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- Javan S, Tabesh M. Action of carbon dioxide on pulmonary vasoconstriction. *J Appl Physiol.* In press 2005

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## ORIGINAL ARTICLE

## Platelet Factor 3 Based-clotting Time Assay as a Quality Marker for Long-term Storage of Platelet Concentrates

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## ABSTRACT

**Background:** Platelets rapidly lose their qualities usually after 5 day of storage. Different standard methods have been recommended to check the quality of platelets during storage which some of them show better correlation with other quality markers during storage. The purpose of this study was to demonstrate if platelet factor 3 (PF3) assay could be an indicator of storage lesion and provide a significant correlation with other quality markers during long-term storage of platelet concentrates (PC) up to 11 days.

**Methods:** Twelve random units of PC were placed in a standard platelet incubator under continuous agitation at 22-24°C for eleven days. Samples were taken on days 1, 3, 5, 8 and 11. Parameters such as pH, glucose, lactate dehydrogenase (LDH), platelet count of the bags, mean platelet volume (MPV) and platelet distribution width (PDW) and PF3 were measured. The correlation coefficient of PF3 and pH with the abovementioned parameters was evaluated.

**Results:** The mean percentage of changes for PF3, pH, glucose, LDH, platelet count, MPV and PDW on day 11 compared to the first day were found to be 61, 15, 52, 440, 19, 18 and 39%, respectively. After LDH, PF3 had the highest change relative to the other markers. PF3 demonstrated better correlation with glucose, platelet count, MPV and PDW compared with pH during long-term PC storage.

**Conclusion:** Platelet factor 3 based-clotting time assay could be a potential candidate for monitoring the quality of PC due to apparent trend of its changes during storage with better correlation between the quality markers.

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### Introduction

Platelet storage lesions (PSL) are well-known events during preparation and storage of platelet concentrates (PC). Such changes are associated with decreased hemostatic function of platelets after transfusion and with poor post-transfusion recovery.<sup>1</sup> Various factors are responsible for the PSL and hence poor quality of PCs. There are numerous standards to check the quality of platelets during storage that have been published by various authorities, such as the European Council and American

Association of Blood Banks (AABB). Various tests are available varying from the simplest tests such as swirling to complex platelet function tests. Though swirling is a simple and non-invasive test, it is prone to observer bias.<sup>2,3</sup> The swirling was observed in 94% of cases with a pH value in the range of 6.7-7.5.<sup>2</sup> This pH range is associated with adequate in vivo survival.<sup>4,5</sup> Unfortunately, in practice there is no single laboratory test than can accurately predict the efficacy of a platelet transfusion and in other words its appropriate recovery following transfusion.<sup>6</sup> Previous

studies have reported that pH, as a major quality marker, shows the highest correlations with recovery and survival of platelets in healthy subjects.<sup>7,8</sup>

Platelet factor 3 (PF3), as a phospholipid lipoprotein blood coagulation factor derived from platelets acts with certain plasma thromboplastin factors to convert prothrombin to thrombin. The PF3 assay relies on the principle that incubation of platelet-rich plasma (PRP) with kaolin activates the procoagulant activity of platelets, resulting in a progressive shortening of both the recalcification time and Russell viper venom time and can be used specifically for assessing platelet procoagulant activity.<sup>9,10</sup> This novel test may also be used to monitor procoagulant activity of platelet membrane in platelet substitutes such as lyophilized intact platelets<sup>11</sup> or lyophilized infusible platelet membranes<sup>12-16</sup> in the future as well. We aimed to assess if PF3 assay indicates higher correlation compared with other quality markers such as pH during long-term storage of platelet concentrates up to 11 days and if it could be applied to monitor quality control of platelet concentrates in the future.

## Materials and Methods

### Study Design

In this study, 12 PCs were collected from healthy volunteers in Tehran Blood Transfusion Center as a routine procedure according to the platelet rich plasma method from whole blood with soft and hard spin centrifugation steps, respectively. After collection, standard PCs were kept undisturbed for one hour. The sterility test were performed to show no bacterial contamination of PCs and then they were placed in a standard platelet incubator with shaker (Danesh Pajooesh Fajr Co, Iran) under continuous agitation at 22-24°C for eleven days.

### Sampling

Sampling was done aseptically with a large bore needle.

Three milliliter samples of each PC unit were taken on 1, 3, 5, 8 and 11 days and the following tests were performed by biochemical and hematological analyzers.

### Biochemical and Hematological Tests

Parameters such as pH, glucose as a main catabolic cell pathway (glycolysis), lactate dehydrogenase (LDH) as an enzyme marker of cell viability, pre-donation platelet count of donor, total platelet count of the bags, platelet yield extraction, mean platelet volume (MPV) and platelet distribution width (PDW) as morphostructural platelet indices were measured.

### PF3 Assay

PF3 as a major platelet function based-clotting time assay was performed on the samples during storage of platelet concentrates according to the kaolin clotting time method.<sup>9</sup> Fresh platelets and physiological normal saline were used as positive and negative controls, respectively. The clotting time was converted to percent of activity by use of a standard curve.

### Statistical Analysis

True values was converted to mean percentage values with regard to initial value of 100 percent on day 1 and correlation coefficient (r) analysis was performed to compare the ability of PF3 and pH as quality markers during a 11-days storage period.

## Results

The relevant data of twelve PCs in terms of quality control parameters of platelets such as pH, glucose, LDH, platelet count, MPV and PDW during eleven days storage are reported as mean percentage changes in table 1. Mean percentage platelet yield extraction of PCs during storage up to eleven days was shown in table 2. During storage, metabolic activity of platelets continued

**Table 1:** Mean percentage changes in the quality control markers of twelve platelet concentrates during standard storage up to eleven days

Day of storage	PF3	Glucose	pH	LDH	Platelet Count	MPV	PDW
1	100* (0)**	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)
3	90*** (10)	90 (10)	99 (1)	171 (71)	98 (2)	108 (8)	113 (13)
5	77 (23)	80 (20)	98 (2)	253 (153)	92 (8)	110 (10)	115 (15)
8	60 (40)	65 (35)	93 (7)	377 (277)	85 (15)	114 (14)	123 (23)
11	39 (61)	48 (52)	85 (15)	540 (440)	81 (19)	118 (18)	139 (39)

\*Percent of initial value was assumed 100 on day 1 for all quality control markers; \*\*Data shows mean percentage of increase or decrease relative to initial value of 100 percent; \*\*\*Mean Percentage: Each true value was converted into percent with regarding to initial value of 100 percent on day 1

**Table 2:** Mean percent of platelet yield extraction\* of twelve platelet concentrates (PCs) during standard storage up to eleven days

Day of storage	Total platelet count of PCs/bag (×10 <sup>4</sup> )	Platelet yield extraction/bag (%)	Platelet yield extraction/bag** (%)
1	6862002	61	100
3	6711816	60	98 (2)
5	6309816	56	92 (8)
8	5803638	52	85 (15)
11	5544546	49	81 (19)

\*Total pre donation platelet count/bag was calculated as 11149184×10<sup>4</sup>; \*\*Percent of initial value of platelet yield was assumed 100 on day 1 for PCs

leading to glucose consumption that led to a trend of decreasing pH due to glycolytic energy generation and lactate production. Better trend was observed in LDH production, PF3 activity decline, glucose consumption, increasing of PDW and MPV, platelet count depletion and decreasing pH, respectively (figure 1). Our results showed that platelet counts per bag do decrease slowly during storage, albeit with the acceptable platelet count ( $>55 \times 10^9/\text{bag}$ ) according to the conventional standards even at the end of the storage (table 2).

The coefficient correlation of pH and PF3 versus other quality markers was calculated in table 3. Strong correlation was observed between pH and LDH, PF3, glucose, PDW, platelet count and MPV, respectively. High correlation was also observed between PF3 and glucose, platelet count, PDW, pH, MPV and LDH, respectively.

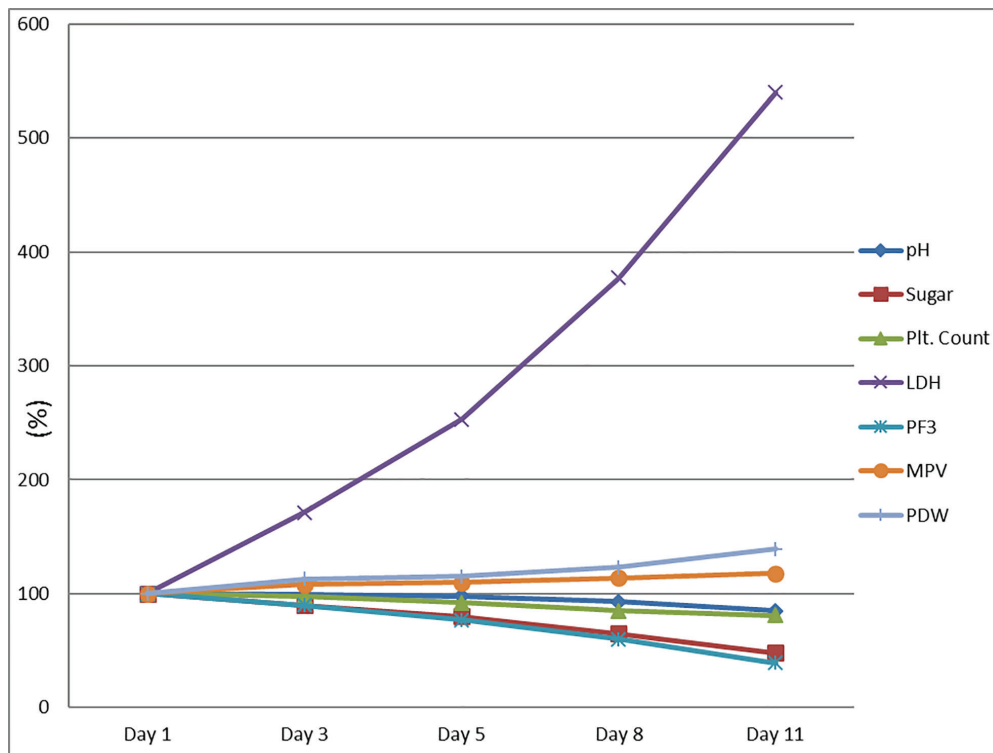
### Discussion

We found that PF3 could be altered as a result of platelet storage and hence be associated with storage lesions during long-term storage of PCs similar to other quality parameters such as pH, glucose, LDH, platelet count,

MPV and PDW. Similar results have been reported by previous studies for pH,<sup>7,17-26</sup> glucose,<sup>7,19,20,22-26</sup> LDH,<sup>20,22,23,25</sup> platelet count,<sup>7,20,21,23-26</sup> MPV<sup>20,21,23</sup> and PDW<sup>21</sup> of PCs that confirm their roles in monitoring the quality of platelets during storage. Minor differences in these studies may be related to the preparation method of the platelets,<sup>27,28</sup> the plastic material of the storage bag,<sup>4</sup> the ability of bags to exchange gas across its surface,<sup>5</sup> storage temperature,<sup>27,29</sup> the type of anticoagulant used, the platelet concentration in the bag and the agitation.<sup>27</sup>

On the other hand, PF3 which has phospholipid procoagulant activity of platelet membrane, as a clotting time assay, has not been used as a quality marker in the literature. Chao<sup>30</sup> used PF3 assay to show the functional properties of infusible platelet membrane product. Recently, studies have shown that flow cytometric analysis of CD41/CD61 and CD42b platelet receptors with PF3 based-clotting time assay may also show the status of platelet concentrates during storage.<sup>31</sup>

Accordingly, Bode<sup>32</sup> analyzed platelet factor 3 in platelet concentrates during PC storage and demonstrated higher PF3 and LDH activity that were significantly correlated



**Figure 1:** Trend in pH, glucose, platelet count, LDH, PF3, MPV and PDW. Each point represents the mean percentage value of twelve platelet concentrates stored in standard condition up to eleven days with regarding to initial value of 100 percent on day 1.

**Table 3:** Correlation coefficient values of pH and PF3 with other platelet quality control markers during 11 days storage of platelet concentrates

Parameter	Correlation coefficient of pH (r)	Correlation coefficient of PF3 (r)
Glucose	0.961	-0.999
LDH	-0.977	0.929
Plt. Count	0.934	0.982
MPV	-0.868	0.958
PDW	-0.959	0.978
PF3	-0.969	1.000
pH	1.000	-0.969

together only in standard, manual PC. Measurement of cytoplasmic leakage of LDH may also be used as a quality parameter and reflects platelet membrane damage. LDH has been shown to correlate to platelet survival ( $r=0.64$ ).<sup>33</sup> In our study, LDH and PF3 had trends showing storage lesions more relevant in comparison with other markers (Figure 1).

Our results demonstrated that pH declines steadily from day 1 (7.78) to day 5 (7.61) of storage, but sharper decline was observed between days 5 to 11 of storage with the mean pH of 6.61 at the end of storage which reflects better preservation of platelets during long-term storage with only 15 percent decrease in the pH. There was not an increase in pH between the first and third days of storage as reported by Dekkers<sup>33</sup> which may reflect the temporary changes in gas concentrations. The pH is an important marker for the quality of PCs *in vitro* since at values below 6.8, platelets become spherical; this change in shape becomes irreversible when the pH drops below 6.2. Platelet metabolism ceases completely when pH values drop below 6.0.<sup>34</sup>

### Conclusion

It may be concluded that platelet factor 3 based-clotting time assay can be a potential candidate for monitoring the quality of PCs due to the observed trend of its changes during storage. In comparison with pH, this test may also show better correlation with the other quality markers. However, further investigations are required to find the efficacy and precision of PF3 as quality marker and also look for other markers that help predict precisely status of PCs during storage.

**Conflict of Interest:** None declared.

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## ORIGINAL ARTICLE

## The Evaluation of Immunophenotypes in Diffuse Large B Cell Lymphoma: A Single Center Study

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## ABSTRACT

**Background:** Diffuse large B-cell lymphoma (DLBCL) is an aggressive malignancy of mature B lymphocytes. It is known as a heterogeneous disease with variable therapeutic responses and alternative therapies. Morphological and immunophenotypical evaluation of the biopsy specimens can help diagnose DLBCL.

**Methods:** In the current study, 44 patients were chosen from Shaheed Sadoghi Hospital (2010–2013), central Iran. Immunohistochemical method was used for detecting biomarkers such as CD2, CD3, CD20 and CD45.

**Result:** In this study, 54.5% of the patients were men and 45.5% were women. Most of the patients were 40-50 years old. Moreover, 10 (22.7%) patients had lymph node metastasis and 6 (13.6%) patients had stomach involvement. Positive expression of CD45 and CD20 biomarkers were expressed in 100% and 97.7% of the patients. Positive expression of CD3 and CD2 was expressed in 40.9% and 81.8% of the patients, respectively. C-expression of CD45 and CD20 biomarkers was seen in 43 patients. Moreover, there was no relation between biomarkers and sex and age ( $P>0.05$ ).

**Conclusion:** The result of this study showed that high number of CD45 and CD20 have been seen in Iran's population. Moreover expression of CD20 and CD45 is different as compared with other populations. It seems that these differences can be due to ethnic groups and nature of malignant cells.

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### Introduction

Diffuse large B-cell lymphoma (DLBCL) is an aggressive malignancy of mature B lymphocytes.<sup>1,2</sup> It is known as a heterogeneous disease<sup>3</sup> with variable therapeutic responses and alternative therapies.<sup>4</sup> The causes of diffuse large B cell lymphoma are not well understood yet.<sup>5</sup> Following nodular lymphocyte predominant HL, diffuse large B-cell lymphoma (DLBCL) can be observed as one of the most common lymphoid malignancy in adults diagnosed on basis of morphology and immunophenotype.<sup>6</sup> DLBCL accounts for 30–40%

of adult non-Hodgkin lymphomas.<sup>7</sup> The peak occurrence of diffuse large B-cell lymphoma (DLBCL) arises in the seventh decade of life.<sup>8</sup> The first sign of this disease is rapidly growing mass, fever, weight loss, and night sweats.<sup>9</sup> Diffuse large beta cell lymphoma disorder manufactured of a clinically and pathologically heterogeneous group of lymphoproliferative malignancies, most of which are B-cell origin.<sup>8</sup> Morphological and immunophenotypical evaluation of the biopsy specimens can help to diagnosis of DLBCL.<sup>10</sup>

Diffuse large B cell lymphoma includes three variants

in term of morphology. Centroblastic, Immunoblastic<sup>5,11</sup> and anaplastic. Most DLBCL cases are centroblastic.<sup>5</sup> Each has a diverse clinical presentation and prognosis. However, the usual treatment for each of these is chemotherapy, often in combination with an antibody targeted at the tumor cells.<sup>12</sup> The disease is treatable in most patients, but fewer than half of them attain a durable remission.<sup>1</sup> CD3 is a marker for T cells and natural killer cells.<sup>13</sup> It is specific for T-cell derivation. CD2 is also expressed by T and natural killer (NK) cells and has been reported in T/NK cell lineage neoplasms as well as in immature B-lymphoblastic and myeloid leukemias.<sup>14</sup> CD45 (lymphocyte common antigen) is a receptor-linked protein tyrosine phosphatase<sup>15</sup> and expressed on all leucocytes. It plays a significant role in the action of these cells.<sup>16</sup> CD20 is an activated glycosylated phosphoprotein. It is expressed on the surface of B cells beginning at the pro-B phase (CD45R+, CD117+).<sup>17</sup> We aimed to evaluate CD markers such as CD45, CD20, CD3 and CD2 in patients with diffuse large B cell lymphoma in Yazd city, central Iran.

### Materials and Methods

In this study, 44 patients were enrolled from Shaheed Sadoghi Hospital, Yazd, central Iran during 2010-2013. The specimens were conserved in formalin. Following fixation, the specimens were embedded on wax paraffin and sliced to 4 µm in thickness for staining. The hematoxylin and eosin staining method was used to stain the tissue sections. In immunohistochemical method, Endogenous peroxidase was blocked by 3% hydrogen peroxide in methanol for 10 minutes at room temperature. Heat-induced epitope retrieval was done by heating these

sections in citrate buffer (pH 9.0) using the microwave technique. After cooling, the sections were exposed with primary antibody (table 1).

Then, the specimens were exposed to Horseradish peroxidase rabbit anti-mouse IgG for 30 min and incubated with 3,3-diamino-benzidine tetrahydrochloride. The sections were counterstained with hematoxylin and rinsed in tap water, followed by immersing in graded alcohol, xylene and finally mount. Negative control was done by displacement of the primary antibody with fetal bovine serum in each series.

### Result

In our study, the mean±SD age of the patients was 55.8±9.5 years. 27 (61.2%) out of 44 patients were younger than 60 years old. 54.5% of the patients were men. 10 (22.7%) patients had lymph node metastasis. Table 2 shows the primary tissue involvement.

In this study CD45 was expressed in 100%, CD20 in 97.7%, CD3 in 40.9%, and CD2 in 81.8, respectively. Table 3 shows expression of these biomarkers in the patients.

Table 4 shows co-expression of Biomarkers. No relation between percentage of expression of biomarkers with sex was observed (P>0.05).

### Discussion

In the present study, immunohistochemical staining of the specimens with DLBCL were positive for CD45 and CD20 in almost all of the patients and less than half expressed CD3 and CD2. Asano and colleagues reported negativity for CD20 and CD3 in their patients with DLBCL.<sup>18</sup> Kevin and co-workers reported that some cases of DLBCL with an anaplastic morphology may be rich in

**Table 1:** Antibodies used for immunohistochemical staining of the tumor markers in patients with DLBCL

Antibody	Isotype	Dilution	Source
CD2	Monoclonal mouse antibody AB75	Ready to use	Dako
CD20	Monoclonal mouse antibody L26	Ready to use	Dako
CD3	Polyclonol rabbit	Ready to use	Dako
CD45	Monoclonal mouse 2B11+PD7/26	Ready to use	Dako

**Table 2:** Primary tissue involvement in patients with DLBCL

Primary tissue involvement	Number/Percent
Stomach	6 (13.6%)
Cervix	2 (4.5%)
Thyroid	3 (6.8%)
Breast	1 (2.2%)
Bone marrow	2 (4.5%)
Pelvis	2 (4.5%)
Skin	2 (4.5%)
Colon	2 (4.5%)
Kidney	3 (6.8%)
Testis	2 (4.5%)
Neck	3 (6.8%)
Lymph node	10 (22.7%)
Tonsil	4 (9.09%)
Mesentery	2 (4.5%)
Total	44 (100%)



**Table 3:** Positive expression of biomarkers in patients with DLBCL

Positive expression of biomarkers	Number/Percent
CD45	44 (100%)
CD20	43 (97.7%)
CD2	36 (81.8%)
CD3	18 (40.9%)

**Table 4:** Co-expression of biomarkers in patients with DLBCL

Co-expression of biomarkers	Number	Percent
CD20, CD45	43	97.7
CD20, CD2	36	81.8
CD20, CD3	18	40.9

Reed-Sternberg like cells and thus simulate lymphocyte-depleted classical Hodgkin lymphoma. However, in contrast to Hodgkin lymphoma, these neoplastic cells were uniformly positive for both CD20 and CD79a antigens and were negative for CD15. Therefore, they reported that the majority (65-85%) of the cases of DLBCL in their study were of B-cell type.<sup>11</sup>

Stein et. al reported that CD30 which was constantly expressed in Reed-Sternberg cells, also is expressed by a subset of DLBCL patients.<sup>19</sup> In another study, aberrant expression of a single T-cell-associated antigen (CD5) on specimens of DLBCLs was reported.<sup>20</sup> The researchers also reported aberrant co-expression of 2 T-cell-associated antigens; CD2 and CD7 in patients with diffuse DLBCL. In a study by Toyama et. al, flowcytometric immunophenotyping of the DLBCL cases were negative for CD2, CD3 and positive for CD20.<sup>21</sup> Asano et. al in another study reported that CD30 and CD45 were positive in DLBCL (anaplastic variant), but negative for CD3, CD10, CD20, CD15.<sup>18</sup>

## Conclusion

The result of this study showed high percentage of CD45 and CD20 seen in Iran's positivity in Iranian patients with lymphoma. It seems that these differences could be due to ethnic factors.

**Conflict of Interest:** None declared.

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## ORIGINAL ARTICLE

## The First Discrete Choice Experiment On Usage of Bypassing Agents in Hemophilic Patients in Iran

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## ABSTRACT

**Background:** Bleeding events in hemophilic patients with inhibitors are managed by bypassing agents. Currently available agents in Iran are recombinant activated factor VII (rfVIIa; Aryogen, Aryoseven) and Feiba (factor eight inhibitor bypassing agent). No standardized and accurate assay is currently available for monitoring the effectiveness of bypassing agents. We suggested that history of the patients' response and also their preference could be a reliable method for assessing the efficacy of bypassing agents; therefore, we designed a multi-centric discrete choice experiment study to assess the factors that affect the efficacy of bypassing agents.

**Methods:** Hemophilic patients older than 2 years with inhibitors who required bypassing agents for the treatment of bleeding episodes were eligible to participate in the study. Patients' preference toward treatment with either Feiba or Aryoseven was measured with a DCE (discrete choice experiment) design on a phone interview.

**Results:** 80 patients were enrolled from 5 centers in Iran. At enrollment, the mean age was 18.6 years (range, 2-50 years). 47 patients (58%) preferred to receive FEIBA, 21 patients (21.2%) favored Aryoseven and 12 (14.8%) patients claimed no difference between the two products.

**Conclusion:** Our results indicated that according to the DCE method, patients preferred Feiba to Aryoseven while the main reason was their higher efficacy. In addition, adverse reactions in both groups were almost equal. As a result, it seems that presence of both products in the market for hemophilic patients with inhibitors is absolutely essential.

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### Introduction

Hemophilia A and B are X-linked disorders are the result of low levels or absence of the factor VIII (FVIII) and factor IX (FIX), respectively.<sup>1</sup> The mainstay of treatment in both hemophilia disorders is factor replacement therapy. As a result of replacement therapy,

20 to 35% of patients affected with hemophilia A and 6% of those with hemophilia B develop inhibitory antibodies.<sup>2</sup> Bleeding events in patients with inhibitors used to be treated by bypassing hemostatic agents during the last decades.<sup>1,3</sup> Currently available bypassing agents in Iran are recombinant activated factor VII (rfVIIa;

Aryogen Aryoseven) and Feiba VH (Baxter, Deerfield, IL). Clinical observation of the patients' response to bypassing agents is still a significant task for monitoring the effectiveness of bypassing agents as no standardized and accurate assay is currently available for it. It seems that past history of the patients' response and preference could be a reliable measurement for the effectiveness of the type of the treatment.<sup>4,5</sup>

The cost of managing bleeding episodes in patients with inhibitors is high and the expenses associated with bypassing therapy represents a significant liability to the patients. Considering patient's drug preference can lead to promising consequences in supplying the required products for providing better care for the hemophilic patients with lesser expenditures.<sup>5</sup> The focus on discrete-choice experiment (DCE) in medical research in recent years has heightened the awareness of the patient's perspective of health outcomes.<sup>6</sup> According to this approach we designed a multicentric DCE study to assess the factors that may be associated with hemophilic patients' preferences towards Aryoseven or Feiba.

### Study Design

The main research question of this study was to assess hemophilic patients' preferences over the two available bypassing agents (Feiba or Aryoseven) in patients who have developed inhibitors.

Hemophilic patients older than 2 years with known inhibitors from Mofid children's hospital, "hemophilia comprehensive care center", Tehran and Imam Khomeini hospital of Tehran, Shiraz, Isfahan and Zahedan, who were in need to receive bypassing agents for the treatment of bleeding episodes were eligible to participate in this study. Patients' preferences towards treatment with either Feiba or Aryoseven was measured with a DCE (discrete choice experiment) design through a phone call interview. DCE is a quantitative method for illustrating the individual preferences. It permits researchers to demonstrate how responders value the selected attributes of a program or a product by asking them to state their choice over different hypothetical alternatives (8).

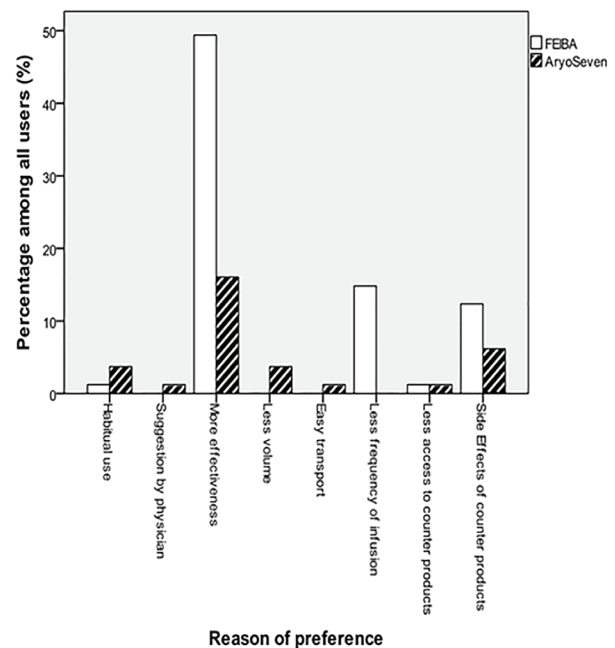
It is noteworthy to mention that Aryoseven or Feiba have been prescribed in the customary manner in accordance with the terms of the marketing authorization. In order to prevent the bias in the mentioned study, assignment of the patients to a particular therapeutic strategy was made retrospectively and decision to prescribe these agents had been made at least 3 months prior to the enrollment in the study. The objective of this study was to reach the following answers in a cross-sectional survey. A) Which aspect of a medication upon patients' view was considered important and B) Are the hemophilic patients qualified to have a preference over Aryoseven or Feiba considering the objective results of their medication.

### Results

80 patients were enrolled from 5 centers in Iran. At enrollment, the mean age was 18.6 years (range, 2-50) years. 47 patients (58%) preferred Feiba, 21 patients (27%) preferred Aryoseven and 12 (15%) patients disclosed no

difference over the two products. In terms of efficacy, 18 out of 47 patients (38%) who preferred Feiba described it as excellent, 21(44%) as very good, 4 (1%) mentioned as good and 1 patient as average, respectively. Data from 3 patients was not available. On the other hand, among patients who favored Aryoseven as drug of choice, 5 patients (24%) described it as excellent, 11 (52%) as very good and 5 (24%) as average. Patients who were satisfied with Aryoseven, mentioned that their bleeding episodes were controlled with more than 3 doses of Aryoseven in 54.5%; whereas in group of patients who preferred Feiba, bleeding events were controlled with 3 doses of Feiba in 46.8% of the cases, respectively.

Features of the products which were considered by the patients to favor each drug is shown in figure 1.



**Figure 1:** Features considered by the patients to assign the preference of bypassing agents in hemophilic patients with inhibitors.

59% of the patients had experienced total relief of their symptoms with 3 or more doses of Feiba, 10.6% with 2 doses and 25.5% with only one dose of it; on the other hand, in Aryoseven group 59% had relief with more than 3 doses, 36.4% with 2 and 4.5% with 1 dose of the drug. In patients who received Feiba, longer injection intervals (22.5%) and side effects (21.3%) were the main reason to prefer this product.

In Aryoseven group, side effects were reported in 22.7%, longer injection intervals in 13.6% and transportability of the drug in 4.5%, respectively.

### Discussion

Interpreting the preferences of the patients by health professionals could be beneficial in the field of policy making and treatment planning.<sup>6</sup> A DCE is a quantitative technique for explaining the individual's preferences in different fields.<sup>7</sup> It allows researchers to unveil how individuals' priorities selected particular attributes of a program or a product by asking them to share their



choices over different hypothetical alternatives. These include the elicitation of views on diagnosis, treatment and supportive care.<sup>7,8</sup> In a DCE point of view, respondents are asked to choose the most-preferred alternative from a set of hypothetical profiles, assuming that these are the only alternatives available.<sup>8</sup>

As a matter of fact, bypassing agents do not restore the normal pathway of hemostasis in hemophilia, hence the routine laboratory coagulation assays do not assess precisely the hemostatic activity of bypassing agents, and also no validated assay is available to measure their in vivo efficacy or predict individual's response to the treatment. As a result, the patient's preference methods could provide an alternative and clinical method for characterizing patients' needs or priorities. In a study from Iran, Golestani et. al reported similar effects in reducing joint bleeding episodes in comparison between the two bypassing agents.<sup>9</sup> Additionally, there were also other features which prompted the patients to make such preferences which included the rate of occurrence of side effects and interval of injections.

In developing countries, the idea of application of DCE to elicit the questions of health policy and treatment planning is relatively recent, but appears to be of growing interest and could be used as a resolving method unless there is more objective measures to determine the efficacy of some therapeutic options.<sup>6</sup>

### Conclusion

To the best of our knowledge this study is the very first one based on DCE method which compares hemophilic patients' preference over the two available bypassing agent product (Aryoseven versus Feiba) for treatment of their bleeding episodes due to high titers of inhibitors. Our results indicated that according to the DCE method analysis, patients preferred Feiba to Aryoseven and the most significant reason was the more efficacy.

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## ORIGINAL ARTICLE

## Mucinous and Non-Mucinous Adenocarcinoma in Colorectal Cancer Patients

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## ABSTRACT

**Background:** The oncologic behavior of mucinous adenocarcinoma (MA) of colorectal differs from non-mucinous adenocarcinoma (NMA). MA is more advanced at diagnosis and has a poorer prognosis than NMA. We aimed to evaluate prognostic factors and survival rate in patients with MA compared with NMA in Western Iran.

**Methods:** During 2008-2015 in a retrospective study, 83 patients with CRC referred to the Oncology Clinic in Kermanshah, Iran. Binary logistic regression analysis was used for the correlation between risk variables with the type of pathology.

**Results:** The mean follow-up was 32 months (range, 12-72 months) and in this interval, there were 26 deaths and 3 patients were lost to follow-up and therefore, were omitted them from survival analysis. There was no significant correlation between NMA and MA with sex, degree of differentiation of tumor, tumor site, tumor size, KRAS mutation and lymph node metastasis, but a significant correlation was observed with age ( $P < 0.05$ ). Binary logistic regression analysis showed there was a significant correlation between age ( $P = 0.01$ , odds ratio 11.93 and 95% CI 1.61-88.46) and type of pathology. The survival rate and mean survival were 54.3% and 23 months for NMA group, versus 80.6% and 25.4 months for MA group, respectively.

**Conclusion:** The prevalence of NMA in CRC was more than MA. In this study, the MA patients had lower age and more risk of recurrence compared to NMA patients and unlike other studies, 5-year survival rate was significantly higher in NMA patients than that in MA patients.

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## Introduction

Colorectal cancer (CRC) is the third most common neoplastic disease worldwide and is the second leading cause of cancer death in men and women in the United States.<sup>1</sup> In the World Health Organization (WHO) classification, mucinous adenocarcinoma (MA) is defined as an adenocarcinoma in which >50% of the lesion is composed of pools of extracellular mucin. Tumor with <50% of the lesion composed of mucin is categorized

as having mucinous component.<sup>2</sup> MA is a histological subtype of CRC and comprises approximately 1-6% of all colorectal epithelial cancers.<sup>3</sup> The oncological behavior of MA differs from non-mucinous adenocarcinoma (NMA).<sup>4</sup> MA is more advanced at diagnosis and has a poorer prognosis than NMA.<sup>3,5</sup> MA is associated with proximal location of tumor, advanced stage at diagnosis, microsatellite instability, and BRAF and KRAS mutations compared with NMA,<sup>2</sup> however, these differences are

not definitely confirmed, yet.<sup>6</sup> KRAS mutations are useful markers for predicting responses to anti-epidermal growth factor receptor (EGFR) monoclonal antibodies in metastatic colorectal cancers (mCRC) and CRC patients with a KRAS mutation do not respond to treatment with cetuximab or panitumumab.<sup>7</sup> Clinical data have proven that mutant RAS genes are negative predictive biomarkers and that patients with a KRAS/NRAS mutation do not benefit from an anti-EGFR antibody-based therapy.<sup>8</sup> We aimed to evaluate prognostic factors and survival rate in patients with MA compared with NMA in Western Iran.

## Materials and Methods

### Patients

During 2008-2015, in a retrospective study, 83 patients with CRC referred to the Oncology Clinic in Kermanshah city, Iran. The patients were divided into two groups based on the type of pathology (51 patients in NMA group versus 32 patients in MA group). Age, sex, degree of tumor differentiation, tumor size, tumor site, lymph node metastasis, KRAS and survival rate were studied in all patients. Overall survival (OS) was defined as the length of the time from diagnosis or the start of the treatment to death due to any cause or the date of the last follow-up.

Binary logistic regression analysis was used for the correlation between risk variables with the type of pathology. The correlation between the variables was analyzed in IBM SPSS version 19 by T-test for the means and Chi-square test for other variables. The survival graph was plotted by GraphPad Prism 5.  $P < 0.05$  was significant statistically.

## Results

The mean duration of follow-up was 32 months (range, 12-72 months) and in this interval, there were 26 deaths and 3 patients lost their follow-up and therefore were omitted from survival analysis. All patients had stage III or IV and there was no significant difference between stage in the two groups. The mean age (range) for NMA was 60 years (28-80 year) versus 54 years (31-78 years) for MA (Table 1). There was no significant correlation between NMA and MA with sex, degree of tumor differentiation, tumor site, tumor size, KRAS mutation and lymph node metastasis, but was a significant correlation with age ( $P < 0.05$ ). Therefore, the mean age of MA group was lower than NMA group.

Binary logistic regression analysis between the variables and pathologic subtype of CRC has been shown in Table 2. There was a significant correlation between age ( $P = 0.01$ , odds ratio (OR) 11.93 and 95% CI 1.61-88.46) and type of pathology.

The 5-year OS in CRC has been shown in Figure 1 based on the type of pathology (MA group vs. NMA group). The survival rate and mean survival were 54.3% and 23 months for NMA group, versus 80.6% and 25.4 months for MA group, respectively. There was a significant correlation between survival in two groups ( $P < 0.05$ ). Therefore, survival in MA group was better than NMA group.

## Discussion

CRC is the fourth most common cancer in men and the third most common in women.<sup>9</sup> Out of 255 CRC

**Table 1:** The correlation between type of pathology and prognostic factors in colorectal cancer (n=83)

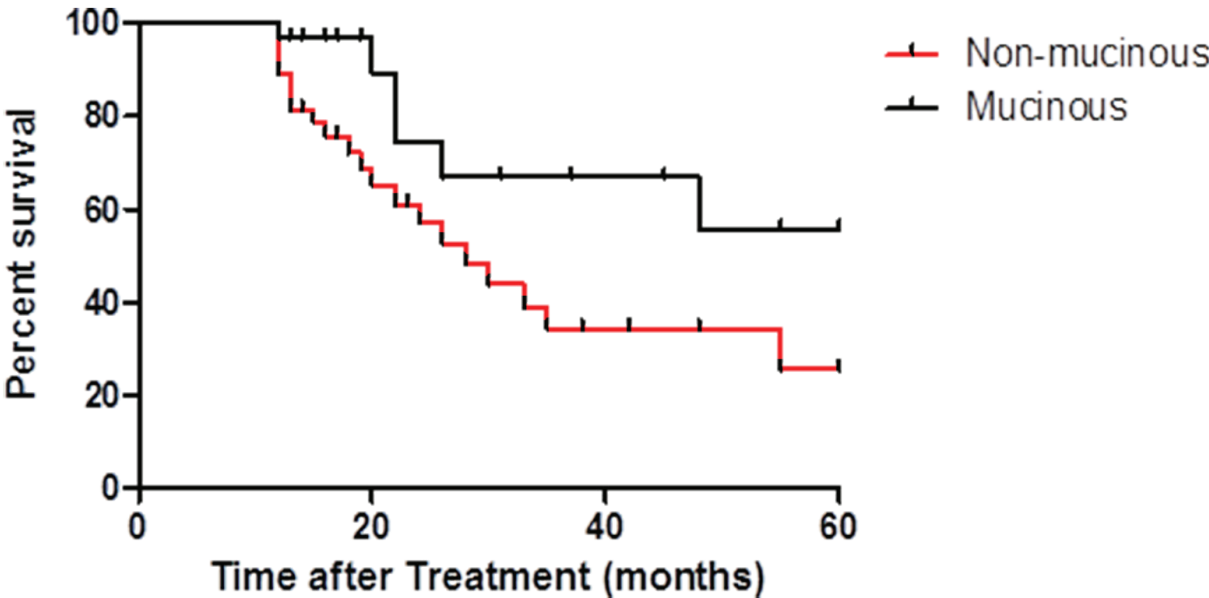
Variables	Non-mucinous adenocarcinoma N=51	Mucinous adenocarcinoma N=32	P value
<b>Age, years</b>			
Mean	60	54	0.039
Range	28-80	31-78	
≥60	26 (51%)	10 (31.3%)	
<b>Sex</b>			
Male	30 (58.8%)	21 (65.6%)	0.061
Female	21 (41.2%)	11 (34.4%)	0.351
<b>Differentiation</b>			
Poorly differentiated	2 (6.9%)	1 (5.9%)	0.136
Moderately differentiated	7 (24.1%)	9 (52.9%)	
Well differentiated	20 (69%)	7 (41.2%)	
Unknown	22	15	
<b>Tumor site</b>			
Rectum	41 (80.4%)	21 (65.6%)	0.107
Colon	10 (19.6%)	11 (34.4%)	
<b>Tumor size, cm</b>			
Mean	5.6	5.5	0.968
Range	1.5-12	1.5-10	
<b>KRAS</b>			
Wild-type	27 (52.9%)	19 (59.4%)	0.365
Mutation	24 (47.1%)	13 (40.6%)	
<b>Lymph node metastasis</b>			
Yes	27 (52.9%)	19 (59.4%)	0.517
No	24 (47.1%)	13 (40.6%)	



**Table 2:** Binary logistic regression analysis between the variables with type of pathology

Variables	P value	OR	95% CI
Age, ≥60 vs <60	0.01	11.93	1.61-88.46
Sex, male vs female	0.44	0.49	0.08-2.96
Grade, well vs moderate or poorly	0.78	0.82	0.21-3.19
Tumor site, rectum vs colon	0.10	7.65	0.66-87.98
KRAS, wild-type vs mutation	0.23	3.01	0.48-18.97
Lymph node metastasis, yes vs no	0.80	0.79	0.12-5.09

A binary logistic regression model was selected using Akaike Information Criteria (AIC) in stepwise selection. Odds ratios are adjusted for all of the factors listed in the table. OR: odds ratio.



**Figure 1:** The 5-year overall survival in colorectal cancer based on type of pathology

patients, 49 (19%) patients had a histologically confirmed diagnosis of MA.<sup>10</sup> MA comprises approximately 1 to 6% of all colorectal epithelial cancers.<sup>5</sup> In our study, out of 83 patients, 51 patients (61.4%) were NMA patients. The results of more studies has shown that the prevalence of NMA in CRC is more than MA.

In one study on CRC patients, the distribution for age at diagnosis, stage and treatment of patients with MA was similar to that of NMA patients and patients with mucinous histology had fewer well or moderately-differentiated tumors than NMA patients ( $P<0.05$ ).<sup>11</sup> In another study, only the tumor recurrence was significantly more common in MA, whereas the TNM stage, metastatic site and pattern of metastasis were not significantly different from each other in the two groups.<sup>3</sup> Maksimović S. and colleagues reported that MA patients compared to patients with NMA were found to have younger age, more lymph node metastases and higher frequency of advanced stage disease ( $P<0.05$ ).<sup>5</sup> As compared with NMA, the tumor diameter of MA was significantly longer and their age was younger. Also, MA had more lymph node metastasis and less recurrence.<sup>12</sup> Tumor stage and histological grading were higher in MA patients ( $P<0.001$ ); whereas rate of lymphatic invasion was higher in NMA.<sup>13</sup> In this study, MA patients had lower age compared to

NMA patients that was a statistical significant finding, but there was no other significant correlation between sex, differentiation of tumor (grade), tumor size, tumor site, lymph node metastasis and KRAS mutations and pathologic subtype of the tumor. Although, the prognostic factors in MA and NMA patients are not the same in different studies, but the majority have reported younger age for MA tumors of colorectal and also a higher risk for recurrence in this group.

Different studies in patients with colorectal cancer has shown that prognosis of MA tumors is poorer than NMA tumors.<sup>3</sup> One study reported that no statistically significant difference was noted in 5-year survival between MA and NMA tumors of CRC.<sup>12</sup> Maksimović S reported that the statistics for 5-year survival had a significant difference in MA patients compared to patients with NMA pathology(39% vs. 60,3%)<sup>5</sup> and in another study, 5-year overall survival was 81.4% in MA versus 87.4% in NMA ( $P=0.005$ ).<sup>4</sup> In patients with stage III and IV (advanced stage), MA was associated with a worse survival compared with NMA.<sup>14</sup> It was recently reported that mucinous histology indicates a poor response to oxaliplatin and/or irinotecan-based protocols and is associated with poor overall survival. In contrast to BRAF mutations, no significant difference has been

observed in clinicopathological parameters based on KRAS genotype in many studies.<sup>15</sup> This shows the impact of KRAS/BRAF mutations on the clinicopathological features and prognosis of the CRC patients, particularly in terms of the type of KRAS mutations; at codons 12 vs. 13.<sup>7</sup> KRAS mutation was not associated with a specific clinicopathological feature including age, sex, ethnicity of the patient, site of the tumor, differentiation of the tumor and mucinous status.<sup>15</sup> In comparison to NMA patients, MA patients had worse outcome and long-term overall survival rates. MAs may have special biological behavior which is an independent prognostic factor for patients with CRC.<sup>13</sup> Five-year cause-specific survival was 67% for NMA and 61% for MA ( $P<0.05$ ).<sup>11</sup> After a median follow-up of 45 months in another study, median OS for MA patients was 14 months compared with 23.4 months for NMA group.<sup>16</sup> In this study, 5-year survival rate was 54.3% (median OS, 23 months) in NMA patients compared with 80.6% (median OS, 25.4 months) for MA patients ( $P<0.05$ ). This result was in contrast to the results in previous studies. Therefore, genetics and probably ethnicity can affect on the metabolism of the drugs, especially anti-EGFRs that in a research,<sup>6</sup> has been reported that MA have distinct clinicopathological and genetic characteristics compared to NMA. Therefore, the differences in the rate of OS in the various studies are presumed to be due to genetic differences. Future studies should focus on genetics of the patients, so can exactly check the impact of these two factors on survival.

### Conclusion

In this study, the prevalence of NMA in CRC was more than MA subtype. MA patients had lower age at diagnosis and had a more risk of recurrence compared to NMA patients and unlike other studies, 5-year survival rate was significantly higher in NMA patients than that in MA patients.

**Conflict of Interest:** None declared.

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## ORIGINAL ARTICLE

## Prevalence of Alloantibodies and Autoantibodies in Transfusion Dependent Thalassemia Patients

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## ABSTRACT

**Background:** The development of anti-red blood cell alloantibodies remains a major problem in transfusion of blood in thalassemia major patients. Also, Autoantibodies can result in clinical hemolysis and difficulty in cross-matching blood. We studied the frequency of red blood cell alloimmunization and autoimmunization among thalassemia patients who received regular transfusions in Ilam province of Iran.

**Methods:** This study was carried out on 110 multiply transfused patients with thalassemia major. The saline method, Albumin method, direct/indirect coombs' and Three cell panel test used for detection of red blood cell alloantibody/ autoantibody.

**Results:** 12 patients out of total 110 patients (10.9 %) developed alloantibodies and 2 (1.81 %) developed autoantibodies. Rh and Kell blood group system alloantibodies were most commonly found, with the majority of patients being transfused with blood matched for ABO and D antigens only.

**Conclusion:** This study suggests screening RBC antigens prior to transfusion. Our findings accentuate the necessity of antigen typing of supposed to be transfused red blood cells and screenings tests before the first transfusion, at least for Rh (Rh system) and Kell (Kell system) antigens.

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### Introduction

Red blood cell (RBC) alloimmunization is an immune response against foreign RBC antigens; this generally occurs after sensitization due to blood transfusions and pregnancies.<sup>1</sup> Rh, Kell, Kidd and Duffy alloantibodies have a high clinical importance; they react at 37°C and cause hemolysis in transfused patients, fetuses and newborns.<sup>1-3</sup> Thalassemia is the most common genetic disorder worldwide and is the most common hereditary hemolytic anemia have been considered.<sup>4,5</sup> The disease has a high prevalence in the Mediterranean in the Middle East (Iran), India, and Southeast Asia is seen.<sup>6,7</sup> There are many forms of thalassemia due to decreased synthesis or lack of synthesis occurs in one or more chains of hemoglobin

molecules.<sup>8,9</sup> The expansion of anti- RBC alloantibodies and autoantibodies can significantly make difficulties transfusion therapy. Various alloantibodies are hemolytic and may cause, however not regularly, hemolytic transfusion reactions and limit the availability of further safe transfusion. Others are clinically insignificant. Erythrocyte autoantibodies appear fewer commonly, but they can result in clinical hemolysis and in difficulty in cross-matching blood. Patients with autoantibodies may have a higher transfusion proportion and often require immunosuppressive drugs, a splenectomy, or alternative managements. Life-long RBC transfusion remnants the chief treatment for major thalassemia.<sup>10-12</sup> In spite of the recognition of autoantibodies as transfusion-related



threats, little is known about the extent and reasons of these occurrences among thalassemia patients or the suitable inhibition procedures. Methods for inhibition or management of alloimmunization are under discussion. They vary from providing of RBCs matched for all the major antigens associated with clinically significant antibodies to blood matched only for antibodies that have already been made. Aims for controversy as to the best approach lay in the fact that numerous alloantibodies are not harmful, and costly inhibition procedures may therefore benefit only some patients.<sup>13-15</sup> Conventional therapy is transfusion life-long regular blood which reduces the severe complications of anemia, maintain growth and increase the survival.<sup>8,9,16,17</sup> However, transfusion will seek out the complications includes iron overload, infections and alloantigens and autoantibodies formed against antigens from red blood cells.<sup>5,6,17</sup> Repeated blood transfusion in these patients, different antigens on red blood cells enter into the patient's body and stimulate the patient's immune system to produce alloantibodies and autoantibodies. Since when choosing a transfusion blood just ABO and RH groups controlled, most alloantibodies formed against other blood group systems which cause delayed hemolytic reactions and limits further safe transfusion and cross-matching is also faced with the problem. Some alloantibodies also are clinically insignificant.<sup>5,9,18</sup> Knowing the side effects of blood transfusion, is a major problem in the treatment of patients with thalassemia. The purpose of this study was to determine the prevalence of alloimmunization and erythrocyte autoimmunization among thalassemia patients who received regular transfusions in Ilam province of Iran.

### Materials and Methods

After obtaining consent from all patients, clinical and transfusion informations of 110 transfusion dependent thalassemia patients (range: 10-35 years) who received regular transfusions were studied. The transfusion informations of all patients were studied for the presence of alloimmunization or autoimmunization, and antibody specificity. 110 patients who initially had negative results for RBC Alloantibodies/autoantibodies, were enrolled in the study. All patients were questioned and filled a questionnaire comprising of kind of thalassemia, date of the first blood transfusion, transfusion blood type (standard, washed, leucoreduced), hematocrite/hemoglobin level, reticulocyte count, direct and indirect Coombs test, total and direct bilirubin, hemoglobinuria and the type of alloantibody, as well as signs and symptoms like infection, dyspnea, chill, jaundice and pain (Table 1).

Sample from patients who received regular transfusions was drained in tubes containing acid citrate dextrose 21 days after the patients received a packed RBC transfusion and just before their next transfusion. Blood group typing done by Anti-A, Anti-B anti-D reagent (Iranian Blood Research and Fractionation Holding Company, Tehran-Iran) in Tube test procedure for each patient. Prior to each transfusion for detection of new Alloantibodies/

**Table 1:** Post-transfusion clinical and laboratory investigations in thalassemia patients with alloimmunization.

Post transfusion assessments	Percent
Positive direct coombs test	82
Increased reticulocyte count	58
Hemoglobinuria	52
Headache	63
Backache	66
Jaundice	63
Chill	62
Infection	42
Dyspnea	41

autoantibodies to RBC antigens, serum was analyzed by means of standard blood bank procedures. The serum was mixed with saline-suspended red cells with the addition of low ionic strength saline (LISS) and incubated at 37°C for 15 minutes. Alloantibody screening and identification was done using 3-cell antigen panel in Ilam blood transfusion organization (Iran blood transfusion organization, Iran, Lot No: 119405), and an anti-IgG reagent was used for the antiglobulin phase (synagen, Lot No: m4051). The panel test which determines 18 blood groups for the following antigens "D, C, E, e, c, K, k, M, N, S, s, Fy<sup>a</sup>, Fy<sup>b</sup>, Jk<sup>a</sup>, Jk<sup>b</sup>, Le<sup>a</sup>, Le<sup>b</sup> and P". Briefly, four drops of patient's serum add to be tested to each labeled tube. Then, two drop of thoroughly mixed reagent RBC add to the appropriate labeled tube. Subsequently, two drops of Albumin 22% added to each tube (Refining and blood research, Lot No: 89.BSA05). After mixing the contents of each tube, incubate all tubes at room temperature and 37°C (±1°C) for 15-30 minutes. Then proceed directly with the antiglobulin test phase following incubation. Wash tubes a minimum of 3 times with isotonic saline. Completely decant saline after final wash to obtain a "dry" red cell button. Add two drops anti-IgG to each tube. Centrifuge tubes in 3000 RPM for 1-3 minute. Immediately re-suspend the cells by gentle agitation; examine the tubes macroscopically for agglutination. If the screen was positive, an extended panel to recognize the antibody and a direct antiglobin (DAT) was implemented. Absorption techniques were employed in patients presenting with a new autoantibody. In cases of a positive DAT, additional study using specific reagents to detect IgG, IgM, or a complement were approved according to manufactory method in room temperature and 37°C. When an antibody was detected, eluates were prepared and confirmed against common sample RBCs. polyethylene glycol was used to enhance the reactivity.

### Results

There were 48 (43.7%) females and 62 (56.3) males. The mean age of patients was 22.5 (1SD±9.5). The mean age for the first blood transfusion was 5 (1SD±3.6) months for thalassemia major. 12 patients out of total 110 patients (10.9%) developed alloantibodies and two (1.81%) developed autoantibodies. The identified alloantibodies contains: D, C, E, c, K, Jk<sup>a</sup>, Fy<sup>a</sup> and Le<sup>a</sup> (Table 2).

**Table 2:** Prevalence of alloantibodies in patients

Blood Groups	Number (%)
RH system	7 (58.3)
D	4 (33.4)
C	1 (8.3)
E	1 (8.3)
c	1 (8.3)
Kell system	2 (16.6)
Kidd system	1 (8.3)
Duffy system	1 (8.3)
Lewis system	1 (8.3)

Anti-D and anti-Le<sup>a</sup>, was the most prevalent clinical significant, and non-significant antibodies, respectively. There was not any significant relationship between sex and prevalence of alloantibody ( $p > 0.05$ ). There was not any significant relationship between age and prevalence of alloantibody ( $p > 0.05$ ) (Table 3).

Prevalence of alloantibody in this study was 10.9% (12 patients) and 89.1% (98 patients) showed no alloantibody in their serum. Of 12 alloimmunized patients, 7 patients (11.3% of male patients) were male and 5 patients (10.4% of female patients) were female. The identified autoantibodies contains: P and N. Anti-P and anti-N were not clinical significant. Results of this study indicate low frequency of RBCs alloimmunization. The incidence of alloimmunization according to blood group was not confirmed. The alloantibody of 4 patients (33.33%) was recognized within six days of the transfusion; of 3 (25%) within ten days; of 2 (16.66%) within Thirty days; of 2 (16.66%) within 35 days and 1 (8.3%) within three months. The autoantibody of 1 patient (50%) was recognized within two months of the transfusion and 1 (50%) within four months. The highest titer was 1:128 (for anti-D) and lowest titer was 1:8 (for anti-Jk<sup>a</sup>). There was no significant correlation between alloimmunization rate and patients who regularly used washed/leukoreduced packed cell.

Out of 12 patients with alloantibodies 10 patients were found with two antibody, and 2 had three antibodies. The antibody screening panel test was positive in 39 patients (35.45%). There was not any significant relationship between sex and prevalence of autoantibody ( $P > 0.05$ ). There was not any significant relationship between age and prevalence of autoantibody ( $P > 0.05$ ) (Table 4). For patients who were on iron chelator (desferrioxamine), the pre-transfusion hemoglobin level was less than 8 gm/dl and post-transfusion hemoglobin level was 13 gm/dl. Patients who were not on iron chelator, the pre transfusion hemoglobin level was less than 8 gm/dl and post transfusion hemoglobin level was 11 gm/dl.

Patients with O negative blood group were the most frequent alloimmunized subjects, while patients with B negative and AB negative blood groups had the lowest frequent of alloimmunized subjects. Nevertheless, no significant risk or safety was well-known by the OR/p-value; the existence of alloimmunization in relation to blood group was not confirmed (Table 5).

### Discussion

In our study, prevalence of alloantibody was 10.9%. The most prevalent antibody was against D antigen. Transfusion of red blood cells (RBCs) is a public method in the management of patients with major thalassemia for two reasons: (1) transfusion of packed cells increases the oxygen-carrying capacity of the blood in the patients with major thalassemia, and (2) the replacement of the non-functional RBCs with functional ones may improve the symptoms or avoid the complications of the disorder. Alloimmunization against blood groups occurs following transfusion, pregnancy and transplantation. In patients who are transfused regularly such as thalassemia and sickle cell anemia patients the frequency of alloimmunization is high.<sup>19</sup> The rate of alloimmunization in the other parts of Iran has been reported as following: 7.4% (Tehran),

**Table 3:** Correlation between sex and age with prevalence of alloantibody

Characteristics	Total	Non-alloimmunized		Alloimmunized		OR (95% CI)	P value
		n	(%)	n	(%)		
Number of Patients, (%)	110	98	(89.1)	12	10.9)	-	-
Age 8-19 years	73	66	(90.41)	7	9.58)	0.909 (0.309-2.495)	0.999
≥20 years	37	32	(86.48)	5	(13.51)		
Sex						0.925 (0.318-2.582)	0.999
Male	62	55	(88.7)	7	11.3))		
Female	48	43	(89.6)	5	(10.4)		

Fisher's Exact Test; OR: Odds Ratio

**Table 4:** Correlation between sex and age with prevalence of autoantibody

Characteristics	Total	Non-autoimmunized		Autoimmunized		OR (95% CI)	P value
		n	(%)	n	(%)		
Number of Patients, (%)	110	108	(98.18)	2	(1.81)	-	-
Age 8-19 years	73	73	(100)	0	-	0.800 (0.056-11.512)	
≥20 years	37	35	(94.6)	2	(5.40)		1.000
Sex						0.800 (0.056-11.512)	1.000
Male	62	62	(100)	0	-		
Female	48	6	(95.9)	2	(4.1)		

Fisher's Exact Test; OR: Odds Ratio

**Table 5:** Incidence of post-transfusion alloimmunization in patients with major thalassemia in relation to the ABO/RhD blood group

Blood group	Total	Non-alloimmunized		Alloimmunized		Antibodies	OR (95% CI)	P value
		n	(%)	n	(%)			
O positive	42	37	88	5	12	Anti-C Anti-K Anti-Lea Anti-Fya	0.701 (0.102-4.989)	0.826
O negative	13	9	70	4	30	Anti-D (x 3) Anti-K	0.356 (0.051-2.098)	0.124
A positive	16	15	94	1	6	Anti-c	0.413 (0.401-1.950)	0.928
A negative	7	6	86	1	14.3	Anti-D	0.572 (0.015-11.201)	0.999
B positive	19	17	89.5	2	10.5	Anti-K Anti-E	0.345 (0.078-8.107)	0.819
B negative	4	4	100	0	0	-	0.417 (0.008-3.207)	0.999
AB positive	7	6	86	1	14.3	Anti-JKa	0.978 (0.008-0.189)	0.0504
AB negative	2	2	100	0	0	-	0.347 (0.123-1.206)	0.0321

Fisher's Exact Test; OR: Odds Ratio

5.34% (Fars province), 18.7% (Southwest Iran) and 2.87% (Northeast Iran).<sup>7,19</sup> Furthermore, the alloimmunization frequency in other countries is 30% (Kuwait), 19.5% (Egypt), 7.4% (Hong Kong), 5% (Italy), 8% (India), and 3.7% (Greece).<sup>7,18,20-22</sup> Compared with Kuwait and Egypt, Results of this study point out low incidence of RBCs alloimmunization. This low alloimmunization frequency indicates that there is homogeneity of red cell antigens in blood donors & recipients. The antibody screening panel test was positive in 45 patients (40.9%). In another study in Southwest Iran (Ahvaz), the antibody screening panel test was positive in 42 patients (32.06%).<sup>19</sup> Also, two patients (4.1%) had autoantibody, while in another study in Southwest Iran (Ahvaz) was 12.7%.<sup>19</sup> Keikhaei, B., et al. Showed that the predominant pattern of alloimmunization was alloantibodies against RH sub-groups system in 55 percent of patients and 33% of patients had alloantibodies against Kell system.<sup>19</sup> In this study, we demonstrated that the predominant pattern of alloimmunization was alloantibodies against RH system (D, C, E and c) in 58.3 percent of patients and 16.6 percent of patients had alloantibodies against Kell system. One cause for the high incidence of anti-D among patients may mostly be due to lack of enough knowledge and education about weak D, partial D and Del• phenotypes in blood bank staff. So it is necessary for blood bank professionals and other healthcare employees involved in blood transfusion, to be well informed about these phenotypes. The D antigen is composed of numerous epitopes (designated by “epD”). Subsequent studies with monoclonal antibodies defined 30 or more epitopes designated as “epDI” to “epD9”.<sup>23</sup> Amino acid changes in intracellular regions of the protein may alter D epitopes. Altered D is organized into four groups: weak D, partial D (including category D), Del• and nonfunctional RHD.<sup>24-27</sup> One of the most important reasons for alloimmunization is the transfusion of some red blood cells with rhesus D incompatible with thalassemia patients due to false negative results in D typing of blood donors. Traditionally, weak D red cells were defined as having a reduced amount of D antigen (formerly called “D<sup>w</sup>”) that required an indirect antiglobulin test (IAT) for detection. Weak D types are the result of an SNP

that encodes a single amino acid change predicted to be located in the intracellular or transmembrane region of the protein, rather than on the outer surface of the red cell. The amino acid changes may affect the insertion of the protein in the membrane and, thus, reflect the reduced number of D antigen sites on the red cells.<sup>25</sup> A weak D phenotype can occur with many partial D phenotypes, Ce in trans with suppression of RHD, in the Rh<sub>mod</sub> phenotype, and via autosomal recessive inheritance of two weak RHD alleles. The latter accounts for the majority of weak D phenotypes present in the general population. Because all the mutations are intramembranous or intracellular, it is assumed they do not significantly alter the presentation of D epitopes on the extracellular loops. This may explain why most weak D individuals do not make alloanti-D when transfused with D positive blood.<sup>25,28</sup> Partial D antigens are RHD proteins with missing D epitopes. Although they type as D-positive, persons with partial D antigens can make alloanti-D antibodies reactive with allogeneic, but not autologous, RBCs. The alloanti-D produced by these individuals recognizes D-specific epitopes missing on their own RBCs. In contrast to weak D types, partial changes are predicted to be located on the exterior membrane surface. Some partial D types are detected only by the IAT. Red cells that express extremely low levels of D antigen that cannot be detected by routine serologic methods are designated as “D-elution” or D<sub>el</sub>. Del cells are found in 10% to 30% of D-negative people of Asian ancestry. It is worth noting that the identification of weak D phenotype in blood recipients is not necessary because they do not develop anti-D, after receiving D-positive blood. But partial D phenotypes need to be identified because transfusion of D-positive blood to partial D subjects will lead to alloimmunization. Hence, it is recommended to consider a separate refrigerator for storing products supposed to be given to patients with major thalassemia. We also recommend to do following experiments on the blood that is supposed to be transfused to patients with major thalassemia:

1- Using anti-D reagents combine a monoclonal IgM, which causes direct agglutination at room temperature, with a monoclonal or polyclonal IgG that is reactive by



IAT for the determination of weak D.

2- Typing Donors for D: The aim of D typing of RBC donors, including the identification of units with weak D or partial D types, is to prevent anti-D immunization of recipients. A unit labeled Rh negative must be confirmed by testing an integrally attached segment before transfusion. Weak D testing is not required. The RhD type of the units labeled Rh positive do not require confirmatory testing.

3- Typing Patients for D: When the D type of a patient is determined, a weak D test is not essential except to assess the red cells of an infant whose mother is at risk of D immunization. Monoclonal IgM reagents type numerous samples as being D positive in direct testing that would have previously been identified only by IAT. Consequently, some of the concerns regarding the unnecessary use of Rh-negative blood and RhIG have been addressed.

4- D typing discrepancies should always be inspected and resolved. An Rh-negative blood transfusion is an appropriate option for patients needing immediate transfusion, but a thorough clerical and serologic investigation should be performed. *RHD* genotyping is also useful to resolve D typing discrepancies. Because donor centers test for weak D, a donor who is correctly classified as Rh positive may be classified as Rh negative as a recipient. This discrepancy should not be considered problematic but, rather, should be communicated to the patient and health-care staff and be noted in the patient's medical record.

5- Inappropriately, serologic reagents cannot certainly be used to differentiate persons with partial D that is reactive only with improved techniques and procedures from D-positive persons. Many partial D red cells type as strongly D positive in direct tests and are known only after the patients produce anti-D. Guidelines regarding D typing techniques and choice of blood components for transfusion should be based on the patient population, risk of immunization to D, and limited supply of D-negative blood components. These guidelines should statement what should be done when a partial D type is found before the patient makes anti-D. Anti-D is a clinically significant antibody, and preventing immunization in females of childbearing potential is important to avoid the complications of HDFN.

6- RH genotyping: RH genotyping is an effective attachment to serologic testing for the typing of transfused patients, RHD zygosity determination, noninvasive fetal D typing, determination of D status, and identification of antigen-matched blood for patients with major thalassemia.

7- Typing multi-transfused patients: In patients receiving chronic or massive transfusions, the existence of donor red cells in the peripheral blood often makes red cell phenotyping by agglutination incorrect or problematic. Genotyping overcomes this restriction because blood grouping can be determined with DNA prepared from a blood sample, though the sample was collected after transfusion.

8- D-negative, first-time donors are screened for RHD

to detect red cells with very weak D.

By several factors, alloimmunization is limited in the traditional practice of transfusion of blood. Many of the antigens present on RBCs uncommonly give rise to alloimmunization even when injected into patients lacking the antigen.<sup>29</sup> The factors for alloimmunization are complex and involve at least 3 main contributing features: The RBC antigenic difference between the blood donor and the recipient; the recipient's immune status; and the immunomodulatory effect of the allogeneic blood transfusions on the recipient's immune system. A low rate of alloimmunization may be expected when there is homogeneity of RBC antigens between the blood suppliers and recipients.<sup>18</sup> The ability to respond to alloantigens differs importantly from person to person. Some individuals will not become immunized to any antigens in spite of continuous transfusion, whereas others will become immunized, when transfused, to many of the antigens that they lack.<sup>30</sup> Blood banks will provide enough compatible blood for patients with thalassemia. Ameen et al. have reported Anti-K in 72% of patients and anti-E in 45.6%<sup>31</sup> and in our study Anti K, anti-E, anti-C, anti-c and anti D were found in 16.6%, 8.3%, 8.3%, 8.3% and 33.4 of patients respectively. In another study, Karimi et al. detected high prevalence of antibodies against Rh system (47.7%).<sup>32</sup> In Europe and the United States, the most commonly reported alloantibody in alloimmunized patients were alloantibody against Rh and Kell antigens. In a study in Minnesota, also anti-E and anti-K had higher rate than the other.<sup>33</sup> RBCs alloantibody formation was not influenced by gender, age at start of transfusions and number of packed cells received.

## Conclusion

This study suggests screening RBC antigens prior to transfusion. Our findings accentuate the necessity of antigen typing of supposed to be transfused red blood cells and screenings tests before the first transfusion, at least for Rh (Rh system) and Kell (Kell system) antigens.

**Conflict of Interest:** None declared.

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## CASE REPORT

# Lineage Switch in Childhood Leukemia: A Case Report and Review of Literature

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## ABSTRACT

Acute leukemia which is the most common cancer in children is a heterogeneous group of clonal malignancies. The conversion of the leukemic cell lineage during the course of the disease or later is termed lineage switch. It has been rarely reported in the literature. In leukemia lineage switch, conversions from lymphoblastic leukemia to myeloid leukemia or vice versa are reported. Herein, we report a 7-year-old child with acute lymphoblastic leukemia which switched to acute myeloid leukemia upon relapse.

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## Introduction

Acute leukaemia is a heterogeneous group of clonal malignancies categorized as acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) or mixed phenotype acute leukemia (MPAL). All subtypes have cytogenetic and molecular alterations that have prognostic implications. Lineage switch which is the conversion of the leukemic clone is known to be associated with poor prognosis.<sup>1-3</sup> There are occasional reports of switch in leukemic cell lineage (lymphoid or myeloid) through the course of the disease in the literature.<sup>1-3</sup> Distinct hypotheses have been considered to explain the lineage switch.<sup>3</sup> Here, we present the clinical and laboratory features of a case with pre B ALL who switched to AML as a relapse.

## Case Presentation

A 7-year-old boy was admitted to Mofid Children's Hospital with a history of pallor, abdominal pain, chemosis and cervical lymphadenopathy. The initial laboratory findings demonstrated a white blood cell count of  $5.3 \times 10^9/L$ , 56% Neutrophils, 44% lymphocytes, Hemoglobin 8 g/dL, and platelet  $149 \times 10^9/L$ . Bone marrow aspiration was cellular with 94% blasts which were medium-sized. Immunophenotyping was as follows: HLA-DR (71.5%), CD10 (10 %), CD19 (90%), CD20 (95 %), CD22 (43%), CD13 (2 %) and CD33 (8 %) which was in favor of B lineage acute leukemia.

Cytogenetic study was positive for t(8,14) and karyotype demonstrated 46 XY with duplication of one segment of long arm of chromosome 1. The patient was treated

with protocol for B-Cell ALL (LMB 89 Protocol). Chemotherapy was continued for three years and the patient was in complete remission. At that time, the patient developed a short period of pancytopenia whom underwent bone marrow aspiration for further evaluation. It revealed complete infiltration of blasts which according to flowcytometry was compatible with AML. Immunophenotyping was positive for CD13 (63%), CD33 (34%), CD15 (43%), HLA-DR (82%), CD117(66%), CD45(89%),c MPO(57%), CD22 (43%), CD10 (1%), CD19 (1%), CD20 (1%) which accordingly diagnosis of AML non-M3 was established. Karyotype was unsuccessful at this time. The patient received AML directed chemotherapy and also was scheduled to go through allogeneic stem cell transplantation; however, remission was not achieved and the patient died of the disease.

### Discussion

Although rarely, switches between lymphoid and myeloid lineages may occur during treatment of acute leukemias.<sup>4</sup> Among suggested hypotheses for lineage switch is a deviation in the initial leukemic clone which actually had been MPAL; hence, the first diagnosed lineage would be converted to another at a later time.<sup>4-6</sup> In other words, lineage switch could be a part of the biologic spectrum of mixed-lineage leukemias.<sup>4-7</sup> Rossi and colleagues proposed the involvement of early bipotential B-macrophage progenitors in the course of lineage switch raising the possibility that the lineage switching event would be the conclusion of the leukemogenic mutations targeting this early bi-potential progenitor cell.<sup>4-8</sup> Additionally another explanation by Strass and co-workers could be the selection of a pre-existing chemotherapy-insensitive minor population of cells of different lineage among the predominant population of leukemic blasts at diagnosis; (biphenotypic or bilineage leukemias) resulting in a resistant subclone with expression of different antigens.<sup>4,5,9</sup>

In our patient, immunophenotyping showed a new myeloid population. The emergence of chemo-resistant subclones that are undetectable by regular methods or substitution of a new leukemic clone are probably possible and neither of them could be ruled out for our patient. Cytotoxic drugs are responsible for the process of phenotypic conversion by inducing a multipotential clone or as a cause of inducing drug-resistant clones in case of multiclonal leukemia.<sup>7</sup> In a study from Argentina published in 2012, frequency of lineage switch in childhood leukemia was 0.6% ( 9 patients), which was from lymphoid to myeloid in 7 and from myeloid to lymphoid in 2 cases.<sup>4</sup> Although, the principle mechanisms involved in lineage switch continue to be unclear, plasticity of leukemic progenitors which could be multidirectional and reversible is suggested as a possible hypothesis.<sup>10</sup>

### Conclusion

We described a case of lineage switch leukemia from ALL to AML which is a rare event. Correct diagnosis

relies upon confirmation by immunophenotyping of the lineage conversion and certification that the same cytogenetic/molecular alterations remain despite the phenotypic changes. Prognosis of lineage switch leukemia is poor as our patient could not achieve a remission and dies of his disease.

**Conflict of Interest:** None declared.

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## LETTER TO EDITOR

# Metronomic Effect as A New Hypothesis in Maintenance Therapy of Acute Lymphoblastic Leukemia

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## Dear Editor

Growing evidence indicates that the innate and adaptive immune system has an important role in both development and treatment of cancer. The concept of metronomics therapy and mechanism of chemotherapeutic agents during maintenance phase of acute lymphoblastic leukemia (ALL) is an obscure topic for oncologists compared with treatment of other phases of this malignancy.

Some new immunological mechanisms have been proposed for the treatment of ALL during the maintenance phase of chemotherapy in addition to traditional irradiation mechanisms. Using regular continuous low-dose chemotherapeutic drugs during the maintenance phase is similar to metronomic strategy that may restore or mediate anticancer immune responses or antiangiogenic effects.<sup>1</sup> Anti-pyrimidines (6-mercaptopurine or 6-thioguanine), dexamethasone or prednisone, oral methotrexate and vincristine are standard approaches in maintenance therapy of ALL.

Malignant cells can escape from immune surveillance in different phases of tumor interaction with the host's immune system. Regulatory T cells (Treg) are CD4<sup>+</sup> CD25<sup>+</sup> lymphocytes, enriched in FoxP3, glucocorticoid-induced TNF receptor and cytotoxic T-lymphocyte-associated antigen-4 that can inhibit antigen-specific immune response both in a cytokine-dependent and cell contact-dependent method.<sup>2</sup> Treg cells can inhibit

anti-tumor immune response by suppressing the activity of both tumor-specific effector cells (CD8<sup>+</sup> cytotoxic T lymphocytes and CD4<sup>+</sup> T helper cells) and tumor-nonspecific effector cells (natural killer [NK] and NK T cells).<sup>1</sup> Also, Treg cells have been shown to be increased in a variety of human cancers. Increased frequency of Treg cells is associated with tumor progression and loss of treatment response.<sup>3</sup> Moreover, suppression of Treg cell activity by either specific blockade or depletion of them can enhance immune response against tumor-associated antigens.<sup>1</sup>

During the last decade novel mechanisms has been found for methotrexate in the treatment of rheumatoid arthritis. It restores defective Treg cell function through demethylation of the FOXP3 locus, leading to subsequent increase in FoxP3 and CTLA-4 expression.<sup>4</sup> Dexamethasone improves the function of regulatory T-cells and sets up a new balance of Th1/Th2 that markedly enhances FOXP3 expression and generates CD25(high) cells with phenotypic characteristics attributable to natural Treg cells.<sup>5</sup>

6-Thioguanine inhibits different steps of the angiogenesis process in vitro and uses a potent anti-angiogenic activity in vivo. Its anti-angiogenic ability together with its antimetabolite activity towards tumor cells may contribute to its action during maintenance therapy in acute myeloid leukemia (AML). These results

suggest a new rationale for the use of purine analogs in the management of AML.<sup>6,7</sup>

6-mercaptopurine (6-MP) specifically inhibits both the early and the late phases of the angiogenesis process in vitro and exerts a potent anti-angiogenic activity in vivo.<sup>6</sup> Vincristine also suppresses angiogenesis and the anti-angiogenic activity may be enhanced by combination with 6-TG.<sup>8</sup>

In summary we consider low dose continuous conventional chemotherapy as a metronomic mechanism of action with induction of antitumor immune response and antiangiogenic effects in ALL patients. This hypothesis needs to be more investigated specially in pediatric ALL.

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## PHOTO CLINIC

# Portal Vein Thrombosis Following Splenectomy in $\beta$ -thalassemia Major

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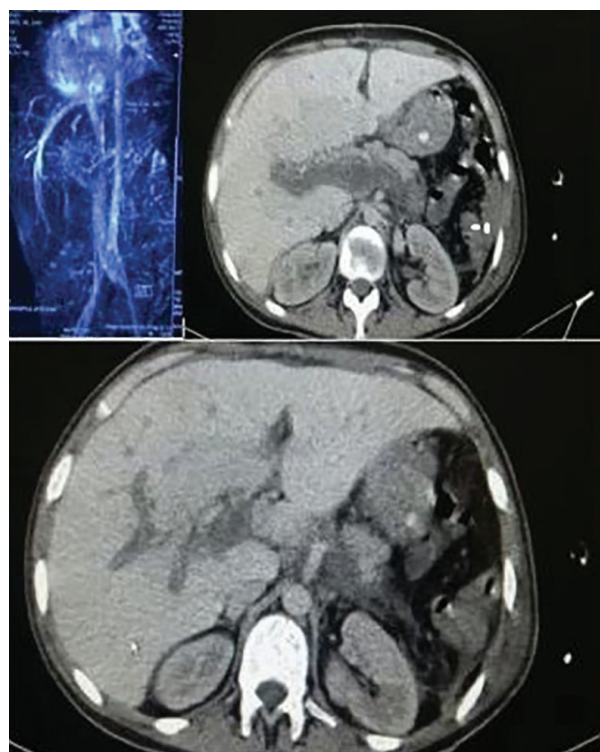
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A 25-year-old man with thalassemia major underwent cholecystectomy and splenectomy due to persistent colicky abdominal pain and increased blood requirements. He was febrile during the week after splenectomy, so that a thoraco-abdominal CT scan was scheduled for the patient which revealed evidence of thrombosis in main portal vein, superior mesenteric vein and splenic vein. Ultrasound color Doppler confirmed extensive area of thrombosis in portal vein. MRV images did not show any flow in portal and splenic veins (figure 1).

Thromboembolic (TE) events have been frequently reported in  $\beta$ -thalassemic patients along with risk factors such as diabetes, cardiopulmonary dysfunction, hypothyroidism, liver function abnormalities and post splenectomy thrombocytosis.<sup>1</sup> Although a high prevalence of thromboembolic events in thalassemia intermedia, particularly in splenectomized patients has been reported (29%),<sup>2</sup> incidence of TE has been reported to be from 1.1-5.3% in thalassemia major patients in different studies.<sup>1</sup>

Portal vein thrombosis is a well-known complication following splenectomy in beta thalassemia major.<sup>1</sup> Female gender, decreased levels of coagulation inhibitors, thrombocytosis and huge splenomegaly are predisposing factors for developing portal vein thrombosis. However, it is recommended that Doppler ultrasonography be performed in all patients after splenectomy to screen portal vein thrombosis.<sup>1</sup> We suggest that prophylactic



**Figure 1:** MRV images shows evidence of lack of flow in portal venous system. Abdominal CT-scan shows extensive thromboses in portal, mesenteric and splenic veins with cavernous transformation.



antiplatelet and antithrombotic therapy be considered in all thalassemic patients even before splenectomy.

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