von dem Borne AE, Zola H. Update of CD antigens. J Immunol. 1996; Apr 1, 158 (7): 3035-3036.

11- Fischer FR, Luo Y, Luo M, Santambrogio L, Dorf ME. RANTES-induced chemokine cascade in dendritic cells. J Immunol. 2001; Aug 1, 167 (3): 1637-1643.

12- Singh H, Chaudhary R, Ray V. A platelet index as quality markers of platelet concentrates during storage. Clin Lab Haematol. 2003; Oct, 25 (5): 307-310.

13- Shanwell A, Diedrich B, Falker C, Jansson B, Sandgren P, Sundkvist L, Svensson L, Vesterinen M, Gulliksson H. Paired in vitro and in vivo comparison of aphersis platelet concentrates stored in platelet additive solution for 1 versus 7 days. Transfusion. 2006; Jun, 46 (6): 973-979.

14- Vasconcelos E, Figueiredo AC, Seghatchian J. Quality of platelet concentrates derived by platelet rich plasma, buffy coat and Apheresis. Transfus Apher Sci. 2003; Aug, 29 (1): 13-16.

15- Kaufman J, Spinelli SL, Schultz E, Blumberg N and Phipps RP. Release of biologically active CD154

during collection and storage of platelet concentrates prepared for transfusion. J Thromb Haemost. 2007; Apr, 5 (4): 788-796.

16- Santos-Martinez MJ, Medina C, Jurasz P, Radomski MW. Role of metalloproteinase in platelet function. Thromb Res. 2008; 121 (4): 535-542.

17- Seghatchian J. Platelet storage lesion: An update on the impact of various leukoreduction processes on the biological response modifiers. Tranfus Apher Sci. 2006; Feb, 34 (1): 125-130.

18- Yuasa T, Ohto H, Yasunaga R, Kai T, Shirahama N, Ogata T. Improved extension of platelet storage in a polyolefin container with higher oxygen permeability. Br J Haematol. 2004; Jul, 126 (1): 153-159.

19- Dijkstra-Tiekstra MJ, Kuipers W, Setroikromo AC, de Wildt-Eggen J. Overnight or fresh buffy coat-derived platelet concentrates prepared with various platelet pooling systems. Transfusion. 2008; Apr, 48 (4): 723-730.

20- Singh RP, Marwaha N, Malhotra P, Dash S. Quality assessment of platelet concentrates prepared by platelet rich plasma-platelet concentrate, buffy coat poor-platelet concentrate (BC-PC) and aphersis-PC methods. Asian J Transfus Sci. 2009; Jul, 3 (2): 86-94.

21- Reikvam H, Marschner S, Apelseth TO, Goodrich R Hervig T. The Mirasol Pathogen Reduction Technology system and quality of platelets stored in platelet additive solution. Blood Transfus. 2010; Jul, 8 (3): 186-192.

22- Shooshtari MM, Pourfathollah AA. Supply of donated blood in the Islamic Republic of Iran. East Mediterr Health. 2007; Jul-Aug, 13 (4): 980-981.

23- Dumont LJ, AuBuchon JP, Whitley P, Herschel LH, Johnson A, McNeil D, Sawyer S, Roger JC. Seven-day storage of single-donor platelets: Recovery and survival in an autologous transfusion study. Transfusion. 2002; Jul, 42 (7): 847-854.

24- Dijkstra-Tiekstra MJ, Pietersz RN, Huijgens PC. Correlation between the extent of platelet activation in platelet concentrates and in vitro and in vivo parameters. Vox Sang. 2004; Nov, 87 (4): 257-263.

25- Vassallo RR, Murphy S. A critical comparison of platelet preparation methods. Curr Opin Hematol. 2006; Sep, 13 (5): 323-330.

26- Rock G, Neurath D, Cober N, Freedman N, Leduc M, Nelson E, Lu M. Seven-day storage of random donor PLT concentrates. Transfusion. 2003; Oct, 43 (10): 1374-1377.

27- Andreu G, Vasse J, Sandid I, Tardivel R, Semana G. Use of random versus apheresis platelet concentrates. Transfus Clin Biol. 2007; Dec, 14 (6): 514-521.

28- Apelseth TO, Hervig TA, Wentzel-Larsen T, Bruserud O. Cytokine accumulation in photochemically treated and gamma-irradiated platelet concentrates during storage. Transfusion. 2006; May, 46 (5): 800-810.

29- Wadhwa M, Seghatchian MJ, Dilger P, Sands D, Krailadisiri P, Contreras M, Thorpe R. Cytokines in WBC-reduced apheresis PCs during storage: A comparison of two WBC-reduction methods. Transfusion. 2000; Sep, 40 (9): 1118-1126.

30- Grellier P, Benach J, Labaied M, Charneau S, Gil H, Monsalve G, Alfonso R, Sawyer L, Lin L, Steiert M, Dupuis K. Photochemical inactivation with amotosalen and long-wavelength ultraviolet light of Plasmodium and Babesia in platelet and plasma components. Transfusion. 2008; Aug, 48 (8): 1676-1684.

31- Zaki Mel S, Fouda M, Sharaf Eldeen OA, Zaghloul MH. Extracellular accumulation of bioactive substances; interleukin-1beta (IL-1ß) and plasminogen activator inhibitor-1 (PAI-1) in stored blood units and relation to bacterial contamination. Hematology. 2006; Aug, 11 (4): 301-305.

32- Dunne WM Jr, Case LK, Isgriggs L, Lublin DM. In-house validation of the BACTEC 9240 blood culture system for detection of bacterial contamination in platelet concentrates. Transfusion. 2005; Jul, 45 (7): 1138-1142.

33- Riedel S, Siwek G, Beekmann SE, Richter SS, Raife T, Doern GV. Comparison of the BACTEC 9240 and BacT/Alert blood culture systems for detection of bacterial contamination in platelet concentrates. J Clin Microbiol. 2006; Jun, 44 (6): 2262-2264.

Journal of the Egyptian Society of Haematology & Research, Vol. 7, No. 1, March: 11-16, 2011

Analysis of G71D Mutation of HAMP Gene and H63D Mutation of HFE Gene in ß-Thalassemia Major Patients

HEBA H. ABOU-ELEW, M.D.[1]; SAMAH ABD EL-HAMID, M.D.[1]; RANIA I.M. GODA, M.D.[2]; RANIA FAWZY, M.D.[1]; SHADIA RAGAB, M.D.[3]; NAGWA ABDALLAH, M.D.[4]; AMIRA FARHAN, M.Sc.[3] and HANAA R. MOHAMED, M.D.[3]

The Departments of Clinical & Chemical Pathology[1], Pediatrics[2], Kasr El-Aini Faculty of Medicine, Cairo University, Clinical & Chemical Pathology[3] and Pediatrics[4], National Research Center

ABSTRACT

Background: Hepcidin encoded by HAMP gene plays a key role in modulating iron absorption in ß-thalassemia. Hepcidin deficiency due to either mutations in HFE gene encoding the hemochromatosis protein (HFE) or in the HAMP gene have been implicated in iron overload.

Aim of the Work: To establish the presence of G71D mutation of HAMP gene and H63D mutation of HFE gene in ß-thalassemia major patients as well as to assess their impact on iron overload in these patients.

Material and Methods: This study included 52 ß-thalassemia major patients and 46 age- and sex- matched healthy controls. Genotyping of G71D of HAMP and of H63D of HFE variants was performed by polymerase chain reaction-restriction fragment length polymorphism analysis. Estimation of iron overload was based on serum ferritin and transferrin saturation.

Results: Among the studied ß-thalassemia patients, 30 (57.7%) carried the wild-type profile, 13 (25%) carried G71D mutation of HAMP gene, 12 (23.1%) carried the H63D mutation of HFE gene and 3 (5.8%) carried both mutations. Both HAMP-G71D and HFE-H63D mutations observed among patients were in the heterozygous condition. Patients with either HAMP-G71D or HFE-H63D variants did not show significant difference in iron overload parameters in relation to wild-type patients.

Conclusion: The G71D mutation of HAMP gene and H63D mutation of HFE gene are common variants detected in about one fourth of the studied ß-thalassemia major patients. Neither the HAMP-G71D mutation nor the HFE-H63D mutation is a major determinant of iron overload in patients with ß-thalassemia major.

Key Words: *ß-thalassemia major – HAMP – G71D – HFE – H63D.*

INTRODUCTION

Hepcidin, encoded by HAMP gene, is a 25 amino acid peptide that, in addition to being involved in innate immunity **[1]**, appears to play a crucial role in iron homeostasis in humans, regulating both iron absorption from the intestine and its recycling by macrophages **[2-5]**. Hepcidin is down-regulated by erythropoiesis **[2]**, anemia, and hypoxia **[3]**, whereas it is up-regulated by iron overload **[4]** and inflammation **[3,5-7]**. Hepcidin deficiency due to either mutations in HFE gene encoding the hemochromatosis protein (HFE) or in the HAMP gene is the cause of iron overload in most forms of hereditary hemochromatosis (HH) **[8]**. Furthermore, hepcidin deficiency is the main or contributing factor of iron overload in iron-loading anemias such as ß-thalassemia **[9]**.

In ß-thalassemia major, transfusions rather than dietary iron absorption are the predominant cause of iron overload. In chronically transfused patients, hepcidin concentrations are significantly higher than in nontransfused patients, presumably due to both increased iron load and the alleviation of ineffective erythropoiesis. However, hepcidin concentrations decrease in the intervals between transfusions, as the effect of each transfusion wears off **[10-12]**. During those periods, decreased hepcidin and the resulting increase in intestinal iron absorption may be significant contributors to patients' iron load **[9]**.

A missense mutation in HAMP gene that leads to substitution of glycine 71 by aspartic acid (G71D) due to a G→A substitution at nucleotide 212 in exon 3 changes the charge of amino acid 71 and is likely to affect the activity of hepcidin **[13]**. H63D is a variant of the HFE

gene characterized by a G→C change at nucleotide 187 that results in a change in histidine at position 63 to aspartic acid. This mutation alters dietary iron absorption **[14]**. The interaction of the mutations of genes influencing iron homeostasis with thalassemias may have a synergistic effect, increasing the iron absorption and storage **[15,16]**. Knowledge of mutation prevalence of the genes influencing iron overload would ensure preventive treatment for iron overload. The aim of the present study was to establish the presence of G71D mutation of HAMP gene and H63D mutation of HFE gene in ß-thalassemia major patients as well as to assess their impact on iron overload in these patients.

MATERIAL AND METHODS

Subjects:

This study included 52 ß-thalassemia major cases regularly attending the Hematology Clinic of the New Children Hospital, Cairo University and 46 healthy age- and sex-matched control subjects. Patients' age ranged from 2 to 25 years with a mean age of 12 years. They were 23 (44.2%) males and 29 (55.8%) females. The age of the control subjects ranged between 2 and 23 years with a mean age of 9.4 years. They were 28 males and 18 females. The diagnosis of ß-thalassemia was based on clinical presentation, hematological indices, iron overload and hemoglobin electrophoresis. For each subject of patients and controls, 4mL peripheral venous blood was collected in EDTA vials for molecular studies, and 2mL of blood without anticoagulant was collected for evaluation of iron overload parameters. An informed consent approved by the institutional Ethics Committee was obtained from all participants or their parents.

Clinical parameters:

All patients were on regular blood transfusion; 19/52 (36.5%) patients received 50 or less transfusions per life (ranging from 12 to 49 and median of 25) while 33/52 (63.5%) patients received more than 50 transfusions in life (ranging from 52 to 294 and median of 120).

The studied patients experienced thalassemia-related complications in the form of skull deformities and mongoloid facies in 45/52 (86.5%) and bony aches in 30/52 (57.6%). Hepatitis C virus infection was detected in 16/52 (30.8%) patients and hepatitis B virus infection in 1/52 (1.9%) patient. No cardiovascular or endocrinal complications were reported in any of the studied patients.

All patients were on iron chelation therapy: 26/52 (50%) patients received oral deferiprone alone, 24/52 (46%) patients were on S.C. desferrioxamine therapy and 2/52 (4%) patients received combined desferrioxamine and deferiprone treatment. Compliance to iron chelation therapy was verified in 25/52(48%) of chelated patients whereas 27/52 (52%) received irregular iron chelation.

Iron overload parameters:

Iron profile was assessed for both ß–thalassemia patients and controls. Serum iron and total iron binding capacity (TIBC) were measured colorimetrically and serum transferrin saturation was calculated. Serum ferritin was determined by Microparticle Enzyme Immunoassay (AxSYM, Abbott, USA) after an overnight fast.

Genotypic analysis:

Genomic DNA was extracted from peripheral blood leukocytes by QIAamp DNA Blood Mini Kit (#51104, QIAGEN). Genotyping of G71D of HAMP and of H63D of HFE variants was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis according to Merryweather-Clarke et al. **[13]** and Feder et al. **[17]**, respectively. For each individual, we systematically amplified two PCR fragments surrounding both mutations in two separate reactions, using the following pairs of primers: for G71D mutation; sense primer 5'-ATGCAGGGAGGTGTGTTA GGAGGCT- 3' and antisense primer 5'-TGCAAGGC-AGGGTCAGGACAAGCTCTT AGC- 3', for H63D mutation; sense primer 5'-ACATGGTTAA-GGCCTGTTGC-3' and antisense primer 5'-GCCACATCTGGCTTG AAATT-3'. PCR was performed in reactions containing 3µL of extracted DNA in the presence of 1µL of each primer (10 pmole/µL), 12.5µL of Taq PCR Master Mix (QIAGEN) and 7.5µL nuclease-free water in a total volume of 25µL. The PCR conditions consisted of an initial melting temperature of 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 1 minute. A final extension step of 10 minutes at 72°C terminated the reaction.

The sizes of the amplified fragments were 714bp for G71D (exons 2 and 3) and 207bp for H63D (exon 2). Ten microliters of the amplified products was subjected to separate digestion with 10 units of Aci I (FastDigest® AciI, #FD1794 - Fermentas Life Sciences) for the G71D mutation at 37°C for 15 minutes, as well with10 units of Bcl I (#R0160S - New England Biolabs) for the H63D substitution at 50°C for 1 hour, according to the manufacturer's recommendations. The digested products were then run on a 2.5% agarose gel for 1 hour and photographed under UV light (Fig. 1).

Statistical methods:

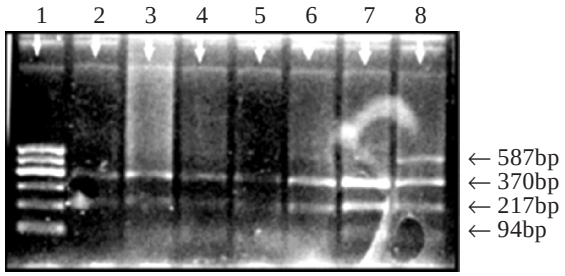
Statistical calculations were done using computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows. Comparison of numerical variables was done using Student *t*-test in comparing 2 groups when normally distributed and Mann Whitney U test when not normally distributed. For comparing categorical data, Chi square (χ^2) test was performed. Multivariate analysis model was used to test for the preferential effect of important variable(s) on ferritin level. *p*-values less than 0.05 was considered statistically significant **[18]**.

RESULTS

Among the studied ß-thalassemia patients, 30 (57.7%) carried the wild-type profile, 13 (25%) carried G71D mutation of HAMP gene, 12 (23.1%) carried the H63D mutation of HFE gene and 3 (5.8%) carried both mutations. Both HAMP-G71D and HFE-H63D mutations observed among patients were in the heterozygous condition. The allelic frequency of G71D and H63D variants among the studied patients were 12.5% and 11.5%, respectively (Table 1).

Of the healthy controls studied, 37/46 (80.4%) carried a wild-type genetic profile in both genes, 6/46 (13.0%) had a variation in HAMP-G71D, 4/46 (8.7%) in HFE-H63D, and 1/46 (2.2%) in both. Both HAMP-G71D and HFE-H63D mutations observed in the control group were in the heterozygous condition. The allelic frequency of G71D and H63D variants among controls were 6.5% and 4.3%, respectively. Compared to controls, ß-thalassemia patients showed borderline significant higher frequency of H63D mutation (23.1% vs. 8.7%, p=0.062). No statistically significant difference in gene frequencies of G71D mutation was observed between studied patients and controls (Table 1).

A: G71D mutation



B: H63D mutation

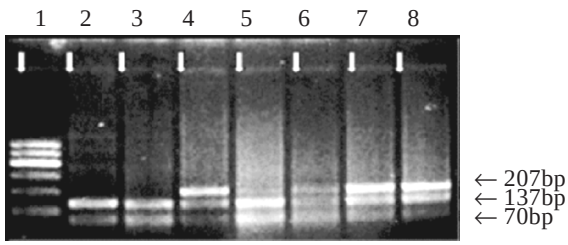


Fig. (1): Agarose gel electrophoresis of PCR fragments digested by restriction enzymes. (A) Diagnosis of G71D mutation: Aci I digestion of a 714bp PCR product containing HAMP exons 2 and 3. Wild-type digestion product sizes are 370, 217, 94 and 33bp; digestion products from the mutant allele are 587, 94 and 33bp. Lane 1: PCR marker of 100bp, Lanes 2, 3, 4, 5, 6, 7: Wild type individuals, Lane 8: heterozygous individual. (B) Diagnosis of H63D mutation: Bcl I digestion of a 207bp PCR product containing HFE exon 2. Wild-type digestion product sizes are 137 and 70bp. The mutation abolishes the restriction site. Lane 1: PCR marker of 100bp, Lanes 2, 3, 5 wild type individuals, Lanes 4, 6, 7, 8 heterozygous individuals.

Table (1): Comparison of genotype and allele frequencies of G71D and H63D mutations between ß-thalassemia patients and controls.

Mutation	No. / Genotype frequency (%)		*p* value	Allele frequency (%)
	Wild-type	Heterozygous		
G71D:				
Thalassemic patients (n=52)	39 (75.0)	13 (25.0)	0.200	12.5
Controls (n=46)	40 (86.0)	6 (13.0)		6.5
H63D:				
Thalassemic patients (n=52)	40 (76.9)	12 (23.1)	0.062	11.5
Controls (n=46)	42 (91.3)	4 (8.7)		4.3

$p<0.05$ is statistically significant.

Patients carrying either HAMP-G71D or HFE-H63D variants did not show statistically significant difference in iron overload parameters in relation to wild-type patients (Table 2).

Table (2): Comparison of iron profile parameters between wild-type patients and patients with HAMP-G71D variant or patients with HFE-H63D variant.

ß-Thalassemia patients	Transferrin saturation (%)	Serum ferritin (ng/mL)
G71D variant:		
Wild-type patients (n=39)	79.3±18.7	2406.7±1214.5
Heterozygous patients (n=13)	77.8±20.9 (p=0.775)	2376.8±1165.4 (p=1.00)
H63D variant:		
Wild-type patients (n=40)	78.7±19.7	2295.5±1260.8
Heterozygous patients (n=12)	79.9±17.6 (p=0.853)	2744.8±878.6 (p=0.140)

Statistical analyses are all related to the wild-type group, $p<0.05$ is statistically significant.

Multivariate regression analysis was done to reveal the independent association of factors that may significantly affect serum ferritin level among the study sample. It included age of onset of disease, number of transfusions in life, compliance to iron chelation therapy, presence of HCV antibody, presence of G71D mutation of HAMP gene, presence of H63D mutation of HFE gene and number of mutations harbored by the studied patients. Analysis revealed that only the number of blood transfusions per life significantly increases serum ferritin level (p=0.003).

Table (3): Multivariate regression analysis of independent factors that may affect serum ferritin level among the studied patients.

Variable	Coefficient	p-value	95% Confidence Interval	
			Upper limit	Lower limit
Age at onset	469.200	0.149	–174.433	1,112.834
No. of Transfusions/life	7.753	0.003*	2.864	12.643
Compliance to chelation	184.672	0.655	–641.102	1,010.446
HCV Ab	436.714	0.395	–587.958	1,461.386
H63D of HFE	400.658	0.570	–1,009.060	1,810.375
No. of mutations	502.243	0.286	–433.792	1,438.279

*p-value <0.05 is statistically significant.

DISCUSSION

In the present study, we analyzed the frequency of G71D mutation of HAMP gene and H63D mutation of HFE gene in 52 TM patients and 46 control subjects. Both mutations were found in about one fourth of the studied TM patients in a heterozygous condition. Their allelic frequencies were 12.5% and 11.5% for G71D and H63D variants, respectively. A lower frequency of both mutations was observed among control group (13.0% and 8.7% for G71D and H63D, respectively) with a borderline statistically significant difference regarding H63D mutation compared to the studied patients (p=0.062).

The frequency of G71D mutation of HAMP gene detected in the present study is higher than that reported by earlier studies. G71D mutation of HAMP gene was detected in the general north European population at an allele frequency of 0.3% **[13]** and has been identified in France **[19]**, Italy **[20]** and UK **[13]**. The H63D mutation has a prevalence of approximately 16% in the European population **[17,21]**. In accordance to our results, earlier Egyptian studies reported that the allele frequency of H63D mutation ranged from 13 to 30% in thalassemic patients and between 10 and 11% in controls **[22,23]**.

In the current study, the presence of either G71D mutation of HAMP gene or H63D mutation of HFE gene did not seem to influence iron overload in ß-thalassemia major patients. The functional relevance of the G71D amino acid substitution is not clear. However, it must be emphasized that this missense mutation is lo-

cated between 4 of the 8 structural cysteines of the 25-amino acid mature hepcidin peptide, and that the change of a neutral amino acid to an acidic residue frequently leads to crucial protein structure modifications **[19]**. Conflicting results were reported regarding the impact of this mutation as a possible modifier in iron overload diseases **[13,19,20,24]**.

It has been reported that the H63D mutation has an impact on iron overload in patients with beta thalassemia trait and thalassemia major **[25-27]**. Nevertheless, among the studied TM patients, no statistically significant difference in iron overload parameters was found between patients carrying HFE-H63D variant and wild-type patients. In literature, the significance of H63D was uncertain. H63D homozygosity has been found in association with an iron overload genotype and a study in mice showed that H63D mutation altered the normal HFE pathway, increasing iron overload **[28]**. Our data are in agreement with different groups who reported that the presence of H63D heterozygous state does not influence iron overload in ß-thalassemia major or minor **[29,30]**. In contrast, when the H63D mutation is found in the homozygote state, it may influence the ferritin levels of ß-thalassemia carriers **[31]**.

Multivariate regression analysis of independent factors that may significantly affect serum ferritin level was done in the present study and revealed that only the number of blood transfusions taken in life significantly increased serum ferritin level (p=0.003). Iron overload is an inevitable consequence of regular blood transfusion and can be seen after only 10-20 transfusions in patients with thalassemia **[32]**. The number of mutations harbored by the studied patients whether single or double mutations did not affect serum ferritin level in the multivariate regression model (p=0.286). This contrasts the hypothesis proposed by Duca and colleagues who suggested that the iron burden could be aggravated by the co-existence of mutations in HFE and HAMP genes in ß-thalassemia major patients poorly responsive to chelation therapy **[33]**.

Serum ferritin evaluation is a simple and convenient approach for assessment of iron overload that is widely used in clinical practice. Limitation of our study was inability to assess liver iron concentration (LIC) which remains the reference standard for determining iron load in patients at risk of iron overload. Many studies have assessed the correlation between serum ferritin levels and LIC, demonstrating a good correlation in patients with thalassemia major, mainly at lower LIC values **[34,35]**.

Conclusion:

The G71D mutation of HAMP gene and H63D mutation of HFE gene are common among ß-thalassemia major patients. Neither the HAMP-G71D mutation nor the HFE-H63D mutation is a major determinant of total body iron status in patients with ß-thalassemia major. The frequent occurrence of ß-thalassemia major and HAMP-G71D and HFE-H63D gene mutations raises the possibility of genetic interactions and emphasizes the value of screening for other HAMP and/or HFE mutations in thalassemias to modify treatment modalities of iron overload.

REFERENCES

1- Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science. 2004; 306: 2090-2093.

2- Pak M, Lopez MA, Gabayan V, et al. Suppression of hepcidin during anemia requires erythropoietic activity. Blood. 2006; 108: 3730-3735.

3- Nicolas G, Chauvet C, Viatte L, et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest. 2002; 110: 1037-1044.

4- Pigeon C, Ilyin G, Courselaud B, et al. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. J Biol Chem. 2001; 276: 7811-7819.

5- Nemeth E, Valore EV, Territo M, et al. Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. Blood. 2003; 101: 2461-2463.

6- Nemeth E, Rivera S, Gabayan V, et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest. 2004; 113: 1271-1276.

7- Wrighting DM, Andrews NC. Interleukin-6 induces hepcidin expression through STAT3. Blood. 2006; 108: 3204-3209.

8- Le Gac G, Férec C. The molecular genetics of haemochromatosis. Eur J Hum Genet. 2005; 13: 1172-1185.

9- Nemeth E. Hepcidin in beta-thalassemia. Ann N Y Acad Sci. 2010; 1202: 31-35.

10- Kearney SL, Nemeth E, Neufeld EJ, et al. Urinary hepcidin in congenital chronic anemias. Pediatr. Blood Cancer. 2007; 48: 57-63.

11- Nemeth E, Ganz T. Hepcidin and iron-loading anemias. Haematologica. 2006; 91: 727-732.

12- Origa R, Galanello R, Ganz T, et al. Liver iron concentrations and urinary hepcidin in beta-thalassemia Haematologica. 2007; 92: 583-588.

13- Merryweather-Clarke AT, Cadet E, Bomford A, et al. Digenic inheritance of mutations in HAMP and HFE results in different types of haemochromatosis. Hum Mol Genet. 2003; 12 (17): 2241-2247.

14- Bomford A. Genetics of haemochromatosis. Lancet 2002; 360: 1673-1681.

15- Rees DC, Singh BM, Luo LY, et al. Nontransfusional iron overload in thalassemia. Association with hereditary hemochromatosis. Ann N Y Acad Sci. 1998; 850: 490-494.

16- Jazayeri M, Bakayev V, Adibi P, et al. Frequency of HFE gene mutations in Iranian beta-thalassaemia minor patients. Eur J Haematol. 2003; 71: 408-411.

17- Feder JN, Gnirke A, Thomas W, et al. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nat Genet. 1996; 13 (4): 399-408.

18- Saunders DB, Trapp GR. Basic and Clinical Biostatistics. 3rd ed. Connecticut: Appleton & Lang. 2001.

19- Jacolot S, Le Gac G, Scotet V, et al. HAMP as a modifier gene that increases the phenotypic expression of the HFE pC282Y homozygous genotype. Blood. 2004; 103: 2835-2840.

20- Biasiotto G, Roetto A, Daraio F, et al. Identification of new mutations of hepcidin and hemojuvelin in patients with HFE C282Y allele. Blood Cells Mol Dis. 2004; 33: 338-343.

21- Lyon E, Frank E. Hereditary hemochromatosis since discovery of the HFE gene. Clin Chem. 2001; 47: 1147-1156.

22- El-Rashidi FH, Elshafey AE, Ragab SM, et al. Haemochromatosis gene mutation H63D is a risk factor for iron overload in egyptian beta-thalassemic children. Egypt. J Med Hum Genet. 2008; 9 (2): 149-59.

23- Madani HA, Afify RA, Abd El-Aal AA, et al. Role of HFE gene mutations on developing iron overload in beta-thalassaemia carriers in Egypt. East Mediterr Health J. 2011; 17 (6): 546-551.

24- Altès A, Bach V, Ruiz A, et al. Mutations in HAMP and HJV genes and their impact on expression of clinical hemochromatosis in a cohort of 100 Spanish patients homozygous for the C282Y mutation of HFE gene. Ann Hematol. 2009; 88 (10): 951-955.

25- Fargion S, Sampietro M, Cappellini MD, et al. Thalassemias and their interaction with hemochromatosis. In: Barton JC,Wards CQ (eds) Hemochromatosis: genetics pathophysiology, diagnosis and treatment. Cambridge University Press, Cambridge, United Kingdom. 2000; pp 435-441.

26- Guillermo J Ruiz-Argüelles. Heterozygosity for the H63D mutation in the hereditary hemochromatosis (HFE) gene may lead into severe iron overload in b-thalassemia minor: Observations in a thalassemia kindred. La Revista de Investigacion Clinica. 2001; 53: 117-120.

27- Melis MA, Cau M, Deidda F, et al. H63D mutation in the HFE gene increases iron overload in beta thalassemia carriers. Haematologica. 2002; 87: 242-246.

28- De Diego C, Opazo S, Murga MJ, et al. H63D homozygotes with hyperferritinaemia: Is this genotype, the primary cause of iron overload? Eur J Haematol. 2006; 78: 66-71.

29- Piperno A, Mariani R, Arosio C, et al. Haemochromatosis in patients with beta-thalassaemia trait. Br J Haematol. 2000; 111: 908-914.

30- Yamsri S, Sanchaisuriya K, Fucharoen S, et al. H63D mutation of the hemochromatosis gene and serum ferritin levels in Thai thalassemia carriers. Acta Haematol. 2007; 118: 99-105.

31- Politou M, Kalotychou V, Pissia M, et al. The impact of the mutations of the HFE gene and of the SLC11A3 gene on iron overload in Greek thalassemia intermedia and beta(s)/beta(thal) anemia patients. Haematologica. 2004; 89 (4): 490-492.

32- Porter JB. Practical management of iron overload. Br J Haematol. 2001; 115: 239-252.

33- Duca L, Delbini P, Nava I, et al. Hepcidin mutation in a beta-thalassemia major patient with persistent severe iron overload despite chelation therapy. Intern Emerg Med. 2010; 5 (1): 83-85.

34- Telfer PT, Prestcott E, Holden S, et al. Hepatic iron concentration combined with long-term monitoring of serum ferritin to predict complications of iron overload in thalassaemia major. Br J Haematol. 2000; 110: 971-977.

35- Olivieri NF, Brittenham GM, Matsui D, et al. Iron-chelation therapy with oral deferiprone in patients with thalassemia major. N Engl J Med. 1995; 332: 918-922.

Journal of the Egyptian Society of Haematology & Research, Vol. 7, No. 1, March: 17-23, 2011

A Study of Potassium Channel Genes Human Ether a-go-go (heag) and Human ether a-go-go Related Gene (HERG) in Acute Leukemia: Potential Role as a Risk Factor and Impact on Response to Therapy

RANIA M. GAWDAT, M.D.* and RAAFAT M. ABDELFATTAH, M.D.**

The Departments of Clinical & Chemical Pathology, Beni Suef University and Clinical Oncology, National Cancer Institute, Cairo University***

ABSTRACT

Background: Human ether a-go-go (heag) and Human ether a-go-go related gene (HERG) are two K+ channel encoded genes, have been related to cancer biology, especially in solid tumors while few studies demonstrate their role in leukemia.

Objectives: Our main aim was to detect the expression of heag mRNA in de novo acute myeloid leukemia (AML) and HERG mRNA in de novo acute leukemia patients, focusing on their prevalence as a risk factor for acute leukemia (AL), their association to FAB subtypes and their response to induction chemotherapy.

Patients and Methods: The study included 84 newly diagnosed acute leukemia patients (64 AML and 20 ALL cases) with 20 healthy individuals as a control group using Reverse Transcriptase-Polymerase Chain Reaction.

Results: The mRNA of HERG was detected in 32/64 (50%) of AML patients and 7/30(35%) of the acute lymphocytic leukemia (ALL) while heag was present in 11/44 (25%) of the AML patients. The expression of both genes were absent in the control group. The highest poor clinical outcome rates were achieved in HERG + patients while the clinical response to the initial chemotherapy was not affected by the presence of heag.

Conclusion: Our findings indicate that HERG and heag genes have an oncogenic potential indicating that they may be necessary for leukemic proliferation. HERG+ is important for risk estimation and prognosis of acute leukemia, while heag gene plays an important role in the pathogenesis of AML.

Key Words: *K+ ion channels – Human ether a go-go gene – Human ether a go-go related gene – Acute leukemia.*

INTRODUCTION

Voltage-gated Potassium K+ channels comprise the largest family of ion channels encoded by ~300 genes with phenotypic diversity. They are not just the main determinants of the cell resting membrane potential, but have also been linked to cell volume control, cell cycle progression, and cardiac repolarization **[1]**.

The ether a-go-go (EAG) family is one of the voltage gated K+ channels that comprises three subfamilies: Human ether a-go-go (heag), Human ether a-go-go related gene (HERG) and ether a-go-go like gene (ELK) **[2]**.

heag channels are expressed exclusively in brain, slightly in placenta and transiently in fusing myoblasts **[3,4]**. The function of heag channels in neurotransmitter release at the neuromuscular junction to initiate action potential in Drosophila melanogaster larvae is well known but their physiological function is unknown in mammals **[5,6]**.

HERG channels are more widely expressed and their functions differ according to their localization. They have a dominant presence in normal human myocardium where being involved in the repolarization phase of the cardiac action potential **[2]**.

In recent years, the role of several voltage gated K+ channels had been described in plethora of malignancies, especially of heag and HERG channel genes. However it is not clear whether these channels play causal role in oncogenesis or whether the oncogenic process result in aberrant expression and activation of EAG families **[7]**.

Several theories have been advanced as how heag and HERG channels could promote malignant transformation. One of these explanations is that cancer cell is more depolarized than normal cell. Accordingly, the overexpression of K+ channels on cell membrane results in hyperpolarization which evokes calcium ions influx resulting in increased transition of cells through G1/S phase of the cell cycle, thereby facilitating cell cycle progression **[8]**.

Another explanation revealed that the presence of hypoxia enables the channels to release hypoxia inducing factor and vascular growth endothelial factor which leads to increased angiogenesis and subsequent invasion and metastasis of tumors **[9]**.

Furthermore, on activation the nuclear localization sequence of the K+ channel results in its perinuclear localization which leads to mitogen activated protein kinase (MAPK) pathway activation resulting in increased cell proliferation **[10]**. Interestingly, the promotor region of HERG harbors multiple binding sites of oncoproteins as the nuclear factor kappa light chain enhancer of activated B cells (NFkB) and there was hypothesis that the mutations of these oncoproteins activate HERG expression **[11,12]**.

The aim of our study was to examine the prevalence of HERG and heag mRNA, focusing on their prevalence as a risk factor for acute leukemia (AL), their association to FAB subtypes and their response to induction chemotherapy.

MATERIAL AND METHODS

Patients:

The study comprised 64 AML and 20 ALL newly diagnosed cases. AML cases included 36 males and 28 females with an age range of 6-75, mean of 40.2±15.6 and median of 41 years while ALL cases included 13 males and 7 females with an age range of 3-54, mean of 19.3±12.2 and median of 17 years. They were provided by both the medical Oncology Department of National Cancer Institute, Cairo University and Beni Suef hospital, Beni Suef University. None of the patients had received any prior treatment and the diagnosis of acute leukemia was confirmed on the basis of complete history taking, clinical examination for hepatosplenomegaly and lymphadenopathy, in addition to the laboratory investigations which included complete blood picture, cytochemical features and immunophenotyping. Informed consent was obtained from all patients involved in the study and from the control group which consisted of twenty healthy individuals with an age range of 5-50, mean of 27.7±13.1 and median of 27.5 years. The patient's characteristics are shown in Table (1).

Methods:

The study of HERG and heag mRNA gene expression was performed by RT-PCR.

1- *Sampling:*

Five ml of venous blood were collected under aseptic conditions in EDTA vacutainer from every patient and control individual.

2- *RNA extraction:*

Total cellular RNA was extracted from the mononuclear cells using the QIA amp RNA blood Mini kit (QIAGEN, Catalogue number 52304) according to the manufacturer's protocol, to be followed by cDNA preparation using ReveRT™ First strand cDNA synthesis kit [Bio Life Scientific products, Catalog#: 5201]. cDNA samples were checked for integrity by PCR detection of human GAPDH using the same conditions for HERG as described below. The primers of GAPDH comprise a sequence between nucleotide 457 to 595 of GAPDH giving rise to a 138bp band. The obtained cDNA was then used as template for the subsequent PCR amplifications of the HERG and heag genes.

3- *PCR reaction:*

Amplifications were performed in a reaction mixture of 3µl cDNA, 12.5µl Master Mix (Fermentas, K 0171 which contains TaqDNA polymerase in reaction buffer, Mg Cl2 and dNTPs), 1µl of 25pmol of each of the forward and the reverse specific primers of HERG and GAPDH, with the addition of water (nuclease-free) to reach a final volume of 25µl in one multiplex PCR reaction assay. The same PCR reaction was used for heag.

The oligonucleotide primers for HERG and GAPDH were as reported by Chernubi et al. **[13]**, HERG primers: Forward 5´ TCC AGC GGC TGT ACT CGG GC 3´; Reverse 5´ TGG ACC AGA AGT GGT CGG AGA ACT C 3´ and GAPDH primers: Forward 5´ AAC AGC

CTC AAG ATC ATC AGC AA; Reverse 5´ CAG TCT GGG TGG CAG TGA T 3´. While the primers used for heag were as reported by Meyer et al. **[14]**, Forward 5´ TCC TCG TTG TAT TTC ACA ATG ACC 3´; Reverse 5´ ATG GGC AAG GGT GGT TTC C 3´.

PCR conditions used for HERG amplification: Thirty-five cycles of amplification were carried out after 2min of enzyme activation at 94ºC; each composed of denaturation at 94ºC; for 30sec, annealing at 56ºC for 1min, and extension at 72ºC for 1min. PCR products were run on a 2% agarose gel stained with ethidium bromide using the 50bp DNA ladder (Fermentas™ Egypt) as a molecular weight marker, and bands were visualized on a UV trans-illuminator. The primers for HERG encompass a nucleotide region from 2171 to 2746 of the HERG cDNA sequence giving rise to 575bp band.

PCR conditions used for heag amplification: An initial enzyme activation step at 94ºC for 3min; 32 cycles of denaturation at 94ºC for 1min, annealing at 59ºC for 1min, and extension at 72ºC for 1min then final extension at 72ºC for 7min. The amplified products were analyzed on 2% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. The positive samples for heag gene were detected at the specific molecular weight of 617bp.

Statistical methods:

Data was analyzed using SPSSwin statistical package version 17 (SPSS Inc., Chicago, IL). Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann-Whitney test. Comparison between 3 groups was done using Kruskal-Wallis test then post-Hoc "Schefe test" was used on rank of the variables for pair-wise comparison. Odds ratio (OR) with it 95% confidence interval (CI) were used for risk estimation. A *p*-value <0.05 was considered significant.

RESULTS

RT-PCR was used to detect mRNA transcripts for HERG K+ channels encoding gene in leukemia cells obtained from 64 AML and 20 ALL patients, meanwhile only 44 out of 64 AML samples were analyzed for the presence of heag expression, as ALL and the remaining AML samples were excluded due to low mRNA content. Both genes expression were examined in 20 healthy control individuals.

HERG mRNA expression was detected in 32 cases (50%), while only 7 cases (35%) of ALL samples expressed HERG; Healthy PB cells did not show detectable HERG expression (Table 2 and Fig. 1).

heag gene m RNA expression was detected in 11/44 (25%) AML cases, significantly higher in patients than control group that did not express heag (*p*–=0.027) (Table 2 and Fig. 2). Patients were identified as HERG+ / heag + or HERG– / heag - according to their expression.

The risk estimate for developing acute leukemia in the presence of HERG mRNA was (Odds ratio 1.44 and 95% CI 1.23-1.70) while the prevalence of heag in AML shows an Odds ratio of 1.6 and 95% CI of 1.3-1.9).

According to the FAB classification, HERG+ expression showed a subtype dependent expression pattern in AML (Table 3). However, the limited availability of samples in each group of FAB subtype was an obstacle for statistical analysis to compare between HERG + and HERG-patients. In ALL, 6/13 (46%) of B-ALL were HERG + patients and 1/7 (14.2%) of T-ALL was positive. heag was positive in 8/20 (40%) M2, 2/7(28.5%) M4, only 1/8 (12.5%) M1.

No association between HERG or heag expression and any of the clinical or laboratory findings parameters was encountered.

The impact of HERG and heag expression on clinical response to the initial chemotherapy

As regards the response of AML patients to induction chemotherapy, the highest remission rate was achieved in HERG– patients, while HERG+ patients had a higher rate of poor response (*p*-value <0.001, odds ratio 33; 95 CI 6.56-165). Similar pattern was shown in ALL (*p*=0.003, odds ratio 4.33; 95 CI 1.60-11.69) (Table 4).

On the contrary, there was no statistically significant difference between patients with heag+ and heag- in AML as regards response to treatment (*p*=1).

Table (1): Clinical and laboratory characteristics of 64 AML and 20 ALL patients.

Patient characteristics	AML patients	ALL patients
Gender:		
Male	36 (56.3%)*	13 (65%)
Female	28 (43.8%)	7 (35%)
Age at diagnosis:		
Years	40.2±15.5 (6-75)**	19.3±12.2 (3-54)
Median	41	17
Blood cell count:		
Hb gm/dl	7.5±1.8 (2.7-11.6)	7.6±2.4 (3.5-11.8)
TLC x 10^9/L	74.9±10.5 (16.9-705)	96.7±90.7 (25-335)
Plt x 10^9/L	50±52.5 (4.0-338)	42.6±24.0 (6.0-96)
BM Blasts %	65±29 (1-99)	80±21.2 (29-99)
Hepatomegaly	43 (67.2%)	13 (65%)
Splenomegaly	26 (40.6%)	12 (60%)
LN enlargement	12 (18%)	16 (80%)
FAB classification:		
M0	3 (4.7%)	
M1	11 (17.2%)	
M2	33 (51.6%)	
M3	6 (9.4%)	
M4	9 (14.1%)	
M6	2 (3.1%)	
T-ALL		7 (35%)
B-ALL		13 (65%)
CR achieved:		
Achieved	24 (37.5%)	10 (50%)
Not achieved	40 (62%)	10 (50%)

Hb : Hemoglobin.
TLC : Total leucocytic count.
Plt : Platelets.
LN : Lymph node.
FAB : French-American-British classification of AML.
CR : Complete remission.
*No(%) **Mean±SD (range).

Table (2): Comparison between AML, ALL and control group as regards HERG and heag expression.

Gene expression	Group			*p*-value
	AML	ALL	Controls	
HERG +ve	32/64* (50%)	7/20 (35%)	0/20 (0%)	<0.001 HS
heag +ve	11/44 (25%)		0/20 (0%)	0.027 S

*No. (%)

Table (3): HERG / heag gene expression in AML in relation to FAB subtype.

FAB type	HERG	heag
M0	2/3 (66.7%)*	0/2 (0%)
M1	4/11 (36.4%)	1/8 (12.5%)
M2	17/33 (51.5%)	8/20 (40%)
M3	1/6 (16.7%)	0/6 (0%)
M4	7/9 (77.8%)	2/7 (28.5%)
M6	1/2 (50%)	0/1 (0%)

*No. (%)

Table (4): The impact of HERG expression achieving complete remission (CR) in AL patients.

Parameter	CR rate		*p*-value
	HERG+	HERG–	
AML	2/32* (63%)	22/32 (68.8)	<0.001
ALL	0/7 (0%)	10/13 (76.9%)	0.003

*No. (%)

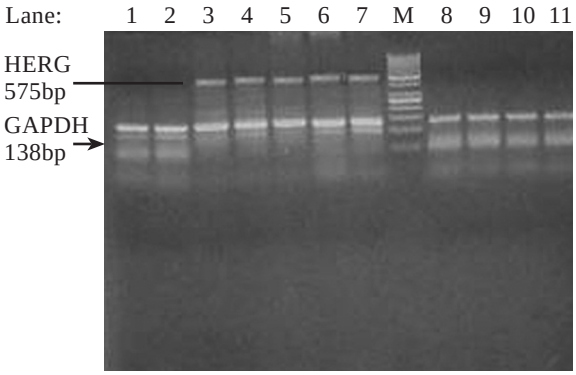


Fig. (1): Electropherogram of HERG expression.
M: 50-1000 bp ladder.
Lane 1 and 2: Negative AML and ALL cases.
Lane 3 to 7: Positive AML cases.
Lane 8 to 11: Control cases.

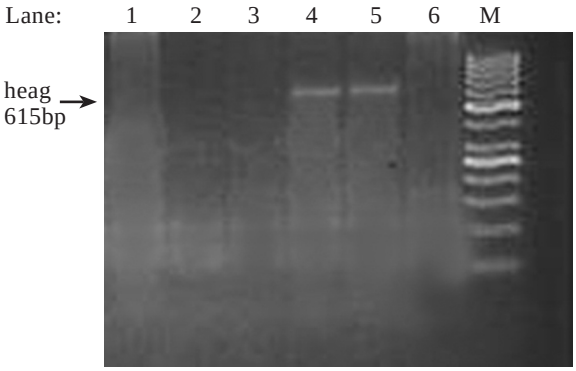


Fig. (2): Electropherogram of heag expression.
M: 50-1000bp ladder
Lane 1-3: Negative AML cases.
Lane 4,5: Positive AML cases.
Lane 6: Control case.

DISCUSSION

Ion channels have been implicated in many diseases, either in a primary etiologic role (Channelopathies) or as mediators in pathogenesis **[15]**. The identification of K+ ion channels and characterization of their functions in tumor cells have stimulated interest in the roles of K+ channels in tumorigenesis and cancer therapy **[16]**.

In the current study, the expression of HERG genes was analyzed in 64 AML and 20 ALL patients. The results showed that HERG expression was detected in both AML, ALL samples but not in normal peripheral blood cells; which this is in accordance with the early previous researches **[17,18,19]**.

The initial study reporting a potential link between the EAG family K+ channels and cancer showed that high HERG mRNA was present in 17 cancer cell lines of different species including human and murine **[20]**. Following this discovery, another group showed that Chinese Hamster ovary cells when transfected with heag gene exhibited a transformed cancer phenotype **[4]**. Many studies showed that HERG gene was overexpressed in many solid tumors **[21-24]**.

It had been reported that HERG K+ channels show differential expression patterns in tumor cell lines. Its overexpression had been suggested to represent a selective advantage for these tumor cells **[25]**.

Two recent studies, provided evidence that HERG mRNA was expressed in almost all the primary leukemia cells, CD34+/ CD38-/ CD123+ leukemic stem cells but not in circulating CD 34+ cells or normal PB mononuclear cells; they reported that HERG expression was not associated with the clinical features of leukemia and its blockage induces growth arrest of leukemia cells in G1 phase which suggests that leukemia cells need HERG channels to proceed with the cell cycle **[26,27]**.

The involvement of heag K+ channels in leukemia has not been systematically studied, although its relevance in many solid tumors is well established **[28]**. Smith et al. **[19]** did not detect heag in chronic lymphocytic leukemia, while Agarwal et al. **[29]** did not detect it in ALL; therefore, authors concluded that heag is not relevant in lymphatic leukemia **[29]**.

In the current study, we examined heag expression in 44 AML patients; heag gene was detected in 11/44 (25%) of AML and none of the control cases. The positive cases included 8/20 (40%) M2, 2/7 (28.5%) M4 and 1/8 (12.5%) case was M1. Using REAL time PCR, Agarwal et al. **[29]** detected heag expression in 47/118 (40%) of AML cases. The expression has showed a subtype dependent pattern; M2 and M4 were the most common subtypes that expressed heag, This is similar to our study taking in consideration that M2 and M4 were the most frequent subtypes analyzed.

The current study showed that the prevalence of HERG or heag may play an important role as a risk estimate factor in leukemia. Our study also showed that HERG+ expression in acute leukemia patients had adverse response to the initial induction chemotherapy which reflects poor prognosis.

The prognostic value of HERG expression in tumor cells has been evaluated in several tissues. Studies showed that HERG K+ channels were involved in different aspects of tumor establishment, progression and mediating tumor invasion **[30,31]**. In AML patients, HERG+ expression is associated with a higher probability of relapse, shorter time to relapse and shorter overall survival time compared with HERG- AML patients **[32]**.

It was hypothesized that HERG K+ channels regulated different aspects of acute leukemia pathophysiology such as survival and proliferation in the bone marrow **[24]**, cell motility and transendothelial migration possibly through PIK/AKT **[32]** or stromal cell-derived factor-1 (SDF-1/CXCR4) pathway **[33]**.

In the current study, HERG+ ALL cases included 6/13 (46%) B-ALL and 1/7 (14.2%) T-ALL. In a recent study, Pillozzi et al. **[34]** examined both HERG m RNA and protein expression in ALL by using real time PCR and Flowcytometry, both methods detected overexpression of HERG transcript in ALL blasts, also, it was shown that HERG expression correlated with that of CD10+ precursor B-ALL. Authors reported that leukemic cells are known to be protected from chemotherapy by mesenchymal stem cells (MSC) and HERG channel function appears to be important for this effect. Thus, agents that exclusively target HERG may

be adequate to overcome MSC-induced drug resistance **[34]**.

heag could be used as an additional prognostic factor in AML as it significantly correlates with a bad prognosis **[29]**, this was in contrast to our study as heag positivity in AML cases did not affect their initial response to chemotherapy, but this finding would require the support of prospective studies in a large sample population.

Blocking heag and HERG channels inhibits cell proliferation and disease progression, but since both heag and HERG belong to the same family of K+ channels and shares 47% of the amino acid sequence, both channels are affected by the same drugs. The use of different drugs to block these genes as monoclonal antibodies **[35]** and small interfering RNA **[36]** are expected to provide such an advantage in cardiac cases. The same might apply to acute leukemia.

In conclusion, both HERG and heag have an oncogenic potential, by affecting the proliferation of leukemia cells which suggests that both K+ channels are molecular markers for human neoplastic haematopoietic cells. HERG+ is important for risk estimation and prognosis of AL, while heag gene has more important role in leukomogenesis of AML rather than its prognosis which needs more justification by a large prospective study. Both K+ channels are considered as a potential pharmacological targets for cancer therapy.

REFERENCES

1- Perrin MJ, Subbiah RN, Vandenberg JI, et al. Human ether-a-go-go related gene(HERG) K+ channels: Function and dysfunction. Prog Biophys Mol Biol. 2008; 98 (2-3): 137-148.

2- Camacho J. Ether -a-go-go potassium channels and cancer. Cancer Letters. 2006; 233: 1-9.

3- Occhiodoro T, Bernheim L, Liu JH, et al. Cloning of a human ether a-go-go potassium channel expressed in myoblasts at the onset of fusion. Fed Eur Bioch Soc Lett. 1998; 434: 177-182.

4- Pardo LA, Camino DD, Sanchez A, et al. Oncogenic potential of EAG K+ channels. EMBO J. 1999; 18: 5540-5547.

5- Martin S, De Liveria L, De Queriroz M, et al. Eag1 potassium channel immunohistochemistry in the CNS of adult rat and selected regions of human brain. Neuroscience. 2008; 155 (3): 833-844.

6- Bauer CK, Schwarz JR. Physiology of EAG K+ channels. J Membr Biol. 2001; 182 (1): 1-15.

7- Asher V, Sowter H, Shaw R, et al. Eag and HERG potassium channels as novel therapeutic targets in cancer. World Journal of Surgical Oncology. 2010; 8: 113-119.

8- Wonderlin WF, Strobi JS. Potassium channels, proliferation and G1 progression. J Membr Biol. 1996; 154: 91-107.

9- Downie BR, Sanchez A, Knotgen H, et al. Eag1 expression interferes with hypoxia homeostasis and induces angiogenesis in tumors. J Biol Chem. 2008; 283 (52): 36234-36240.

10- Hegle AP, Marble DD, Wilson GF. A voltage-driven switch for ion-independent signaling by ether-a-go-go K+ channels. Proc Natl Acad Sci USA. 2006; 103 (8): 2886-2891.

11- Lin H, Xiao J, Luo X, et al. Overexpression HERG K+ channel gene mediates cell growth signals on activation of oncoproteins SP1 and NK-kappa B and inactivation of tumor suppressor Nkx3.1. J Cell Phisiology. 2007; 212: 137-147.

12- Jehle J, Schweizer PA, Katus HA, et al. Novel roles for HERG K+ channels in cell proliferation and apoptosis. Cell death and disease. 2011; 2,e 193.

13- Cherubini A, Taddel GL, Crociani O, et al. HERG potassium channels are more frequently expressed in human endometrial cancer as compared to non-cancerous endometrium. British Journal of Cancer. 2000; 83 (12): 1722-1729.

14- Meyer R, Schonherr R, Gavrilova-Ruch O, et al. identification of Ether a` go-go and calcium-activated potassium channels in human melanoma cells. J of membrane Biol. 171: 107-115.

15- Pardo LA, Contreras-Jurado M, Zientkowska M, et al. Role of voltage potassium channels in cancer. J Memb Biol. 2005; 205: 115-124.

16- Wang Z. Role of K+ channels in regulating tumor cell proliferation and apoptosis. Eur J Physiol. 2004; 448: 274-286.

17- Hofmann G, Bernabi O, Crociani A, et al. HERG K+ channels activation during beta(1) integrin-mediated adhesion to fibronectin induces up-regulation of alpha (v) beta (3) integrin in the preosteoclastic leukemia cell line FLG 29.1. J Biol Chem. 2001; 5: 42.

18- Pillozzi S, Brizzi, Balzi M, et al. HERG potassium channels are constitutively expressed in primary human acute myeloid leukemia and regulate cell proliferation of normal and leukemic hemopoietic progenitors. Leukemia. 2002; 16: 1791-1798.

19- Smith GA, Tsui H-W, Newell EW, et al. Functional up-regulation of HERG K+ channels in neoplastic hemopoietic cells. The Journal of Biological Chemistry. 2002; 277 (21): 18528-18534.

20- Bianchi L, Wible B, Acrangeli A, et al. HERG encodes a K+ Current highly conserved in tumors of different histogenesis: A selective advantage for cancer cells. Cancer Research. 1998; 8: 815-8222.

21- Suzuki T, Takimoto K. Selective expression of HERG and kv2 channels influences proliferation of uterine cancer cells. Int J Oncol. 2004; 25 (1): 153-159.

22- Shao XD, Wu KC, Guo XZ, et al. Expression and significance of HERG protein in gastric cancer. Cancer Biol Ther. 2008; 7: 45-50.

23- Roy J, Vantol B, Cowley EA, et al. Pharmacological separation of hEag and HERG channel function in in the human mammary carcinoma cell line MFC-7. Oncol Report. 2008; 19: 1511-1516.

24- Dolderer JH, Schuldes H, Bockhom H, et al. HERG1 gene expression as a specific tumor marker in colorectal tissues. Eur J Surg Oncol. 2010; 36 (1): 72-77.

25- Masi A, Becchetti A, Restano-Cassulini R, et al. HERG1 channels are overexpressed in glioblastoma multiforme and modulate VEGF secretion in glioblastoma cell lines. Br J Cancer. 2005; 93: 781-792.

26- Li H, Liu L, Guo T, et al. Expression and functional role of HERG1, K+ channels in leukemic cells and leukemic stem cells. Journal of Huazhong University of Science and Technology. 2007; 27 (3): 257-260.

27- Li H, Liu L, Guo L, et al. HERG K+ channel expression in CD34+/CD38^{-}/CD 123 cells and primary leukemia cells and analysis of its regulation in leukemia cells. Internal Journal of Hematology. 2008; 87: 387-392.

28- Hemmerlein B, Weseloh RM, Mello de Queiroz F, et al. Overexpression of Eag1 potassium channels in clinical tumors. Molecular Cancer. 2006; 5: 4153.

29- Agarwal JR, Griesinger F, Stuhmer W, et al. The potassium channel Ether a` go-go is a novel prognostic factor with functional relevance in acute myeloid leukemia. Molecular Cancer. 2010; 9: 18-33.

30- Wang H, Zhang Y, Cao L, et al. HERG K+ channel a regulator of tumor cell apoptosis and proliferation. Cancer Research. 2002; 62: 4843-4848.

31- Kunzelmann K. Ion channel and cancer. J Mem Biol. 2005; 205: 159-173.

32- Pillozzi S, Brizzi MF, Bernabei PA. VEGFR-1 (FLT-1), ß1 integrin, and HERG K+ channel for a macromolecular signaling complex in acute myeloid leukemia: Role in cell migration and clinical outcome. Blood. 2007; 110: 1238-1250.

33- Li H, Du Y-M, Guo L, et al. The role of HERG K+ channels and a functional link between HERG K+ channels and SDF-1 in acute leukemia migration. Experimental Cell Research. 2009; 315: 2256-2264.

34- Pillozzi S, Masselli M, De Lorenzo E, et al. Chemotherapy resistance in acute lymphoblastic leukemia requires HERG1 channels and is overcome by HERG1 blockers. Blood. 2011; 117: 902-914.

35- Pardo LA, Stuhmer W, et al. Eag an emerging oncological target. Cancer Research. 2008; 68 (6): 1611-1613.

36- Weber C, De Queiroz FM, Downie BR, et al. Silencing the activity and proliferative properties of the human Eag1 potassium channel by RNA interference. J Biol Chem. 2008; 281 (19): 13030-13037.

Journal of the Egyptian Society of Haematology & Research, Vol. 7, No. 1, March: 25-33, 2011

CXCL12 G801A Gene Polymorphism and the Risk of Tissue Infiltration in Acute Leukaemia

SHEREEN M. ELHOSEINY, M.D.*; RAAFAT M. ABDELFATTAH, M.D.** and KHALED S. ELHADIDY, M.D.***

The Departments of Clinical Pathology, Faculty of Medicine, Beni Suef University, Clinical Oncology, National Cancer Institute, Cairo University** and Internal Medicine, Faculty of Medicine, Beni Suef University****

ABSTRACT

Background: In acute leukemia blasts invade the blood stream and may localize in extramedullary sites. Stromal cell-derived factor-1 (SDF-1), coded by CXCL12 gene, is a chemokine that plays an important role in stem cell homing and malignant cell trafficking. CXCL12 G801A polymorphism could influence blast dissemination and tissue infiltration in acute leukemia.

Aim of Work: To characterize the expression pattern of CXCL12 G801A gene polymorphism in patients with acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) in relation to clinical features and laboratory findings at diagnosis, and its correlation with disease progression and outcome in an Egyptian patient's cohort.

Patients and Methods: CXCL12 G801A was analyzed in 65 AML and 35 ALL patients together with 30 normal controls using a polymerase chain reaction-restriction fragment length polymorphism assay [PCR-RFLP].

Results: CXCL12 801A/A, and 801A/G were associated with a higher PB blasts, BM blasts, compared to 801G/G homozygous patients (p=0.001 and 0.03 respectively) in AML and (p=0.005, 0.008 respectively) in ALL. The number of extramedullary tumor sites was higher in the A allele carriers (p=0.001) in AML, but in ALL the difference was near statistical significance (p=0.006). Patients with 801A allele relapsed and died more frequently (62.5%, 76.9%) than those with 801G/G (37.5%, 23.1%) in AML and ALL respectively; yet the difference was not statistically significant (p=0.08 in both AML and ALL).

Conclusion: CXCL12-G801A polymorphism is associated with higher mobilization of BM blasts, its infiltration to extramedullary tumor sites; it is also associated with treatment resistance, early relapse and mortality. CXCL12-G801A is an adverse prognostic factor and may define an important risk group in acute leukemia patients.

Key Words: *SDF-1 – CXCL12 G801A – ALL – AML – Extramedullary infiltration.*

INTRODUCTION

Acute myelogenous leukemia (AML) is characterized by uncontrolled proliferation within the bone marrow of myeloid progenitors arrested in their maturation process **[1]**. In contrast to normal hematopoiesis, it is usually associated with egress of immature cells from the bone marrow into the circulation and in some AML subtypes, these cells may anchor in extramedullary locations, such as in the liver and spleen **[2,3]**. Peripheral blood blast (PBB) count and the number of extramedullary tumor sites are extremely variable from one patient to another and depend, in part, on AML subtype **[3,4]**.

Stromal cell-derived factor-1 (SDF-1), which now is designated as CXCL12, is a homeostatic chemokine that signals through its receptor CXCR4, which in turn plays an important role in hematopoiesis, development, and organization of the immune system **[3]**. They also contribute to stem cell homing and may play a role in the trafficking of leukemic cells **[1]**.

SDF-1 is constitutively produced in the bone marrow by immature osteoblasts lining the endosteum region and by stromal and endothelial cells. SDF-1 is also produced by different hematopoietic cells as well as by AML blast cells, which express varying amounts of functionally active CXCR4 **[4,5,6]**. It is also secreted by stromal and endothelial cells of other organs such as heart **[7]**, skeletal muscle **[8]**, liver **[9]**, brain **[10]**, and kidney **[11]**. Moreover, its secretion increases during tissue damage such as heart infarct **[12]**, limb ischemia **[13]**, toxic liver

damage **[9]**, excessive bleeding **[14]**, total body irradiation, and after-tissue damage related to chemotherapy **[15,16]**.

CXCR4 and its ligand SDF-1 were also shown to have an important role in breast **[17]**, prostate **[18]**, and sympathetic nervous system cancer metastasis **[19]**, as well as in the migration assays of malignant cells from pancreatic cancers **[20]**, non-Hodgkin B cell lymphomas **[21]**, chronic lymphocytic leukemia **[22]**, chronic myeloid leukemia **[23]**, and acute leukemia **[4,5,6]**. However, the role of SDF-1/CXCR4 interactions in the control of human AML cell trafficking and disease progression is poorly understood **[2]**.

Previous studies have reported an association between the mobilizing capacity of haematopoietic progenitor cells (HPCs) and a single nucleotide polymorphism (SNP) in CXCL12 **[24]**, the SDF-1-encoding gene. This polymorphism is located at nucleotide position 801 (G to A transition, counting from the ATG start codon) in the 3' untranslated region (3'UTR) of the SDF-1 transcript. The ability of blasts to exit from the bone marrow microenvironment, circulate in the peripheral blood, and anchor in extramedullary locations might thus depend on the CXCL12 genotype **[4]**.

In this study, we analyzed the expression of the CXCL12 (G801A) gene polymorphism in 100 patients with acute leukemia, 65 with de novo AML and 35 with acute lymphoid leukemia (ALL), aiming to determine whether CXCL12. G801A polymorphism is important for the dissemination of malignant cells in acute leukemia and also to determine its role in the progression of the disease.

PATIENTS AND METHODS

Patients: One hundred Egyptian patients with newly diagnosed acute Leukemia referred to the National Cancer Institute (NCI)) and Beni Suef University hospital were included in this study. They were 65 AML and 35 ALL patients.

Diagnosis of Acute leukemia was based on (1) morphologic findings from Giemsa stained smears of bone marrow (BM) aspirates, (2) cytochemical stains criteria such as negativity for myeloperoxidase (MPO) and sudan black B (SBB) in cases of Acute Lymphoblastic Leukemia [ALL] or their positivity in cases of Acute Myeloid Leukemia [AML] and positivity for acid phosphatase in T-cell Acute Lymphoblastic Leukemia [T-ALL] and (3) immunophenotyping criteria as CD10 +/-,CD19+,CD20+,CD22+ for B-ALL,CD2+/-,CD3+,CD5+/-,CD7+ for T-ALL, and positivity of CD13 and CD33 for AML cases. Follow-up of patients was carried out for one year to study any possible association between CXCL 12 G801A polymorphism and the response of patients to therapy.

Concerning the 65 AML patients, they were 37 males and 28 females with a mean age of 43.2±16.6 (26-75) and median 46 years. Patients were classified according to the French-American-British (FAB) classification into: 11 M0, 15 M1, 18 M2, 12 M4, 4 M5, 4 M6 and only 1 case of M7.

As for the 35 ALL patients, they were 21 males and 14 females with a mean age of 23.5±11 (21-54) and median 24 years. They were classified according to immunophenotyping into 22 B-lineage (B-ALL) and 13 T-lineage (T-ALL) ALL.

Thirty age and sex matched individuals were included as a control group.

DNA isolation and CXCL12 genotype analysis:

Mononuclear cells (MNCs) were isolated from 2ml BM aspirate or peripheral blood at diagnosis by Ficoll density gradient centrifugation. Genomic DNA was extracted using QIAamp DNA Mini Blood kit (cat. no. 51304) (Qiagen, Germany) according to the manufacturer's instructions.

CXCL12 G801A polymorphism was determined with a polymerase chain reaction-restriction fragment length polymorphism assay [PCR-RFLP]. The PCR primers were:

- 5'-CAGTCAACCTGGGCAAAGCC-3'(F)
- and 5'-AGCTTTGTGCCTGAGAGTCC-3'(R) **[4]**.

PCR assay was performed for each sample in a final reaction volume of 25µL, using 5µL genomic DNA, 12.5µL universal master mix, 1µL CXCL12 G801A forward primer, 1µL CXCL12 G801A reversed primer, together with 5.5µL distilled water (DW).

The PCR conditions were as follows: 35 cycles of amplification consisting of, denaturation at 95°C for 30 seconds, annealing at 58°C for 1 minute, extension at 72°C for 30 seconds then a final extension at 72°C for 10 minutes was performed **[4]**. All reactions were done using the thermal cycler Applied Biosystems (Berkin Elmer 9600).

The PCR product was digested with the restriction endonuclease MspI **[4]** (Fermentas, Fast Digest ® MspI # FD 0544) and put at 37°C for 30 minutes. The products were then resolved on 2% agarose gel electrophoresis containing ethidium bromide, then visualized using UV transilluminator. DNA molecular weight marker (QIAGEN GelPilot 50 bp Ladder (100) (cat no. 239025) was used to assess the size of the PCR-RFLP products.

Because of the elimination of the MspI restriction site, MspI digestion of the PCR product, results in two fragments of 100 and 202bp for the 801G allele and in one fragment of 302bp for the 801A variant **[4]**. So the homozygous G/G results in two fragments of 100 and 202bp, the homozygous variant A/A results in one fragment of 302bp, while the heterozygous variant A/G results in three fragments of 100, 202bp and 302bp (Fig. 1).

Remission status was assessed after completion of induction chemotherapy. Complete remission (CR) was defined as follows: Granulocyte count of $\geq 1.5 \times 10^9$/L, platelet count of $\geq 100 \times 10^9$/L, no PB blasts, BM cellularity of ≥20% with maturation of all cell lines and <5% blasts, and no extramedullary leukemia. Relapse was defined as reappearance of PB blasts, >5% blasts in BM, or appearance of extramedullary manifestations after CR was achieved.

Statistical methods:

Data were analyzed using SPSS version 17. The data were summarized using descriptive statistics: Mean, standard deviation, median, range (minimal and maximum values) for quantitative variables and number and percentage for qualitative values. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann-Whitney test (non parametric *t*-test). Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA) then post-Hoc "Schefe test" on rank of variables was used for pair-wise comparison. Spearman-rho method was used to test correlation between numerical variables. A *p*-value ≤0.05 was considered significant **[25]**.

RESULTS

The present study included 100 patients with acute leukemia, 65 AML and 35 ALL together with 30 healthy controls.

Table (1) represents CXCL12 G801A among all studied groups. The frequency of 801 A allele does not differ significantly between AML, ALL patients and controls.

Tables (2,3) represent comparison between AML and ALL patients. There was no statistically significant difference between AML and ALL patients as regards gender, WBCs, Hb, platelets count, hepatomegaly, splenomegaly (HSM) or any extramedullary sites. After follow-up of patients for one year, there was no statistically significant difference between AML and ALL patients as regards CR (50.8% in AML versus 62.9% in ALL patients). There was a statistically significant difference between AML and ALL patients as regards age, (median 46 vs. 24 years), PB blasts and BM blasts were higher in ALL than AML patients. ALL patients presented with higher frequency of lymph nodes (LN) enlargement than AML patients (42.9% vs. 20%).

Tables (4,5) compare between characters of AML patients as regards CXCL12 G801A polymorphism. There was no statistical significant difference between wild type G/G and mutant type (A/A or A/G) as regards gender, age, WBCs, Hb, or platelets count. There was a statistical significant difference between the wild and the mutant type as regards HSM, LN enlargement, and extramedullary tumor sites being higher in patients harboring the A allele. Also PB blasts and BM blasts were significantly associated with the A allele. Although AML patients with bad outcome (relapse or mortality) showed higher frequency of the mutant type (A/A or A/G) than in the wild type (G/G) (62.5% vs. 37.5%), yet the difference did not reach statistical significance (p=0.08).

Tables (6,7) compare between characters of ALL patients as regards CXCL 12 G801A polymorphism. There was no statistically significant

difference between wild type G/G and mutant type (A/A or A/G) as regards gender, age, WBCs, Hb, or platelets count. Although A allele was more frequent in patients with HSM (70% vs. 30%) and with LN enlargement (73.3% vs. 26.7%); yet the difference was not statistically significant (p=0.09 and 0.1 respectively). The same applied to extramedullary tumor sites (68% vs. 32%); yet the difference was near statistical significance (p=0.06). As for the immunophenotyping, there was no statistically significant difference between B lineage (40.9% in G vs. 59.1% in A allele) and T lineage ALL (46.2% in G vs. 53.8% in A allele). The only statistically significant difference between the wild and the mutant type in ALL patients was in PB blasts and BM blasts which both were significantly higher in A allele than G allele (p=0.005 and 0.008 respectively). As for response to treatment, ALL patients with mutant type had bad response (relapse or mortality) more frequent than those with wild type (76.9% vs. 23.1%), but the difference did not reach statistical significance (p=0.08).

Table (1): Comparison between acute leukemia patients and control as regards CXCL 12 G801A polymorphism.

Group	No.	Wild Type Homozygous G/G	Mutant type		*p*-value
			Heterozygous A/G	Homozygous A/A	
		No. (%)	No. (%)	No. (%)	
AML	65	32 (49.2%)	30 (46.2%)	3 (4.6%)	
ALL	35	15 (42.9%)	18 (51.4%)	2 (5.7%)	0.75
Control	30	18 (60%)	11 (36.7%)	1 (3.3%)	

Table (2): Clinical and haematological parameters of AML and ALL patients.

Parameter	AML (65 patients)	ALL (35 patients)	*p*
Gender:			
Male: No. (%)	37 (56.9%)	21 (60%)	
Female: No. (%)	28 (43.1%)	14 (40%)	0.9
Age at diagnosis (years)	43.2±16.6 (26-75)*	23.5±11 (21-54)	
	46**	24	<0.001
Total leucocytic count x10^9/L	62.8±87.8 (2.5-340)	79.4±84.3 (1.4-274.7)	
	32	41.3	0.3
Hemoglobin gm/dl	7.8±1.8 (2.7-11.6)	7.3±2.5 (2.3-11.6)	
	8.1	7.9	0.8
Platelets x10^9/L	58.2±49 (7-200)	74.5±71.7 (5-296)	
	45	54	0.4
Peripheral Blood blasts	26±21.2 (0-78)	47.9±24.7 (12-95)	
	21	45	<0.001
Bone Marrow blasts	55.3±31.2 (21-99)	86.5±14.4 (29-99)	
	57	89	<0.001
Hepatomegaly: No. (%)	35 (53.8%)	20 (57.1%)	0.8
Splenomegaly: No. (%)	36 (55.4%)	20 (57.1%)	0.8
LN enlargement: No. (%)	13 (20%)	15 (42.9%)	0.02
Extramedullary sites: No. (%)	40 (61.5%)	25 (71.4%)	0.3

*Mean ± SD (range), **Median.

Table (3): Comparison between AML and ALL patients as regards clinical outcome.

Parameter	AML (65 patients)	ALL (35 patients)	*p*-value
Complete Remission (CR)	33 (50.8%)*	22 (62.9%)	0.2
No CR (relapsed or death)	32 (49.2%)	13 (37.1%)	
Death	17 (26.2%)	6 (17.1%)	0.3

* No. (%).

Table (4): Clinical and Hematological findings in 65 AML patients in relation to CXCL 12 G801A polymorphism.

Parameter	Wild Type G/G (32 cases)	Mutant Type A/G + A/A (33 cases)	*p*
Gender:			
Male: No. (%)	17 (45.9%)	20 (54.1%)	0.6
Female: No. (%)	15 (53.6%)	13 (46.4%)	
Age at diagnosis: years	39.1±17.9 (26-75) *	35.3±15.3 (27-62)	
	36.5**	35	0.4
Total leucocytic count: $x10^9$/L	71.8±97.9 (2.7-40)	54.2±77.3 (2.5-327)	
	35.7	32	0.6
Hemoglobin: gm/dl	7.9±1.7 (3.5-11.6)	7.7±2 (2.7-11.4)	
	8	8.1	0.7
Platelets: $x10^9$/L	59.3±52.1 (7-198)	57.1±46.5 (8-200)	
	40	50	0.8
Peripheral Blood blasts: %	14.2±12.2 (0-50)	37.5±21.9 (0-78)	
	12	38	<0.001
Bone Marrow blasts: %	47.3±29.9 (23-98)	63.1±30.9 (21-99)	
	43.5	70	0.03
Hepatomegaly: No. (%)	13 (37.1%)	22 (62.9%)	0.04
Splenomegaly: No. (%)	13 (36.1%)	23 (63.9%)	0.02
LN enlargement: No. (%)	0 (0%)	13 (100%)	<0.001
Extramedullary sites: No. (%)	13 (32.5%)	27 (67.5%)	0.001
FAB subtype: No (%)			
M0	2 (18.2%)	9 (81.8%)	
M1	5 (33.3%)	10 (66.7%)	
M2	11 (61.1%)	7 (38.9%)	
M4	6 (50%)	6 (50%)	
M5	4 (100%)	0 (0%)	
M6	3 (75%)	1 (25%)	
M7	1 (100%)	0 (0%)	

*Mean ± SD (range).
FAB: French American British classification.

Table (5): Impact of CXCL 12 G801A polymorphism on clinical outcome of AML patients.

Parameter	Wild Type G/G (32 cases)	Mutant Type A/G + A/A (33 cases)	*p*-value
Complete Remission (CR)	20 (60.6%)*	13 (39.4%)	0.08
Not in CR (relapse or death)	12 (37.5%)	20 (62.5%)	
Death	7 (41.2%)	10 (58.8%)	0.5

* No. (%).

Table (6): Clinical and Hematological findings in 35 ALL patients in relation to CXCL 12 G801A polymorphism.

Parameter	Wild Type G/G (15 cases)	Mutant Type A/G + A/A (20 cases)	*p*
Gender:			
Male: No. (%)	11 (52.4%)	10 (47.6%)	
Female: No. (%)	4 (28.6%)	10 (71.4%)	0.2
Age at diagnosis: years	28.9±10.1 (24-38) *	29.9±11.8 (21-54)	
	26**	28.5	0.9
Total leucocytic count: $x10^9$/L	69.4±82.6 (1.4-270)	86.9±86.9 (2.1-274.7)	
	39.8	42.5	0.5
Hemoglobin: gm/dl	7.1±2.8 (2.3-10.5)	7.5±2.2 (2.5-11.6)	
	7.3	8.1	0.7
Platelets: $x10^9$/L	88.6±87.5 (5-296)	63.9±57.3 (6-257)	0.5
	54	54	
Peripheral Blood blasts: %	34.8±19.6 (12-92)	57.8±23.8 (13-95)	0.005
	34	62.5	
Bone Marrow blasts: %	79.5±18.3 (29-98)	91.9±7.4 (70-99)	0.008
	85	94	
Hepatomegaly: No. (%)	6 (30%)	14 (70%)	0.09
Splenomegaly: No. (%)	6 (30%)	14 (70%)	0.09
LN enlargement: No. (%)	4 (26.7%)	11 (73.3%)	0.1
Extramedullary sites: No. (%)	8 (32%)	17 (68%)	0.06
Immunophenotype:			
B lineage: 22 cases	9 (40.9%)	13 (59.1%)	0.7
T lineage: 13 cases	6 (46.2%)	7 (53.8%)	

*Mean ± SD (range). **Median.

Table (7): Impact of CXCL 12 G801A polymorphism on clinical outcome of ALL patients.

Characteristic	Wild Type G/G (15 cases)	Mutant Type A/G + A/A (20 cases)	*p*-value
Complete Remission (CR)	12 (54.5%)*	10 (45.5%)	0.08
No CR (relapse or death)	3 (23.1%)	10 (76.9%)	
Death	2 (33.3%)	4 (66.7%)	0.6

*No. (%)

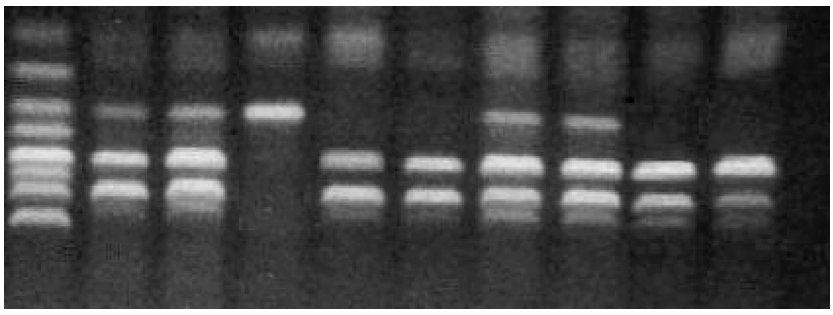


Fig. (1): PCR-RFLP analysis of CXCL 12 G801A polymorphism using MspI restriction enzyme:
M: DNA molecular weight marker: 500 bp.
Lane 1, 2, 6, 7: Heterozygous (A/G): 3 bands 100, 202 & 302bp.
Lane 3: Homozygous (A/A): One band at 302bp.
Lane 4, 5, 8, 9: Wild type (G/G): 2 bands at 100 and 202 bp.

DISCUSSION

In contrary to solid tumors that invade into the BM usually in the late stage of the disease, acute leukemias originate in the BM **[2]**. In the marrow microenvironment, acute leukemia cells are in close contact with marrow stromal cells that provide growth and survival signals through surface-bound or secreted factors **[3]**.

Some studies emphasized the idea that tumor cell migration and organ-specific metastasis are critically regulated by chemokines and their receptors **[2]**. SDF-1 is constitutively produced in the BM by immature osteoblasts lining the endosteum region, stromal and endothelial cells **[26]**.

SDF-1 was claimed to have numerous biological roles. In addition to controlling cell motility, SDF-1 can regulate cell proliferation, induce cell cycle progression, and act as a survival factor for both human and murine stem cells **[27,28]**.

Previous studies have indicated that the chemokine SDF-1a encoded by the CXCL12 gene and its receptor CXCR4 play an important role in metastatic cancers **[4,29]**. CXCL12 secretion by stromal cells attracts cancer cells, acting through its cognate receptor, CXCR4, which is expressed by both hematopoietic and non-hematopoietic tumor cells **[3]**. SDF-1/CXCR4 signaling is active in many cancer cells, including those of solid tumors and hematological malignancies **[4]**.

Previous studies have reported an association between the mobilizing capacity of normal hematopoietic progenitor cells and polymorphism at position 801 (G to A transition) in CXCL12, the SDF-1-encoding gene **[4,24,30]**. The ability of leukemic cells to exit from the bone marrow microenvironment, circulate in the peripheral blood and anchor in extramedullary locations might thus depend on the CXCL12 genotype **[30]**.

In this study, CXCL12 (G801A) gene polymorphism was determined in 65 AML and 35 ALL patients, together with 30 controls.

We found that the mutant type (A/A or A/G) was associated with higher PB and BM blasts in both AML and ALL patients compared with the wild type G/G. This finding of PB blasts in AML patients is in accordance with previous studies **[4,30]**. Up to our best knowledge, there are no reported studies for this polymorphism in ALL patients till know; this is the first study.

Also, we found that the homozygous and heterozygous 801A carriers (A/A or A/G) had higher frequency of extramedullary tumor sites compared with the 801G/G wild type patients.

The functional significance of this polymorphism has not been characterized **[31]**. It was hypothesized in 1998 that this mutation could be associated with increased marrow stromal cell secretion of SDF-1 **[32]**, without confirmation to date. On the other hand, it could be associated with lower secretion of SDF-1, a hypothesis supported by the lower SDF-1 level observed in the plasma of normal homozygous 801A subjects **[33]**. This decreased production of SDF-1 might explain the increased capability of malignant cells to egress from the bone marrow microenvironment **[4]**.

As for the response to treatment, we found that the 801A carriers (A/A or A/G) were associated with poor response to treatment as they relapsed and died more frequently than the 801G/G AML or ALL patients; yet the difference was not statistically significant. This might be explained by the finding that SDF-1 enhanced in vitro survival of normal human CD34+ cells and murine stem cells in the absence of growth factors **[27,28]**. This effect of SDF-1 in stress deprivation conditions may give an advantage in the growth and dissemination of blast cells, especially in view of the fact that BM SDF-1 levels increase after irradiation and chemotherapy **[15]**. Because VLA-4 activation was shown to be involved in the pathogenesis of AML minimal residual disease **[34]** and SDF-1 can activate the major integrins VLA-4 and VLA-5 **[35]**, therefore SDF-1 may be important for the persistence of BM minimal residual disease that causes AML relapse after chemotherapy **[2]**.

AML cells express SDF-1 and in vitro treatment of AML cells with neutralizing anti-CXCR4 Abs, anti-SDF-1 Abs, decrease cell survival, implying autocrine regulation of AML cell survival by endogenous SDF-1 **[2]**. However, the fact that blocking CXCR4 or SDF-1 reduced but did not completely inhibit the proliferation and survival of AML cells suggest that factors

and pathways other than CXCR4/SDF-1 interactions are also involved in the regulation of these processes **[2].**

Different studies found that the CXCL12-3'A allele was associated with higher yield of CD34+ cells **[36]**, faster recovery of both granulocytes and platelets after BM transplantation **[37]** and good mobilization capacity **[38]**.

In conclusion we found that the CXCL12 G801A gene polymorphism is a genetic determinant involved in the clinical presentation of leukemia. It is associated with increased release of blasts from the BM to the blood and higher frequency of distal dissemination. It is also associated with resistance to treatment, more frequent relapse and early mortality. So it could be a risk factor for extramedullary dissemination. Accordingly assessment of CXCL12 G801A polymorphism might help in identifying patients at risk of early relapse and mortality.

REFERENCES

1- Voermans C, van Heese WP, de Jong I, et al. Migratory behavior of leukemic cells from acute myeloid leukemia patients. Leukemia. 2002; 16: 650-657.

2- Tavor S, Petit I, Porozov S, et al. CXCR4 regulates migration and development of human acute myelogenous leukemia stem cells in transplanted NOD/SCID mice. Cancer Res. 2004; 64: 2817-2824.

3- Burger JA, Kipps TJ. CXCR4: A key receptor in the crosstalk between tumor cells and their microenvironment. Blood. 2006; 107: 1761-1767.

4- Dommange F, Cartron G, Espanel C, et al. CXCL12 polymorphism and malignant cell dissemination/tissue infiltration in acute myeloid leukemia. FASEB J. 2006; 20: E1296-1300.

5- Mohle R, Bautz F, Rafii S, et al. The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell derived factor-1. Blood. 1998; 91: 4523-4530.

6- Mohle R, Schittenhelm M, Failenschmid C, et al. Functional response of leukaemic blasts to stromal cell-derived factor-1 correlates with preferential expression of the chemokine receptor CXCR4 in acute myelomonocytic and lymphoblastic leukaemia. Br J Haematol. 2000; 110: 563-572.

7- Askari AT, Unzek S, Popovic ZB, et al. Effect of stromal-cell-derived factor-1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet. 2003; 362: 697-703.

8- Ratajczak MZ, Majka M, Kucia M, et al. Expression of functional CXCR4 by muscle satellite cells and secretion of SDF-1 by muscle-derived fibroblasts is associated with the presence of both muscle progenitors in bone marrow and hematopoietic stem / progenitor cells in muscles. Stem Cells. 2003; 21: 363-371.

9- Kollet O, Shivtiel S, Chen YQ, et al. HGF, SDF-1, and MMP-9 are involved in stress-induced human CD34+ stem cell recruitment to the liver. J Clin Invest. 2003; 112: 160-169.

10- Lazarini F, Tham TN, Casanova P, et al. Role of the alpha-chemokine stromal cell-derived factor (SDF-1) in the developing and mature central nervous system. Glia. 2003; 42: 139-148.

11- Schrader AJ, Lechner O, Templin M, et al. CXCR4/CXCL12 expression and signalling in kidney cancer. Br J Cancer. 2002; 86: 1250-1256.

12- Abbott JD, Huang Y, Liu D, et al. Stromal cell-derived factor-1alpha plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. Circulation. 2004; 110: 3300-3305.

13- Iwaguro H, Yamaguchi J, Kalka C, et al. Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. Circulation. 2002; 105: 732-738.

14- Ratajczak MZ, Kucia M, Reca R, et al. Stem cell plasticity revisited: CXCR4-positive cells expressing mRNA for early muscle, liver and neural cells “hide out” in the bone marrow. Leukemia. 2004; 18: 29-40.

15- Ponomaryov T, Peled A, Petit I, et al. Induction of the chemokine stromal derived factor-1 following DNA damage improves human stem cell function. J Clin Invest. 2000; 106: 1331-1339.

16- Kucia M, Reca R, Miekus K, et al. Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: pivotal role of the SDF-1-CXCR4 axis. Stem Cells. 2005; 23: 879-894.

17- Liang Z, Yoon Y, Votaw J, et al. Silencing of CXCR4 blocks breast cancer metastasis. Cancer Res. 2005; 65: 967-971.

18- Hirata H, Hinoda Y, Kikuno N, et al. CXCL12 G801A polymorphism is a risk factor for sporadic prostate cancer susceptibility. Clin Cancer Res. 2007; 13: 5056-5062.

19- Russell HV, Hicks J, Okcu MF, et al. CXCR4 expression in neuroblastoma primary tumors is associated with clinical presentation of bone and bone marrow metastases. J. Pediatr. Surg. 2004; 39: 1506-1511.

20- Koshiba T, Hosotani R, Miyamoto Y, et al. Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: A possible role for tumor progression. Clin. Cancer Res. 2000; 6: 3530-3535.

21- Arai J, Yasukawa M, Yakushijin Y, et al. Stromal cells in lymph nodes attract B-lymphoma cells via production of stromal cell-derived factor-1. Eur J Haematol. 2000; 64: 323-332.

22- Burger JA, Burger M, Kipps TJ. Chronic lymphocytic leukemia B cells express functional CXCR4 chemok-

ine receptors that mediate spontaneous migration beneath bone marrow stromal cells. Blood. 1999; 94: 3658-3667.

23- De Oliveira CE, Cavassin GG, Perim Ade L, et al. Stromal cell-derived factor-1 chemokine gene variant in blood donors and chronic myelogenous leukemia patients. J Clin Lab Anal. 2007; 21 (1): 49-54.

24- Benboubker L, Watier H, Carion A, et al. Association between the SDF1–3'A allele and high levels of CD34(+) progenitor cells mobilized into peripheral blood in humans. Br J Haematol. 2001; 113: 247-250.

25- Dawson B, Trapp RG. Basic and clinical biostatistics; Mcgraw-Hill Inc. Third edition. 2001.

26- Imai K, Kobayashi M, Wang J, et al. Selective secretion of chemoattractants for haemopoietic progenitor cells by bone marrow endothelial cells: A possible role in homing of haemopoietic progenitor cells to bone marrow. Br J Haematol. 1999; 106: 905-11.

27- Lee Y, Gotoh A, Kwon H-J, et al. Enhancement of intracellular signaling associated with hematopoietic progenitor cell survival in response to SDF-1/CXCL12 in synergy with other cytokines. Blood. 2002; 99: 4307-17.

28- Broxmeyer HE, Kohli L, Kim CH, et al. Stromal cell-derived factor-1/CXCL12 directly enhances survival/antiapoptosis of myeloid progenitor cells through CXCR4 and Galphai proteins and enhances engraftment of competitive, repopulating stem cells. J Leukoc Biol. 2003; 73: 630-8.

29- Balkwill F. Cancer and the chemokine network. Nat Rev Cancer. 2004; 4: 540-50.

30- Herault O, Dommange F, Espanel C, et al. Tumor cell dissemination and tissue infiltration are associated with CXCL12-G801A polymorphism in de novo acute myeloid leukemia. Blood (ASH Annual Meeting Abstracts). 104: Abstract 66, 2004.

31- Kimura, R, Nishioka, T, Soemantri, A, et al. Allele-specific transcript quantification detects haplotypic variation in the levels of the SDF-1 transcripts. Hum Mol Genet. 2005; 14: 1579-1585.

32- Winkler C, Modi W, Smith MW, et al. Genetic restriction of AIDS pathogenesis by an SDF-1 chemokine gene variant. ALIVE Study, Hemophilia Growth and Development Study (HGDS), Multicenter AIDS Cohort Study (MACS), Multicenter Hemophilia Cohort Study (MHCS), San Francisco City Cohort (SFCC). Science. 1998; 279: 389-393.

33- Soriano A, Martinez C, Garcia F, et al. Plasma stromal cell-derived factor (SDF)-1 levels, SDF1–3'A genotype, and expression of CXCR4 on T lymphocytes: Their impact on resistance to human immunodeficiency virus type 1 infection and its progression. J Infect Dis. 2002; 186: 922-931.

34- Matsunaga T, Takemoto N, Sato T, et al. Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. Nat Med. 2003; 9: 1158-65.

35- Peled A, Kollet O, Ponomaryov T, et al. The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: Role in transendothelial/stromal migration and engraftment of NOD/SCID mice. Blood. 2000; 95: 3289-96.

36- Bogunia-Kubik K, Gieryng A, Dlubek D, et al. The CXCL12-3'A allele is associated with a higher mobilization yield of CD34 progenitors to the peripheral blood of healthy donors for allogeneic transplantation. BM Transplant. 2009 Sep; 44 (5): 273-8.

37- Gieryng A, Bogunia-Kubik K, Lange A. CXCL12 gene polymorphism and hematologic recovery after transplantation of peripheral blood progenitor cells. Transplant Proc. 2010 Oct; 42 (8): 3280-3.

38- Ben Nasr M, Reguaya Z, Berraies L, et al. Association of stromal cell-derived factor-1-3'A polymorphism to higher mobilization of hematopoietic stem cells CD34+ in Tunisian population. Transplant Proc. 2011 Mar; 43 (2): 635-8.