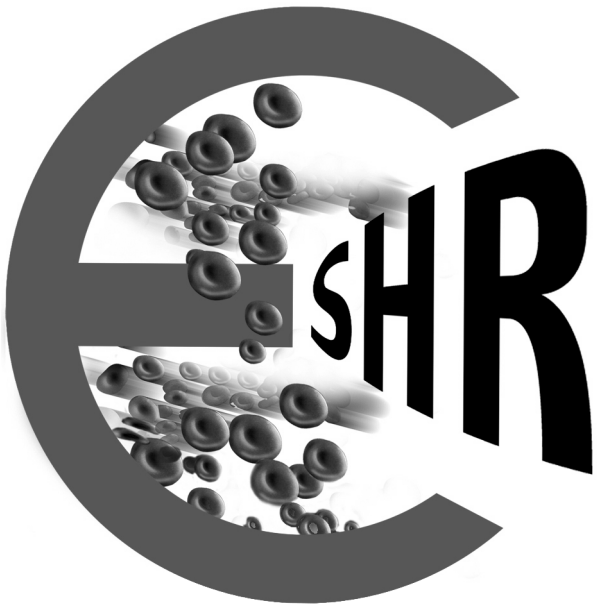
The Journal of the Egyptian Society of Haematology & Research



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Prognostic Significance of FLT-3-ITD Mutations in Adult Patients with Acute Myeloid Leukemia (AML)

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ABSTRACT

Background: FLT3 is a receptor tyrosine kinase with important roles in hematopoietic stem/progenitor cell survival and proliferation. Recently, it emerged as a possible prognostic factors in AML patients especially those with normal cytogenetics who constitute a heterogeneous group of patients requiring individualization of treatment.

The Aim of the Study: Is to test for the presence of FLT3-ITD mutation in exon 11 and to correlate it with other prognostic factors.

Patients and Methods: A total of 75 patients with newly diagnosed AML were included in this study between January 2004 and January 2006. Diagnosis was established by bone marrow examination, and immunophenotyping. Mononuclear cells (MN) were obtained from bone marrow samples at presentation by Ficoll-Hypaque density gradient centrifugation method and stored at –80ºC until use. All samples were analyzed for ITD mutation in exon 11 of the FLT 3 gene after extraction of genomic DNA from MN cells using PCR technique.

Results: Our patient group included 37 females (49.3%) and 38 males (50.7%) with a median age of 33 years. Blood indices analysis revealed a mean total leucocytic count of 43.42 x 10^9/L ±53.45, mean hemoglobin level of 6.7gm/dL ±2 and mean platelet count of 39.24 x 10^9/L ±31.46. The median percentage of blasts in peripheral blood was 46% and in marrow was 63%. The most commonly encountered FAB subgroup was M2 (44%), followed by M1 (36%), M4 (12%), M5 (6.7%) and finally M0 (1.3%). The FLT3-ITD mutation was tested for the 75 patients; 17 were found to be positive (22.7%) and 58 (77.3%) were negative. An attempt to correlate the clinical, hematological and immunophenotypic findings with the likelihood of positive FLT3/ITD mutation failed to find a correlation between the possibility of FLT3/ITD mutation and any of these variables except high percentage of blasts cells ≥50% in bone marrow. Molecular genetics testing for inv 16 and t(8,21) was available in 47 patients. Six/47 patients (12.7%) were positive, however, there was no correlation with FLT3/ITD status. Complete remission was achieved in 54/75 of patients (72%). Forty out of the 75 patients (53.3%) achieved CR after one course of induction, while 14 out of the 20 patients who received a second induction achieved CR (18.7%). Among those who were FLT3/ITD+ve, 10/17 (59%) achieved CR compared to 44/58 (76%) among those who were FLT3/ITD –ve (p=0.22). Three out of seventeen patients (17.64%) with FLT3/ITD +ve required a second course of induction to achieve CR compared to 17/58 (29.3%) with FLT3/ITD –ve. After a minimum follow up period of 12 months, the overall median duration of complete remission was 8.59 months (95% confidence interval 6.64, 10.55), [8.13] months for FLT3/ITD –ve patients (95% confidence interval 7.13, 11.69) and 3.93 months for patients who are FLT3/ITD +ve (95% confidence interval 2.51, 7.69 p0.0258)]. After a follow up period of 38 months the median survival was 7.4 month with a mean of 4.8 month (95% confidence interval 2.7-6.8). (4.1 month FLT3/ITD +ve with a mean of 10.1 months (95% confidence interval 7.9-12.3,8.9 month FLT3/ITD –ve p=0.0064).

Statistical analysis of the possible prognostic factors showed that only high TLC and age showed statistically significant influence on incidence of CR rate. Whereas age and FLT3/ITD demonstrated statistically significant longer duration of CR and survival (p=0.000 and 0.025) respectively.

Conclusion: FLT-3 ITD mutations are correlated with adverse prognosis particularly in patients with AML. This genetic marker either alone or in combination with others might serve to tailor treatment for some heterogenous AML patient population like those with normal cytogenetics.

Key Words: *AML – FLT3.*

INTRODUCTION

Karyotyping is still recognized as the most important prognostic factor in patients with AML. However, more than 50% of these patients have normal karyotype and are allocated in the intermediate risk group **[1,2]**. The results of

recent studies showed that they represent a heterogenous group and that molecular differences might help for further prognostication and tailoring of treatment **[3,4]**.

FLT3 (fms-like tyrosine kinase 3) is a class III tyrosine kinase receptor (RTK) involved in signaling pathways regulating the proliferation of hematopoietic stem cells and early progenitor cells. Like other class 3 RTKs (e.g., fms, kit, PDGF), FLT3 consists of 5 extracellular immunoglobulin-like domains, a transmembrane domain, a juxta-membrane (JM) domain, 2 intracellular tyrosine kinase (TK) domains separated by a kinase insert domain and an intracellular C-terminal domain **[5]**. The gene encoding FLT3 maps to chromosome band 13q12 and comprises 24 exons that span a genomic region of approximately 100kb **[6,7]**.

In patients with AML two types of activating FLT3 mutations have been identified in two functional domains of the receptor, the juxtamembrane (JM) domain and the split TKD. The JM domain which is crucial for kinase autoinhibition is disrupted by ITDs of various size and insertion sites in 28% to 34% of cytogenitically normal AML (CN-AML). FLT3-ITDs result in ligand-independent dimerization and tyrosine auto-phosphorylation as well as activation of the RAS/MAPK, STAT5 and PI3K/AKT pathways **[8,9]**. The activation loop (AL) in the carboxy-terminal lobe of the TKD is affected by point mutations, small insertions or deletions mainly involving codon 835 and 836 in 11% to 14% of CN-AML. In vitro studies and results from global gene expression profiling revealed that there are similarities but also important differences in signal transduction properties between FLT3-ITDs and FLT3 TKD mutations that may explain differences in clinical phenotypes **[10]**.

The aim of this study is to estimate the incidence of FLT-3 ITD mutation in Egyptian patients with be novo AML other than M3, and to correlate it with potential prognostic factors.

PATIENTS AND METHODS

This study involved 75 patients with newly diagnosed AML presenting to the Medical Oncology Department, National Cancer Institute, Cairo University, in the period between January 2004 and January 2006. Patients fulfilled the following criteria: Age between 18-60 years, ECOG performance status ≤2, all FAB subtypes except M3, no other malignancy, no prior chemotherapy or radiotherapy, no medical contraindications, normal ejection fraction (as assessed by echocardiography). Pretreatment evaluation: All patients underwent the following: Full history and physical examination, complete and differential blood count, bone marrow analysis including cellularity, morphology, cytochemistry. Flowcytometry for immunophenotyping, cytogenetics, serum chemistry including hepatic and renal profiles, calcium level as well as uric acid. All cases were classified according to the French, American, British [FAB] classification. CSF examination was performed to those with symptoms of CNS involvement. Baseline Chest X-ray and abdominal sonar were obtained. All patients signed informed consent before starting treatment.

FLT3 ITD detection: Mononuclear cells were obtained from bone marrow samples at presentation and stored at –80ºC until use. Genomic DNA of all samples were analyzed for mutation of exon 11 of the FLT3 gene using genomic PCR method. The use of exon 11 specific primers allowed covering the whole JM & the first part of TK-1 domain where most of the reported mutations are located.

Fifty µg genomic DNA was amplified in a 50µl reaction containing 10µl TisHcl (PH 8.3), 50µl Kcl, 1.5µl Mgcl, 200µl of each d NTP, 2.5U Tag polymerase, 40pmol of each primer and 6% dimethylsulphate. Amplification process consisted of 40 cycles of 4ºc for 30 seconds (denaturation), 50ºc for 45 seconds (annceling) and 72ºc for 1 minute (extension). One more step of final extension at 72ºc for 7 minutes was added.

The sequence of the primer used is:

- 11F (Sense) 5'' CAATTTAGGTATGAAAGCC –3''.
- 11R (Antisense) 5' C AAA CTCT AAATTTT CTCT–3''.

Ten ul of the PCR product were electrophoresed on 2% agarose gel, stained with ethidium bromide and photographed. Wild type band size was 133bp whereas an extra PCR band (mutant) appeared in case of FLT ITD.

Fusion genes detection by RT-PCR:

RNA was extracted from 300µl peripheral blood or bone marrow sample using a salting out procedure (Purescript, Gentra, Minneapolis, MN, USA) according to manufacturer's instructions A visible, translucent RNA pellet was then performed, washed by 70% ethanol and rehydrated for 50min in an ice bath. Reverse transcription was done using Multiscribe Reverse Transcriptase enzyme in a final mix of 40µl volume with a Gold RNA PCR kit (Applied Biosystems, USA). Cyclic conditions consisted of 25ºC for 10min and 42ºC for 1 hour. PCR was performed to detect the core binding factor fusion genes t(8;21) (q22;q22) and inv (16) (p13q22)/t(16;16) (p13;q22).

Primers used for detection of fusion genes were taken from the European BIOMED-1 concerted action Investigation of minimal residual disease in acute leukemia **[11]**.

Five µl c DNA was amplified in a 50µl reaction volume containing 20µml TrisHcl, 50µml Kcl (pH 8.3), 2.5µml Mgcl, 400µml final concentration of each primer, 200µml of each deoxyribonucleotide triphosphate (d NTPs) and 1.5U Tag polymerase.

Cyclic conditions consists of 95ºc initial molting for 10 minutes then 35cycles of 94ºc for 30 seconds (denaturation), 65ºc for 60 seconds (annealing) and 72ºc for 60 seconds (extension). Nested PCR consisted of same volume, reagents and cycle conditions as for first round using internal (nested) primers and using 1µl DNA template from the first round.

Primer sequence for t(8;21) (q22;q22) AML1/ETO:

- AML1-A: CTACCGCAGCCATGAAGAACC.
- ETO-B: AGAGGAAGGCCCATTGCTGAA.
- AML1-C: ATGACCTCAGGTTTGTCGGTCG.
- ETO-D: TGAACTGGTTCTTGGAGCTCCT.

Primer sequence for Inv 16 (p13,q22) CBFB-MYM11:

- CBFB-A: GCAGGCAAGGTATATTTGAAGG.
- MYM11-B2: TCCTCTTCTCCTCATTCTGCTC.
- MYM11-D2: CTTGAGCGCCTGCATGTT.
- CBFB C: GGGCTGTCTGGAGTTTGATG.

Product size for AML1-ETO was 395bp for first round PCR and 260bp for second round whereas amplicans size for CBFB-MYM11 ranged from 418 to 1345bp for first round and from 175-1200bp for second round.

All PCR products obtained through individualized RT-PCR reactions were separated on a 2% ethidium bromide agarose gel for 30 minutes. Fragments size was determined by running a molecular weight marker of known size and comparing the distance of unknown fragment in relation to the ladder (ø, Phi X DNA-HAE III, 500µg/ml; Cat. 302-61, New England Biolab).

Treatment plan:

Patients received standard induction chemotherapy using cytosine arabinoside and anthracycline as 7 and 3 protocol. Patients who achieved 1st CR were consolidated by the same regimen then HLA typing was done for those below 40 years of age with good general condition, –ve inv(16) & t(8,21). Those with HLA identical donor were referred for allogenic BMT as soon as possible. Those with no HLA identical donor were given 4 cycles of HAM regimen, patients who were +ve for inv (16) or t(8,21) i.e. favorable risk or >40 years were given 4 cycles of HAM consolidation.

Intrathecal prophylaxis was given only for cases with AML M5 (high risk of CNS disease) after reaching CR by induction chemotherapy and for patients with CNS disease at presentation. Triple intrathecal prophylaxis was given every 8 weeks for a total of 6 injections using methotrexate 15mg, Ara-C 40mg and dexamethazone 4mg.

Those with CNS disease at presentation were given triple intrathecal injection simultaneously with induction treatment until CSF was free. This was followed by craniospinal irradiation 24Gy following recovery then double intrathecal injections in cases of CR with Ara-c 40mg and dexamethazone 4mg every 8 weeks for 7 doses without MTX to avoid leukoencephalopathy.

Statistical analysis:

Statistical analysis was done using IBM compatible computer and according to the following tests:

Descriptive statistics was presented in frequency tables, means, and standard deviations whenever appropriate.

Analytical tests used included:

- Chi-square test for comparing two quantitative variables.
- Survival analysis and analysis of duration of complete remission were done using Kaplan Meier analysis.
- Significance level of 0.05 was used in all statistical tests.
- Disease-free survival (DFS): Included time to an event (death or relapse) measured from the end of induction for patients who achieved CR (induction deaths & non-responders were excluded).
- º Overall survival (OS): Included time from diagnosis to death.

RESULTS

Patient characteristics:

A total of 75 patients were included in this study, 37 females (49.3%) and 38 males (50.7%) with an age range of 18-60 years and a median of 33 years.

The mean total leucocytic count was 43.42 x 10^9/L ±53.45 with a range of 1.7-328 x 10^9/L and a median of 20 x 10^9/L. Blasts in peripheral blood were detected in 62 patients (82.6%) and the mean percentage of blasts in peripheral blood was 49%; while their mean percentage in marrow was 61.96±20.33 with 70.7% of patients having ≥50% blast cells.

All the studied cases were classified according to FAB classification. The most commonly encountered FAB subgroup was M2 (44%), followed by M1 (36%), M4 (12%), M5 (6.7%) and finally M0 (1.3%). The details of patients' characteristics are shown in Tables (1,2).

FLT3-ITD mutation:

The FLT3-ITD mutation was tested for the total 75 patients; 17 were found to be positive (22.7%) and 58 (77.3%) were negative. An attempt to correlate the clinical, hematological and immunophenotypic findings with the mutational status of FLT3/ITD failed to find a correlation between FLT3/ITD mutation with any of theses variables except high total leucocytic count although it did not reach statistical significance, Table (3).

Table (1): Characteristics of adult patients with AML (n=75).

Parameter	Total No. (%)
Age (Y):	
Mean±SD	32.84±10.5
<45	63 (84%)
≥45	12 (16%)
Sex:	
Female	37 (49.3)
Male	38 (50.7)
Symptoms:	
Fatigue	63 (84%)
Fever	21 (28%)
Bone aches	31 (41.3%)
Bleeding	19 (25.3%)
Signs:	
PS	
I	11 (14.6%)
II	64 (85.4%)
Lymphadenopathy	12 (16%)
Splenomegaly	20 (26.7%)
Hepatomegaly	26 (34.7%)
Gum hypertrophy	6 (8%)
Mucositis	18 (24%)
CNS infiltration	3 (4%)

Table (2): Characteristics of 75 adult patients with AML (Hematological and Biological parameters).

Parameter	Total No. (%)
WBCs (x 10^9/L):	
Mean±SD	43.41±53.45
<25	39 (52%)
25-100	28 (37.3%)
>100	8 (10.7%)
HB (gm/dL):	
Mean±SD	6.7±2
<8	58 (77.3%)
≥8	17 (22.7%)
Platelets (x 10^9/L):	
Mean±SD	39.24±31.46
<50	55 (73.3%)
≥50	20 (26.7%)
Peripheral blood blasts:	
+ve	62 (82.6%)
–ve	13 (17.4%)
B.M cellularity:	
Hypercellular	63 (84%)
Normocellular	12 (16%)
FLT3-ITD:	
+ve	17 (22.7%)
–ve	58 (77.3%)
T(8,21):	
+	3 (6.4%)
–	44 (93.7%)
Not done	28 (37.3%)
Inv(16):	
+	3 (6.4%)
–	44 (93.7%)
Not done	28 (37.3%)

Table (3): Correlation of hematological parameters with FLT3/ITD status in 75 adult AML cases.

Variable	FLT3/ITD+	FLT3/ITD-	*p*-value
WBCs:			
<25	6 (35.3%)	33 (56.9%)	
25-100	9 (52.9%)	19 (32.8%)	0.23
>100	2 (11.8%)	6 (10.3%)	
HB:			
<8	13 (76.5%)	45 (77.6%)	1.0
≥8	4 (23.5%)	13 (22.4%)	
Platelets:			
<50	13 (76.5%)	42 (72.4%)	1.0
≥50	4 (23.5%)	16 (27.6%)	
% of leukemic cells in marrow:			
<50	9 (52.9%)	13 (22.4%)	0.03
≥50	8 (47.1%)	45 (77.6%)	
FAB:			
M0	8 (47.1%)	1 (5.9%)	
M1	8 (47.1%)	19 (32.8%)	
M2	1 (5.9%)	25 (43.1%)	0.98
M4		6 (10.3%)	
M4eo		2 (3.4%)	
M5		3 (5.1%)	
M5b		2 (3.4%)	

Molecular genetics:

The expression of t(8;21) & inv 16 was tested in 47 patients. Three out of the 47 patients (6.4%) were found to be positive for t(8;21) and 3 patients for inv (16). An attempt to correlate FLT3/ITD with t(8;21) and inv (16) failed to find a statistically significant correlation.

Toxicity of induction:

Scoring of treatment toxicity was done according to WHO criteria.

Hematological toxicity: The mean duration of neutropenia (neutrophils <500mm^3) was 8.1 days ±2.7. On the other hand, the mean duration of neutropenia (neutrophils 500-1000mm^3) was 14.9 days ±5.2. The mean duration of hemoglobin recovery (Hb >8gm/dl) was 12.9 days ±7.2. The mean duration of platelet count recovery (Plt >50 x10^9/L) was 15 days ±6.8. Clinically observed refractoriness to platelet transfusion was encountered in 3 patients.

Non-hematological toxicity: Mucositis was the most common complication, encountered in 65 cases (86.67%) with 38.67% of patients experiencing G3 mucositis. Nausea and vomiting was recorded in 45 cases (60%) with 37.33% having G2. No grade III or IV were encountered.

Other toxicities include diarrhea in 15 cases (20%); mostly GII (10.67%), infectious complications was found in 63 (84%) patients; 38 (50%) had chest infection, while 19 (25.3%) had line related infections, and 4 (5.3%) had perianal infection. Hepatic toxicity was generally mild, with GIII hyperbilirubinemia in 1 patient (1.3%).

Alopecia was observed in 45 (60%) patients with 33.3% developing G2 alopecia.

Response rate:

Complete remission was achieved in 54 out of 75 patients (72%). Forty out of the 75 patients (53.3%) achieved CR after one course of induction, while 14 out of the 20 patients who received a second induction achieved CR (18.7%). Among those who are FLT3/ITD +ve, 10/17 (59%) achieved CR compared to 44/58 (76%) among those who are FLT3/ITD –ve (p=0.22). Three/17 (17.64%) with FLT3/ITD +ve required a second course of induction to achieve CR while 17/58 (29.3%) with FLT3/ITD –ve required a second course of induction to achieve CR. On the other hand, among those who are FLT3/ITD +ve, 2/17 (11.76%) failed to achieve CR compared to 4/58 (6.9%) of the FLT3/ITD –ve patient. Other hematological and clinical factors that might affect CR are shown in Table (4). Only high TLC and age showed statistically significant influence on incidence of CR rate.

Early death:

Early death was encountered in 15 out of 75 patients (20%). Early death in the group of FLT3/ITD +ve patients was encountered in 5/17 (29.4%) while it was encountered in 10/58 (17.2%) of the FLT3/ITD-ve patients. Septicemia (39%) was the leading cause of death in our patients followed by bleeding (21%).

Duration of complete remission:

After a minimum follow-up period of 12 months, the overall mean duration of complete remission i.e disease free survival (DFS) was 7.23 months with a mean duration of 8.59 months (95% confidence interval 6.64,10.55). Those who are FLT3/ITD+ve had a shorter DFS [the median duration of DFS is 3.93 months with a mean duration of 5.1 months (95% confidence interval 2.51, 7.69)] compared with FLT3/ITD–ve patients [median duration of DFS is 8.13 months with a mean duration of 9.41

months (95% confidence interval 7.13, 11.69)]. The difference in DFS between the 2 groups was statistically significant (p=0.0258) Figs. (1,2). Other factors that might have had an effect on duration of CR are shown in Table (5). Beside FLT3 status, age was the only variable affecting CR duration.

Table (4): Factors affecting complete remission (CR) rate in 75 adult AML cases.

	Complete remission No. (%)	p value
Age:		
<45	50/63 (79.4%)	0.003
≥45	4/12 (33.3%)	
Sex:		
Females	23/37 (62.2%)	0.075
Males	31/38 (81.6%)	
TLC:		
<25	32/39 (82.1%)	0.004
25-100	20/28 (71.4%)	
>100	2/8 (25.0%)	
HB:		
<8	43/58 (74.1%)	0.45
≥8	11/17 (64.7%)	
PLT:		
<50	40/55 (72.7%)	1.0
≥50	14/20 (70.0%)	
LDH:		
Normal	19/25 (86.4%)	0.09
High	34/50 (65.4%)	
FAB:		
M1,M2	45/60 (75%)	0.34
Others	9/15 (60%)	
FLT3:		
+ve	10/17 (58.8%)	0.22
–ve	44/58 (75.9%)	
T(8;21):		
+ve	3/3 (100%)	1.0
–ve	34/44 (77.3%)	
Inv (16):		
+ve	2/3 (66.7%)	0.49
–ve	36/44 (81.8%)	

Overall survival:

After a follow-up period of 38 months the median survival was 7.4 month with a mean survival of 8.9 months (95% confidence interval 7.0-10.7), with 5 patients (7.8%) remaining alive at 24 month and only 2 (1.6%) remaining alive at 38 months. The median survival was 4.1 months with a mean of 4.8 months (95% confidence interval 2.7-6.8) for FLT3/ITD +ve patients compared with a median survival of 8.9 months and a mean of 10.1 months (95% confidence interval 7.9-12.3) for FLT3/ITD –ve patients and the difference was statistically significant (p=0.0064), Figs. (3,4).

An attempt to find the influence of t(8;21) and inv (16) on overall survival failed to find a significant improvement. Patients positive for t(8;21) had a median overall survival of 12.7 month compared to 8.2 months for those who did not show this expression (p=0.350). The same applied for inv (16). Patients positive for inv (16) had a median survival of 6.8 months, compared to 8.3 months. (p=0.368) in patients who are negative for inv (16).

Prognosis:

Statistical analysis of the possible prognostic factors showed that among the factors listed in Table (4) only high TLC and age showed statistically significant influence on incidence of CR rate. Also the difference in age showed statistically significant influence on CR (p=0.003). CR rate was 79.4% below 45 years and 33.3% in patients aged ≥45 years. On the other hand, age and FLT3/ITD demonstrated statistically significant longer duration of CR (p=0.000 and 0.025, respectively). The latter also affected survival significantly.

Table (5): Factors affecting duration of CR in 75 adult AML cases.

Parameter	Mean duration of CR	95% confidence interval	Significance
Age:			
<45	9.16	(7.12,11.2)	0.000
≥45	1.66	(0.92,2.39)	
TLC:			
25	8.7	(6.23,11.24)	0.994
25-100	8.18	(5.01,11.35)	
>100	8.57	(5.95,11.18)	
HB:			
<8	9.13	(6.76,11.49)	0.238
≥8	6.57	(4.08,9.05)	
Platelets:			
<50	8.01	(6.12, 9.91)	0.446
≥50	9.88	(5.18, 14.57)	
LDH:			
Normal	9.45	(5.53,13.36)	0.513
High	8.21	(6.19,10.23)	
% of leukemic cells in marrow:			
<50	7.63	(4.65,10.62)	0.954
≥50	8.74	(6.49,10.98)	
BM cellularity:			
Hypercellular	9.33	(7.09,11.57)	0.108
Normocellular	5.71	(1.56,9.87)	
Blasts % on D14:			
≤40%	8.6	(6.06,11.15)	0.65
>40%	7.06	(.00,17.07)	
FLT3:			
+ve	5.10	(2.51,7.69)	0.025
–ve	9.41	7.13,11.69)	

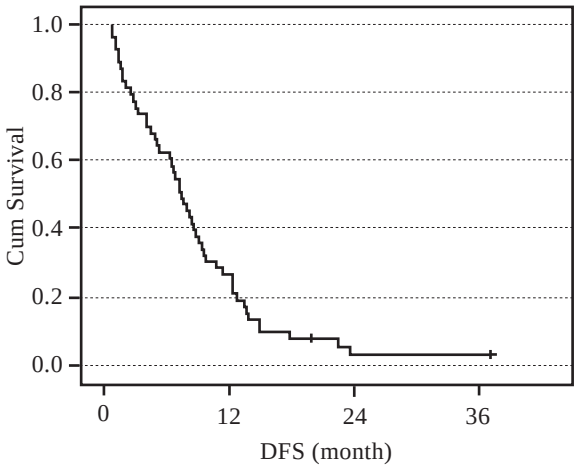

Fig. (1): Duration of complete remission in 75 adult AML cases.

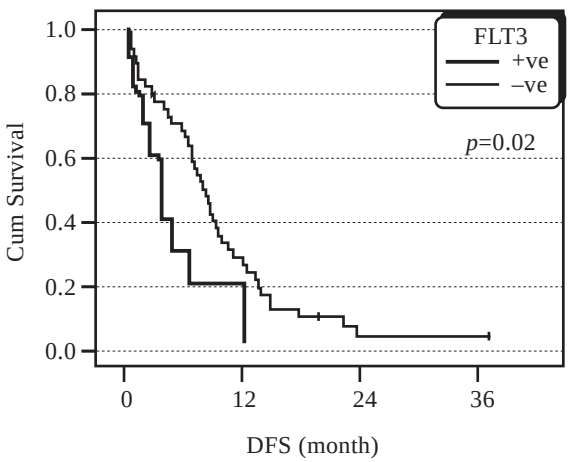

Fig. (2): Duration of complete remission according to FLT3/ITD in 75 adult AML cases.

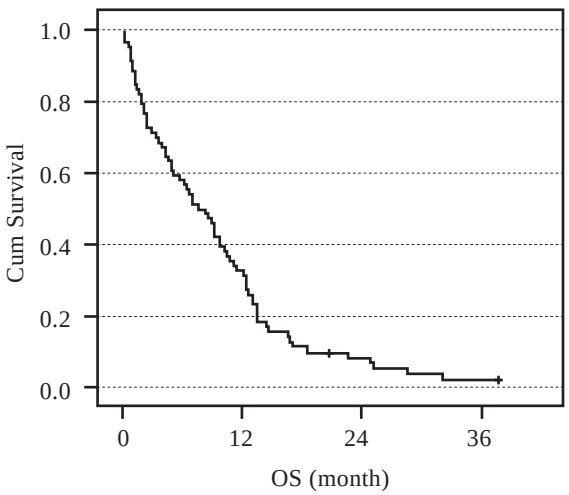

Fig. (3): Overall survival in 75 adult AML cases.

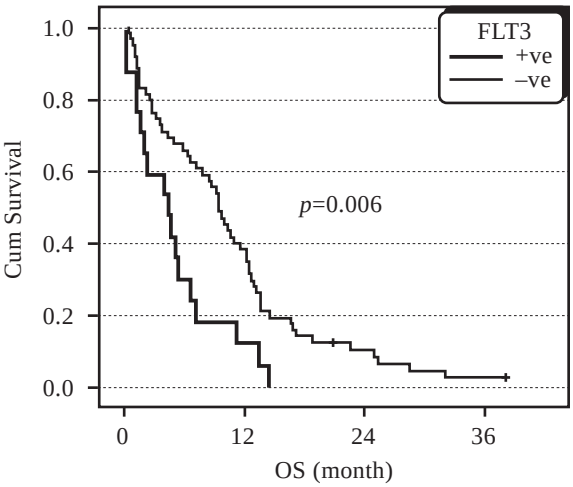

Fig. (4): Overall survival according to FLT3/ITD in 75 adult AML cases.

DISCUSSION

A variety of well-defined factors including age, intensity of post remission therapy (in younger adults), karyotype at diagnosis and P-glycoprotein affect outcome of treatment of adult patients with AML **[12]**.

In this study we investigated mutation of the FLT3-ITD gene by genomic PCR method in 75 newly diagnosed adult AML cases other than M3. The incidence of FLT3/ITD mutation was found to be 22.7% (17/75). This finding is in the range reported by other investigators (17-28%) **[13-17]**.

There was no age or gender preference. Furthermore, patients with FLT3/ITD had higher WBCs at diagnosis, although it did not reach significant value. This concurs with previous studies **[15,18,19]**. The number of bone marrow blast cells, and the presence of peripheral blasts showed no correlation. On the contrary, higher blasts in the bone marrow or peripheral blood were reported in positive cases by some investigators **[18,19]**. Furthermore, Thiede et al. **[20]**, and Munoz et al. **[21]**, showed that FLT3/ITD was significantly increased in patients with the FAB M5, M4, respectively with 40-50% positive cases. More than 60% of our patients were M1, M2, with a 45% incidence of positive cases.

The FLT-3ITD mutation adversely affected the outcome with a significantly shorter disease free survival and overall survival in positive patients confirming the results of previous studies in our patient population **[15,18,19]**. On the

other hand, while some studies **[20,22]** observed no effect of the FLT3/ITD on the overall survival for the whole group of positive patients; yet, survival was significantly affected in those who had both alleles positive for the mutation suggesting that not only the existence of the mutation will affect the bad outcome but this is also related to the level of mutant allele emphasizing the need for use of a'quantitation assay to determine the mutant/wild type ratio.

Complete remission was achieved in 54/75 of patients (72%). Although FLT3/ITD positive cases showed lower response rate, yet it did not reach statistical significance. This concurs the with studies conducted by other investigators **[15,18,19,20,22,23].**

From a clinical perspective, FLT3 mutations are relevant because of their prognostic impact and because constitutively active FLT3 is an attractive target for molecular therapy. Although the value of intensification of treatment (e.g. stem cell transplant) based on the FLT-3 status remains controversial, yet a number of FLT3 inhibitors at various stages of clinical development are available with promising results when combined with conventional chemotherapy **[24].**

In conclusion, there is widespread evidence that the presence of a FLT-3 mutation is a powerful prognostic and potentially predictive factor. Furthermore, it might abolish the good prognostic significance of other genetic markers as NPM1. The biological heterogeneity of AML has started to be unreavled with the wider use of genomic technologies contributing to refined diseases classification and tailoring of therapy.

REFERENCES

1- Slovak ML, Kopecky KJ, Cassileth PA, et al. Karyotypic analysis predicts outcome of pre-remission and post-remission therapy in adult acute myeloid leukemia: A Southwest Oncology Group/Eastern Cooperative Oncology Group study. Blood. 2000; 96: 4075-4083.

2- Mrózek K, Heinonen K, Bloomfield CD. Clinical importance of cytogenetics in acute myeloid leukaemia. Baillieres Best Pract Res Clin Haematol. 2001; 14: 19-47.

3- Döhner K, Tobis K, Ulrich R, et al. Prognostic significance of partial tandem duplications of the MLL gene in adult patients 16 to 60 years old with acute myeloid leukemia and normal cytogenetics: A study of the AML Study Group Ulm (AMLSG ULM). J Clin Oncol. 2002; 20: 3254-3261.

4- Schnittger S, Kinkelin U, Schoch C, et al. Screening for MLL tandem duplication in 387 unselected patients with AML identify a prognostically unfavorable subset of AML. Leukemia. 2000; 14: 796-804.

5- Matthews W, Jordan CT, Wiegand GW, et al. A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. Cell. 1991; 65: 1143-1152.

6- Abu-Duhier FM, Goodeve AC, Wilson GA, et al. Genomic structure of human FLT3: Implications for mutational analysis. Br J Haematol. 2001; 113: 1076-1077.

7- Agnès F, Shamoon B, Dina C, Rosnet O, et al. Genomic structure of the downstream part of the human FLT3 gene: exon/intron structure conservation among genes encoding receptor tyrosine kinases (RTK) of subclass III. Gene. 1994; 145: 283-288.

8- Nakao M, Yokota S, Iwai T, et al. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia. 1996; 10: 1911-1918.

9- Hayakawa F, Towatari M, Kiyoi H, et al. Tandem-duplicated Flt3 constitutively activates STAT5 and MAP kinase and introduces autonomous cell growth in IL-3–dependent cell lines. Oncogene. 2000; 19: 624-631.

10- Small D. FLT3 mutations: Biology and treatment. Hematology Am Soc Hematol Educ Program. 2006; 178-184.

11- van Dongen JJ, Macintyre EA, Gabert JA, Delabesse E, et al. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: Investigation of minimal residual disease in acute leukemia. Leukemia. 1999 Dec; 13 (12): 1901-28. Review.

12- Tallman MS, Gilliland DG, Rowe JM. Drug therapy for acute myeloid leukemia. Blood. 2005 Aug 15; 106 (4): 1154-63.

13- Abu-Duhier FM, Goodeve AC, Wilson GA, Gari MA, et al. FLT3 internal tandem duplication mutations in adult acute myeloid leukaemia define a high-risk group. Br J Haematol. 2000 Oct; 111 (1): 190-5.

14- Rombouts WJ, Löwenberg B, van Putten WL, Ploemacher RE. Improved prognostic significance of cytokine-induced proliferation in vitro in patients with de novo acute myeloid leukemia of intermediate risk: Impact of internal tandem duplications in the Flt3 gene. Leukemia. 2001 Jul; 15 (7): 1046-53.

15- Kottaridis PD, Gale RE, Frew ME, Harrison G, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: Analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood. 2001 Sep 15; 98 (6): 1752-9.

16- Schnittger S, Schoch C, Dugas M, Kern W, et al. Analysis of FLT3 length mutations in 1003 patients

with acute myeloid leukemia: Correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. Blood. 2002 Jul 1; 100 (1): 59-66.

17- Gale RE, Green C, Allen C, Mead AJ, et al. Medical Research Council Adult Leukemia Working Party. The impact of FLT3 internal tandem duplication mutant level, number, size, and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. Blood. 2008 Mar 1; 111 (5): 2776-84.

18- Fröhling S, Schlenk RF, Breitruck J, Benner A, et al. AML Study Group Ulm. Acute myeloid leukemia. Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with acute myeloid leukemia and normal cytogenetics: A study of the AML Study Group Ulm. Blood. 2002 Dec 15; 100 (13): 4372-80.

19- Bienz M, Ludwig M, Leibundgut EO, Mueller BU, et al. Risk assessment in patients with acute myeloid leukemia and a normal karyotype. Clin Cancer Res. 2005 Feb 15; 11 (4): 1416-24.

20- Thiede C, Steudel C, Mohr B, Schaich M, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: Association with FAB subtypes and identification of subgroups with poor prognosis. Blood. 2002 Jun 15; 99 (12): 4326-35.

21- Muñoz L, Aventín A, Villamor N, Juncà J, et al. Immunophenotypic findings in acute myeloid leukemia with FLT3 internal tandem duplication. Haematologica. 2003 Jun; 88 (6): 637-45.

22- Whitman SP, Archer KJ, Feng L, Baldus C, et al. Absence of the wild-type allele predicts poor prognosis in adult de novo acute myeloid leukemia with normal cytogenetics and the internal tandem duplication of FLT3: A cancer and leukemia group B study. Cancer Res. 2001 Oct 1; 61 (19): 7233-9.

23- Marzac C, Teyssandier I, Calendini O, Perrot JY, et al. Flt3 internal tandem duplication and P-glycoprotein functionality in 171 patients with acute myeloid leukemia. Clin Cancer Res. 2006 Dec 1; 12 (23): 7018-24.

24- Dohner H. Implications of the molecular characterization of AML. In Hematology, ASH Educational Book. 2007; 412-419.

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Review Article:
Therapeutic Potential of Stem Cells

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INTRODUCTION

The use of hemopoietic stem cells derived from the bone marrow (BM) or from the peripheral blood after mobilization with growth factors, for the treatment of benign and malignant hematological disorders, is well established. This review is meant to cover the use of stem cells in regenerative therapy to repair or substitute other organs e.g. Liver, cardiac or skeletal muscles, neurological, pancreatic, renal tissue or others. In fact, it is the experience with BM transplantation (BMT) that opened the way for the potential use of stem cells to regenerate organs other than the BM. This was derived from an observation of hepatocytes carrying the Y chromosome in a female patient receiving a male BM allograft. Unless cell fusion has occurred, there would be no other explanation but the transformation of one or the other cell types of the BM graft into hepatocytes (vide infra).

Several diseases are characterized by depletion of the stem cell pool such as BM failure due to malignancy of the hematopoietic stem cell (HSC) or genetic defects in the HSC itself (e.g. Fanconi's Anemia); these diseases have been successfully treated by BMT. Other diseases involve destruction of tissues that may not be robustly replenished from stem cell pools e.g. liver failure due to cirrhosis; these diseases are dealt with by direct organ transplantation. However, organ transplantation has much limitation with the first and most prominent being inadequate availability of organs **[1]**. Besides, organ transplantation does not apply in other situations e.g. CNS diseases or muscular dystrophy.

It has to be clearly stated, however, that the results of stem cell therapy for regenerative medicine are still, largely, controversial. It has to be clearly stated, as well, that this should not hinder clinical trials as long as the fundamental criteria supporting ongoing trials have been met **[2]**. The declaration of Helsinki states. "In the treatment of a patient, where proven prophylactic, diagnostic and therapeutic methods do not exist or have been ineffective, the physician, with informed consent of the patient, must be free to use unproven or new prophylactic, diagnostic and therapeutic measures, if in the physician's judgment it offers hope of saving life, re-establishing health or alleviating suffering" **[3]**.

Historically, it was only 40 years ago that two types of stem cells were identified, namely "hematopoietic" stem cells and BM "stromal" cells. In 1981 "embryonic" mouse stem cells (mESC) were isolated and described **[4,5]**. In the 90s, stem cells were discovered in the brain and more recently in the liver, heart, skin, GIT…etc. In 1998, human embryonic stem cells (hESC) were isolated **[6]**.

By definition, stem cells should be capable of self-renewal, differentiation into at least one mature cell type as well as functionally repopulate the tissue of origin when transplanted in a damaged recipient. Stem cells are subclassified based on their species of origin, tissue of origin and their potential to differentiate into specific type(s) of mature cells. This latter character is referred to as stem cell potency. The hierarchies of stem cells include totipotent, pluripotent capable of producing the three germinal layer cells, multipotent giving rise to cells of close

family e.g. hemopoietic cells, oligopotent e.g. giving rise to a limited number of blood cell lineages, bipotent e.g. giving rise to B cells and macrophages and unipotent giving rise to one cell type. The only totipotent stem cell is the zygote and its immediate daughter cells. The embryonic stem cells are harvested from the inner cell mass of the blastocyst 7-10 days after fertilization and early differentiation; they are supposed to keep this totipotency. After birth, the stem cells are called adult stem cells. Adult (postnatal) stem cells, while still pluripotent, have been thought to have more limited differentiation ability and to be organ specific e.g. HSC that give rise to all types of blood cells, neural stem cells (NSC) that give rise to neurons, astrocytes, and oligodendrites, mesenchymal stem cells (MSC) that differentiate into fibroblasts, osteoblasts, chondrocytes and adipose tissue, endothelial progenitor cells (EPC) that give rise to endothelial cells of blood vessels, oval cells that can give rise to hepatocytes and biliary tract, pneumocytes that can give rise to lung cells….etc. **[7]**. However, this concept of specificity of adult stem cells has been recently challenged and adult stem cells proved to show some flexibility; this flexibility is termed "plasticity". A widely accepted definition of "plasticity" has yet to be established but, in general, it refers to the ability of adult stem cells to cross lineage barriers and adopt the expression profile and functional phenotype of cells that are unique to other tissues **[7]**.

Possible mechanisms for plasticity:

Normal body homeostasis is kept by a balance between the rate of cell loss and regeneration. Under physiological conditions, this latter is secured by proliferation of progenitor cells; the tissue stem cells may be quiescent or undergo a very low rate of proliferation. In case of tissue injury, the stem cells may be called upon. If the injury is mild, the local stem cells will take care of the repair. But if the injury is too much, the local stem cells in the tissue may not cope; the cytokines released as a result of the injury will mobilize the BM stem cells, push them in circulation where ultimately they are going to home to the injured tissue through receptor ligand interaction. All the studies documenting stem cell plasticity used models of tissue injury to induce homing and differentiation of transplanted stem cells. Tissue damage likely creates a favorable environment for the crossing of lineage barriers. Probably tissue injury, through apoptosis and/or necrosis, creates a microenvironment (e.g. cytokine milieu or extracellular matrix characteristics) that enables efficient engraftment of circulating stem cells **[7]** Tissue injury may, probably create a situation comparable to the physiological situation necessitating continuous proliferation and production of new hemopoietic cells by the BM. Translineage differentiation of BM stem cells may occur either through differentiation or fusion. The proof of one mechanism does not exclude the others.

Direct and indirect differentiation:

Several mechanisms may be involved:

1- BM cells that differentiate into diverse cell types represent a previously unsuspected and unrecognized population of high pluripotent uncommitted stem cells located in the BM; in this case this is not true plasticity.

2- Committed HSCs undergo transdifferentiation. "Transdifferentiation" refers to ability of one committed cell type to change its gene expression pattern to that of a completely different cell type. This may be indirect through dedifferentiation going back to a stem cell phenotype and then redifferentiate along a different cell lineage. Alternatively it could be direct transdifferentiation involving direct transition in the gene expression pattern.

Fusion: An alternative mechanism would be the fusion of BM derived cell with an organ specific somatic cell to form a heterokaryon, thereby having the specific gene expression pattern of the organ in a cell capable of dividing and differentiation into new cells belonging to that organ. Cell fusion per-se is a known phenomenon e.g. in vitro fusion of fibroblasts with myoblasts is known to result in expression of muscle specific mRNA by the fibroblast nuclei **[8]**. However its contribution to plasticity is controversial.

Several studies have used chromosomal analysis to show that BM derived lung, muscle **[9]**, and kidney **[10]** are 2N, suggesting but not proving that they do not result from fusion but rather transdifferentiation. Cell ploidy, however, is not an absolute proof as some tetraploid cells can be present in normal tissue and the other

way round a tetraploid cell resulting from fusion could subsequently be a 2N particularly if the 2 nuclei did not fuse. In contrast to the previous studies other workers reported the opposite in the case of severely injured liver **[11,12]**. In both studies, donor derived BMSC were transplanted into FAH–/– mice, and engraftment into hepatocytes was proved by weaning of the FAH–/– mice off NTBC, the drug which allows them to survive in the absence of the FAH enzyme. In those mice, the majority of the hepatocytes that were FAH+ (donor derived) also had markers of the recipient cells suggesting that fusion had occurred. Whether fusion occurs or not and the magnitude of its contribution to plasticity still awaits final answers. Fusion may be a naturally occurring phenomenon, or a response to intense tissue injury. If the resulting cells are healthy and functional, the phenomenon will have a great physiological significance. Of course, the concern that the resulting cells may carry high potential for malignant transformation will always be in the background.

Stem cell sources:

Currently the main source of stem cells for regenerative therapy is embryonic stem cells and BM derived Adult stem cells. However other types of stem cells are used mainly fetal stem cells, cord blood stem cells and placental stem cells. Also in special situation like CNS, specialized stem cells may be specifically collected.

EMBRYONIC STEM CELLS

Mouse ESCs were first isolated in 1981 **[4]**. Human ESCs were first reported in 1998 **[6]**. Although in its infancy, hESC is thought to represent a theoretically inexhaustible source for regenerative medicine, a research tool to study development, both normal and abnormal and also to provide a platform to develop and test new therapies. When ESC are removed from culture conditions that block differentiation, they aggregate and develop into cystic structures called embryoid bodies containing derivatives of all three embryonic germ layers **[13]**. However human development is a complex choreography of events, each taking place in a critical temporal and spatial pattern. Unraveling the developmental pathways that specify formation of specific tissue within the embryo, so the possibility that these pathways can be recapitulated in vitro, is one of the main challenges in hESC research **[1]**. Many cell types have been derived from hESC in vitro including neural tissue **[14-17]**, insulin secreting cells **[18]**, cardiomyocytes **[18-21]**, hematopoietic cells **[22-24]**, endothelial cells **[15]**, osteoblasts **[25]** and hepatocytes **[26]**.

The strategy to produce these specialized cells from hESC involves 3 factors:

1- A combination of culture conditions that favors differentiation towards one or the other cell type.

2- Transgenic approaches that exploit factors known to direct differentiation.

3- Reporter systems to identify and allow isolation of that cell type.

The transgenic approach is helpful in defining developmental pathways, and may allow development of culture conditions that would enrich for a specific cell type without reliance on transgenes. However, it would be preferable to develop methods to purify the cells of interest relying on their endogenous and surface marker characteristics without introducing a reporter gene. If transgenes have to be introduced, safety measures are of absolute necessity including for instance the introduction of a suicide gene that would specifically and selectively destroy the transplanted cells if necessary **[27]**.

BONE MARROW DERIVED STEM CELLS

Types of BM derived stem cells:

Hematopoietic stem cells (HSC):

The only true assay for the presence of HSCs is their ability to reconstitute the hematopoietic system of a myeloablated host. If BM is depleted from cells bearing lineage specific markers, the resultant populations called "lin-" is 10-100 times enriched for HSC. HSC are characterized by a number of markers; in human they are generally CD34+. A special subset deserves mentioning that is the side population (SP). They are so called because they have a unique ability to extrude Hoechst dye, and when examined by FACS analysis they fall within a separate population that is to the "side" of the rest of the cells on a dot plot of emission data in the blue Vs. red spectrum. This ability to extrude the dye is attributed to the expression of ABCG2 transporter. SP cells are also present in other tissues. There is controversy whether these SP

cells are tissue specific stem cells within these organs **[28]** or if they are actually BM derived SP cells lodged within these tissues **[29-31]**.

Marrow stromal cells or Mesenchymal stem cells (MSCs):

MSCs are multipotent adult stem cells that reside within the bone marrow microenvironment **[32,33]**. These cells are characterized by growing and expanding in culture as an adherent layer with finite life span. They have the ability to differentiate not only into osteoblasts, chondrocytes, neurons, skeletal muscle cells, but also into vascular endothelial cells **[34]** and cardiomyocytes **[35-38]**. MSCs are present as a rare population of cells in BM, representing perhaps 0.001% to 0.01% of the nucleated cells; ~ 10 fold less abundant than HSC. In contrast to HSCs which are elusive, difficult to isolate and grow in culture, MSCs can be readily grown in culture. They can be cloned and expanded in vitro ≥ one million fold and retain the ability to differentiate to several mesenchymal lineages **[33,39]**. Although perhaps quiescent, bone marrow MSCs can divide rapidly once cell division begins; the cell population becomes very homogenous with time in culture and remains so for many passages **[40]**. A rapidly dividing population termed recycling stem (RS) cells was isolated from the BM as a subpopulation of MSC. RS cells were termed RS1 for small agranular rapidly proliferating cells and RS2 for small granular cells whereas the more typical fibroblastic MSCs were considered mature MSCs in culture **[41,42]**. In a series of articles, Verfiaille and collaeuges **[43-46]** described marrow progenitor cells or multipotent adult progenitor cells (MAPKs). The cultured cells have many of the attributes of MSCs; they lack MHC class I and II on their surface, so presumably, they may be used allogeneically. MSCs have been isolated from adipose tissue **[47-50]**, a source that is readily available and easily accessible in many patients (vide infra); their characteristics and behavior are virtually indistinguishable from BM-derived MSCs **[48]**. Although MSCs express a number of surface molecules that have cognate legends on T cells as well as they can be induced to express class II MHC by incubating the cells with INFγ, yet they lack the B7 costimulatory molecules CD80 and CD86 and these are not induced by INFγ treatment **[51,52]**. MSCs have been shown to inhibit T cell proliferation; when cultured with responder T cells in mixed lymphocyte reaction, they do not generally cause T cell proliferation but usually reduce the response of T cells to other stimulators. This effect is mediated via secretion of hepatocyte growth factor and transforming growth factor β **[52]**. The inhibition is dose dependent, independent of MSC source **[53]** and affects as well memory T cells **[54]**. Whereas autologous cell-base therapy poses no risk of rejection, an "off shelf" allogeneic cell product would be much more cost effective and much easier to administer and could potentially allow delivery of greater number of cells than autologous cell therapy **[2]**. MSCs appear to avoid the problem of rejection by being hypoimmunogenic (vide supra). As such, MSCs may allow allogeneic cell therapy while avoiding rejection. The advantages of all MSCs are many. Besides being readily accessible, easy to handle, can be expanded in culture and maintain their multipotentiality, the donor can be chosen ahead, qualified and tested for the absence of different disease organisms. Allogeneic MSCs can be ready in advance so they are immediately available when needed by a patient.

Endothelial progenitor cells (EPCs):

Cells with phenotypic and functional characteristics similar to the fetal angioblast are also present in adult human bone marrow. 6 These cells, known as EPCs, express some, but not all, cell surface markers characteristic of mature endothelium, certain surface markers of hematopoietic cells, and transcription factors that identify them as precursor cells **[55,56-58]**. In addition to endothelial cell surface markers, EPCs also express markers of immature cells, including AC133, a novel hematopoietic stem cell marker **[59]** not expressed on mature endothelial cells **[60]**.

UMBILICAL CORD BLOOD STEM CELLS

Umbilical cord blood (UCB) contains both hematopoietic stem cells and mesenchymal precursor cells **[61]**. Because stem cells in UCB exist in higher numbers than in adult human blood or bone marrow **[62]**, several populations of cells derived from UCB are possible sources of stem cells for tissue repair. Kogler and colleagues **[63]** have described a population of cells from human UCB called unrestricted somatic stem cells. These cells, which are fibroblast like in appearance, adhere to culture dishes; are negative for c-kit, CD34, and CD45; and are

capable of differentiating, both in vitro and in vivo, into a variety of tissues **[2]**.

STEM CELL APPLICATIONS IN REGENERATIVE THERAPY

Stem cell therapy for cardiac repair:

Coronary heart disease and heart failure continue to be significant burdens to health care systems all over the world. It is also one of the leading causes of death even if the standard therapeutic measures are followed especially in cases with congestive heart failure. Therefore any new treatment modality that benefits heart failure patients has the potential to result in dramatic improvement in health outcomes and substantial cost savings for communities.

Animal studies for regenerative cardiac repair dates back to early 90s **[64,65]**. Menasche et al. **[66]** described the first group of patients to receive skeletal myoblasts for cardiac repair. An elegant review of the current situation has been published by Boyle et al. **[2]**. Currently, results on more than 400 patients have been published. Though most of them are small pilot studies that lack randomization or control groups, yet all of them have proved that cell therapy is safe and feasible as well as they provide encouraging, albeit preliminary, signs of efficacy.

Administered stem cells may improve cardiac functions through different mechanisms:

1- Active myocardial regeneration resulting from transdifferentiation of the stem cells **[67]**.

2- Development of new blood vessels of donor origin due to transdifferentiation of the stem cells into endothelial cells **[68]** or of host origin due to growth factor-mediated paracrine effect **[69]**.

3- Production of cytokines and other factors that promote myogenic repair and prevent fibrosis **[70]**.

4- Cellular therapy contributes to the restoration of stem cell niches, facilitating the ability of the heart to regenerate itself **[71]**.

Of course understanding the mechanism is essential and would lead to improvement in the approach to therapy and hence the outcome. Yet the more important is that the patient will benefit whatever the mechanism is.

Various types of stem cells have been used including embryonic stem cells, resident cardiac stem cells, skeletal myoblasts, adult BM-derived stem cells, mesenchymal stem cells, endothelial stem cells and umbilical cord stem cells.

When transplanted into infracted myocardium, embryonic stem cell-derived cardiomyocytes engraft and improve cardiac function in rodent models **[72]**. Embryonic stem cells have the advantage of being capable to differentiate, as well, into vascular endothelium thus improving blood supply. Directed differentiation of hESC is based on protocols used in mESC **[73,74]**. However, these hESC-derived cardiomyocytes are immature, and have structural and functional properties consistent with fetal cardiomyocytes **[75]**. ESC-derived cardiomyocytes constitute a mixed population; they were transplanted as such in animal experiments. In a clinical setting, however, one would prefer to engraft a specific type of cardiomyocytes. In the setting of chronic heart failure or myocardial infarction, for instance, the cell type needed is ventricular cardiomyocytes, not a sinus-nodal type; the latter could be arrythmogenic and might cause considerable morbidity **[76]**. To-date, no human clinical studies have been initiated because of both ethical issues and also the possibility of teratoma formation suggested by a study injecting ES cells in skeletal muscles **[77]**.

In recent years, evidence has accumulated suggesting that the heart has endogenous regenerative potential. Undifferentiated colonogenic cells have been separated from both human and murine hearts. These cells have been separated and phenotyped **[78,79]**. They are responsible for replacement of ongoing turnover and for minor repair. Moreover they may represent a therapeutic target that, if enhanced, could induce cardiac self-repair **[2]**. Clusters of self-adherent cells (cardiospheres) that grew from cultured adult cardiac tissue derived from both human and murine hearts have been recently described **[80]**. These cells have been shown to be colonogenic and capable of transdifferentiation in vitro and to induce both myocardial and vascular regeneration **[81]**. A side population cells capable of differentiating into cardiac and hematopoietic lineages in vitro was separated from both developing and adult heart **[82]**. Cardiac stem cells are reported to increase in number immediately

after myocardial infarction **[83]** and it has been suggested that BM may represent a reservoir for cardiac stem cells, the depletion of which might contribute to diminished cardiac repair **[84]**. To date there are no clinical trials of human cardiac stem cells.

Autologous skeletal myocytes or satellite cells are another potential source for cardiac repair. They are the reservoir of regenerative cells for skeletal muscle tissue and are solely committed to myogenic lineage. Experimental studies and initial clinical trials have shown engraftment of donor cells and improvement in global cardiac pump function **[66,85,86]**.

One of the commonly used sources for stem cells is the BM. In humans, after orthotopic transplantation of female hearts into males, up to 15% of cardiac myocytes can be donor derived. The only possible source is BM-derived stem cells. Apparently, there is an intrinsic repair mechanism for minor cardiac damage within the BM but it is inadequate to repair larger damage **[2]**. Therapeutic benefit has been demonstrated in mice with experimentally induced myocardial infarcts that receive intracardiac injection of whole marrow (or Kit+ BM cells) during the initial post infarct period **[70]**. The use of green florescent protein (GFP)-positive donor BM demonstrated that the donor cells contributed to both cardiomyocytes and endothelial cell formation **[87]**. This regenerative therapy can be executed by either direct injection into the peri-infarct rim or by GCSF-induced mobilization of the stem cells from the BM to home to the site of injury **[67,88]**. Using the latter approach endothelial and smooth muscles were proliferating, but new myocytes growth predominated **[89]**. However differentiation of lineage negative, C-Kit+ cells into myocytes was denied by other workers **[90,91]**. Yet beneficial effect was reported in all studies. Other studies provided evidence that precursors of both cardiomyocytes and endothelial cells exist within the mononuclear cell fraction of bone marrow (BMMNCs). Studies by three groups are of special importance as they included controls. The first group **[92]** included 10 patients who received autologous BMMNCs reinfused into the infarct-related artery 7 days after myocardial infarction (MI); the control group included 10 patients who refused the intervention. The second study group **[93,94]** randomized 59 patients after acute MI to receive either intracoronary infusion of BMMNCs or ex vivo expanded circulating progenitor cells. The cells were delivered in the infarct-related artery 4 days after MI. The third group **[95]** randomized 60 patients after successful percutaneous coronary intervention for acute MI to receive either intracoronary BMMNCs or standard therapy. Taken together, the studies suggested that BMMNCS are safe and may improve cardiac function by a substantial and clinically meaningful degree following MI. In contrast to MI setting, patients with chronic ischemic cardiomyopathy are unlikely to release signals from damaged myocardium to induce stem cell homing. Therefore endomyocardial injection of cells is needed to deliver the cells to the exact location where their effect is required **[96]**.

The other type of stem cells used is MSCs; they are found in BM, muscle, skin and adipose tissue (vide supra). Studies showed that MSCs transdifferentiate in vivo into cardiomyocytes and endothelial cells **[97-100]** with significant increase in capillary formation and improved cardiac function **[101,102]**. In one study performed in pigs, allogeneic MSCs were used with no evidence of rejection.

In an elegant study performed by Miyahara et al., 2006 **[69]** in a rat model, Mesenchymal stem cell sheets were prepared in vitro from adipose tissue. Four weeks after coronary ligation, monolayer mesenchymal cells were transplanted onto the scarred myocardium. To identify the transplanted cells in myocardial sections, the authors used GFP-expressing cell grafts. They grafted monolayer MSCs onto the scar area of the anterior wall. After transplantation, the engrafted sheet gradually grew to form a thick stratum that included newly formed vessels (12.2%±0.6%), undifferentiated cells (57.8%± 2.2%) few smooth muscles (5.0%±0.3%) and few cardiomyocytes (5.3%±0.3%). No evidence of apoptosis was detected in the transplanted cells. Cardiac functions improved in all the mice and they all survived indicating that fatal arrythmogenic problems were not caused by integration of the MSC tissue. These data suggested that the major improvement may be mainly explained by growth factor-mediated paracrine effects leading to improvement of vascular supply rather than direct transdifferentiation into cardiomyocytes. It is worth men-

tioning that the MSC tissue included a large number of newly formed blood vessels. These vessels were composed of graft-derived cells, host-derived cells or both. The MSC sheet secreted a large amount of angiogenic and antiapoptotic cytokines, including VEGF and HGF. These results suggested that MSCs induce neovascularization not only through their ability to differentiate into vascular cells but also through growth factor mediated paracrine regulation. MSCs have also been used clinically **[103]**; 69 patients were randomized after acute MI to receive intracoronary autologous MSCs or placebo. Significant improvement in global and regional left ventricular function and significant reduction in the size of the perfusion defect was observed.

Another type of cells used in cardiac regenerative therapy is EPCs. After MI, injected EPCs homed to the infarct region within 48 hours **[88]**; at 14 days there was a marked increase in the number of capillaries in the infarct zone and the peri-infarct rim resulting from the induction of both vasculogenesis and angiogenesis leading to prevention of apoptosis as well as to some degree of cardiomyocytes regeneration **[104]**. EPCs can be obtained from BM using CD34+ cells which are enriched in EPCs and they can be expanded ex vivo and reinfused via the coronary artery **[105]**.

Umbilical cord stem cells were used in few experimental animal studies **[106-108]** but no clinical studies have been reported.

Stem cell therapy for neurological disorders:

The mammalian central nervous system (CNS) is an enormously complex organ. In the adult CNS, this complexity presents profound complications for useful regeneration in disease or damage; diverse insults are repaired with non-functional astrocytic scarring **[109]**. At present not a single neurodegenerative disorder can be reversed, none halted, and the evidence that any can even be slowed down is very slight. The modest and frequently inadequate or abortive replacement of CNS cells lost by ageing or disease falls far short of the regenerative capacity observed in most other organs. This has stimulated the search for more imaginative, regenerative treatments, generally based on cell implantation **[110]**. Scientists and clinicians, however, recognize the need to move cautiously towards cell implantation goals, as damaging results due to premature clinical testing would be devastating to patients as well as to the emerging stem cell neural repair field **[111]**. Three main challenges have to be considered. First the timing of implantation bearing in mind the reversibility of acute deficits and the irreversible axon loss of chronic lesions **[112]**. Second, the site of implantation could be problematic in widely disseminated diseases. Third the optimum cell type to be used in different situations **[110]**. Neuronal tissue is a vast collection of highly specialized cell types, each with unique roles. Directed differentiation and isolation of specific neuronal subtypes will need to be achieved **[1]**. Even if directed differentiation is able to yield specific neuronal subtypes, another concern in their use remains. Unlike cells of the hemopoietic system, there is no evidence that when delivered via the circulation, neurons can home to their appropriate location in the nervous system; thus they must be delivered to the correct site surgically **[1]**. However Akiyama et al. **[113,114]** have shown that intravenous infusion of BM cells can lead to myelination in the spinal cord. Also an opposing statement was delivered by Sanberg, **[115]** in the Lifecell conference at Chennai, Jan-2006 "It is now evident that the circulatory system can be used as a route to deliver specific cells (stem cells) to the damaged brain to facilitate repair and recovery". Furthermore, in order to properly function, neurons require synaptic input from neighboring neurons; regenerated neurons will need to integrate properly with existing, fully developed neurons to re-establish functional neural network. In addition to not restoring function, failure to integrate properly could result in epileptiform activity **[1]**.

Many cell types have been used for the treatment of neurological diseases including ESC, embryonic neural precursor cells, adult stem cells and BM stem cells. In addition to these, other cell types have been used in repair of spinal cord injury mainly, Schwann cells and olfactory nervous system cells.

ESCs were first isolated in 1981. Their substantial proliferative potential carries the advantage that numerous grafts can be prepared from a single sample. The significant risk of teratoma formation **[116]** was denied by Zhang et al. **[117]** who reported wide migration and appropriate differentiation of these cells in the neonatal

brain without teratoma formation. However, this is the general outcome when cells are implanted in the developing brain and cannot be extrapolated to cell therapy in the adults **[118]**. Away from the ethical problems, hESC have been grown in culture **[119]** and differentiated to neuronal cells **[120]**.

Embryonic neuronal precursor cells develop from ESC; they are a mixed population of stem and progenitor cells with a more limited proliferation and differentiation capacity confined to different types of neural cells. Adult neural stem cells (NSC) are also thought to be capable of dividing asymmetrically to produce a more committed progenitor and an identical daughter cell **[109]**. Neural stem cells isolated from human fetuses have been differentiated, in vitro, into oligodendrocytes [the myelinated cells lost in multiple sclerosis (MS)] **[121,122]** and dopaminergic neurons (lost in Parkinson's disease) **[123]**. Improved functional benefit has been observed in animal models of Parkinson's disease following, in vitro, expansion and differentiation of neural progenitors **[124]**. Early clinical trials using unselected human embryonic mesencephalic tissue demonstrated practical problems; several fetuses are required to secure enough cells for each transplant **[125]**.

Adult neural stem cells have been found in the brains of both rodents and humans **[126,127]**. Precursors of oligodendrites have been differentiated from adult rat brain and have demonstrated the capacity to produce myelin in vitro **[122]**. Adult neuronal progenitor cells (NPC) have also been differentiated into neurons when they engraft in certain areas of the CNS **[128]**.

Bone marrow stem cells provide a relatively accessible source of different types of stem cells. BM-derived cells can be easily expanded in vitro and manipulated to express markers of neuroectodermal lineage **[129-132]**. Although the expression of neural markers does not imply functionality, rodent studies have demonstrated that focal implantation or intravenous infusion of BM cells can lead to myelination in the spinal cord **[13,114]**. Similarly, BM-derived cells injected into focal areas of cerebral ischemia **[132]** or infused peripherally **[133]** led to functional improvements. Also, appropriate neural differentiation and amelioration of neurological deficit was reported in an animal model of Parkinson's disease following transplantation of BM stromal cells **[134]**.

Like with other systems, the transplanted cells may not exert their influence merely by transdifferentiation into functional cells replacing lost, damaged or dysfunctional cells but may act on the host environment to increase plasticity or resistance to disease. The possibility of grafting cells capable of secreting inhibitory neurotransmitters into foci of epileptic activity has been explored **[135]**.

Self-evidently, if stem cell transplantation is to be a useful therapeutic modality, the transplanted cells must produce a therapeutic benefit without significant harm. A balance must be struck between a primitive stem cell capable of multilineage differentiation and proliferation but which has a risk of malignancy and a cell with reduced differentiation and lineage potential but which is still capable of providing sufficient numbers of the appropriate cell phenotype and/or functional benefit. The recent appreciation that adult stem cells have much of the differential potential previously associated only with embryonic stem cells has encouraged those who have ethical objections to the use of human embryonic or fetal material, and such cells may have the additional advantage of being easier to control in vivo. Adult stem cells, particularly those originating outside the CNS, could be a source of autologous transplant material that is relatively easy to obtain and may also have increased resistance to CNS pathology **[109]**.

Stem cells in specific neurological diseases:

The specificity of cell types damaged by the pathological process has a significant impact on how amenable the disease is likely to be to cell replacement therapy. The more diffuse the damage, the more invasive the replacement strategy may need to be **[109]**. To follow are some examples of neurological diseases amenable to treatment with stem cells.

Parkinson's disease (PD): The symptoms of idiopathic PD result from the focal degeneration of dopaminergic neurons in the substantia nigra. A focal approach to replace a small number of spatially discrete neurons might be clinically beneficial. Clinical trials originally suggested that intrastriatal transplantation of fetal dopaminergic neurons could be beneficial

[136,137] but results of more robust randomized trial were generally disappointing **[138,139]**. However, transplantation of adult BM stromal cells into an animal model of Parkinson's disease showed some appropriate differentiation and amelioration of the deficit **[134]**.

Multiple sclerosis (MS): Neurological function is impaired in MS because of damage to myelin and the myelin-producing cells (oligodendrocytes), resulting in the disruption of the electric signaling. Spontaneous remyelination is known to occur in MS but it is inadequate and unsustained **[140]**. MS is, by definition, multifocal and injection of cells into each and every lesion is not practical. However, only small percentage of lesions is largely contributing to the disability. Targeted implantation at the site of symptomatic spinal cord or brain stem plaques might be initially beneficial but a more global treatment would be needed in the long term. This will necessitate a more complex strategy for cell delivery unless the cells themselves retain tropism for areas of pathology and can migrate to sites of damage following intraventricular or intrathecal delivery. Intravenous delivery of stem cells would also be effective due to the breakdown of the blood-brain barrier **[109]**. Oligodendrocytes are the cells responsible for most spontaneous remyelination. They would be the candidate of choice but the number of these cells is limited and their migration through normal brain is considerably impeded **[141]**. An alternative cell type is fetal neuronal stem cells but adult neuronal stem cells may be used preferentially. These can be directed to start differentiating along the oligodendrocyte lineage prior to transplantation **[109]**, a commitment that appears necessary for efficient myelination **[142]**. Another cell source is autologous BM; cells of BM origin can be induced to express oligodendrocyte antigens in vitro **[143]**. Myelination has been shown to occur in the rodent spinal cord following both focal implantation and IV infusion of BM-derived cells **[113,114]**.

Huntington's disease (HD): HD is characterized by spatial disruption and loss of complex connections of the medium spiny neurons. Attempts to reverse deficit using implanted fetal striatal neurons were reported as early as 1983 **[144]**. Several studies have now reported that fetal striatal neurones engraft, survive **[144-147]**, establish afferent **[148]** and efferent connections **[149]**, restore striatal sensitivity to dopamine **[146]** and reverse behavioral deficits in animal models of HD **[150,151]**. Encouragingly, primate studies suggest that the resultant benefit might extend beyond improvement in locomotor deficit and that there may also be amelioration of cognitive dysfunction **[152]**.

Alzheimer's disease (AD): The physiologic cause of AD is loss of neurons and neuronal dysfunction in the frontal and parietal association neocortex. Many neurotransmitter systems are involved in the pathology of this disease, but the most affected are the cholinergic, noradrenergic and serotonergic systems. Drug therapies and other intervention strategies to prevent or delay the progression of AD have been limited, at best **[153]**. Neuroreplacement therapy will undoubtedly become more feasible. It is well established that the olfactory sensory pathway is pathologically affected in AD. Severe loss (as much as 75%) of the anterior olfactory nuclei neurons in early-onset AD has been reported. Because of their vulnerability to toxic substances in the environment, olfactory sensory neurons readily degenerate and are replenished continuously from a population of stem cells at the base of the olfactory epithelium. Stem cells originating from the subventricular zone are known to migrate into the olfactory system **[154]**. Furthermore, these NSCs migrate into the hippocampus **[155]** and other parts of brain **[156]**, which may be important for proper maintenance of cognitive function. Thus, deficits in normal neurogenesis and differentiation of NSCs may be implicated in a cascade of impairment in olfactory function and cognitive function, as observed in AD. Mutations in amyloid-b precursor protein (APP) and the presenilins (PS1 and PS2) are evidenced in familial early-onset AD. It has been reported that the adult human brain retains multipotent progenitors **[157]**, suggesting that regeneration of CNS cell types may occur throughout life. Since NSCs carrying defected PS may be found in the familial AD brain, and because these NSCs may not properly respond to differentiation factors released from damaged neurons (due to deficits in the Notch signaling pathway), it was therefore suggested that AD patients may have impaired olfactory and cognitive functions as a combined consequence of progressive neuronal loss coupled with a defective neuroreplacement system **[153]**. Thus, for familial AD, a therapeutic strategy

by which HNPs carrying defected presenilin are replaced by HNPs carrying wild type presenilin was proposed by Sugaya and Brenen, **[153]**. As a result of this therapy, healthy HNPs respond to endogenous differentiation factors, and migrate and differentiate in the affected brain to locales where they are needed.

Amyotrophic lateral sclerosis (ALS): ALS or classical motor neurone disease is a progressive condition that results in widespread muscle denervation due to the loss of both central and peripheral motor neurones. Both hematogenous and intrathecal delivery of stem cells have been considered, and the latter used in clinical trials with peripheral blood stem cells **[158]**. This is particularly ambitious given that axons will be expected to extend distances measured in tens of centimeters.

Stroke: Stroke is a common cause of neurological disability and death. The recovery that occurs following ischemic damage to the brain may be attributed both to neuronal plasticity, as well as neurogenesis following ischemia **[159,160]**. This has encouraged attempts to supplement endogenous repair using stem cells. Beneficial effects could potentially be mediated through the exogenous supply of cells capable of neurogenesis and/or neovascularization or via modulation of the environment to enhance plasticity or the survival and differentiation of host cells. Intracerebral transplantation of cells derived from neural cell lines have been reported to show some benefit in rat models of stroke **[161,162]** and in humans **[163,164]**. Animal studies have suggested that BM cells are recruited to ischemic brain **[165]**. BM-derived cells have also been demonstrated to contribute to functional improvements in animal models of stroke when injected focally **[132]** or delivered intravenously **[133]**. The exact mechanism underlying this remains unclear, but the transplanted bone marrow cells have been shown to be associated with new vessel formation **[122]**, increased levels of brain-derived neurotrophic factor and nerve growth factor **[128]**, as well as the expression of neuronal markers **[132,166]**.

Cell Therapy for Spinal Cord Injury (SCI):

Different approaches with different cell types have been used in a trial to repair SCI.

Peripheral nerve grafts with various combinations of therapy were reported to promote recovery with regeneration of supraspinal axons into, through and beyond grafts in adult rats **[167,168]**. This approach has also shown some success in treatment of chronic incomplete human SCI **[169]** but it did not prove successful in people with complete SCI **[170]**.

Schwann cells from peripheral nerves have been transplanted into rat models of SCI. After contusion and implantation of Schwann cells, cavitation is reduced and sensory and spinal axons extend into grafts, and many are remyelinated **[171]**. Recovery of hind limb function was reported in some **[171]** but not all studies **[172]**. Human Schwann cells have also been transplanted into the transected spinal cord of rats with attenuated immune systems; functional improvement was also reported **[173]**. So far, there have been no peer-reviewed reports of clinical trials involving the transplantation of Schwann cells after SCI **[174]**.

Olfactory nervous system cells from the embryonic and adult olfactory bulb or mucosa have been transplanted after SCI. Functional recovery and/or CNS axon regeneration has been reported when cells are transplanted immediately or up to 2 months after SCI in adult rats **[175-177]**, although whether olfactory cells directly myelinate axons after SCI remains controversial **[178]**. Transplantation of cells from the olfactory nervous system does not, however, promote CNS axon regeneration and functional recovery under all circumstances **[171,179-181]**. Transplants from fetal olfactory bulbs or adult mucosa were reported to be performed in more than 400 humans in China, Portugal and Colombia **[170,182,183]**. Improvement in motor and sensory functions was reported **[170,182]** but it is difficult to gauge safety and efficacy of this intervention **[174]**.

Embryonic CNS tissue was also tried as a source of cells in SCI. After contusion and transplantation of fetal spinal cord into the lesion site, small but significant improvement of function was observed in rats **[184,185]** and cats **[186]**. The difficulty of obtaining fetal tissue for transplantation is a limiting factor for that approach in humans.

Embryonic stem/progenitor cells transplantation for SCI faces three major challenges namely controlling the survival, integration and

differentiation of transplanted cells **[174]**. Different types of stem cells were tried to repair injured adult rodent spinal cord including stem cells **[187,188]** or progenitor cells **[189-191]** derived from rodent embryonic or human umbilical cord; modest improvement in functional recovery was reported by some **[187,192,193]**. Neural progenitors derived from human fetuses have been transplanted into immunosuppressed mice **[194]** and non-human primates **[195]** after contusion. In both cases, the transplanted cells survived and differentiated into cells with characteristics of oligodendrocytes and neurons, and were associated with locomotor improvements **[194,195]**. The best approach is to use progenitor cells that have been pre-differentiated to a desired lineage before transplantation. Transplantation of hESC-derived oligodendrocyte-restricted progenitor cells into the adult rat spinal cord 7 days after injury enhanced remyelination and promoted improvement of motor function. The cells survived, migrated over short distances and differentiated into oligodendrocytes. By contrast, when cells were transplanted 10 months after injury, there was no enhanced remyelination or locomotor recovery **[196,197]**.

Adult stem/progenitor cells are now being considered for CNS transplantation. Transplantation of HSCs promotes functional recovery after compression-induced SCI in mice **[198,199]** and transplantation of BMSCs significantly improves hind limb function after SCI in mice and rats **[199-201]**. A small scale human trial was conducted in which autologous BMSCs were intravenously delivered to nine patients with SCI **[202]**. The improvements observed appeared to fall within an expected range of spontaneous recovery, and one participant advanced from ASIA category B to D. However it can be concluded only that a measure of procedural safety was demonstrated. Adult neural progenitor cells (NPCs), isolated from the dentate gyrus, the subventricular zone or spinal cord, have been shown to self-renew, and to be multipotent in vitro and after transplantation into the CNS **[203,204]**. After transplantation of adult NPCs into the intact and injured murine spinal cord, differentiation into only astrocytes or oligodendrocytes is observed **[205-206]**. NPCs transplanted 2 weeks post-injury survived, migrated, integrated in the injured spinal cord tissue, generated mature oligodendrocytes that remyelinated the injured axons, and promoted some functional recovery. However, NPCs transplanted 8 weeks post-injury did not survive, and failed to exert similar effects **[207]**. Therefore, there is a need to find and neutralize the inhibitory obstacles present in chronic SCI that interfere with NPC survival after transplantation **[174]**.

Damage to the spinal cord often results in progressive tissue loss and subsequently in cavity formation. These cavities may be of substantial diameter leaving only a small rim of white matter **[208]**.

However to bridge a large gap in the injured tissue may be difficult if not impossible without tissue engineering. A scaffold grafted into the site of injury may provide necessary mechanical support for the transplanted cells, guide axonal growth and promote better integration with host tissue. Different compounds were used **[193,209]** but the potential problem may be based on the type of cells used to populate the scaffold as well as on the development of a glial scar around the injury. An alternative approach utilizing enzyme chondroitinase ABC has been reported **[210]**. It is well-known that at the site of the spinal cord injury a glial scar forms containing extracellular matrix molecules including chondroitin sulphate proteoglycans which are inhibitory to axonal growth. In a recent study the investigators have used specific enzyme chondroitinase ABC to degrade chondroitin sulphate **[211]**.

Stem cell therapy for liver diseases:

Orthotopic liver transplantation has proven to be effective in the treatment of a variety of life-threatening liver diseases; however, significant morbidity and mortality remains. In addition, the growing disparity between the number of donated organs and the disproportionately large number of patients awaiting transplantation has provided an impetus for developing alternative therapies for the treatment of liver failure **[212]**. Novel strategies designed to increase the number of organs transplanted, such as the use of adult living donors, are not without significant risk to both the donor and recipient **[213]**.

The hepatic parenchyma is made up of hepatocytes and cholangiocytes. Unlike other organs, liver cell mass is restored primarily through division of the majority of mature hepatocytes

and not via a dedicated stem cell population. At times of overwhelming cell loss with long standing iterative injury (e.g. chronic viral hepatitis), or when hepatocytes replication is impeded, regeneration seems to occur via a second cell compartment **[214,215]**. In rodents, these are oval cells but in human they are more aptly called hepatic progenitor cells **[216]**; attempts to identify the originating stem cell are hampered by lack of markers **[217]**.

Many cell sources have been tried for hepatic regeneration including fetal and adult hepatocytes, embryonic cells and BM-derived cells.

Hepatocyte transplantation has several advantages over whole liver transplantation. Intact liver has to be transplanted within a short time; isolated liver cells may be cryopreserved for later use **[218]**. However it is still unclear whether cryopreserved cells can engraft and function as well as fresh cells **[219]**. Another advantage of hepatocyte transplantation is that a single donor could potentially provide hepatocytes for several patients. However, despite unequivocal evidence of function in some patients, the efficacy of hepatocyte transplantation has been difficult to prove **[219]**. Although transplanted hepatocytes become integrated into host parenchyma, function, and survive, they proliferate poorly in the host liver. Animal models have shown that extensive repopulation by transplanted hepatocytes requires exposure of the transplanted hepatocytes to proliferation stimuli and selective loss of the host parenchyma **[220-222]**. The liver and spleen are the most reliable sites for hepatocyte engraftment and function. The peritoneal cavity may also be a site for transplantation of encapsulated or matrix-attached hepatocytes **[216]**. The expanded extracellular matrix associated with liver cirrhosis increases the endothelial barrier to engraftment in the liver. However, transplanted hepatocytes can migrate into cirrhotic nodules and integrate into liver plates following intraportal infusion in rodents. Furthermore, transplanted hepatocytes express enzymes associated with normal liver function, such as glucose-6-phosphatase and glycogen, and are capable of significant expansion following transplantation, as long as there is no ongoing injury to the liver **[223]**. Data indicates that transplanted hepatocytes that are resistant to the underlying disease could potentially repopulate a severely diseased cirrhotic liver. Several issues, however, may limit hepatocyte transplantation into the cirrhotic liver. Portal-systemic shunts will result in translocation of hepatocytes to the pulmonary circulation. While hepatocytes do not engraft in this location and are rapidly cleared, translocation of a large number of transplanted cells may produce pulmonary emboli with resultant cardiopulmonary compromise. More importantly, the presence of portal hypertension increases the risk of portal vein thrombosis; potentially further compromising host liver function. Finally, it is unclear whether the transplanted cells can function within cirrhotic nodules when there is ongoing injury or whether enough cells can engraft in the decompensated cirrhotic liver to significantly affect overall liver function. Transplantation experiments in urokinase-type plasminogen activator (uPA) transgenic mice and fumarylacetoacetate hydrolase (FAH)-deficient mice have suggested that the proliferative potential of adult hepatocytes is infinite **[224,225]**. However, treatment for a number of acute liver failure or end-stage liver disease is limited. Several experiments in animal models of liver failure have shown that hepatocyte transplantation resulted in a significant prolongation of survival time **[226-228]**. Clinical trials of hepatocyte transplantation to treat acute or chronic liver failure and inherited liver disorders have been performed **[229-231]**. Although the clinical efficacy of hepatocyte transplantation varies with the case of the liver diseases, hepatocyte transplantation is considered a potential treatment for metabolic liver diseases and a bridge for patients awaiting a donor liver for liver transplantation. Living or cadaveric livers as well as livers not used for liver transplantation are possible sources of hepatocytes. However, their availability is limited due to the shortage of donors **[232]**. Accordingly if hepatocytes can be generated, in vitro, from various types of stem cell; this might constitute a more available source for hepatocyte transplantation.

Embryonic cells would, logically, be the best candidate. Differentiation of ES in vitro seems to recapitulate early embryonic development **[220]**. When mouse embryonic cells are cultured in Petri dishes, embryoid bodies (EBs) form within few days **[233]**. Cultured EBs start to express the hepatocyte-related genes within a couple of weeks **[234-238]**. Albumin-expressing cells are observed in cultured EBs as clusters

in a multilayered structure **[234-238]**. Combinations of HGF with other growth factors, including oncostatin M and nerve growth factor (NGF) are used to induce hepatocyte-related gene expression in cultured EBs **[239,240]**. Generally, the effect of growth factors and the extracellular matrices on hepatocyte differentiation in EBs is limited, suggesting the difficulty of inducing ESCs to differentiate into hepatocytes in cultured EBs. A group reported spontaneous differentiation of functional hepatocytes in cultured EBs in the absence of exogenous growth factors **[234,236]**. It seems likely that hepatic differentiation proceeds by cytokines secreted by other cells in the developing EBs, and presumably via cell-cell interactions. Animal experiments have proved that ESC, per se or after in vitro manipulation, can differentiate into hepatocytes in vivo. Teratomas resulting from transplantation of mouse ESCs were shown to contain cells with mature hepatocyte phenotype **[241,242]**. When hepatocytes were isolated from the teratoma and transplanted into injured mouse liver, they integrated without forming teratoma **[242]**. Transplantation of EBs obtained from in vitro culture of mouse ESCs resulted in the formation of hepatocytes expressing albumin, however, teratoma formation was frequently observed **[243]**. Thus, elimination of tumorigenic cells from EBs is an important task for ES cell-based cell replacement therapy to become feasible. This can be achieved by Percoll discontinuous gradient centrifugation **[244]**, with antibodies or with a suicidal gene **[245]**. The Hepatocyte-rich fraction was found to improve the prothrombin time and total bilirubin markers **[244]**, to suppress fibrosis **[246]** and hence improve liver injury **[220]**. Human and Monkey ESCs were also shown to be capable of differentiation into hepatocytes in vitro under the effect of growth factors **[247-250]**. Inspite of all the progress, hepatocyte differentiation by ESCs is inefficient, and the mechanism of liver development needs to be understood to direct the hepatocyte differentiation from ESCs **[220]**.

Bone marrow-derived cells constitute an appealing source of stem cells for regenerative therapy of liver diseases as it is with other organs. Using Y chromosome tracking, a sparse number of hepatocytes seemed to be originating from the BM in male recipients of female orthotopic liver transplants, and in females who had received BMT from male donors **[251,252]**. In perhaps the most exciting demonstration of BMSC plasticity, transplantation of Lin-$Kit^{+}Sca^{+}Thy1^{lo}$ (KTLS) BM cells to irradiated hosts was used to treat an inborn error of metabolism. This was performed on an animal model of hereditary type I tryosinaemia, the fumarylacetoacetate hydrolase knockout mouse [FAH (-/-)]. With time, it became apparent that these initial observations were difficult to reproduce, and later elegant studies in the same FAH (-/-) mouse model conclusively showed that monocyte-hepatocyte fusion was the explanation for the restored normal phenotype to the FAH-deficient liver, in which hepatocytes formed by fusion expanded rapidly owing to a considerable survival advantage **[253,254]**. Unfortunately, in the absence of a strong selective pressure, it seems that stable long-term engraftment of BM-derived parenchymal cells is unusual. In the hepatitis B surface antigen transgenic mouse, the BM contributed to hepatocyte repopulation through cell fusion, but only at a very modest rate. In this model, constitutive HBsAg expression induces chronic low-grade hepatocyte turnover with nodule formation, and inhibition of hepatocyte replication with retrorsine provokes an oval cell response. Here, the contribution from BM-derived cells to hepatocyte repopulation waned to just 1.6% by 6 months, presumably owing to lack of a sustained selection advantage **[255]**. Low level repopulation was also documented in other animal models including C Cl4-induced liver damage **[256]**. The current balance of evidence therefore suggests that, under circumstances of severe or repeated injury, BM cells can contribute to only a minor amount of liver parenchymal regeneration, primarily through cell fusion. In therapeutic terms, cell fusion may be a powerful tool to correct metabolic disorders of hepatic origin. This has been exploited in a number of isolated clinical scenarios. For example, sequential healthy donor hepatocyte transplantation was able to moderate the clinical phenotype of argininosuccinate lyase deficiency, an inborn error of metabolism, in an affected child for periods of one year. Histological engraftment through cell fusion of over 10% was detectable together with an improvement in clinical and metabolic indices **[257]**. The evidence as to which type of BMSC is responsible for liver repopulation is conflicting. In early studies looking at BM contribution to hepatocytes in the FAH mouse, it seemed that HSCs were the stem cell fraction

involved **[258]**. The HSCs seemed to be the key cell in BMT experiments of CCl4 liver injury in irradiated C57/B6 mice **[259]**. In vitro, HSCs can be induced to differentiate into hepatocyte-like cells, given the appropriate medium containing HGF. More importantly, when cocultured with injured hepatocytes across a barrier through which soluble mediators can pass, HSCs could be induced to differentiate into hepatocytes **[260]**. Conversely, when human BMSC fractions were directly xenografted into rat liver damaged with allyl alcohol, only the MSC fraction seemed to give rise to hepatocyte-like progeny, positive for mRNA albumin expression **[261]**. Also in vitro transdifferentiation of MSCs into hepatocytes can be demonstrated when co-cultured with fetal liver cells **[262]**. Whether it is the HSC or the MSC compartment that contributes to BM-derived hepatocytes, or whether it can be both, remains unresolved **[217]**. Monocytes, though not stem cells, were also claimed to contribute. When treated with macrophage-colony stimulating factor and interleukin-3 and subsequently conditioned with hepatocyte medium, cells with the morphology, marker gene expression and metabolic function of hepatocytes were found. On transplantation into NOD/SCID mice, these cells showed liver integration and albumin expression. One study has shown that rodent and human multipotent adult MAPCs can be induced to adopt a hepatocyte phenotype in vitro and can display limited hepatocyte function (e.g., secrete urea, cytochrome P450 activity) **[263]**. MAPCs can also apparently differentiate into hepatocytes when infused in vivo into nonirradiated mice, although function was not determined **[264]**. However, it is worth noting that other laboratories have found it notoriously difficult to propagate MAPCs from BM **[265]**.

In contrast to hepatocytes, where derivation from the BM is limited, there is a significant contribution from BMSCs to the non–parenchymal cells within the liver. The sinusoidal endothelium seems to have BM origins. Circulating EPCs, which are of BM origin, participate in the formation of new blood vessels at ischemic sites throughout the body including the liver. These EPCs may have extra beneficial effects on hepatocyte regeneration, and fibrosis resolution **[217]**. There is also evidence that fibrogenic cells in the liver originate from BM. Hepatic damage during chronic liver disease is usually accompanied by progressive fibrosis. As a consequence of liver inflammation, hepatic stellate cells (HpSCs) become activated, proliferate and synthesize collagen. They display a myofibroblast phenotype histologically distinguished by expression of a smooth muscle actin, and are thought to be central to the pathogenesis of liver fibrosis; there is, therefore, much interest in being able to clinically modify their activity. It has been suggested that HpSCs have their embryological origins in the septum transversum mesenchyme. There is in fact a growing body of evidence to indicate that the myofibroblast population, at least in part, derives from BMSCs. In gender crossover BMT experiments using CCl4 and thioacetamide models of liver injury, up to 70% of HpSCs and myofibroblasts associated with septal scars were BM derived **[266]**. In contrast, a recent study, using CCl4 induced fibrosis model, transplantation of MSC separated from BM and propagated in culture for 4 weeks was associated with decreased fibrosis and improved liver function **[267]**. Certainly in the liver, it is likely that there is more than one population of collagen-producing cell disparate in derivation. A similar situation was encountered in human. In across gender transplantations, 6-22% of hepatic scar associated myofibroblasts were derived from BM. Recurrence of hepatitis C, accompanied by rapid and aggressive liver fibrosis, is a major cause of graft dysfunction and failure. The implication here is that a significant proportion of the fibrotic response is attributable to the recipient's cells rather than a property of the donor organ. Human BM-derived myofibroblasts have also been found in other tissues including the intestine, the lung **[268,269]**, the skin and kidney, the location being dependent on the site of injury **[270-272]**. The BM cell that gives rise to the myofibroblast is controversial but the suggestion is that the main protagonist is the MSC. In effect, more than one BMSC compartment may be contributing to the scar-forming cells within the damaged liver. Likewise, different studies have collectively shown that both HSCs and MSCs may repopulate the liver or ameliorate liver disease by promoting regeneration or attenuating fibrosis. At present, the specific role of each BMSC is incompletely defined and the validity of future work is crucially dependent on exactly how donor BMSCs are isolated and characterized.

The mechanisms of homing of BM cells to the liver are extensively investigated. HSCs express the cellular receptor CXCR4, to which the natural ligand is stromal derived factor-1 (SDF-1). When the SDF-1 concentration within the BM is reduced, HSCs are recruited into the circulation and migrate along a concentration gradient **[273,274]**. It has been shown that injurious stimuli such as irradiation and inflammation upregulate hepatic SDF-1 production **[275]**. Inoculation of human SDF-1 increases homing of HSCs to the liver, and blockade of CXCR4 abrogates it. The CXCR4 receptor has also been shown on oval cells, which in vitro seem to migrate along a SDF-1 gradient. HGF, upregulated during hepatic regeneration, can augment CXCR4 expression on HSCs and potentiate SDF-1-induced migration. Stem cell factor, the production of which localizes to the same area in the liver, acts synergistically with SDF-1 to induce HSC migration in vitro. HSCs express c-kit, the receptor for stem cell factor. Other factors such as matrix metalloproteinase-9 (MMP-9), which augments HSC release from the BM, and IL-8, which is upregulated in liver disease and stimulates granulocyte production of MMP-9, are also likely to be important. The literature on what determines MSC homing is more conflicting. It seems at best that only a small proportion of MSCs can express functionally active CXCR4 **[276]**. Using green fluorescent protein (GFP) as a cell marker, MSC migration to pancreatic islets in response to SDF-1 has been demonstrated, but no in vivo experiments have investigated MSC homing to the liver **[277]**. Clearly, the clarification of the factors controlling BMSC migration has important implications for future treatment in liver disease. In particular, if the precise precursor of the BM-derived myofibroblast is identified and its migration pathway elucidated, then the development of liver-specific anti-fibrotic therapies may become possible **[217]**. Proper homing of exogenously applied stem cells is likely to depend on whether they can integrate into their respective niches. This may depend on whether the existing stem cells within the niche have been disrupted or depleted. In the BM, myeloablation through irradiation will have this effect. In the liver, toxic damage (e.g., with CCl4) can alter the local niche. The fate of transplanted BM cells may thus be determined by whether they are introduced locally into the liver or whether their inoculation is peripheral, via the BM. The prior manipulation of the stem cell niche in the recipient is likely to be an important factor in the outcome.

The therapeutic potential of BM-derived cells is documented in animal models; whether or not engraftment and organ reconstitution continues in the long term has not been answered. One pathway by which recovery can occur in chronic liver disease is through a reduction in hepatic fibrosis. When MSCs in vitro were induced to adopt a hepatocyte phenotype and then transplanted intravenously into non-irradiated CCl4-damaged recipients, a histological decrease in hepatic fibrosis and a rise in serum albumin were noted **[278]**. Likewise in a similar animal model and experimental paradigm, the transplantation of a BM mononuclear MSC subpopulation led to a reduction in liver fibrosis when infused early enough after the onset of injury **[279]**. It may be, at least in part, that the anti-fibrotic property of BM cells is conferred by the infusion of macrophages (which express MMPs central to the degradation of collagen bands) (280). It has been clearly shown that BM-derived macrophages are crucial to the resolution of CCl4-induced liver fibrosis during the recovery phase after injury **[281]**. Another possible explanation for the reduction in fibrosis is that migrating BM cells increase hepatocyte proliferation and suppress fibrogenesis by supplying growth factors and cytokines critical to the recovery process. Amelioration of liver fibrosis was also achieved with EPC treatment, in the presence of increased HGF and vascular endothelial growth factor, and a reduction in the pro-fibrotic mediator transforming growth factor-β **[282]**. The application of BM cell treatment in liver is not as advanced as it is in cardiac diseases. In patients with chronic liver disease, there does not seem to be an increase in circulating BM-derived stem cells (defined as CD34+) at times of acute decompensation **[283]**. Nor does there seem to be a consistent improvement in liver function when G-CSF is given to patients with cirrhosis to increase the CD34+ cell count in peripheral blood, though isolated improvements in some biochemical indices are noted **[284]**. There are only a handful of clinical trials, all of which are small-scale, uncontrolled feasibility studies. The first study looked at patients with liver cancer undergoing portal vein embolization to induce contralateral lobe hypertrophy and there-

by increase the size of the future remnant liver volume before an extensive partial hepatectomy **[285]**. Accelerated hepatic regeneration was demonstrated in three of these patients after the infusion of autologous CD133+ BM cells. By CT criteria, the left lateral segments hypertrophied by two and a half times more than in non-BM cell-treated controls. Another preliminary uncontrolled study in five patients with cirrhosis showed a transient improvement in clinical parameters such as serum bilirubin and albumin over 60 days after portal vein or hepatic artery infusion of $1x10^6$ to $2x10^8$ autologous CD34+ BMSCs. Again feasibility and safety were demonstrated **[286]**. The only other published clinical trial involved nine patients with cirrhosis who received portal vein infusion of 5.2×10^9 autologous unsorted BM cells **[287]**. Follow-up was longer, at 24 weeks, and patients showed some improvement in Child-Pugh score and albumin. Liver biopsies, when taken, showed increases in proliferating cell nuclear antigen staining, an indirect marker of hepatocyte turnover; however, there was no control arm. A recent case report describes the use of autologous BMSCs as rescue treatment for hepatic failure in a 67-year-old man ineligible for liver transplantation **[288]**. The portal venous infusion of 5×10^6 CD34+ cells, obtained from peripheral blood after G-CSF induction, led to an apparent rapid improvement in hepatic synthetic function in this patient, although BMSCs were not identifiable as they were not labeled with markers before transplantation. In none of the clinical trials so far has colonization or even engraftment of transplanted cells been demonstrated in recipient livers. It is conceivable that the variable change in parameters of hepatic function may be occurring through the supply of growth factors promoting liver regeneration and fibrosis resolution. This in itself may be a sufficiently satisfactory end point.

Thus, currently, there is very little evidence that BMSCs can make hepatocytes at a level that could be clinically useful, nor has stable or long term engraftment been demonstrated. It is more probable that a realistic goal of BMSC treatment is to stimulate the regeneration of endogenous parenchymal cells or enhance fibrous matrix degradation. It appears that BMSC treatment can create a milieu conductive to liver regeneration through the transient supply of growth factors, but it is likely that repeated treatment would be required in clinical practice; this has not yet been studied. It is important to take into account the potential that stem cells may have for malignant transformation. It has become increasingly evident that the cellular origin of HCC is the oval cell or hepatic progenitor cell. This raises a theoretical concern that BMSC treatment may accelerate carcinogenesis in patients with liver disease. There is already a well-documented incidence of HCC in patients with cirrhosis, the precise cohort for which stem cell treatment may be most needed. There is of course the theoretical potential to exploit the BM-hepatic fibrogenic axis to influence and deliver antifibrotic treatments through the BM. This is an area in which future investigation may prove rewarding.

Stem cell therapy for skeletal muscle repair:

There are more than 20 types of muscular dystrophy and numerous other muscle disorders, but treatment options are almost nonexistent **[289]**.

Many cell types have been used in animal models of Duchenne's muscular dystrophy (DMD) including BM-derived cells, synovial membrane-derived MSC and mesoangioblasts.

Injection of marrow cells into damaged muscles leads to marrow derived cells with myocytes-specific gene expression **[290]**; functionality of the marrow derived myocytes is as yet unclear. An elegant study using transplantation of GFP+ marrow cells documents the engraftment kinetics of BM derived myocytes after transplantation of whole marrow contributing to approximately 3.5% of the muscle fibers in response to exercise **[291]**. A case report is that of a boy who was diagnosed with relatively mild DMD at the age of 12. The boy had received Allogeneic BM transplantation at the age of one year. It was suggested that healthy muscle fibers forming from the donor BM might have decreased the severity of the disease. Rare donor derived nuclei expressing normal dystrophin (0.5-0.9) were detected in the skeletal muscle fibers **[292]**.

Adult human mesenchymal stem cells isolated from synovial membrane (hSM-MSCs) were shown to have myogenic potential in vitro **[293]**. In a later study, by the same group, their myogenic differentiation was characterized in a nude mouse model of skeletal muscle regen-

eration and their therapeutic potential was tested in the mdx mouse model of DMD. Differentiation was sensitive to environmental cues, since hSM-MSCs injected into the blood stream engrafted in several tissues, but acquired the muscle phenotype only within skeletal muscles.

In a recent study Sampaolesi et al. **[294]** used a novel type of stem cells, termed mesoangioblast which can be harvested fro small blood vessels **[295]**. These cells have a number of advantages; they are relatively easy to isolate, and their number can be expanded greatly in tissue culture without losing the ability to form muscles. A dog model of DMD was used. The mesoangioblast cells transplanted were either healthy cells or genetically corrected autologous cells. The cells were infused in a major hind limb artery and the dogs received 5 injections at monthly interval. One dog received the infusion into the aorta. All dogs showed marked improvement that was especially evident in the last one that was able to walk 5 months after the last injection. In general dogs receiving donor cells improved more than those receiving corrected autologous mesoangioblasts. This method is promising for potential clinical application in DMD as well as a variety of other muscle diseases.

Stem cell therapy for renal regeneration:

Most researchers agree that the kidney should likely possess stem cells but evidence for functional renal stem cells within adult mammals remains elusive and their regenerative ability is incomplete **[296]**. A non-hematopoietic population of CD133 cells has been isolated from human kidney, cloned in vitro and found able to contribute to tubular regeneration in severe combined immune deficiency (SCID) mice **[297]**. In addition, cells with attributes of mesenchymal stem cells (including differentiation into fat and bone) have been cultured from glomeruli and whole kidneys of mice **[298]** although their ability to generate epithelial cell types was not explored. The contribution of extra-renal stem cells is suggested by the presence in tubules of appropriately differentiated epithelial cells that are of extra-renal origin, e.g. the epithelial nucleus bears an unexpected Y-chromosome in either a male recipient of a female renal allograft, or in a female recipient of a male bone marrow graft **[299]**.

There is evidence that circulating endothelial precursors originating from the BM can contribute to repairing the damage in kidney graft **[300]**. There is also evidence of extra-renal origin of mesangial cells **[301]**, myofibroblasts **[302,303]** and podocytes **[299,304-305]**. BM transplantation weeks or months before induction of renal injury was shown to result in differentiation of some of the transplanted cells into renal tubular cells capable of division though their long term survival is not known **[306]**. Other studies have shown engraftment of BMSC into nonepithelial mesangial cells and interstitial cells within the kidney **[307,309]**. In an elegant study, Held et al. **[310]** were able to generate a renal phenotype in the FAH (–/–) mouse mentioned earlier; up to 50% of the tubular cells expressed the FAH+ donor phenotype. Sugimoto et al. **[311]** recently reported that grafting whole wild type BM into young mice lacking the expression of the a3 chain of procollagen IV leads to an astonishing partial restoration of expression of the missing collagen chain (with incorporation of α3, α4, α5 triple helices in renal basement membranes), expression of α3 chain mRNA by podocytes, accompanied by improved architecture of the glomerular basement membrane (GBM) and, importantly, improved renal function **[311]**. Their results are especially remarkable in that renal injury was already established at the time of rescue with unaffected BM. By comparison, results of Poulsom et al. **[312]**, using an Alport model indicated that renal function and survival was better in recipients of normal BM than Alport BM. The authors concluded that any benefits of BM in Alport mice are derived from the HSC compartment as they have demonstrated that MSC are ineffective. The data collectively show that BM-derived stem cells could be potentially helpful in treatment of renal diseases.

Stem cell therapy for type 1 diabetes:

Type 1 diabetes is a T cell-mediated, organ specific autoimmune disorder, in which the body's own immune system attacks β cells and damages them sufficiently resulting in reduced insulin production. Recently it was shown that liver stem cells/hepatocytes can transdifferentiate into insulin-producing cells **[313]**. Such liver-derived insulin-producing cells may overcome immunosuppression. Moreover cells transfected with the human insulin gene produce insulin **[314]** including human BM MSC **[315]**.

A wide range of cell sources are being used to grow β cells for the treatment of diabetes including embryonic stem cells, BM stem cells, pancreatic ductal cells, mature β cells and hepatic cells.

Both derivatives of embryonic and adult stem cells are shown to secrete insulin in vitro. Human embryonic cells were reported to be induced to form islet-like clusters similar to immature pancreatic cells **[316]** with the possibility of reversal of hyperglycemia through the transplantation of embryonic stem cells derived insulin-producing cells **[317]**. Intraperitoneal transplantation of encapsulated ESC may protect them from immune attack **[318]**, however the effect of encapsulation on differentiation of stem cells needs to be addressed **[319]**.

Various types of adult stem cells were shown to transdifferentiate in vitro and/or in vivo into insulin producing cells; these include brain-derived neuronal progenitor cells **[320]**, rat neural stem cells **[321]**, umbilical cord blood cells **[322]**, BM stem cells **[323]**, and multipotent pancreatic progenitor cells **[324]**. The use of adult stem cells to produce insulin-secreting β cells for improving the disease condition in type 1 diabetes offers a new window of opportunity for effective treatment and cure. The major hurdle, however, is autoimmunity which could be overcome by engineering stem cells to escape recognition by the immune system **[319]**. Stem cell therapy for diabetes, however, is still in the early animal phases and its premature use in human may put patients at risk.

Currently the american diabetes association recommends:

- Genetic engineering of nonpancreatic cells into glucose-sensitive insulin-producing cells.
- Transforming stem cells or pancreatic endocrine cell lines into glucose-sensitive insulin-producing cells.
- Xenograft of nonhuman islet cells **[320]**.

In Conclusion:

The field of regenerative therapy is extremely promising. The progress in experimental applications is tremendous. Clinical progress, however, still faces many limitations. Apart from ethical considerations, results in experimental animals are still controversial in many situations; these results may not be reproducible in human. A lot of questions about which cells, how much and where to be injected have not yet been satisfactorily answered. The mechanisms of stem cell transdifferentiation or those by which they can ameliorate disease are far from fully understood. However efficacy and safety have been proved in many situations and clinical trials are going on. Currently academic laboratory work, experimental animal studies and clinical trials are going hand in hand.

REFERENCES

1- Lerou PH, Daley GQ. Therapeutic potential of embryonic stem cells. Blood Rev. 2005 Nov; 19 (6): 321-331.

2- Boyle AJ, Schulman SP, Hare JM, et al. Stem cell therapy for cardiac repair: Ready for the next step. Circulation. 2006; 114: 339-352.

3- World Medical Association. Declaration of Helsinki. Available at: http://www.wma.net/e/policy/b3.htm. Accessed December 3, 2005.

4- Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981; 292: 154-6.

5- Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA. 1981; 78: 7634-8.

6- Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998; 282: 1145-7.

7- Herzog EL, Chai L, Krause DS. Plasticity of marrow derived stem cells. Blood. 2003; 102: 3483-3493.

8- Hardeman EC, Chiu CP, Minty A, Blau HM. The pattern of actin expression in human fibroblast X mouse muscle heterokaryons suggests that human muscle regulatory factors are produced. Cell. 1986; 47: 123-130. Petersen BE, Bowen WC, Patrene KD, et al. Bone marrow as a potential source of hepatic oval cells. Science. 1999; 284: 1168-1170.

9- LaBarge MA, Blau HM. Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. Cell. 2002; 111: 589-601.

10- Masuya M, Drake CJ, Fleming PA, et al. Hematopoietic origin of glomerular mesangial cells. Blood. 2003; 101: 2215-2218. Ianus A, Holz GG, Theise ND, Hussain MA. In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. J. Clin. Invest. 2003; 111: 843-850.

11- Vassilopoulos G, Wang PR, Russell DW. Transplanted bone marrow regenerates liver by cell fusion. Nature. 2003; 422: 901-904.

12- Wang X, Willenbring H, Akkari Y, et al. Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature. 2003; 422: 897-901. Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J, Langer R. Endothelial cells derived from human embryonic stem cells. Proc Natl Acad Sci USA. 2002; 99: 4391-4396.

13- Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R. The in vitro development of blastocyst-derived embryonic stem cell lines: Formation of visceral yolk sac, blood islands and myocardium. J Embryol Exp Morphol. 1985; 87: 27-45.

14- Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature. 2004; 429: 41-6.

15- Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol. 2001; 19: 1129-33.

16- Schuldiner M, Eiges R, Eden A, et al. Induced neuronal differentiation of human embryonic stem cells. Brain Res. 2001; 913: 201-5.

17- Reubinoff BE, Itsykson P, Turetsky T, et al. Neural progenitors from human embryonic stem cells. Nat Biotechnol. 2001; 19: 1134-40.

18- Assady S, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M. Insulin production by human embryonic stem cells. Diabetes. 2001; 50: 1691-1697.

19- He JQ, Ma Y, Lee Y, Thomson JA, Kamp TJ. Human embryonic stem cells develop into multiple types of cardiac myocytes: Action potential characterization. Circ Res. 2003; 93: 32-39.

20- Kehat I, Kenyagin-Karsenti D, Snir M, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest. 2001; 108: 407-14.

21- Boheler KR, Czyz J, Tweedie D, Yang HT, Anisimov SV, Wobus AM. Differentiation of pluripotent embryonic stem cells into cardiomyocytes. Circ Res. 2002; 91: 189-201.

22- Itskovitz-Eldor J, Schuldiner M, Karsenti D, et al. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med. 2000; 6: 88-95.

23- Chadwick K, Wang L, Li L, et al. Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. Blood. 2003; 102: 906-915.

24- Kaufman DS, Hanson ET, Lewis RL, Auerbach R, Thomson JA. Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc Natl Acad Sci USA. 2001; 98: 10716-21.

25- Sottile V, Thomson A, McWhir J. In vitro osteogenic differentiation of human ES cells. Cloning Stem Cells. 2003; 5: 149-155.

26- Rambhatla L, Chiu CP, Kundu P, Peng Y, Carpenter MK. Generation of hepatocyte-like cells from human embryonic stem cells. Cell Transplant. 2003; 12: 1-11.

27- Schuldiner M, Itskovitz-Eldor J, Benvenisty N. Selective ablation of human embryonic stem cells expressing a suicide gene. Stem Cells. 2003; 21 (3): 257-265.

28- Wang X, Ge S, McNamara G, Hao QL, Crooks GM, Nolta JA. Albumin expressing hepatocyte-like cells develop in the livers of immune-deficient mice transmitted with highly purified human hematopoietic stem cells. Blood. 2003; 101: 4201-4208.

29- McKinney-Freeman SL, Jackson KA, Camargo FD, Ferrari G, Mavilio F, Goodell MA. Muscle-derived hematopoietic stem cells are hematopoietic in origin. Proc Natl Acad Sci USA. 2002; 99: 1341-1346.

30- Kawada H, Ogawa M. Bone marrow origin of hematopoietic progenitors and stem cells in murine muscle. Blood. 2001; 98: 2008-2013.

31- Majka SM, Jackson KA, Kienstra KA, Majesky MW, Goodell MA, Hirschi KK. Distinct progenitor populations in skeletal muscle are bone marrow derived and exhibit different cell fates during vascular regeneration. J Clin Invest. 2003; 111: 71-79. Toma C, Pittenger M, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation. 2002; 105: 93-98.

32- Makino S, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest. 1999; 103: 697-705.

33- Pittenger MF, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999; 284: 143-147.

34- Reyes M, Dudek A, Jahagirdar B, et al. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest. 2002; 109: 337-346.

35- Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation. 2002; 105: 93-98.

36- Wang JS, Shum-Tim D, Galipeau J, et al. Marrow stromal cells for cellular cardiomyoplasty: Feasibility and potential clinical advantages. J Thorac Cardiovasc Surg. 2000; 120: 999-1005.

37- Jiang Y, Jahagirdar BN, RL Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 2002; 418: 41-49.

38- Nagaya N, Kangawa K, Itoh T, et al. Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. Circulation. 2005; 112: 1128-1135.

39- Muraglia A, Cancedda R, Quarto R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci. 2000; 113: 1161-1166.

40- Pettinger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res. 2004; 95: 9-20.

41- Prockop DJ. Further proof of the plasticity of adult stem cells and their role in tissue repair. J Cell Biol. 2003; 160: 807-809.

42- Prockop DJ, Sekiya I, Colter DC. Isolation and characterization of rapidly self-renewing stem cells from cultures of human marrow stromal cells. Cytotherapy. 2001; 3: 393-396.

43- Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Exp Hematol. 2002; 30: 896-904.

44- Reyes M, Lund T, Lenvik T, et al. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood. 2001; 98: 2615-2625.

45- Verfaillie CM, Schwartz R, Reyes M, Jiang Y. Unexpected potential of adult stem cells. Ann N Y Acad Sci. 2003; 996: 231-234.

46- Verfaillie CM. Adult stem cells: Assessing the case for pluripotency. Trends Cell Biol. 2002; 12: 502-508.

47- Rangappa S, Fen C, Lee EH, et al. Transformation of adult mesenchymal stem cells isolated from the fatty tissue into cardiomyocytes. Ann Thorac Surg. 2003; 75: 775-779.

48- Zuk PA, Zhu M, Ashjian P, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002; 13: 4279-4295.

49- Gaustad KG, Boquest AC, Anderson BE, et al. Differentiation of human adipose tissue stem cells using extracts of rat cardiomyocytes. Biochem Biophys Res Commun. 2004; 314: 420-427.

50- Planat-Benard V, Silvestre JS, Cousin B, et al. Plasticity of human adipose lineage cells toward endothelial cells: Physiological and therapeutic perspectives. Circulation. 2004; 109: 656-663.

51- Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: Implications in transplantation. Transplantation. 2003; 75: 389-397.

52- Di Nicola M, Carlo-Stella C, Magni M, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood. 2002; 99: 3838-3843.

53- Le Blanc K, Tammik L, Sundberg B, et al. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol. 2003; 57: 11-20.

54- Krampera M, Glennie S, Dyson J, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood. 2003; 101: 3722-3729.

55- Vittet D, Prandini MH, Berthier R, et al. Embryonic stem cells differentiate in vitro to endothelial cells through successive maturation steps. Blood. 1996; 88: 3424-3431.

56- Eichmann A, Corbel C, Nataf V, et al. Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. Proc Natl Acad Sci USA. 1997; 94: 5141-5146.

57- Sato TN, Qin Y, Kozak CA, Audus KL. Tie-1 and Tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. Proc Natl Acad Sci USA. 1993; 90: 9355-9358.

58- Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell. 1996; 87: 1171-1180.

59- Yin AH, Miraglia S, Zanjani ED, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood. 1997; 90: 5002-5012.

60- Peichev M, Naiyer AJ, Pereira D, et al. Expression of VEGFR-2 and AC133 by circulating human CD34 cells identifies a population of functional endothelial precursors. Blood. 2000; 95: 952-958.

61- Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol. 2000; 109: 235-242.

62- Mayani H, Lansdorp PM. Biology of human umbilical cord blood derived hematopoietic stem/progenitor cells. Stem Cells. 1998; 16: 153-165.

63- Kogler G, Sensken S, Airey JA, et al. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med. 2004; 200: 123-135.

64- Marelli D, Desrosiers C, El-Alfy M, Kao RL, Chiu RC. Cell transplantation for myocardial repair: An experimental approach. Cell Transplant. 1992; 1: 383-390.

65- Koh GY, Klug MG, Soonpaa MH, Field LJ. Differentiation and long-term survival of C2C12 myoblast graft in heart. J Clin Invest. 1993;92: 1548-1554.

66- Menasche P, Hagege AA, Scorsin M, et al. Myoblast transplantation for heart failure. Lancet. 2001; 357: 279-280.

67- Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. Nature. 2001; 410: 701-705.

68- Schuster MD, Kocher AA, Seki T, et al. Myocardial neovascularization by bone marrow angioblasts results in cardiomyocyte regeneration. Am J Physiol Heart Circ Physiol. 2004; 287: H525-H532.

69- Miyahara Y, Nagaya N, Kataoka M, et al. Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nature Medicine. 2006.

70- Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci USA. 2001; 98: 10344-10349.

71- Moore KA, Lemischka IR. Stem cells and their niches. Science. 2006; 311: 1880-1885.

72- Min J-Y, Yang Y, Converso KL, et al. Transplantation of embryonic stem cells improves cardiac function in postinfarcted rats. J Appl Physiol. 2002; 92: 288-296.

73- He JQ, Ma Y, Lee Y, Thomson JA, Kamp TJ. Human embryonic stem cells develop into multiple types of cardiac myocytes: Action potential characterization. Circ Res. 2003; 93: 32-9.

74- Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. Nature. 2001; 410: 701-705.

75- Kehat I, Kenyagin-Karsenti D, Snir M, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest. 2001; 108: 407-14.

76- Fijnvandraat AC, Lekanne Deprez RH, Moorman AF. Development of heart muscle-cell diversity: A help or a hindrance for phenotyping embryonic stem cell-derived cardiomyocytes. Cardiovasc Res. 2003; 58 (2): 303-12.

77- Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998; 282: 1145-1147.

78- Beltrami AP, Barlucchi L, Torella D, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell. 2003; 114: 763-776.

79- Oh H, Bradfute SB, Gallardo TD, et al. Cardiac progenitor cells from adult myocardium: Homing, differentiation, and fusion after infarction. Proc Natl Acad Sci USA. 2003; 100: 12313-12318.

80- Messina E, De Angelis L, Frati G, et al. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res. 2004; 95: 911-921.

81- Laugwitz K-L, Moretti A, Lam J, et al. Postnatal cardioblasts enter fully differentiated cardiomyocytes lineages. Nature. 2005; 433: 647-653.

82- Martin CM, Meeson AP, Robertson SM, et al. Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. Dev Biol. 2004; 265: 262-275.

83- Urbanek K, Torella D, Sheikh F, et al. Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. Proc Natl Acad Sci USA. 2005; 102: 8692-8697.

84- Mouquet F, Pfister O, Jain M, et al. Restoration of cardiac progenitor cells after myocardialinfarction by self-proliferation and selective homing of bone marrowderived stem cells. Circ Res. 2005; 97: 1090-1092.

85- Ghostine S, Carrion C, Souza LCG, et al. Long-term efficacy of myoblast transplantation on regional structure and function after myocardial infarction. Circulation. 2002; 106 (Suppl I): I-131–I-136.

86- Hagege AA, Carrion C, Menasche P, et al. Viability and differentiation of autologous skeletal myoblast grafts in ischaemic cardiomyopathy. Lancet. 2003; 361: 491-492.

87- Murry CE, Wiseman RW, Schwartz SM, Hauschka SD. Skeletal myoblast transplantation for repair of myocardial necrosis. J Clin Invest. 1996; 98: 2512-2523.

88- Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocytes apoptosis, reduces remodelling and improves cardiac function. Nat Med. 2001; 7: 430-436.

89- Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci USA. 2001; 98: 10344-10349.

90- Murry CE, Soonpaa MH, Reinecke H, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature. 2004; 428: 664-668.

91- Balsam LB, Wagers AJ, Christensen JL, et al. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature. 2004; 428: 668-673.

92- Strauer BE, Brehm M, Zeus T, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation. 2002; 106: 1913-1918.

93- Assmus B, Schachinger V, Teupe C, et al. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). Circulation. 2002; 106: 3009-3017.

94- Schachinger V, Assmus B, Britten MB, et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: Final one-year results of the TOPCARE-AMI Trial. J Am Coll Cardiol. 2004; 44: 1690.

95- Wollert KC, Meyer GP, Lotz J, et al. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: The BOOST randomised controlled clinical trial. Lancet. 2004; 364: 141-148.

96- Perin EC, Dohmann HFR, Borojevic R, et al. Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. Circulation. 2003; 107: 2294-2302.

97- Shake JG, Gruber PJ, Baumgartner WA, et al. Mesenchymal stem cell implantation in a swine myocardial infarct model: Engraftment and functional effects. Ann Thorac Surg. 2002; 73: 1919-1926.

98- Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation. 2002; 105: 93-98.

99- Rangappa S, Reddy V, Bongso A, Lee EH, Sim EKW. Transformation of the adult human mesenchymal stem cells into cardiomyocyte-like cells in vivo. Cardiovasc Eng. 2002; 2: 7-14.

100- Gojo S, Gojo N, Takeda Y, et al. In vivo cardiovasculogenesis by direct injection of isolated adult mesenchymal stem cells. Exp Cell Res. 2003; 288: 51-59.

101- Mangi AA, Noiseux N, Kong D, et al. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med. 2003; 9: 1195-1201.

102- Davani S, Marandin A, Mersin N, et al. Mesenchymal progenitor cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a rat cellular cardiomyoplasty model. Circulation. 2003; 108 (Suppl II): II-253–II-258.

103- Chen S-l, Fang W-W, Ye F, et al. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol. 2004; 94: 92-95.

104- Schuster MD, Kocher AA, Seki T, Martens TP, Xiang G, Homma S, Itescu S. Myocardial neovascularization by bone marrow angioblasts results in cardiomyocyte regeneration. Am J Physiol Heart Circ Physiol. 2004; 287: H525-H532.

105- Erb S, Linke A, Adams V, et al. Transplantation of blood-derived progenitor cells after recanalization of chronic coronary artery occlusion: First randomized and placebocontrolled study. Circ Res. 2005; 97: 756-762.

106- Kim B-O, Tian H, Prasongsukarn K, et al. Cell transplantation improves ventricular function after a myocardial infarction: A preclinical study of human unrestricted somatic stem cells in a porcine model. Circulation. 2005; 112 (Suppl I): I-96 –I-104.

107- Ma N, Stamm C, Kaminski A, et al. Human cord blood cells induce angiogenesis following myocardial infarction in NOD/scid-mice. Cardiovasc Res. 2005; 66: 45-54.

108- Hirata Y, Sata M, Motomura N, et al. Human umbilical cord blood cells improve cardiac function after myocardial infarction. Biochem Biophys Res Commun. 2005; 327: 609-614.

109- Rice CM, Halfpenny CA, Scolding NJ. Stem cells for the treatment of neurological disease. Transfusion Medicine. 2003; 13: 351-361.

110- Scolding N. New cells, new brain. Practical Neurology. 2002; 2: 128-129.

111- Lowry NA, Temple S. Making human neurons from stem cells after spinal cord injury. PLoS MEDICINE 2007; 4: 0236-0238.

112- Charles P, Hernandez MP, Stankoff B, et al. Negative regulation of central nervous system myelination by polysialylated-nerual cell adhesion molecue. Proceedings of the National Academy of Science USA. 2000; 97: 7585-90.

113- Akiyama Y, Radtke C, Honmou O, Kocsis JD. Remyelination of the spinal cord following intravenous delivery of bone marrow cells. Glia. 2002; 39: 229-236.

114- Akiyama Y, Radtke C, Kocsis JD. Remyelination of the rat spinal cord by transplantation of identified bone marrow stromal cells. The Journal of Neuroscience. 2002; 22: 6623-6630.

115- Sanberg PR. Stem cell therapy in the treatment of neurological disorders. Lifecell conference at Chennai. Jan-2006.

116- Bjorklund LM, Sanchez-Pernaute R, Chung S, et al. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. Proceedings of the National Academy of Sciences of the United States of America. 2002; 99: 2344-2349.

117- Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nature Biotechnology. 2001; 19: 1129-1133.

118- Olsson M, Bentlage C, Wictorin K, Campbell K, Bjorklund A. Extensive migration and target innervation by striatal precursors after grafting into the neonatal striatum. Neuroscience. 1997; 79: 57-78.

119- Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al.Embryonic stem cell lines derived from human blastocysts. Science. 1998; 282: 1145-1147.

120- Carpenter MK, Inokuma MS, Denham J, et al. Enrichment of neurons and neural precursors from human embryonic stem cells. Experimental Neurology. 2001; 172: 383-397.

121- Carpenter MK, Cui X, Hu ZY, et al. In vitro expansion of a multipotent population of human neural progenitor cells. Experimental Neurology. 1999; 158: 265-278.

122- Zhang SC, Ge B, Duncan ID. Tracing human oligodendroglial development in vitro. Journal of Neuroscience Research. 2000; 59: 421-429.

123- Storch A, Paul G, Csete M, et al. Long-term proliferation and dopaminergic differentiation of human mesencephalic neural precursor cells. Experimental Neurology. 2001; 170: 317-325.

124- Studer L, Tabar V, McKay RD. Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats. Nature Neuroscience. 1998; 1: 290-295.

125- Hagell P, Brundin P. Cell survival and clinical outcome following intrastriatal transplantation in Parkinson disease. Journal of Neuropathology and Experimental Neurology. 2001; 60: 741-752.

126- Johansson CB, Svensson M, Wallstedt L, Janson AM, Frisen J. Neural stem cells in the adult human brain. Experimental Cell Research. 1999; 253: 733-736.

127- Arsenijevic Y, Villemure JG, Brunet JF, et al. Isolation of multipotent neural precursors residing in the cortex of the adult human brain. Experimental Neurology. 2001; 170: 48-62.

128- Lie DC, Dziewczapolski G, Willhoite AR, et al. The adult substantia nigra contains progenitor cells with neurogenic potential. The Journal of Neuroscience. 2002; 22: 6639-6649.

129- Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR. Turning blood into brain: Cells bearing neuronal antigens generated in vivo from bone marrow. Science. 2000; 290: 1779-1782.

130- Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. Journal of Neuroscience Research. 2000; 61: 364-370.

131- Bonilla S, Alarcon P, Villaverde R, et al. Haematopoietic progenitor cells from adult bone marrow differentiate into cells that express oligodendroglial antigens in the neonatal mouse brain. The European Journal of Neuroscience. 2002; 15: 575-582.

132- Zhao LR, Duan WM, Reyes M, et al. Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. Experimental Neurology. 2002; 174: 11-20.

133- Chen J, Li Y, Wang L, et al. Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. Stroke. 2001; 32: 1005-1011.

134- Lie DC, Dziewczapolski G, Willhoite AR, et al. The adult substantia nigra contains progenitor cells with neurogenic potential. The Journal of Neuroscience. 2002; 22: 6639-6649.

135- Schacter SC, Schomer DL, Blume H, et al. Porcine fetal GABA-producing neural cell transplants for human partial-onset seizures: Safety and feasibility. Epilepsia. 1998; 39 (Suppl. 6), 67.

136- Lindvall O, Brundin P, Widner H, et al. Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. Science. 1990; 247: 574-577.

137- Hagell P, Schrag A, Piccini P, et al. Sequential bilateral transplantation in Parkinson's disease: Effects of the second graft. Brain. 1999; 122: 1121-1132.

138- Svendsen CN, Caldwell MA. Neural stem cells in the developing central nervous system: Implications for cell therapy through transplantation. Progress in Brain Research. 2000; 127: 13-34.

139- Freed CR, Greene PE, Breeze RE, et al. Transplantation of embryonic dopamine neurons for severe Parkinson's disease. The New England Journal of Medicine. 2001; 344: 710-719.

140- Ozawa K, Suchanek G, Breitschopf H, et al. Patterns of oligodendroglia pathology in multiple sclerosis. Brain. 1994; 117: 1311-1322.

141- Roy NS, Wang S, Harrison-Restelli C, et al. Identification, isolation, and promoter-defined separation of mitotic oligodendrocyte progenitor cells from the adult human subcortical white matter. The Journal of Neuroscience. 1999; 19: 9986-9995.

142- Smith PM, Blakemore WF. Porcine neural progenitors require commitment to the oligodendrocyte lineage prior to transplantation in order to achieve significant remyelination of demyelinated lesions in the adult CNS. The European Journal of Neuroscience. 2000; 12: 2414-2424.

143- Deng W, Obrocka M, Fischer I, Prockop DJ. In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. Biochemical and Biophysical Research Communications. 2001; 282, 148-152.

144- Deng W, Obrocka M, Fischer I, Prockop DJ. In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. Biochemical and Biophysical Research Communications. 2001; 282: 148-152.

145- Isacson O, Brundin P, Gage FH, Bjorklund A. Neural grafting in a rat model of Huntington's disease: Progressive neurochemical changes after neostriatal ibotenate lesions and striatal tissue grafting. Neuroscience. 1985; 16: 799-817.

146- Chen GJ, Jeng CH, Lin SZ, et al. Fetal striatal transplants restore electrophysiological sensitivity to dopamine in the lesioned striatum of rats with experimental Huntington's disease. Journal of Biomedical Science. 2002; 9: 303-310.

147- Rosser AE, Barker RA, Harrower T, et al. Unilateral transplantation of human primary fetal tissue in four patients with Huntington's disease: NEST-UK safety report ISRCTN no 36485475. Journal of Neurology, Neurosurgery, and Psychiatry. 2002; 73: 678-685.

148- Pundt LL, Kondoh T, Conrad JA, Low WC. Transplantation of human striatal tissue into a rodent model of Huntington's disease: Phenotypic expression of transplanted neurons and host-to-graft innervation. Brain Research Bulletin. 1996; 39: 23-32.

149- Nakao N, Ogura M, Nakai K, Itakura T. Embryonic striatal grafts restore neuronal activity of the globus pallidus in a rodent model of Huntington's disease. Neuroscience. 1999; 88: 469-477.

150- Isacson O, Dunnett SB, Bjorklund A. Graftinduced behavioral recovery in an animal model of Huntington disease. Proceedings of the National Academy of Sciences of the United States of America. 1986; 83: 2728-2732.

151- Pundt LL, Kondoh T, Conrad, JA, Low WC. Transplantation of human fetal striatum into a rodent model of Huntington's disease ameliorates locomotor deficits. Neuroscience Research. 1996; 24: 415-420.

152- Palfi S, Conde F, Riche D, et al. Fetal striatal allografts reverse cognitive deficits in a primate model of Huntington disease. Nature Medicine. 1998; 4: 963-966.

153- Sugaya K, Brannen CL. Stem cell strategies for neuroreplacement therapy in Alzheimer's disease. Medical Hypothesis. 2001; 00 (0) 1-5 2001 Harcourt

Publishers Ltd doi: 10.1054/mehy.2001.1424, available online at http://www.idealibrary.com on 1.

154- Wu W, Wong K, Chen J, et al. Directional guidance of neuronal migration in the olfactory system by the protein Slit. Nature. 1999; 400: 331-336.

155- Alonso G. Neuronal progenitor-like cells expressing polysialylated neural cell adhesion molecule are present on the ventricular surface of the adult rat brain and spinal cord. J Comp Neurol. 1999; 414: 149-166.

156- Fricker RA, Carpenter MK, Winkler C, et al. Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. J Neurosci. 1999; 19: 5990-6005.

157- Kukekov VG, Laywell ED, Suslov O, et al. Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain. Exp Neurol. 1999; 156: 333-344.

158- Janson CG, Ramesh TM, During MJ, Leone P, Heywood J. Human intrathecal transplantation of peripheral blood stem cells in amyotrophic lateral sclerosis. Journal of Hematotherapy & Stem Cell Research. 2001; 10: 913-915.

159- Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. Nature Medicine. 2002; 8: 963-970.

160- Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell. 2002; 110: 429-441.

161- Sinden JD, Rashid-Doubell F, Kershaw TR, Nelson A, Chadwick A, Jat PS, Noble MD, Hodges H, Gray JA. Recovery of spatial learning by grafts of a conditionally immortalized hippocampal neuroepithelial cell line into the ischaemia-lesioned hippocampus. Neuroscience. 1997; 81: 599-608.

162- Saporta S, Borlongan CV, Sanberg PR. Neural transplantation of human neuroteratocarcinoma (hNT) neurons into ischemic rats. A quantitative dose-response analysis of cell survival and behavioral recovery. Neuroscience. 1999; 91: 519-525.

163- Kondziolka D, Wechsler L, Gebel J, et al. Neuronal transplantation for motor stroke: From the laboratory to the clinic. Physical Medicine and Rehabilitation Clinics of North America. 2003; 14: S153-S160, xi.

164- Kondziolka D, Wechsler L, Goldstein S, et al. Transplantation of cultured human neuronal cells for patients with stroke. Neurology. 2000; 55: 565-569.

165- Eglitis MA, Dawson D, Park KW, Mouradian MM. Targeting of marrow-derived astrocytes to the ischemic brain. Neuroreport. 1999; 10: 1289-1292.

166- Hess DC, Hill WD, Martin-Studdard A, Carroll J, Brailer J, Carothers J. Bone marrow as a source of endothelial cells and NeuN-expressing cells after stroke. Stroke. 2002; 33: 1362-1368.

167- Cheng H, et al. Gait analysis of adult paraplegic rats after spinal cord repair. Exp Neurol. 1997; 148: 544-557.

168- Lee YS, Lin CY, Robertson RT, Hsiao I, Lin V.W. Motor recovery and anatomical evidence of axonal regrowth in spinal cord-repaired adult rats. J Neuropathol Exp Neurol. 2004; 63: 233-245.

169- Cheng H, Liao KK, Liao SF, Chuang TY, Shih Y. H. Spinal cord repair with acidic fibroblast growth factor as a treatment for a patient with chronic paraplegia. Spine. 2004; 29: E284-E288.

170- Amador MJ, Guest JD. An appraisal of ongoing experimental procedures in human spinal cord injury. J Neurol Phys Ther. 2005; 29: 70-86.

171- Takami T, Oudega M, Bates ML, et al. Schwann cell but not olfactory ensheathing glia transplants improve hindlimb locomotor performance in the moderately contused adult rat thoracic spinal cord. J Neurosci. 2002; 22: 6670-6681.

172- Sasaki M, Hains BC, Lankford KL, Waxman SG, Kocsis JD. Protection of corticospinal tract neurons after dorsal spinal cord transection and engraftment of olfactory ensheathing cells. Glia. 2006; 53: 352-359.

173- Guest JD, Bunge MB, Hesse DH, et al. The ability of cultured human Schwann cells within PAN/PVC guidance channels to support regeneration in the transected nude rat spinal cord. Can J Neurol Sci. 1996; 23: S1-S2.

174- Thuret S, Moon LD, Gage FH. Therapeutic interventions after spinal cord injury. Nature Reviews. 2006; 7: 628-643.

175- Li Y, Decherchi P, Raisman G. Transplantation of olfactory ensheathing cells into spinal cord lesions restores breathing and climbing. J Neurosci. 2003; 23: 727-731.

176- Plant GW, Christensen CL, Oudega M, Bunge M. B. Delayed transplantation of olfactory ensheathing glia promotes sparing/regeneration of supraspinal axons in the contused adult rat spinal cord. J Neurotrauma. 2003; 20: 1-16.

177- Keyvan-Fouladi N, Raisman G, Li Y. Functional repair of the corticospinal tract by delayed transplantation of olfactory ensheathing cells in adult rats. J Neurosci. 2003; 23: 9428-9434.

178- Boyd JG, Doucette, R, Kawaja MD. Defining the role of olfactory ensheathing cells in facilitating axon remyelination following damage to the spinal cord. FASEB J. 2005; 19: 694-703.

179- Ruitenberg MJ. et al. NT-3 expression from engineered olfactory ensheathing glia promotes spinal sparing and regeneration. Brain. 2005; 128: 839-853.

180- Riddell JS, Enriquez-Denton M, Toft A, Fairless R, Barnett SC. Olfactory ensheathing cell grafts have

minimal influence on regeneration at the dorsal root entry zone following rhizotomy. Glia. 2004; 47: 150-167.

181- Ramer LM, Richter MW, Roskams AJ, Tetzlaff W, Ramer M.S. Peripherally-derived olfactory ensheathing cells do not promote primary afferent regeneration following dorsal root injury. Glia. 2004; 47: 189-206.

182- Huang H, et al. Influence of patients' age on functional recovery after transplantation of olfactory ensheathing cells into injured spinal cord injury. Chin Med J (Engl.). 2003; 116: 1488-1491.

183- Dobkin BH, Curt A, Guest J. Cellular transplants in China: Observational study from the largest human experiment in chronic spinal cord injury. Neurorehabil. Neural Repair. 2006; 20: 5-13.

184- Kunkel-Bagden, E, Bregman BS. Spinal cord transplants enhance the recovery of locomotor function after spinal cord injury at birth. Exp Brain Res. 1990; 81: 25-34.

185- Bregman BS, Kunkel-Bagden E, Reier PJ, et al. Recovery of function after spinal cord injury: Mechanisms underlying transplantmediated recovery of function differ after spinal cord injury in newborn and adult rats. Exp Neurol. 1993; 123: 3-16.

186- Reier PJ, Stokes BT, Thompson FJ, Anderson PJ. Fetal cell grafts into resection and contusion / compression injuries of the rat and cat spinal cord. Exp Neurol. 1992; 115, 177-188.

187- McDonald JW, Liu XZ, Qu Y, et al. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured spinal cord. Nature Med. 1999; 5: 1410-1412.

188- Harper JM, Krishnan C, Darman JS, et al. Axonal growth of embryonic stem cell-derived motoneurons in vitro and in motoneuroninjured adult rats. Proc Natl Acad Sci USA. 2004; 101: 7123-7128.

189- Herrera J, Yang H, Zhang SC, et al. Embryonic-derived glial-restricted precursor cells (GRP cells) can differentiate into astrocytes and oligodendrocytes in vivo. Exp Neurol. 2001; 171: 11-21.

190- Han SS, Kang DY, Mujtaba T, Rao MS, Fischer I. Grafted lineage-restricted precursors differentiate exclusively into neurons in the adult spinal cord. Exp Neurol. 2002; 177: 360-375.

191- Hill CE, Proschel C, Noble M, et al. Acute transplantation of glial-restricted precursor cells into spinal cord contusion injuries: Survival, differentiation, and effects on lesion environment and axonal regeneration. Exp Neurol. 2004; 190: 289-310.

192- Ogawa Y, Sawamoto K, Miyata T, et al. Transplantation of in vitro-expanded fetal neural progenitor cells results in neurogenesis and functional recovery after spinal cord contusion injury in adult rats. J Neurosci Res. 2002; 69: 925-933.

193- Teng YD, Lavik, EB, Qu X, et al. Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. Proc Natl Acad Sci USA. 2002; 99: 3024-3029.

194- Cummings BJ, Uchida N, Tamaki SJ, et al. Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. Proc Natl Acad Sci USA. 2005; 102: 14069-14074.

195- Iwanami A, Kaneko S, Nakamura M, et al. Transplantation of human neural stem cells for spinal cord injury in primates. J Neurosci Res. 2005; 80: 182-190.

196- Keirstead HS, Nistor G, Bernal G, et al. Human embryonic stem cell derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. J Neurosci. 2005; 25: 4694-4705.

197- Faulkner J, Keirstead HS. Human embryonic stem cell-derived oligodendrocyte progenitors for the treatment of spinal cord injury. Transpl Immunol. 2005; 15: 131-142.

198- Koshizuka S, Okada S, Okawa A, et al. Transplanted hematopoietic stem cells from bone marrow differentiate into neural lineage cells and promote functional recovery after spinal cord injury in mice. J Neuropathol Exp Neurol. 2004; 63: 64-72.

199- Koda M, Okada S, Nakayama T, et al. Hematopoietic stem cell and marrow stromal cell for spinal cord injury in mice. Neuroreport. 2005; 16: 1763-1767.

200- Hofstetter CP, Holmström NAV, Lilja JA, et al. Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. Nature Neurosci. 2005; 8: 346-353.

201- Wu S, Suzuki Y, Ejiri Y, et al. Bone marrow stromal cells enhance differentiation of cocultured neurosphere cells and promote regeneration of injured spinal cord. J Neurosci Res. 2003; 72: 343-351.

202- Reier PJ. Cellular transplantation strategies for spinal cord injury and translational neurobiology. NeuroRx. 2004; 1: 424-451.

203- Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science. 1992; 255: 1707-1710.

204- Gage FH, Coates PW, Palmer TD, et al. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. Proc Natl Acad Sci USA. 1995; 92: 11879-11883.

205- Shihabuddin LS, Horner PJ, Ray J, Gage FH. Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus. J Neurosci. 2000; 20: 8727-8735.

206- Vroemen M, Aigner L, Winkler J, Weidner N. Adult neural progenitor cell grafts survive after acute spinal cord injury and integrate along axonal pathways. Eur J Neurosci. 2003; 18: 743-751.

207- Karimi-Abdolrezaee S, Eftekharpour E, Wang J, Morshead CM, Fehlings MG. Delayed transplantation

of adult neural precursor cells promotes remyelination and functional neurological recovery after spinal cord injury. J Neurosci. 2006; 26: 3377-3389.

208- Oudega M, Gautier SE, Chapon P, et al. Axonal regeneration into Schwann cell grafts within resorbable poly (alpha-hydroxyacid) guidance channels in the adult rat spinalcord. Biomaterials. 2001; 22: 1125-36.

209- Novikov LN, Novikova LN, Mosahebi A, et al. A novel biodegradable implant for neuronal rescue and regeneration after spinal cord injury. Biomaterials. 2002; 23: 3369-3376.

210- Bradbury EJ, Moon LD, Popat RJ, et al. Chondroitinase ABC promotes functional recovery after spinal cord injury. Nature. 2002; 416: 636-40.

211- Murachov's labpage, Development of a Method for Generation of Spinal Cord Neurons from Embryonic Stem Cells for Treatment of Spinal Cord Injury Copyright © 2006 Signaling in the Nervous System. Murashov's Labpage. Last modified: 09/06/2006 Downloaded 0n 7/23/2007.

212- United Network for Organ Sharing. http://www.unos.org.

213- Goldstein MJ, Salame E, Kapur S, et al. Analysis of failure in living donor liver transplantation: Differential outcomes in children and adults. World J Surg. 2003; 27 (3): 356-364.

214- Tatematsu M, Ho RH, Kaku T, et al. Studies on the proliferation and fate of oval cells in the liver of rats treated with 2-acetylaminofluorene and partial hepatectomy. Am J Pathol. 1984; 114: 418-430.

215- Evarts RP, Nagy P, Nakatsukasa H, et al. In vivo differentiation of rat liver oval cells into hepatocytes. Cancer Res. 1989; 49: 1541-7.

216- Roskams TA, Theise ND, Balabaud C, et al. Nomenclature of the finer branches of the biliary tree: Canals, ductules, and ductular reaction in human livers. Hepatology. 2004; 39: 1739-45.

217- Kallis YN, Alison MR, Forbes SJ. Bone marrow stem cells and liver disease. Gut. 2007; 56: 716-724; originally published online 4 Dec 2006; doi: 10.1136/gut.2006.098442.

218- Moshage HJ, Rijntjes PJ, Hafkenscheid JC, Roelofs HM, Yap SH. Primary culture of cryopreserved adult human hepatocytes on homologous extracellular matrix and the influence of monocytic products on albumin synthesis. J Hepatol. 1988; 7: 34-44.

219- Fox IJ, Chowdhury JR. Hepatocyte transplantation. American Journal of Transplantation. 2004; 4 (Suppl. 6) 7-13.

220- Asahina K, Twramoto K, Teraoka H. Embryonic stem cells: Hepatic differentiation and regeneratve medicine for the treatment of liver disease. Current Stem Cell Research & Therapy. 2006; 1.

221- Gupta S, Rajvanshi P, Aragona E, Lee CD, Yerneni PR, Burk RD. Transplanted hepatocytes proliferate differently after CCl4 treatment and hepatocyte growth factor infusion. Am J Physiol. 1999; 276: G629-38.

222- Laconi E, Oren R, Mukhopadhyay DK, et al. Long-term, neartotal liver replacement by transplantation of isolated hepatocytes in rats treated with retrorsine. Am J Pathol. 1998; 153: 319-329.

223- Gagandeep S, Rajvanshi P, Sokhi RP, et al. Transplanted hepatocytes engraft, survive, and proliferate in the liver of rats with carbon tetrachloride-induced cirrhosis. J Pathol. 2000; 191 (1): 78-85.

224- Rhim JA, Sandgren EP, Degen JL, Palmiter RD, Brinster RL. Replacement of diseased mouse liver by hepatic cell transplantation. Science. 1994; 263: 1149-52.

225- Overturf K, Al-Dhalimy M, Ou CN, Finegold M, Grompe M. Serial transplantation reveals the stem-cell-like regenerativepotential of adult mouse hepatocytes. Am J Pathol. 1997; 151: 1273-1280.

226- Sutherland DER, Numata M, Matas AJ, Simmons RL, Najarian JS. Hepatocellular transplantation in acute liver failure. Surgery. 1977; 82: 124-132.

227- Hillan KJ, Burt AD, George WD, MacSween RNM, Griffiths MR, Bradley JA. Intrasplenic hepatocyte transplantation in rats with experimental liver injury: Morphological and morphometric studies. J Pathol. 1989; 159: 67-73.

228- Xiangdong W, Ar'Rajab A, Ahrén B, Andersson R, Bengmark S. Improvement of the effects of intrasplenic transplantation of hepatocytes after 90% hepatectomy in the rat by cotransplantation with pancreatic islets. Transplantation. 1991; 52: 462-6.

229- Habibullah CM, Syed IH, Qamar A, Taher-Uz Z. Human fetal hepatocyte transplantation in patients with fulminant hepatic failure. Transplantation. 1994; 58: 951-952.

230- Grossman M, Rader DJ, Muller DWM, et al. A pilot study of ex vivo gene therapy for homozygous familial hypercholesterolaemia. Nat Med. 1995; 1: 1148-54.

231- Ohashi K, Park F, Kay MA. Hepatocyte transplantation: Clinical and experimental application. J Mol Med. 2001; 79: 617-30.

232- Horslen SP, Fox IJ. Hepatocyte transplantation. Transplantation. 2004; 77: 1481-6.

233- Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R. The in vitro development of blastocyst-derived embryonic stem cell lines: Formation of visceral yolk sac, blood islands and myocardium. J Embryol Exp Morphol. 1985; 87: 27-45.

234- Chinzei R, Tanaka Y, Shimizu-Saito K, et al. Embryoid-body cells derived from a mouse embryonic stem cell line show differentiation into functional hepatocytes. Hepatology. 2002; 36: 22-9.

235- Miyashita H, Suzuki A, Fukao K, Nakauchi H, Taniguchi H. Evidence for hepatocyte differentiation from embryonic stem cells in vitro. Cell Transplant. 2002; 11: 429-34.

236- Asahina K, Fujimori H, Shimizu-Saito K, et al. Expression of the liver-specific gene Cyp7a1 reveals hepatic differentiation in embryoid bodies derived from mouse embryonic stem cells. Genes Cells. 2004; 9: 1297-308.

237- Kania G, Blyszczuk P, Jochheim A, Ott M, Wobus AM. Generation of glycogen- and albumin-producing hepatocyte-like cells from embryonic stem cells. Biol Chem. 2004; 385: 943-53.

238- Ogawa S, Tagawa YI, Kamiyoshi A, et al. Crucial roles of mesodermal cell lineages in a murine embryonic stem cellderived in vitro liver organogenesis system. Stem Cells. 2005; 23: 903-13.

239- Hamazaki T, Iiboshi Y, Oka M, et al. Hepatic maturation in differentiating embryonic stem cells in vitro. FEBS Lett. 2001; 497: 15-9.

240- Kuai XL, Cong XQ, Li XL, Xiao SD. Generation of hepatocytes from cultured mouse embryonic stem cells. Liver Transplant. 2003; 9: 1094-9.

241- Choi D, Oh HJ, Chang UJ, et al. In vivo differentiation of mouse embryonic stem cells into hepatocytes. Cell Transplant. 2002; 11: 359-68.

242- Yamamoto H, Quinn G, Asari A, et al. Differentiation of embryonic stem cells into hepatocytes: Biological functions and therapeutic application. Hepatology. 2003; 37: 983-93.

243- Teramoto K, Hara Y, Kumashiro Y, et al. Teratoma formation and hepatocyte differentiation in mouse liver transplanted with mouse embryonic stem cell-derived embryo bodies. Transplant Proc. 2005; 37: 285-6.

244- Kumashiro Y, Asahina K, Ozeki R, et al. Enrichment of hepatocytes differentiated from mouse embryonic stem cells as a transplantable source. Transplantation. 2005; 79: 550-7.

245- Schuldiner M, Itskovitz-Eldor J, Benvenisty N. Selective ablation of human embryonic stem cells expressing a "suicide" gene. Stem Cells. 2003; 21: 257-65.

246- Teratani T, Yamamoto H, Aoyagi K, et al. Direct hepatic fate specification from mouse embryonic stem cells. Hepatology. 2005; 41: 836-46.

247- Lavon N, Yanuka O, Benvenisty N. Differentiation and isolation of hepatic-like cells from human embryonic stem cells. Differentiation. 2004; 72: 230-8.

248- Rambhatla L, Chiu CP, Kundu P, Peng Y, Carpenter MK. Generation of hepatocyte-like cells from human embryonic stem cells. Cell Transplant. 2003; 12: 1-11.

249- Ginis I, Luo Y, Miura T, et al. Differences between human and mouse embryonic stem cells. Dev Biol. 2004; 269: 360-80.

250- Suemori H, Tada T, Torii R, et al. Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. Dev Dyn. 2001; 222: 273-9.

251- Alison MR, Poulsom R, Jeffery R, et al. Hepatocytes from non-hepatic adult stem cells. Nature. 2000; 406: 257.

252- Theise ND, Nimmakayalu M, Gardner R, et al. Liver from bone marrow in humans. Hepatology. 2000; 32: 11-6.

253- Cantz T, Sharma AD, Jochheim-Richter A, et al. Re-evaluation of bone marrowderivedcells as a source for hepatocyte regeneration. Cell Transplant. 2004; 13: 659-66.

254- Willenbring H, Bailey AS, Foster M, et al. Myelomonocytic cells are sufficient for therapeutic cell fusion in liver. Nature Med. 2004; 10: 744-8.

255- Vig P, Russo FP, Edwards RJ, et al. The sources of parenchymal regeneration after chronic hepatocellular liver injury in mice. Hepatology. 2006; 43: 316-24.

256- Sharma AD, Cantz T, Richter R, et al. Human cord blood stem cells generate human cytokeratin 18-negative hepatocyte-like cells in injured mouse liver. Am J Pathol. 2005; 167: 555-564.

257- Stephenne X, Najimi M, Sibille C, et al. Sustained engraftment and tissue enzyme activity after liver cell transplantation for argininosuccinate lyase deficiency. Gastroenterology. 2006; 130: 1317-23.

258- Lagasse E, Connors H, Al-Dhalimy M, et al. Purified haematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med. 2000; 6: 1229-34.

259- Quintana-Bustamante O, Alvarez-Barrientos A, Kofman AV, et al. Hematopoietic mobilization in mice increases the presence of bone marrowderived hepatocytes via in vivo cell fusion. Hepatology. 2006; 43: 108-16.

260- Jang YY, Collector MI, Baylin SB, et al. Haematopoietic stem cells convert into liver cells within days without fusion. Nat Cell Biol. 2004; 6: 532-9.

261- Sato Y, Araki H, Kato J, et al. Human mesenchymal stem cells xenografted directly to rat are differentiated into human hepatocytes without fusion. Blood. 2005; 106: 756-63.

262- Lange C, Bruns H, Kluth D, et al. Hepatocyte differentiation of mesenchymal stem cells in cocultures with fetal liver cells. World J Gastroenterol. 2006; 12: 2394-7.

263- Schwartz RE, Reyes M, Koodie L, et al. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J Clin Invest. 2002; 109: 1291-302.

264- Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 2002; 418: 41-9.

265- Giles J. The trouble with replication. Nature. 2006; 442: 344-7.

266- Russo FP, Alison MR, Bigger BW, et al. The bone marrow functionally contributes to liver fibrosis. Gastroenterology. 2006; 130: 1807-21.

267- Abdel Aziz MT, Atta HM, Mahfouz S, et al. Therapeutic potential of bone marrow-derived mesenchymal

cells on experimental liver fibrosis. Clin Biochem. 2007; 40: 893-899.

268- Brittan M, Hunt T, Jeffery R, et al. Bone marrow derivation of pericryptal myofibroblasts in the mouse and human small intestine and colon. Gut. 2002; 50: 752-7.

269- Broker V, Langer F, Fellous TG, et al. Fibroblasts of recipient origin contribute to bronchiolitis obliterans in human lung transplants. Am J Respir Crit Care Med. 2006; 173: 1276-82.

270- Moore BB, Kolodsick JE, Thannickal VJ, et al. CCR2-mediated recruitment of fibrocytes to the alveolar space after fibrotic liver injury. Am J Pathol. 2005; 166: 675-84.

271- Direkze N, Forbes SJ, Brittan M, et al. Multiple organ engraftment by bone marrow-derived myofibroblasts in bone marrow-tranplanted mice. Stem Cells. 2003; 21: 514-20.

272- Yamaguchi Y, Kubo T, Murakami M, et al. Bone marrow cells differentiate into wound myofibroblasts and accelerate healing of wounds with exposed bones when combined with an occlusive dressing. Br J Dermatol. 2005; 152: 616-22.

273- Kim CU, Broxmeyer HE. In vitro behavior of hematopoietic progenitor cells under the influence of chemoattractants: Stromal cell-derived factor-1, steel factor, and the bone marrow environment. Blood. 1998; 91: 100-10.

274- Petit I, Szyper-Kravitz M, Nagler A, et al. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. Nat Immunol. 2002; 3: 687-94, Epub 2002 Jun 17. [Erratum in: Nat Immunol. 2002; 3: 787].

275- Kollet O, Shivtiel S, Chen Y-Q, 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: 160-9.

276- Wynn RF, Hart CA, Corradi-Perini C, et al. A small proportion of mesenchymal stem cells strongly express functionally active CXCR4 receptor capable of promoting migration to bone marrow. Blood. 2004; 104: 2643-2645.

277- Sordi V, Malosio ML, Marchesi F, et al. Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. Blood. 2005; 106: 419-427.

278- Oyagi S, Hirose M, Kojima M, et al. Therapeutic effect of transplanting HGFtreated bone marrow mesenchymal cells into CCL4-injured rats. J Hepatol. 2006; 44: 742-8.

279- Fang B, Shi M, Liao L, et al. Systemic infusion of FLK1+ mesenchymal stem cells ameliorates carbon tetrachloride-induced liver fibrosis in mice. Transplantation. 2004; 78: 83-88.

280- Sakaida I, Terai S, Yamamoto N, et al. Transplantation of bone marrow cells reduces CCL4- induced fibrosis in mice. Hepatology. 2004; 40: 1304-1311.

281- Duffield JS, Forbes SJ, Constandinou CM, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. J Clin Invest. 2005; 115: 29-32.

282- Ueno T, Nakamura T, Torimura T, et al. Angiogenic cell therapy for hepatic fibrosis. Med Mol Morphol. 2006; 39: 16-21.

283- Di Campli C, Piscaglia AC, Giuliante S, et al. No evidence of hematopoietic stem cell mobilization in patients submitted to hepatectomy or in patients with acute on chronic liver failure. Transplant Proc. 2005; 37: 2707-2710.

284- Gaia S, Smedile A, Omede P, et al. Feasibility and safety of G-CSF administration to induce bone marrow-derived cells mobilization in patients with end stage liver disease. J Hepatol. 2006; 45: 13-19.

285- am Esch JS 2nd, Knoefel WT, Klein M, et al. Portal application of autologous CD133+ bone marrow cells to the liver: A novel concept to support hepatic regeneration. Stem Cells. 2005; 23: 463-470.

286- Gordon MY, Levicar N, Pai M, et al. Characterisation and clinical application of human CD34+ stem/progenitor cell populations mobilized into the blood by granulocyte colony-stimulating factor. Stem Cells. 2006; 24: 1822-1830.

287- Terai S, Ishikawa T, Omori K, et al. Improved liver function in liver cirrhosis patients after autologous bone marrow cell infusion therapy. Stem Cells. 2006; 24: 2292-2298.

288- Gasbarrini A, Rapaccini GL, Rutella S, et al. Rescue therapy by portal infusion of autologous stem cells in a case of drug-induced hepatitis. Dig Liver Dis. 2006 Jul 26; [Epub ahead of print].

289- Chamberlain JS. A move in the right direction. NATURE/AOP/doi: 10.1038/nature 05406/Published online 15 november 2006.

290- Ferrari G, Cusella-DeAngelis G, Coletta M, et al. Muscle regeneration by bone marrow-derived myogenic progenitors. Science. 1998; 279: 1528-1530.

291- LaBarge MA, Blau HM. Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. Cell. 2002; 111: 589-601.

292- Gussoni E, Bennett RR, Muskiewicz KR, et al. Long-term persistence of donor nuclei in a Duchenne muscular dystrophy patient receiving bone marrow transplantation. J Clin Invest. 2002; 110: 807-814.

293- De Bari CF, Dell'Accio F, Tylzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum. 2001; 44: 1928-1942.

294- Sampaolesi, M. Blot S, D'Antona G, et al. Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs Nature. 2006; 444: 574-579 (30 November 2006)/doi:10.1038/nature05282.

295- Minasi MG, Riminucci M, De Angelis L, et al. The meso-angioblast: A multipotent, self-renewing cell

that originates from the dorsal aorta and differentiates into most mesodermal tissues. Development. 2002; 129, 2773-2783.

296- Al-Awqati Q, Oliver JA. Stem cells in the kidney. Kidney Int. 2002; 61: 387-395.

297- Bussolati B, Bruno S, Grange C, et al. Isolation of renal progenitor cells from adult human kidney. Am J Pathol. 2005; 166: 545-555.

298- da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci. 2006; 119: 2204-2213.

299- Poulsom R, Forbes SJ, Hodivala-Dilke K, et al. Bone marrow contributes to renal parenchymal turnover and regeneration. J Pathol. 2001; 195: 229-235.

300- Poulsom R, Alison MR, Forbes SJ, Wright NA. Adult stem cell plasticity. J Pathol. 2002; 197: 441-456.

301- Cornacchia F, Fornoni A, Plati AR, et al. Glomerulosclerosis is transmitted by bone marrow-derived mesangial cell progenitors. J Clin Invest. 2001; 108: 1649-1656.

302- Grimm PC, Nickerson P, Jeffery J, et al. Neointimal and tubulointerstitial infiltration by recipient mesenchymal cells in chronic renal-allograft rejection. N Engl J Med. 2001; 345: 93-97.

303- Roufosse C, Bou-Gharios G, Prodromidi E, et al. Bone marrowderived cells do not contribute significantly to collagen I synthesis in a murine model of renal fibrosis. J Am Soc Nephrol. 2006; 17: 775-782.

304- Poulsom R, Alison MR, Cook T, et al. Bone marrow stem cells contribute to healing of the kidney. J Am Soc Nephrol. 2003; 14: S48-S54.

305- Stokman G, Leemans JC, Claessen N, Weening JJ, Florquin S. Hematopoietic stem cell mobilization therapy accelerates recovery of renal function independent of stem cell contribution. J Am Soc Nephrol. 2005; 16: 1684-1692.

306- Fang TC, Alison MR, Cook HT, Jeffery R, Wright NA, Poulsom R. Proliferation of bone marrow-derived cells contributes to regeneration after folic acid-induced acute tubular injury. J Am Soc Nephrol. 2005; 16: 1723-1732.

307- Grimm PC, Nickerson P, Jeffery J, et al. Neointimal and tubulointerstitial infiltration by recipient mesenchymal cells in chronic renal-allograft rejection. N Engl J Med. 2001; 345: 93-97.

308- Ito T, Suzuki A, Imai E, Okabe M, Hori M. Bone marrow is a reservoir of repopulating mesangial cells during glomerular remodeling. J Am Soc Nephrol. 2001; 12: 2625-2635.

309- Cornacchia F, Fornoni A, Plati AR, et al. Glomerulosclerosis is transmitted by bone marrow-derived mesangial cell progenitors. J Clin Invest. 2001; 108: 1649-1656.

310- Held PK, Al-Dhalimy M, Willenbring H, et al. In vivo genetic selection of renal proximal tubules. Mol Ther. 2006; 13: 49-58.

311- Sugimoto H, Mundel TM, Sund M, Xie L, Cosgrove D, Kalluri R. Bone-marrow-derived stem cells repair basement membrane collagen defects and reverse genetic kidney disease. Proc Natl Acad Sci USA. 2006; 103: 7321-7326.

312- Poulsom R, Prodromidi EI, Pusey CD, Cook HT. Cell therapy for renal regeneration-Time for some joined-up thinking. Nephrol. Dial Transplant. 2006; 21: 3349-3353.

313- Yang LJ. Liver stem cell-derived B-cell surrogates for treatment of type 1 diabetes. Autoimmune Research. 2006; 5: 409-413.

314- Cheung AT, Dyanandan B, Lewis JT, et al. Glucose dependent insulin release from genetically engineered K cells. Science. 2000; 290: 1959-1962.

315- Lu Y, Wang Z, Zhu M. Human bone marrow mesenchymal stem cells transfected with insulin gene can secrete insulin stably. Ann Clin Lab Sci. 2006; 36: 127-136.

316- Seveg H, Fishman B, Ziskind A, Schulman MItskovitz-Eldor J. Differentiation of human embryonic stem cells into insuli-producing clusters. Stem Cells. 2004; 22: 265-274.

317- FijikawaT, Oh SH, Pi L, Haech HM, et al. Teratoma formation leads to failure of treatment for type 1 diabetes using embryonic stem cells-derived insulin-producing cells. Am J Pathol. 2005; 166: 1781-1791.

318- Dean SK, Yulyana Y, Williams G, Sidhu KS, Tuch BE. Differentiation of encapsulatedembryonic stem cells after transplantation. Transplantation. 2006; 82: 1175-1184.

319- Krishna KA, Rao GV, Rao KS. Stem cell-based therapy for the treatment of Type 1 diabetes mellitus. Regen Med. 2007; 2: 171-7.

320- Hori Y, Gu X, Xie X, Kim SK. Differentiation of insulin-producing cells from human neural progenitor cells PloS Med. 2005; 2: E103.

321- Burns CJ, Minger SL, Hall S, et al. The in vivo differentiation of rat neural progenitor cells into insulin-expressing phenotype. Biochem Biophys Res Commun. 2005; 326: 570-577.

322- Zhao Y, Wang H, Mazzone T. Identification of stem cells from human umbilical cord blood with embryonic and hematopoietic characteristics. Exp Cell Res. 2006; 312: 2454-2464.

323- Tayaramma T, Ma B, Rhode M, Mayer H. Chromatin remodeling factors allow differentiation of bone marrow cels into insulin-producing cells. Stem cells. 2006; 24: 2858-2867.

324- Todorov I, Nair I, Ferreri K, et al. Multipotent progenitor cells isolated from adult human pancreatic tissue. Transplant Proc. 2005; 37: 3420-3421.

325- Campbell S. Request for applications: Islet cell replavement in Type 1 diabetes. Cell Biochem Biophys. 2004; 40: 23-24.

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Factor V Leiden and Antiphospholipid Antibodies in Pregnancies Complicated by Preeclampsia

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ABSTRACT

Background: Several studies have linked inherited and acquired thrombophilia to adverse pregnancy outcome including preeclampsia. Factor V leiden (FVL) is one of the most frequent thrombophilic mutations. Most of the studies about Factor V Leiden mutation in women were done in developed countries while little is known about the incidence and prevalence in developing countries where preeclampsia is still a major cause of maternal mortality.

Objective: The purpose of our study is to investigate the presence of FVL mutation and antiphospholipid antibodies (APA) in cases with preeclampsia and their relation to the maternal and fetal outcome.

Patients and Methods: We performed a prospective case-control study enrolling 116 preeclamptic and 40 normotensive pregnant women. Complete blood count, urea, creatinine and urine for proteinuria as well as APA IgG & IgM and FVL evaluation were done for cases and controls.

Results: FVL mutation was found in 8.6% of preeclamptic pregnancies and 5% of normotensive pregnancies and APA IgM was 22.4% and 5% in preeclamptic and normotensive pregnancies respectively while APA IgG was 31% and 10% in preeclamptic and normotensive pregnancies respectively. Eight fetuses out of ten (80%) had bad outcome in the heterozygous FVL mothers while 24 out of 106 (23%) had bad outcome in wild FVL mothers and the difference is statistically significant (p=0.001).

Conclusions: The incidence of FVL mutation is higher in preeclampsia than normal pregnancy and this may reflect unfavourable fetal outcome.

Key Words: *Preeclampsia – Factor V leiden mutation – Antiphospholipid antibodies.*

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INTRODUCTION

Preeclampsia (PE) affects 6-8% of all pregnancies and is a major cause of maternal and fetal morbidity and mortality. The pathogenesis of preeclampsia is complex and incompletely understood. Many investigators suggest an association with abnormal trophoblast invasion, coagulopathy, genetic and immunological predisposition, dietary abnormalities and vascular endothelial damage **[1]**.

Prochazka et al., suggest an increased prevalence of obstetric complications in female carriers of hereditary or acquired thrombophilias **[2]**. The G (guanine) to A (adenine) substitution at nucleotide 1691 of factor V gene results in resistance to activation by protein C, causing a pro-thrombotic state in FVL carriers **[3]**. The FVL mutation and other hereditary thrombotic risk factors can moderately increase the risk of preeclampsia, the vasculopathy and secondary thrombosis from hypercoagulopathy may result in inadequate perfusion of intervillous space and preeclampsia **[4]**. Most of the studies about FVL mutation in women were done in developed countries while little is known about the incidence and prevalence in developing countries where preeclampsia is still a major cause of maternal mortality **[15]**.

Several reports suggest that quantification of known APA is not prognostic in the assessment of the risk for PE, others suggest that inherited and/or acquired thrombophilia may play a role in the pathogenesis of PE and/or intrauterine growth retardation (IUGR), as their frequencies are increased in women with histories of the

early-onset PE compared to those with normal pregnancies **[5]**.

The aim of this study was to investigate the frequency of FVL mutation and APA in cases with preeclampsia and to evaluate the maternal and fetal outcome in these cases.

PATIENTS AND METHODS

Beginning in November 2006 and ending in October 2007, women with preeclmpsia who were matched for gestational age to normotensive pregnant controls were approached for participation. One hundred and sixteen women with preeclampsia and 40 normotensive women agreed to participate and provided their signed informed consent. This study was done in the Women's Health Centre, Faculty of Medicine, Assiut University. The APA and FVL were done in Clinical Pathology Department, South Egypt Cancer Institute, Assiut University. The institutional ethics committee at Faculty of Medicine, Assiut University approved this study.

Study groups:

Pre-eclampsia group:

All the patients were in their late 3rd trimester of pregnancy. Preeclampsia was considered when a pregnant woman developed arterial hypertension after the 20th week of pregnancy associated with proteinuria. Arterial hypertension was defined when the blood pressure was at least 140/90 mm Hg detected twice with a time difference of 6 hours between them. Proteinuria was defined as the presence of 30 mg/dl in the urine analysis. Exclusion criteria for study participation included chronic hypertension, preeclampsia superimposed on chronic hypertension, prior thromboembolism, transient hypertension or multifetal pregnancy.

Control group:

The control group included women who were in their late 3rd trimester and normotensive throughout pregnancy and had no history of thromboembolic event, abnormalities in blood pressure or proteinuria.

Methods:

The following evaluation data were collected: Age, parity, gestational age, blood pressure, proteinuria, maternal outcome, mode of birth, fetal outcome, birth weight of the babies and their Apgar scores.

The following routine laboratory investigations were done for patients and controls: Complete blood count, urea, creatinine and urine for proteinuria. APA IgG & IgM and FVL evaluation were also done.

For specific investigations, FVL and APA, 3ml venous blood were collected in a tube containing EDTA (ethylene diamine tetra acetic acid) for DNA isolation and 5 ml were collected in plain tubes for serum preparation. The samples were centrifuged within 30 minutes at 3000 rpm for 10 minutes and serum collected and stored at -20ºC for APA.

Antiphospholipid antibodies:

The ORGENTEC Anti-Phospholipid Screen IgG/IgM assay is a quantitative enzyme immunoassay (EIA) intended to screen for the presence of IgG and IgM class autoantibodies against Cardiolipin, Phosphatidyl Serine, Phosphatidyl Inositol, Phosphatidic acid and ß2-Glycoprotein, in human serum or plasma.

Principle of the test:

A mixture of highly purified Cardiolipin, Phosphatidyl Serine, Phosphatidyl Inositol, Phosphatidic acid and human ß2-Glycoprotein I is bound to microwells. Antibodies against these antigens if present in serum will bind to the respective antigens. Washing of the microwells removes nonspecific serum and plasma components. The addition of an acid stops the reaction forming a yellow end-product. The intensity of this yellow color is measured photometrically at 450 nm.

The cut off value for APA IgG is <10 GPL/ml and for APAIgM is <10 MPL/ml.

Factor V leiden:

DNA extraction: DNA is extracted from EDTA blood by high pure template isolation kit (Roche diagnostic).

PCR: Using factor V leiden kit (Light Cycler real time PCR, Roche Diagnostics, Mannheim, Germany). The factor V leiden kit allows the detection and genotyping of a single point mutation (G to A at position 1691) of the human factor V gene, from human whole peripheral blood. The test was performed on the Light

Cycler 1.2 instrument. The hybridization probes are used to determine the genotype by performing a melting curve analysis after the amplification cycles are completed and the amplicon is present at increased concentration. The Red 640-labeled hybridization probe hybridizes to a part of the target sequence that is not mutated and functions as an anchor probe. The fluorescein-labeled hybridization probe spans the mutation site (mutation probe).

During the melting curve analysis, increasing temperature causes the fluorescence to decrease because the shorter of the two probes (mutation probe) dissociates first and the two fluorescent dyes are no longer in close proximity. If the FVL mutation is present the mismatch of the mutation probe with the target destabilizes the hybrid so the decrease in fluorescence will occur at a lower temperature. With the wild type genotype mismatch will not occur, and therefore, the heteroduplex DNA has a higher melting temperature (Tm). The heterozygous genotypes exhibits a distinctive combination of properties. The resulting melting peaks allow discrimination between the homozygous (wild type or mutant) as well as the heterozygous genotype (Fig. 1).

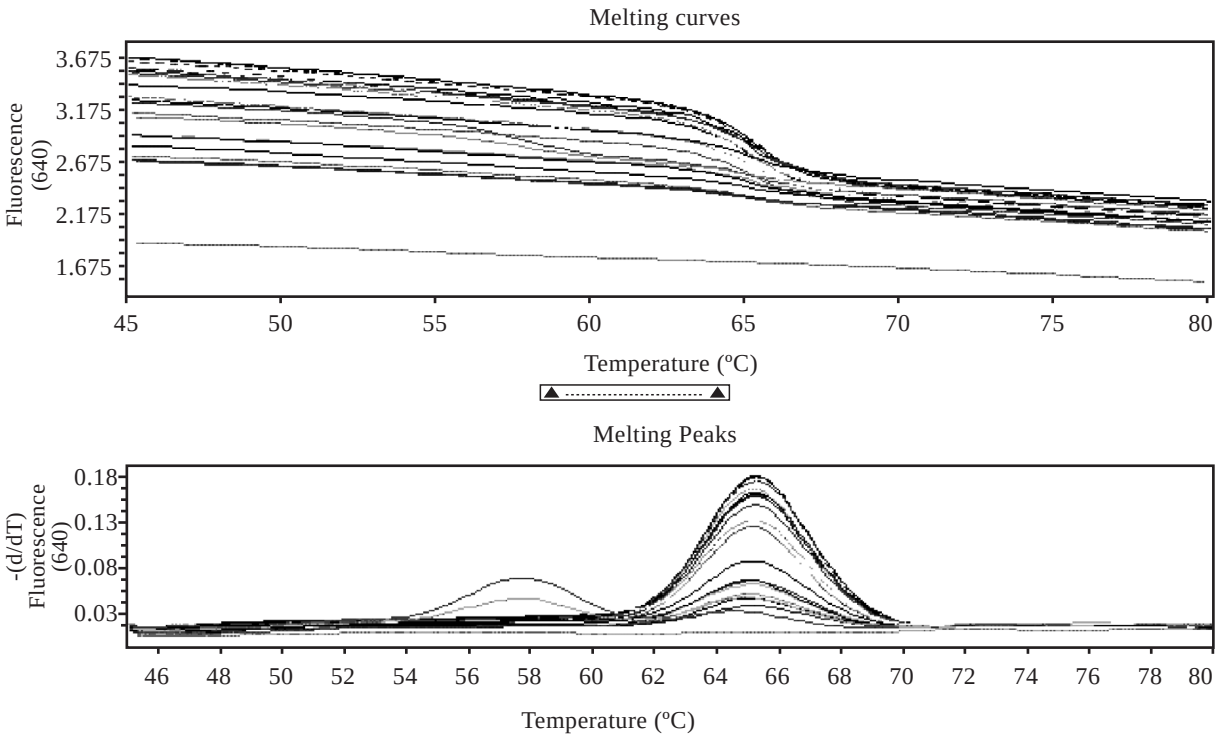

Fig. (1): Melting curve analysis for Factor V Leiden.

This picture reveals 2 peaks at two different melting temperature (one at 58ºC [mutant] and the other at 66ºC [wild]) indicating heterozygous genotype of FVL.

Statistical analysis:

All data were analyzed using SPSS (Statistical Program for Social Sciences) version 11 for windows, 2001, SPSS Inc., Chicago, IL, USA. Comparisons between means for continuous variables were done using independent sample *t*-test. Values are represented as mean ± SD. The relation between values and the outcome was undertaken using the X^2 test. Relationships in variables were assessed by correlation test. A p value <0.05 is considered to be significant. All p values were two-tailed.

RESULTS

One hundred and sixteen preeclamptic women and 40 normotensive pregnant women as a control were enrolled in this study.

Demographic and clinical characteristic of PE and control groups are shown in Table (1).

The laboratory parameters in the PE and control groups are shown in Table (2). Haemoglobin level, leucocytic count, platelets, urea and Creatinine showed comparable values in both groups. The incidence of APA IgG, IgM and FVL (heterozygous) were increased in the PE group than control group but the difference is statistically insignificant.

Patients with preeclampsia were more likely to undergo caesarean section and have intrauterine growth restriction than the control group. Newborn admission to neonatal intensive unit was more in PE patients than the control group and the differences are statistically significant (Table 3).

Eight fetuses out of ten (80%) had bad outcome in the heterozygous FVL mothers while 24 out of 106 (23%) had bad outcome in wild FVL mothers and the difference is statistically significant (p=0.001).

The incidence of bad maternal outcome was more (36%) when APA IgM was positive than when it was negative (9%). The difference is statistically significant (p=0.01).

Table (1): Demographic and clinical characteristics of preeclampsia patients and control group.

Clinical features	PE Patients N=116	Control group N=40	*p* value
Age (years)	16-19 y = 16 (13.8%) 20-35 y = 84 (72.4%) >35 y = 16 (13.8%)	16-19 = 4 (10%) 20-35 = 32 (80%) >35 = 4 (10%)	NS
Parity	Primigravida: 66 (56.9%) Multigravida: 50 (43.1%)	Primigravid: 18 (45%) Multigravid: 22 (55%)	NS
Gestational age (Week)	<37 = 62 (53.4%) >37 = 54 (46.6%)	<37 = 12 (30%) >37 = 28 (70%)	NS
Systolic bp (mm Hg)	156.3	118.2	<0.001
Diastolic bp (mm Hg)	94.6	74.4	<0.001

PE: Preeclampsia. bp: Blood pressur. y: Year. N: Number. NS: Non significant. 0.001: Highly significant.

Table (2): Laboratory parameters in preeclampsia patients and control group.

	PE Patients N=116	Control group N=40	*p* value
Hb. (g/dl)	9.9±1.5	10.1±1.1	NS
WBC (109/L)	7.8±2.5	7.6±2.2	NS
Plt (109/L)	214±77	206±78	NS
Urea (umol/L)	4.7±2.2	4.1±1.4	NS
Creatinine (umol/L)	74±1.3.3	72.1±12.7	NS
APA (IgG)	Positive: 36 (31%) Negative: 80 (69%)	Positive: 4 (10%) Negative: 36 (90%)	NS
APA (IgM)	Positive: 26 (22.4%) Negative: 90 (77.6%)	Positive: 2 (5%) Negative: 38 (95%)	NS
FVL	Wild: 06 (91.4%) Heterozygous: 10 (8.6%)	Wild: 38 (95%) Heterozygous: 2 (5%)	NS

PE : Preeclampsia. WBC : White blood cells. IgG : Immunoglobulin. M, FVL : Factor Vleiden.
N : Number. Plt : Platelet count. G, IgM : Immunoglobulin. NS : Non significant.
Hb : Hemoglobin. APA : Antiphospholipid antibodies.

Table (3): Maternal and fetal outcome in preeclampsia patients and control group.

	PE Patients N=116	Control group N=40	*p* value
Maternal outcome	Good: 98 (84.5%) Bad*: 18 (15.5%)	Good: 34 (85%) Bad: 6 (15%)	NS
Mode of birth	VD: 28 (24.1%) C/S: 88 (75.9%)	VD: 28 (70%) C/S: 12 (30%)	*p*=.001
Fetal outcome	Good: 84 (72.4%) Bad#: 32 (2736%)	Good: 40 (100%)	*p*=0.009
Birth wight (gm)	<2500: 44 (37.9%) >2500: 72 (62.1%)	>2500: 40 (100%)	*p*=0.001
Apgar score 5	Mean: 9.1±1.3	Mean: 10	*p*=0.001
Apgar score 10	Mean: 8.1±1.9	Mean: 10	*p*=0.001

PE : Preeclampsia. VD : Vaginal delivery. * : Intensive care unit admission.
N : Number. C/S : Cesarean section. # : Neonatal intensive care unit admission.

DISCUSSION

Our results showed that the frequency of FVL was (8.6%) in PE group which is more than the control group (5%) but the difference is statistically insignificant.

These results agree with the results of Dona et al. **[6]**, who reported that 8.9% of severe PE were heterozygous for FVL compared to 4.2% in the control group but their results were statistically significant and with the results of Rigo et al. **[7]** who reported 18.33% heterozygous carriers of Factor V Leiden mutation among preeclamptic women and 2.97% heterozygous carriers among healthy controls with the differences between the two groups found to be statistically significant. Dudding et al. **[8]**, reported that maternal FVL is significantly associated with preeclampsia, and Tempelhoff et al. **[9]**, also reported that FVL was 20% in their patients compared to 5% in control.

Our results showed that homozygosity for FVL is not found in either group which agrees with the results of Dona et al. **[6]**, but contrasts with the results of Tempelhoff et al. **[9]**, who found one of their patients and one of the controls to be homozygous for FVL.

Nurk et al. **[13]** found that FVL mutation conferred increased risk of preeclampsia, the risk was highest for preeclampsia at less than 37 weeks. So this latter study supports that FVL mutation may be a risk factor for preeclampsia.

Many studies found no associations between FVL variant allele and preeclampsia **[10-12]**. A meta analysis by Kosmas et al. **[10]** including 2742 hypertensive women and 2403 controls suggested that the associations observed in early and small studies may be due to time-lag bias and publications bias.

The association was evident with FVL mutation (heterozygosity), when the presence of moderate and severe forms of preeclampsia is analyzed separately, the existence of heterogeneity in the moderate form is shown which is not found in the studies related to the severe form **[14]**.

In patients with negative histories for thromboembolism the heterozygous FVL mutation is associated with a lower risk of thromboembolism in pregnancy and therefore neither the screening of all pregnant women nor the treatment of the low risk carriers is recommended **[15]**.

The current study also showed that FVL was not associated with adverse maternal outcome which agrees with the results of Van Pumpus et al. **[15]** who reported that FVL are important genetic risk factor associated with thrombotic risks but this mutation is apparently not related to perinatal outcome in women with preeclampsia. It also agrees with the results of Rigo et al. **[7]** who reported that no statistically significant different perinatal outcomes were found between

Factor V Leiden positive and negative preeclamptic women.

On the other hand, FVL may affect the fetal outcome in which adverse fetal outcome is more in the heterozygous FVL than the wild type in PE group. This result agrees with that of Calderwood **[16]**, who observed an association between maternal FVL and fetal or neonatal stroke and with Dena et al. **[17]**, who concluded that women who are carriers of FVL are faced with increased risk of stillbirth, early onset preeclampsia, severe abruption and possibly fetal growth restriction. Our results are contrast with these of Dudding et al. **[8]**, who observed that there was no association between maternal FVL and fetal growth restriction.

In our study we found positive APA IgG in 31% in PE compared to 10% in controls and positive APA IgM in 22.4% in PE compared to 5% in controls but these differences are statistically insignificant. Studies of women with preeclampsia have confirmed the high incidence of antiphospholipid antibody **[18]**. A number of groups have described increased rate of APA ranging between 10% and 20% among women with preeclampsia **[19]**, severe preeclampsia **[20]** or eclampsia **[21]**. However, at least 2 groups of investigators have found no increased rate of APA among women with preeclampsia **[22,23]**.

Katano et al. **[24]** who did prospective study of 800 unselected obstetric patients have found that the rate of preeclampsia is significantly higher among those women with positive results for APA than among those with negative results. In this study 2% to 7% of the tested women had APA with 0.7% to 7% having anticardiolipin antibodies, and preeclampsia developed in 22% to 50% of these women.

The maternal outcome was affected in our series by APA IgM in which adverse maternal outcome was higher when APA IgM is positive than when it is negative.

We conclude from this study that FVL is more evident in preeclamptic pregnancies than normal pregnancies but these differences are statistically insignificant, FVL is not associated with perinatal outcome but is associated with bad fetal outcome and APA IgM is associated with bad perinatal outcome.

REFERENCES

1- González-Quintero VH, Jiménez JJ, Jy W, Mauro LM, Hortman L, O'Sullivan MJ, Ahn Y. Elevated plasma endothelial microparticles in preeclampsia. Am J Obstet Gynecol. Aug. 2003, 189 (2): 589-93.

2- Procházka M, Happach C, Marsál K, Dahlbäck B, Lindqvist PG. Factor V Leiden in pregnancies complicated by placental abruption. BJOG. 2003, 110 (5): 462-6.

3- Lee R. Factor V Leiden: A clinical review. Am J Med Sci. 2001, 322: 88-102.

4- Tormene D, Simioni P, Prandoni P, Luni S, Zerbinati P, Sartor D, et al. Factor V Leiden mutation and the risk of venous thromboembolism in pregnant women. Haematologica. 2001, 86: 1305-9.

5- Sikkema JM, van Rijn BB, Franx A, Bruinse HW, de Roos R, Stroes ES, van Faassen EE. Placental superoxide is increased in pre-eclampsia. Placenta. Apr. 2001, 22 (4): 304-8.

6- MD Donna S, Dizon-Townson, Nelson LM, Easton K, Ward K. The factor V Leiden mutation may predispose women to severe preeclampsia. Am J Obstet Gynecol. Oct. 1996, 175 (4 Pt 1): 902-5.

7- Rigó J Jr, Nagy B, Fintor L, Tanyi J, Beke A, Karádi I, Papp Z. Maternal and neonatal outcome of preeclamptic pregnancies: The potential roles of factor V Leiden mutation and 5,10 methylenetetrahydrofolate reductase. Hypertens Pregnancy. 2000, 19 (2): 163-72.

8- Dudding T, Heron J, Thakkinstian A, Nurk E, Golding J, Pembrey M, Ring SM, Attia J, Scott RJ. Factor V Leiden is associated with pre-eclampsia but not with fetal growth restriction: A genetic association study and meta-analysis. J Thromb Haemost. Nov. 2008, 6 (11): 1869-75.

9- Von Tempelhoff GF, Heilmann L, Spanuth E, Kunzmann E, Hommel G. Incidence of the factor V Leiden-mutation, coagulation inhibitor deficiency, and elevated antiphospholipid-antibodies in patients with preeclampsia or HELLP-syndrome. Thromb Res. Nov. 2000, 15: 1 (4) 363-5.

10- Kosmas IP Tatsioni A, Ioannidis JPA. Association of leiden mutation in factor V gene with hypertension in pregnancy and preeclampsia: A meta-analysis. J Hypertens. 2003, 21: 1221-8.

11- De Maat MPM, Jansen MWJC, Hille ETM, Vos L, Kupferminc MJ. Thrombophilia and preeclampsia: The evidence so far. Clin Obstet Gynecol. 2005, 48: 406-15.

12- Salomon O, Seligsohn U, Steinberg DM, Zalel Y, Lerner A, Rosenberg N, et al. The common prothrombotic factors in nulliparous women do not compromise blood flow in the feto-maternal circulation and are not associated with preeclampsia or intrauterine growth restriction. Am J Obstet Gynecol. 2005, 191: 2002-9.

13- Nurk E, Tell GS, Refsum H, Ueland PM, Vollse SE T. Factor V Leiden pregnancy complications and adverse outcomes: The Hordaland Homocysteine study. 2006, 99: 289-298.

14- De Santis M, Cavaliere AF, Straface G, Di Gianantonio E, Caruso A. Inherited and acquired thrombophilia: Pregnancy outcome and treatment. Reprod Toxicol. Aug. 2006, 22 (2): 227-33.

15- Van Pampus MG, Wolf H, Koopman MM, van der Ende A, Buller HR, Reitsma PH. Prothrombin 20210 G: A mutation and factor V leiden mutation in women with a history of severe preeclampsia and HELLP syndrome, Hypertens pregnancy. 20 (2001), pp. 291-298.

16- Calder wood CJ, Greer IA. The role of factor V Leiden in maternal health and the outcome of pregnancy. Curr Drug Targets. Aug. 2005, 6 (5): 567-76.

17- Dena Bloomenthal, Peter von Dadelszen, Robert Liston, Laura Magee and Peter Tsang. The effect of factor V Leiden Carriage on maternal and fetal health. CMAJ. 2002, 167 (1): 48-54.

18- Backos M, Rai R, Baxter N, Chilcott IT, Cohen H, Regan L. Pregnancy complications in women with recurrent miscarriage associated with antiphospholipid antibodies treated with low dose aspirin and heparin. BJOG. 1999, 106: 102-7.

19- Allen JY, Tapia-Santiago C, Kutteeh WH. Antiphospholipid antibodies in patients with preeclampsia. Am J Reprod Immunol. 1996, 36: 81-5.

20- Van Pampus MG, Dekker GA,Wolf H, Huijgens PC, Koopman MM, Von Blomberg BM, et al. High prevalence of henals mostatic abnormalities in women with history of severe preeclampsia. Am J Obstet Gynecol. 1999, 180: 1146-50.

21- Rao AA, Ananthakrishna NC. Anticardiolipin antibodies in eclampsia. Int J Gynecol Obstet. 1992, 38: 37-40.

22- Martinez-Abundis E, Gonzalez-Ortiz M, Cortes-Liamas V, Salazar-Paramo M. Anticardiolipin antibodies and the severity of preeclampsia-eclampsia. Gynecol Obstet Invest. 1999, 48: 168-71.

23- Scott RAH. Anticardiolipin antibodies and preeclampsia. BJOG. 1997, 94: 604-5.

24- Katano K, Aoki A, Asa H, Ogasawara M. B2 Glycoprotein I -dependent anticardiolipin antibodies as a predictor of adverse pregnancy outcome in healthy pregnant women. Hum Reprod. 1996, 11: 509-12.

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Original Article:

Changes of Activation and Apoptotic Platelet Markers During Apheresis and Storage

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ABSTRACT

Background: Platelets are known to undergo, shape changes, activation, a release reaction and apoptosis/ necrosis during processing and storage. As aphaeresis may have deleterious impact on platelet achievability and functional integrity.

Methods: Platelets concentrates from 50 male volunteers obtained by COBE spectra cell separator were screened for platelet activation (CD62 and CD154) and apoptosis (Phosphatidylserine detected by Annexin V). Base line of donor samples before separation, during aphaeresis and at the third day of storage samples were collected. Platelet aggregation to ADP and collagen was performed.

Results: There was a statistically significant increase in expression of the activation markers in platelet samples at 2 different time points (during separation samples and 3rd day samples). Although the increase in Annexin V expression was slight, it was statistically significant. There was marked significant decline in the platelet aggregation. The correlations between the values of CD62, CD154 and Annexin V were detected in base line samples and were more evident in both during separation and at the 3rd day of platelets storage. Correlation between values of platelets aggregation to collagen and Annexin V was significantly relevant only in the base line samples. No other correlations were encountered between platelet aggregation and markers of activation and apoptosis during apheresis and storage.

Conclusions: Initial platelet activation which is induced by aphaeresis may have an impact on phosphatidylserine expression with no impact on aggregation function of platelets during storage.

Key Words: *Annexin V – Aphaeresis – CD154 – CD62 – Storage.*

INTRODUCTION

Platelets transfusion can be performed using either whole blood or aphaeresis product. In the case of platelet-apheresis, production of platelet concentrates (PCs) with a standard number of platelets and minimal leukocyte contamination, together with well-preserved function of platelets and minimal platelet activation, is the ultimate goal **[1,2]**.

In spite of substantial scientific effort, mechanisms leading to the damage of platelets during their collection and storage are not fully understood **[3]**. Studies have suggested that platelet activation or stimulation may play an important role in the development of the platelet storage lesion.

CD62 (P-selectin) is a member of the selection family of cellular adhesion molecules, and it mediates the binding of activated platelets to neutrophils, monocytes, and endothelial cells **[3]**. P-selectin expression on platelet surfaces correlates with loss of viability upon transfusion of the stored platelet concentrates **[4]**.

CD154 (CD40 ligand CD40L) is a member of the tumor necrosis family. Several important immunological phenomena are attributed to CD154 interaction with its receptor CD40 including B cell immunoglobulin class switching and maturation of dendritic cells **[5,6]**. CD154 has been identified on the surface of platelets **[7]** and its expression on platelets has a key role in inflammation and prothrombotic activity **[8]**.

In vivo, platelet aging is associated with Phosphatidylserine (PS) exposure. Previous reports described the PS as a target molecule for the recognition and removal of activated or damaged platelets. This function is attributed

to professional phagocytes equipped with specific receptors for PS, such as the class B scavenger proteins SR-BI and CD36 **[9-12]**. This phenomenon means that platelets with a high PS content in the outer plasma membrane could be removed after transfusion by the reticuloendothelial system independently of their functional status **[9,10,13,14]**. These events may play an important role in the removal of senescent platelets from the circulation **[11,12]**.

This study aimed to investigate the platelet alterations by detecting activation and apoptotic markers in concentrates obtained by cell separator pre and during separation and after 3 days storage in our transfusion center. Annexin V measurement using flow cytometry was employed for PS detection. We aimed also to study the effects of these changes on the platelets aggregation function.

MATERIAL AND METHODS

Cytapheresis:

Platelet concentrates were collected from 50 male volunteers who met the requirements of the American Association of Blood Bank for healthy blood donors. Segments from Platelet concentrate bags obtained by COBE Spectra blood separators (Gambro BCT Inc., Lakewood, USA) were used to obtain samples. Base line (BL) samples from donors before separation were collected. The second specimen was obtained during aphaeresis (DS) and the third in the third day of storage in platelet agitator.

Determination of Platelet CD62-P, CD154 and Annexin-V by Flow Cytometry:

To minimize and standardize platelet activation during venesection, an atraumatic procedure was used. Whole blood anti-coagulated with EDTA and the aphaeresis products were diluted 1:8 in HEPES buffer. Twenty five µl of diluted blood were directly labeled with 5µl of each CD62P-FITC, CD154-PE, CD41a Per-CP and Annexin-V APC antibodies combination. CD41a (glycoprotein IIb/IIIa complex) is specific to platelets, thus, it can be used to recognize and gate on the stained platelets. All antibodies were purchased from Pharmingen, BD, USA, except Annexin-V, which was purchased from IQ product, Groningen, Netherlands. After 20 minutes incubation at room temperature in the dark, the samples were lysed by ammonium chloride lysing solution and washed with PBS.

Analysis by flow cytometry was performed immediately using FACSCaliber (BD, USA) by identification of platelets according to their characteristics was determined using log forward scatter for size and log side scatter for granularity. Platelet gate was adjusted such that >95% of the particles analyzed were anti CD41a positive. The platelet activation markers and Annexin-V were expressed as geometric mean fluorescence intensity (GMFI) and as percentage of antibody positive cells. The positivity was defined as fluorescence higher than that of the isotypic control (Fig. 1).

Platelet aggregation studies:

Platelet aggregation to ADP (10µM) and Collagen (2µg/ml) had been performed using optical method of measurement of platelet aggregation (light transmitter method) by platelet aggregation profiler model PAP-4 (Bio/data corporation). The reagents were provided by Chrono-Log Company, USA.

Statistical analysis:

The data were collected, categorized and processed by using Statistical Package for Social Sciences (SPSS), version 15 software packages. The quantitative variables were expressed as mean ± standard deviation (SD) and comparison was done using paired students *t*`-test. *p*-value levels of <0.05 was considered statistically significant. Correlations between quantitative variables were done using Pearson correlation.

RESULTS

Fifty donations were studied; their product characteristics are shown in Table (1). There was a statistically significant increase in the mean values of both CD62 & CD154 (% and GMFl) in DS samples compared to BL values. These markers revealed marked rising in the 3rd day of storage samples and showed a statistically significant increase in comparison to those of BL and DS samples (Table 2).

There were minimal differences between the three samples (BL, DS, and 3rd day) in the mean value (% and GMFl) of Annexin V. However there was statically significant increase in DS samples (% and GMFl) when compared to BL samples and in 3rd day samples (% and GMFI) when compared to BL samples. When the comparison was performed between DS and

3rd day samples there was a statistically significant increase in the percentage of cells expressing Annexin V with no significant difference in Annexin V GMFl.

There was marked decline in the platelet aggregation to both ADP and collagen during separation and on the third day of storage; the difference is statistically significant (Table 2).

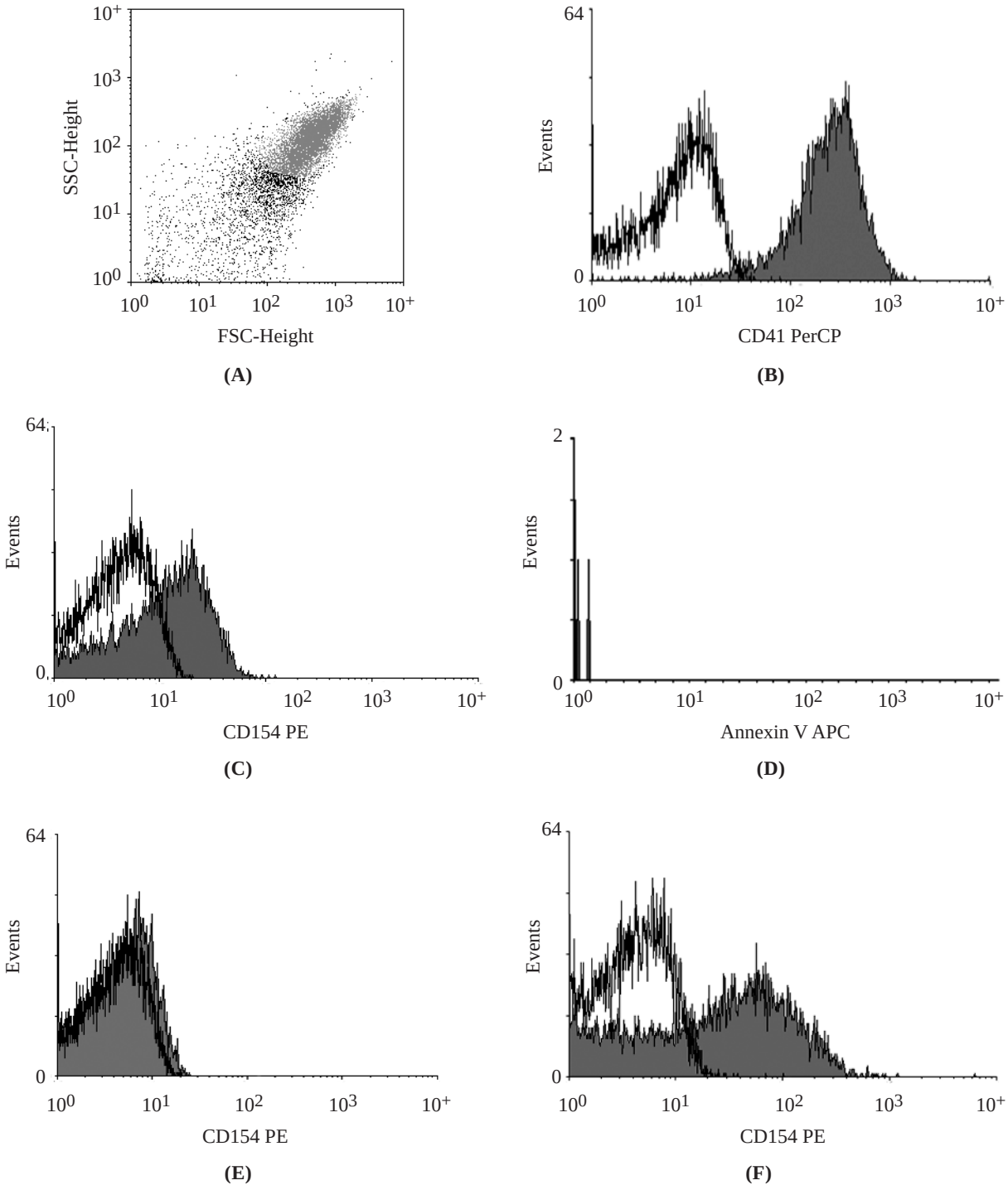

Fig. (1): Platelet gate (A) which adjusted that >95% of the particles were anti CD41a positive (B). CD62 (C) and Annexin-V (D) were expressed as geometric mean fluorescence intensity (GMFI). CD154 were expressed as GMFI in base line (E) and in the 3rd day of storage (F). The positivity was defined as fluorescence (red histogram) higher than that of the isotype control (open histogram).

Positive correlations were observed between percentage values of platelets activating markers CD62 and CD154 in BL samples (r=0.634 and p=0.02) and also between their GMFl values (r=0.684 and p=0.01). These correlations were stronger in D.S and in the 3rd day of platelets storage samples and were observed between % and GMFl of CD62 and CD154 (r=0.905, p<0.001, Fig. 2) and (r=0.919, p<0.001) respectively.

There was a positive correlation between Annexin V percentage expression between of BL and DS samples (r=0.714 and p=0.004), it was also encountered between their GMFl values (r=0.838 and p<0.001). However these correlations decreased when comparing DS and in the 3rd day of platelets storage samples (r=0.646, p=0.043) and (r=0.661, p=0.038) respectively.

On the other hand, correlations between platelets activation and apoptotic markers were performed and revealed positive relation between BL CD62 and BL Annexin V GMFl (r=0.555, p=0.03). This correlation was increased to be r=0.682, p=0.03 in the 3rd day samples. It was also detected also between CD154%, CD154 GMFl and Annexin V GMFl in the 3rd day of storage (r=0.778, p=0.01 and r=0.675, p=0.03 respectively).

Correlation between values of platelets aggregation to collagen and Annexin V in the BL samples was encountered (r=0.535, p=0.04) (Fig. 3). Positive correlations were encountered between DS platelets aggregation to ADP and platelets aggregation to collagen (r=0.688 p=0.002). This correlation was increased after platelets storage in day 3 samples (r=0.775 and p<0.001).

Table (1): Product characteristics for 50 donations.

Procedure time (min)	90±10
Total procedure volume (ml)	2500±340
Platelets volume (ml)	264±18
Platelets yield (plt/10^9)	361±72
Platelet concentration (plt/l)	1150±95

Table (2): Comparison between base line, during separation and 3rd day of storage samples in all tested markers.

Markers	Base line	During Separation	3rd day	*p* value		
				A	B	C
CD62%	1.7±1.7	43.4±16.1	64.3±23.2	0.000	0.000	0.01
CD62 GMFl	3.5±0.47	25.8±34.6	71.2±66.7	0.011	0.011	0.04
CD154%	0.18±0.12	23.8±14.8	47.6±11.4	0.00	0.000	0.04
CD154 GMFl	2.7±0.28	9.9±8.6	19.6±14.5	0.011	0.05	0.04
Annexin V %	0.3±0.17	0.5±0.24	1.0±0.46	0.00	0.001	0.008
Annexin V GMFl	3.15±0.47	3.4±0.6	3.4±0.65	0.00	0.006	0.23
ADP %	67.0±6.7	39.0±4.8	9.0±4.19	0.00	0.000	0.000
Collagen %	60.0±3.4	32.0±5.8	7.0±3.85	0.00	0.000	0.000

GMFI: Geometric mean fluorescence intensity; Quantitative variables are expressed as mean ± standard deviation.
A = *p*-values when base line group compared with DS group.
B = *p*-values when DS group compared with 3rd day group.
C = *p*-values when base line group compared with 3rd day group.

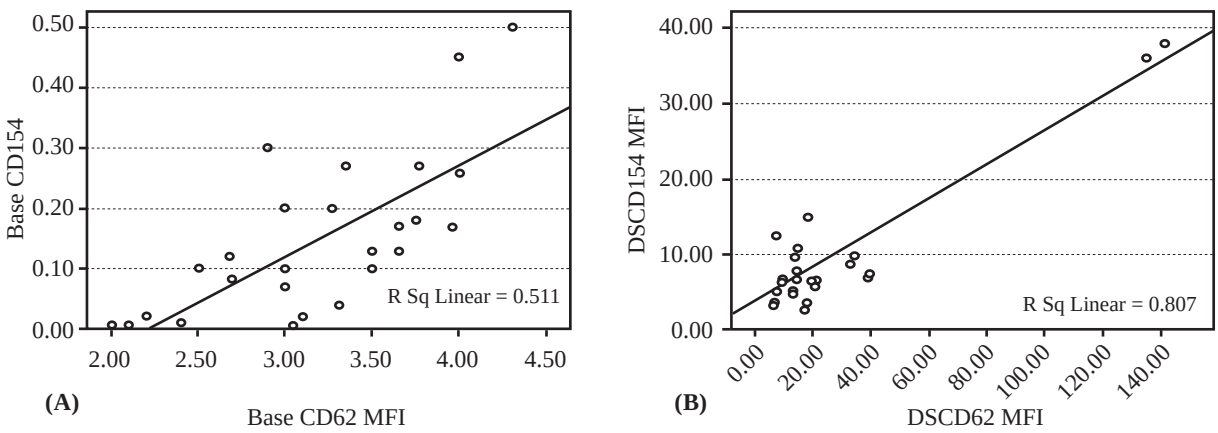

Fig. (2): Correlations between CD62 and CD154 GMFI in base line samples (A) and during separation samples (B).

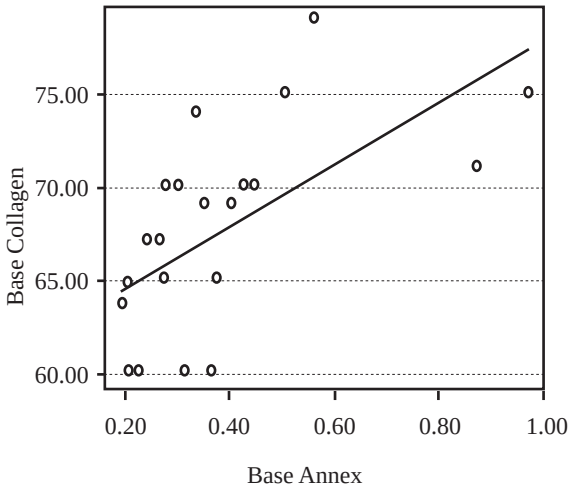

Fig. (3): Correlation between Annexin V % expression and aggregation to collagen in base line samples.

DISCUSSION

The present study was performed to analyze the degree of antigenic alterations in platelets caused by aphaeresis and storage of platelets concentrates, prepared with continuous cell separators. The results of this work demonstrated that platelets activation which was detected by flow cytometric analysis of CD62-P and CD154 begins early during separation and increases during the storage.

A number of authors have demonstrated that platelet activation markers increase during storage of PCs, they suggested some factors which may explain the increase in CD62-P expression such as the manner of agitation, the conditions available in container and the number of polymorphonuclear leukocytes in platelet concentrates **[15,16]**. Other authors **[17]** recognized this increase immediately after collection and after storage compared to pre-donation level in blood donors; they concluded that these changes are may be due to change in pH as a result of acid production which lead to decreased metabolic activity, function and viability of platelets **[18]**. The increased expression of CD62-P during separation may reflect aphaeresis technology; platelets are exposed to both physical forces and artificial surfaces **[19,20]**.

CD154 can activate adherent polymorphnuclear leukocytes and is associated with transfusion-induced acute lung injury **[21]**. Some authors have provided evidence that CD154 is implicated in adverse platelets transfusion reactions **[22]**. Others concluded that CD154 expression on platelets has a key role in inflammation and prothrombotic activity **[8]**. Our findings on platelets changes during and after apheresis are consistent with Kaufman et al. **[23]** who found high level of both membranous and soluble CD154 in stored PCs and provided additional evidence that this increase begins early during separation and increases during storage. This increase is affected by the same factors affecting CD 62-P expression as the current study showed significant positive correlations between the two markers at the three different time points of sampling (pre, during apheresis and after storage).

It is so important to detect the possible links between activation of platelets and their apoptosis, Therefore, in this study, platelet viability was tested by detecting the degree of PS expression on the platelets surface. It showed a minimal increase during separation and storage. There was a significant increase in the percentage of cells expressing Annexin V in 3rd day samples when compared to DS with no significant difference in Annexin V GMFl. Percentage of positive platelets is a qualitative marker to assess the degree of platelet activation but GMFI is a quantitative one **[24]**. It can be concluded that the increase in expression of Annexin V is more during separation and in the storage it is very limited to few platelets. It is possible that enhancement of PS exposure during separation is generated by the stress to which platelets are exposed during the aphaeresis procedure.

On the other hand, correlations were found between the percentage and GMFI of platelets positive for Annexin V during apheresis and the same parameters on third day of storage. These results disagree with Lai et al. **[25]** study who found a correlation between Annexin V value in the 3rd and 5th day of storage with no correlation between the same parameter immediately after apheresis and after storage. This may be attributed to the small size of samples of their study (twenty seven single donor PCs).

Other correlations were found between markers of activation and Annexin V in the 3rd day of storage. Some authors found similar results between P-selectin and Annexin V in PCs collected by continuous apheresis and concluded that the level of P-selectin closely reflects cell damage **[18,26]**. Additionally the results of this

work can suggest that CD154 may reflect cell damage also, and this effect takes some time to be observed as the correlations appear in the 3rd day of storage not during apheresis. To the best of our knowledge no such relations were reported between CD154 and Annexin V before.

A platelet aggregation testing to ADP and collagen results are in good agreement with earlier literatures. It was reported that aggregation response to ADP and collagen decreased gradually from the time of separation of PCs to the storage time in the platelets shaker for three days **[27-29]**. Sloand et al. **[30]** has also compared the platelets aggregation activity to ADP and collagen and postulated that ADP and collagen induced platelet activity of the PCs on the 5th day is much lower than normal. Various authors **[31-34]** demonstrated impaired platelets function through investigation of platelets aggregation with thrombin and collagen. Findings of the current work are in concordance with the results which suggest that PCs should be used as soon as possible, since there is a loss of platelet functions **[34]**.

In the present work, a significant correlation was detected between platelets aggregation to collagen and Annexin V value in BL. This can be explained by the fact that PS in platelets exerts a procoagulant effect, accelerating the enzymatic cleavage of coagulation proteins (e.g., prothrombin) on the platelet membrane. This event triggers the formation of the fibrin clot and the stabilization of the platelet thrombus **[35]**. On the other hand, no other correlations could be detected between platelet aggregation and markers of activation and apoptosis. This means that no one of these factors alone can affect the function of platelets collected by apheresis. Neiva et al. **[36]** found a correlation between pH and aggregation and between PCo_2 and aggregation. Considering that many variations in the composition of platelets membrane can be involved in aggregation, this area needs further work.

It could be concluded that initial platelet activation which is induced by aphaeresis has an impact on PS expression with no impact on aggregation function of platelets during storage.

Conflict of interest statement:

All authors declare that they have no conflict of interest.

REFERENCES

1- Fijhneer R, Modderman PW, Veldman H, Ouwehand WH, Nieuwenhuis HK, Roos D, de Korte D. Detection of platelet activation with monoclonal antibodies and flow cytometry: Changes during platelet storage. Transfusion. 1990; 30: 20-25.

2- Nieuwenhuis HK, van Oosterhout JJG, Rozemuller E, van Iwaarden F, Sixema JJ. Studies with a monoclonal antibody against activated platelets. Blood 1987; 70: 838-845.

3- McEver RP, Martin MN. A monoclonal antibody to a membrane glycoprotein binds only to activated platelets. J Biol Chem. 1984; 259: 9799-9804.

4- Gutensohn K, Alisch A, Geidel K, Crespeigne N, Kuehnl P. Annexin V and platelet antigen expression is not altered during storage of platelet concentrates obtained with AMICUS cell separator. Transfus Sci. 1999; 20: 113-119.

5- Banchereau J, Briere, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. Immunobiology of dendritic cells. Annu Rev Immunol. 2000; 18: 767-811.

6- Kehry MR. CD40-mediated signaling in B cells. Balancing cell survival, growth and death. J Immunol. 1996; 156: 2345-8.

7- Inwald DP, McDowall A, Peters MJ, Callard RE, Klein NJ. CD40 is constitutively expressed on platelets and provides a novel mechanism for platelet activation. Circ Res. 2003; 92: 1041-8.

8- Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G, Kroczek RA. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature. 1998; 391: 591-4.

9- Kuypers FA. Phospholipid asymmetry in health and disease. Curr Opin Hematol. 1998; 5: 122-31.

10- Rinder HM, Murphy M, Mitchell JG, Stocks J, Ault KA, Hillman RS. Progressive platelet activation with storage: Evidence for shortened survival of activated platelets after transfusion. Transfusion. 1991; 31: 409-14.

11- Rigotti A, Acton SL, Krieger M. The class B scavenger receptors SR-Bi and CD36 are receptors for anionic phospholipids. J Biol Chem. 1995; 270: 16221-4.

12- Savill J, Fadok V, Henson P, Haslett C. Phagocyte recognition of cells undergoing apoptosis. Immunol Today. 1993; 14: 131-6.

13- Gutensohn K, Alisch A, Krueger W, Kroeger N, Kuehnl P. Extracorporeal plateletapheresis induces the interaction of activated platelets with white blood cells. Vox Sang. 2000; 78: 101-5.

14- Pereira J, Palomo I, Ocqueteau M, Soto M, Aranda E, Mezzano D. Platelet aging in vivo is associated with loss of membrane phospholipid asymmetry. J Thromb Hemost. 1999; 82: 1318-21.

15- George JN, Pickett EB, Heinz R. Platelet membrane glycoprotein changes during the preparation and storage of platelet concentrates. Transfusion. 1988; 28: 123-6.

16- Kutlay S, Ilhan O, Arslan O, Beksac M. Influence of storage time on activation of platelets collected with CS 3000 Plus and COBE Spectra using platelets storage containers. Ther Apher. 2002; 6: 82-5.

17- Prochazkova R, Andrys C, Hubackova L, Krejesk J. Markers of platelet activation and apoptosis in platelet concentrates collected by aphaeresis. Transfus Apher Sci. 2007; 37: 115-123.

18- Holme S, Sweeney JD, Sawyer S, Elfath MD. The expression of p-selectin during collection, processing, and storage of platelet concentrates: Relationship to loss in vivo viability. Transfusion. 1997; 37: 12-7.

19- Perseghin P, Mascaretti L, Speranza T, Belloti D, Baldini V, Dassi M, et al. Platelet activation during plasma-reduced multicomponent PLT collection: A comparison between COBE Trima and Spectra LRS turbo cell separators. Transfusion. 2004; 44: 125-30.

20- Stohlawetz PO, Hergovich N, Eichler HG, Hocker P, Kapiotis S, Jilma B. Differential induction of P-selectin expression on platelets by two cell separators during platelet apheresis and the effect of gender on the release of soluble P-selectin. Transfusion. 1998; 38: 24-30.

21- Khan SY, Kelher MR, Heal JM, Blumberg N, Boshkov LK, Phipps R, Gettings KF, McLaughlin NJ, Silliman CC. Soluble CD40 ligand accumulates in stored blood components, primes neutrophils through CD40, and is a potential cofactor in the development of transfusion-related acute lung injury. Blood. 2006; 108: 2455-62.

22- Blumberg N, Gettings KF, Turner C, Heal JM, Phipps RP. An association of soluble CD40 ligand (CD154) with adverse reactions to platelet transfusions. Transfusion. 2006; 46: 1813-21.

23- Kaufman J, Spinelli L, Schultz E, Blumberg N, Phipps RP. Release of biologically active CD154 during collection and storage of platelets concentrates prepared for transfusion. J Thromb Hemost. 2007; 5: 788-96.

24- Osborne GW. Flow Cytometry Software Workshop. 2000; www.qbi.uq.edu.au/index.html.

25- Lai M, Rumi C, D"Onofrio G, Puggioni PL, Menichella G, Candido A, Leone G. Phosphatylserine exposure in platelets concentrates during the storage period: Differences between the platelets collected with different cell separators. Transfus Apher Sci. 2002; 27: 239-45.

26- Krailadsiri P, Seghatchian J, Williamson LM. Platelet storage lesion of WBC-reduced, pooled buffy coat-derived platelet concentrates prepared in three in-process filter/storage bag combinations. Transfusion. 2001; 41: 243-50.

27- Koerner K. Thrombocyte function by storage inj PVC bags with increased gas permeability. Beitr Inusionther Klin Ernaehr. 1986; 15: 118-26.

28- Turner VS, Mitchell SG, Kang SK, Hawker RJ. A comparative study of platelets stored in polyvinyl chloride containers plasticized with butyryl triexyl citrate or triethylhexile trimellitate. Vox Sang. 1995; 69: 195-200.

29- Rock G, Tittly P, McCombine N. 5 day storage of platelets collected on a blood cell separator. Transfusion. 1989; 29: 288-91.

30- Sloand ME, Yu M, Klein HG. Comparison of random donor platelet concentrates prepared from whole blood units and platelets prepared from single donor apheresis collections. Transfusion. 1996; 36: 955-9.

31- Wildt-Eggen J, Schrijver JG, Bins M, Gullikson H. Storage of platelets in additive solutions: Effects of magnesium and/or potassium. Transfusion. 2002; 42: 76-80.

32- Gulliksson H, Aubuchon JP, Vesterinen M, Sandgren P, Larsson S, Pickard CA, Herschel I, Roger J, Tracy J, Langweiler M. Storage of platelets in additive solutions: A pilot in vitro study of effects of potassium and magnesium. Vox Sang. 2002; 82: 131-136.

33- Van Der Meer P, Pietersz R, Reesink H. Leukoreduced platelet concentrates in additive solution: An evaluation of filters and storage containers. Vox Sang. 2001; 81: 102-107.

34- Glaser A, Friedlein H, Zingsem J, Zimmermann R, Weisbach V, Ruf A, Eckstein R. Storage of single donor platelet concentrates: Paired comparison of storage as single or double concentrates. J Clinic Apheresis. 2001; 6: 148-154.

35- Tzima E, Walker JH. Platelet annexin V: Rhe ins and outs. Platelets. 2000; 11: 245-251.

36- Neiva T, Machado M, Hoehn M, Hermes E, Vituri J, Ferreira E, D'amico E. Evaluation of platelet aggregation in platelet concentrates: Storage implications. Rev bras Hematol Hemoter. 2003; 25: 207-212.

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Original Article:
Flow Cytometric Platelet Cross-Matching to Predict Platelet Transfusion in Acute Leukemia

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ABSTRACT

Background: A great variety of patient and product related factors influence the outcome of platelet transfusions. Our study assessed the predictive value of a flow cytometric platelet cross match test for the outcome of HLA matched and unmatched platelet transfusions in patients with acute leukemia.

Materials and Methods: Thirty nine patients (26 adults and 13 children), were included in this study. Patients received 60 ABO and RH compatible platelet concentrates ranging from 1 to 4 per patient. We performed flowcytometric platelet cross-matching, HLA class I typing by SSP for patients and complement dependent cytotoxicity (CDC) for donors and screening of HLA class I antibodies by ELISA. Effectiveness of platelet transfusion was evaluated using the corrected count increment (CCI) which was calculated at 60 minutes and 18-24 hours post transfusion. Multivariate analysis was performed to detect which variable, can predict transfusion response more than others.

Results: Cross-matched platelet transfusions associated with good response in 48.6% of transfusion events in adults and 75% in children. The non-crossmatched platelet transfusions associated with poor response in 83.3% in adults and 100% in children. In the presence of clinical factors or HLA alloimmunization in adults, cross-matched platelets were associated with good response in 29.6% and 22.2% respectively. In children this occurred in 81.8% and 66.7% respectively. In presence or absence of HLA matching, flow cytometry platelet cross-matching was the most predictor for transfusion response.

Conclusions: Due to difficulties to find frequent HLA matched donors for acute leukemia patients, flow cytometric platelet cross-matching may provide the most useful way for selecting donors. It is useful even in the presence of alloimunization but only in children.

Key Words: *Flow cytometric platelet cross-matching – HLA alloimunization – Platelet refractoriness.*

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INTRODUCTION

Platelet transfusion is an essential part of the treatment of cancer, hematological malignancies, marrow failure, and hematopoietic stem cell transplantation **[1]**. However, some patients fail to receive the full benefit of platelet transfusions because they do not achieve the appropriate platelet count increment following transfusion **[2]**.

Refractoriness to platelet transfusions is often multifactorial **[3]**. It is most likely due to non-immune factors, although immune factors can sometimes be responsible **[1]**. Non-immune factors associated with decreased post-transfusion platelet count increments include clinical conditions such as splenomegaly, infection, disseminated intravascular coagulation (DIC), females with 2 or more pregnancies, bleeding, fever and use of heparin or amphotericin **[3-5]**.

Platelets express human leukocyte antigens (HLA)-A, HLA-B, and platelet specific antigen (human platelet antigens [HPA]). There is a strong association between the presence of HLA antibodies in the transfusion recipient and platelet refractoriness, but the relation between

platelet-specific antibodies and refractoriness is weaker **[5]**. Before the widespread use of leucocyte-reduced blood components to prevent alloimmunization, 45-70% of chronically transfused patients developed antibodies to HLA class I antigens **[6]**. Chronically transfused patients become alloimmunized to HPA less commonly. The proportion of patients with antibodies to HPA varies, but ranges from 2% to 17% **[7,8]**.

Two main strategies have been used to transfuse alloimmunized patients: Matching donor-recipient HLA antigens and cross-matching platelets. HLA-matching involves identifying the HLA type of the recipient and transfusing platelets from donors with matched antigens. HLA matching requires the availability of large numbers of HLA-typed donors. Alloimmunized patients are often transfused with platelets from donors that are only partially matched **[1]**. Transfusion of HLA-matched platelet, have shortcomings, with up to 20% of HLA-identical platelet transfusions failing to achieve the target platelet increment **[9]**.

A commonly used alternative to HLA-matched platelets is the transfusion of cross-match compatible platelets **[10,11]**. Cross-matching compatibility has been used for identification of candidate platelet donor and may be beneficial in patients in whom refractoriness is due to HPA, so the HLA-matched platelet transfusion has no value **[12]**.

Platelet cross matching by flow cytometry was addressed in many studies **[13,14]**. However, the predictive role of flow cytometric platelet cross-matching on transfusion response and its affection by the presence of clinical factors, HLA alloimmunization and transfusion of HLA-matched platelet were not fully evaluated.

The aim of this study is to assess the predictive value of flow cytometric platelet cross-matching for the outcome of ABO compatible platelet transfusion.

MATERIAL AND METHODS

This study was performed on 39 randomly selected patients with acute leukemia, including 18 female and 21 male, 26 adults and 13 children, all presented to South Egypt Cancer Institute from February 2006 to June 2008. They received 60 platelet transfusions (43 in adults and 17 in children) of ABO and RH compatible leuko-reduced platelet concentrates (PCs) ranging from 1 to 4 per patient. The mean value of transfused platelets was $5.03 \pm 1.13 \times 10^{11}$. PCs were collected by platelet apheresis using cell separator, COBE Spectra, version 7 (Cobe BCT Inc, USA).

In all transfusions, patients were monitored for the presence of splenomegaly, active bleeding, sepsis and fever greater than 38.5ºC. Effectiveness of platelet transfusion was evaluated using the post transfusion corrected count increment (CCI) which was calculated at 60 minutes and 18-24 hours post transfusion according to the formula:

CCI= (Post transfusion platelet count-pre transfusion platelet count $\times 10^9$ x body surface area)/ dose of platelets transfused $\times 10^{11}$ **[15]**.

A poor response was designated as CCI at 60 minutes and at 18-24 hours post-transfusion below 4,500-5,000 and 2,500 platelets per microliter respectively **[11]**.

Flow cytometric platelet cross-matching:

Platelet antibodies in plasma (or serum) were detected by incubating patients' plasma/ serum with donors' platelets. Serum was centrifuged for 3 minutes at 4000 rpm. Patients' serums were diluted 9:1 in 0.3% EDTA-PBS. Twenty µL of diluted patient's serum were incubated with 20µL of PC for 45 minutes at 37ºC in a water bath. EDTA-PBS (0.3%) was added to the tube and centrifugation at 1000 rpm for 10 minutes at room temperature and removal of the supernatant were done. Twenty µL of FITC conjugated antihuman IgG (Becton Dickinson, USA) and PE conjugated CD41 (Diaclone, France) were added. CD41 was used to confirm that we were analyzing platelet population, 95% of the gated cells must be positive for CD41. The samples were incubated for 45 minutes at room temperature to be ready for analysis by flow cytometry.

Positive control: Plasma containing anti HPA-Ia antibodies was treated as a patient sample and was thereafter diluted 1:2 (strong positive) and 1:20 (weak positive) in 0.3% EDTA- PBS.

Negative control: Negative control (donor serum) was treated as a patient sample.

Acquisition and analysis:

Samples analysis was done using FACS Caliber (Becton Dickinson, USA) flow cytometry. Five thousands events in an appropriate gate surrounding the platelet population were collected and data stored.

Fluorescence intensity (FI) from gated population in a histogram (FL1/ counts) with negative control as overlay geometric mean was used for fluorescence ratio (FR) calculation as shown in Fig. (1). The results were visually inspected on the FL1 histogram **[16]**.

FR= FI sample/ FI negative control.

Evaluation of the run:

FR was repeated if FR ≤0.6.

IgG anti platelet antibodies 1:2 positive controls should have FR >6.0.

IgG anti platelet antibodies 1:20 positive controls should have FR ≥1.7.

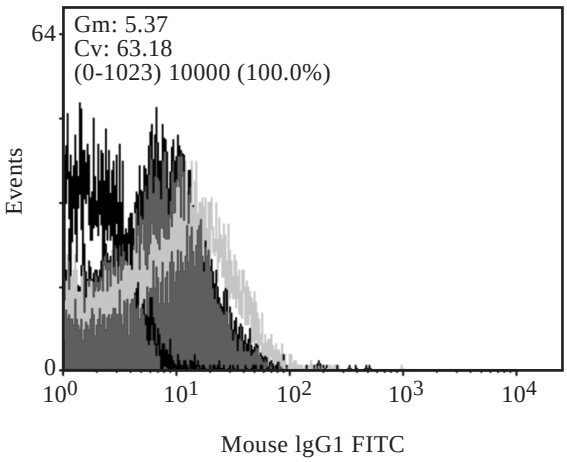

Fig. (1-A): A histogram showing a positive platelet cross-matching (red), negative control (black) and positive control (green).

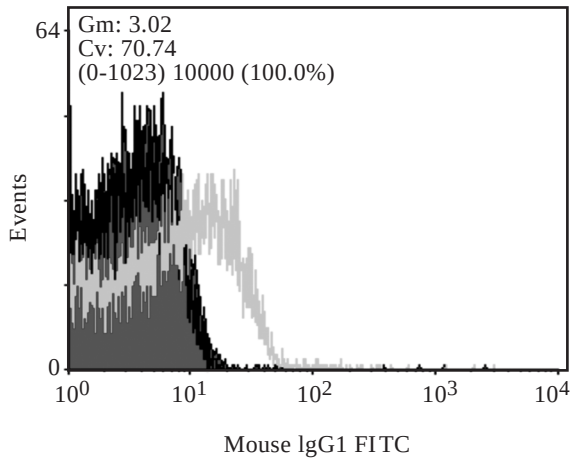

Fig. (1-B): A histogram showing negative platelet cross-matching (red), negative control (black) and positive control (green).

HLA class I typing by complement dependent cytotoxicity (CDC):

HLA class I typing for donors was performed by CDC method using HLA- ready plates ABC 72 (InnoTrain, Kronberg/ Taunus, Diagnostic GmbH).

SSP HLA class I A and B typing:

HLA class I typing for patients was performed by ALLSet+™ Gold SSP (Dynal Biotech Ltd., U.K). It is a PCR based technique, which uses SSP, for DNA based tissue typing. DNA extraction was done by GENE ALL+™ blood SV mini (General biosytsem, Korea).

PCR amplification was done according to manufacturer instructions. Amplicons were electrophoresed on 2% agarose gel. The assignment of alleles merely consists of determining whether amplification has occurred or not, i.e. visualization and detection of the amplification by agarose gel electrophoresis.

Detection of HLA class I antibodies:

Detection of HLA class I antibodies was done by ELISA using Lambda Antigen Tray (LAT^{TM}) (GmbH, Hannover, Grmany). ELISA was performed according to the manufacturer's instruction.

Statistical analysis:

Statistical package for social sciences (SPSS), version 15 was used for data analysis. Mean and standard deviation were used as descriptive value for quantitative data. Chi-square test was used to calculate relation between transfusion response and the presence of clinical factors, HLA alloimmunization in patients, platelet cross-matching and degree of HLA matching between patients and their donors. *p* value is significant when less than 0.05.

Receiver Operator Characteristic (ROC) curve was used for selection of the FR of platelet cross-matching which gives more specific

prediction of transfusion response. Logistic Regression analysis was done to detect which variable can predict transfusion response more than others. Association is present if the odd ratio is more than one. *p* value is significant when less than 0.05.

RESULTS

The clinical and laboratory data of all transfusion events are shown in Table (1). Good response to platelet transfusions was detected in 31 of the 60 transfusion events (51.7%), 19/43 in adults (44.2%) and 12/17 in children (71.3%).

Table (1): Clinical and laboratory data of 60 flowcytometric cross-matched platelet transfusion events.

Data	Adults (43)	Children (17)
Age	39±15.9	8±5.8
Male / Female	22/21	9/8
Splenomegaly	27 (62.8%)	13 (76.5%)
Bleeding	23 (53.5%)	7 (41.2%)
Fever	15 (34.9%)	9 (52.9%)
Infection	12 (27.9%)	5 (29.4%)
HLA antibodies before transfusion	20 (46.5%)	5 (29.4%)
Base line platelet count	17.25 ±12.32	15.69 ±9.28
Platelet count at one hour post transfusion	45.49±35.54	84.53±39.37
Platelet count at 24 hours post transfusion	31.52±26.38	57.35±30.96
Platelet dose (x 10^{11})	5.06±1.19	4.96±0.98
BSA of patients	1.66±0.15	0.87±0.42
CCI at one hour post transfusion	10.16±10.60	10.45±7.54
CCI at 24 hours post transfusion	5.58±6.78	5.98±4.39
Fluorescent ratio	1.33±0.62	1.2±0.31

CCI : Corrected count increment. BSA : Body surface area. HLA : Human leukocyte antigen.

The ROC curve was done to select the cutoff of FR of platelet cross-matching (Fig. 2). The *p* value of the ROC curve was 0.023. The FR less than 1.8 for negative platelet cross-matching (cross-matched platelets) gave the highest significance (p=0.017). So we considered it as the cutoff:

Negative= FR<1.8 Positive= FR≥1.8

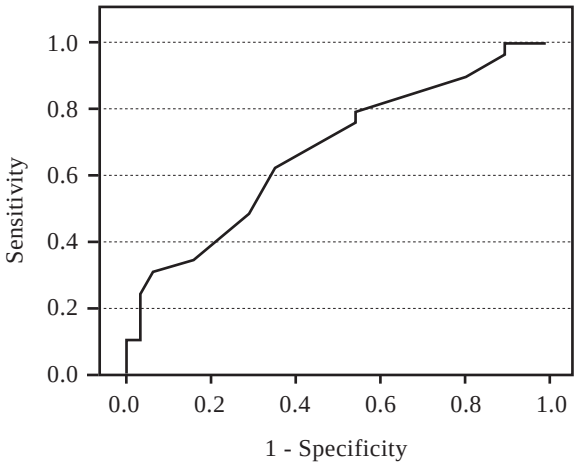

Fig. (2): The ROC curve for selection of the florescent ratio (FR) of platelet cross matching.

Predictive value of flow cytometric platelet cross-matching for platelet transfusion outcome:

Flow cytometric cross-matched platelets were transfused in 52/60 transfusion events (86.7%), 37/43 (86.1%) in adults and 15/17 (88.2%) in children. The difference between good and poor transfusion response regarding flow cytometric platelet cross-matching in the whole group was statistically significant. However, when the patients were divided into adults and children the difference was statistically significant only in the children group (Table 2).

Table (2): Role of flow cytometric platelet cross-matching in prediction of platelet transfusion outcome.

Transfusion response	Flow cytometric platelet cross-matching	
	Cross-matched platelets	Non cross-matched platelets
In all patients:		
Good	30/52 (57.7%)	1/8 (12.5%)
Poor	22/52 (42.3%)	7/8 (87.5%)
p value	0.017	
In Adults:		
Good	19/37 (51.4%)	1/6 (16.7%)
Poor	18/37 (48.6%)	5/6 (83.3%)
p value	0.143	
In Children:		
Good	11/15 (73.3%)	0/2 (0%)
Poor	4/15 (26.7%)	2/2 (100%)
p value	0.041	

p value was calculated by chi-square test

Influence of clinical factors on predictive value of flow cytometric platelet cross-matching:

Thirty eight (27 adults and 11 children) flowcytometric cross-matched platelet transfusions were given in the presence of and 14 (10 adults and 4 children) in the absence of clinical factors that might affect platelet transfusion outcome.

The difference in the predictive value of flow cytometric cross-matched platelets on transfusion response between patients with and without clinical factors is shown in Table (3).

Table (3): Influence of clinical factors on prediction of flow cytometric platelet transfusion response by platelet cross-matching.

Transfusion response	Flow cytometric cross-matched platelets	
	Presence of clinical factors	Absence of clinical factors
In all patients:		
Good	17/38 (44.7%)	12/14 (85.7%)
Poor	21/38 (55.3%)	2/14 (14.3%)
p value	0.008	
In Adults:		
Good	8/27 (29.6%)	9/10 (90%)
Poor	19/27 (70.4%)	1/10 (10%)
p value	0.001	
In Children:		
Good	9/11 (81.8%)	3/4 (75%)
Poor	2/11 (18.2%)	1/4 (25%)
p value	0.770	

p value was calculated by chi-square test

Influence of HLA alloimmunization on predictive value of flow cytometric platelet cross-matching:

Twenty one (18 adults and 3 children) flow-cytometric cross-matched platelet transfusions were given in the presence of and 31 (19 adults and 12 children) in the absence of HLA alloimmunization.

The difference in the predictive value of flow cytometric cross-matched platelets on transfusion response between patients with and without HLA alloimmunization is presented in Table (4).

Influence of using HLA matched platelets on predictive value of flow cytometric platelet cross-matching:

When HLA matched platelets (in 2 or more antigens) were used, cross-matched platelets transfusion was associated with good response in 10/11 (90.9%) while when transfused platelets were not HLA matched, the corresponding fig. was 7/13 (53.8%). The difference was found to be statistically significant ($p<0.05$).

Table (4): Influence of HLA alloimmunization on prediction of platelet transfusion outcome by platelet cross-matching.

Transfusion response	Flow cytometric cross-matched platelets	
	Presence ofHLA alloimmunization	Absence of HLA alloimmunization
In all patients:		
Good	6/21 (28.6%)	23/31 (74.2%)
Poor	15/21 (71.4%)	8/31 (25.8%)
p value	0.001	
In Adults:		
Good	4/18 (22.2%)	13/19 (68.4%)
Poor	14/18 (77.8%)	6/19 (31.6%)
p value	0.005	
In Children:		
Good	2/3 (66.7%)	10/12 (83.3%)
Poor	1/3 (33.3%)	2/12 (16.7%)
p value	0.519	

p value was calculated by chi-square test

Multivariate analysis:

Multivariate analysis was done to detect which variable (clinical factors, HLA alloimmunization and/or flow cytometric platelet cross-matching) can predict transfusion response. Flow cytometric platelet cross-matching was found to be the best predictor of transfusion response with the highest significance followed by HLA alloimmunization then clinical factors. HLA matching showed no predictive value (Table 5).

Table (5): Clinical factors, HLA alloimmunization, flow cytomtric platelet cross-matching and HLA matching as predictors for platelet transfusion response.

Variable	Flow cytometric platelet transfusion response	
	Odd ratio	*p*-value
Clinical factors	2.56	0.466
HLA alloimmunization	2.89	0.312
Platelet cross-matching	19.76	0.050
HLA matching	0.07	0.032

p value was calculated by Logistic Regression test

DISCUSSION

A great variety of patient and product related factors influence the outcome of platelet transfusions. The patient related factors are numerous, as weight, height, splenomegaly, fever, infection, DIC and HLA alloimmunization. Major platelet factors that are associated with poor response are decreased dose of platelets, ABO incompatibility **[17]** and platelets stored more than 48 hours **[18]**. In our study, we tried to minimize the influence of platelet factors by giving the patients ABO-RH compatible-leukoreduced PCs within hours of collection.

In this study flow cytometric platelet cross-matching was found to be a good predictor of transfusion response, transfusion of cross-matched platelets was more successful than transfusion of non cross-matched platelets. The significance of the area under the ROC curve is also indicating that flow cytometric platelet cross-matching is a good technique for platelet cross-matching. Rebulla et al. **[10]** reported the same result although they used another automated technique.

We noticed that flow cytometric platelet cross-matching is a better predictor of transfusion response in children than in adults. It had better prediction of transfusion response when clinical factors were absent than when clinical factors were present. In adults, the predictive role of cross-matched platelets on transfusion response is more affected by the presence of the clinical factors than in children. This reflects the importance of transfusion of flow cytometric cross-matched platelets especially after exclusion of the presence of the clinical factors in adults and even in the presence of clinical factors in children.

Many previous studies **[14,19-21]** stated that the ability of cross-matching to predict transfusion response may be lower in unselected patients with refractoriness to platelet transfusion than in those without associated clinical factors but their patients were with no specific age group.

The same results were observed when HLA alloimmunisation is absent. This better predictive value of platelet cross-matching for transfusion response after exclusion of clinical factors and HLA alloimmunization reflects the importance of transfusing cross-matched platelets to all patients especially in patients who have neither clinical factors nor HLA alloimmunization.

The best predictive role of flow cytometric platelet cross-matching in this study was when HLA matched platelets were used, transfusion of cross-matched platelet was associated with good response in 90.9% when platelets were matched in two or more HLA antigens. This Fig. is higher than that of Sintnicolaas and Löwenberg **[14]**, who found that when flow cytometric platelet cross-matching was negative, platelet transfusion was successful in 75% of transfusion episodes. This may be attributed, at least partially, to the different method used in evaluation of negative cross-matching. They expressed their results in percentage (negative platelet cross-matching less than 25%) while we used FR less than 1.8 as a cutoff for negative cross-matching. This may suggest that using the FR= 1.8 as a cutoff gives more accurate results.

By using multivariate analysis, flow cytometric platelet cross-matching was found to be the best predictor of transfusion response followed by clinical factors and HLA alloimmunization, while HLA matching had no predictive value. To the best of our knowledge, multivariate analysis was not done in previous studies. The lack of predictive value of HLA matching in our study can be explained by lower number of HLA matched donors, but this is not a defect in the present study only, because the finding of HLA matched donors is difficult due to the large number of polymorphisms in the HLA system that complicates the provision of HLA-matched platelets even if a large number of HLA-typed donors are available **[9]**. In a previous study **[22]** on 29 alloimmunized HLA-typed patients, the mean number of potential donors found in a file of 7247 HLA-typed donors, was 6 who were a four antigen match and 33 who were identical at two or three loci.

In addition, the techniques of HLA typing are time-consuming and costly. Also, it has been reported that approximately 40% to 50% of HLA-matched platelet transfusion events do not result in adequate increments **[23]**.

In conclusion, flow cytometric platelet cross-matching is the best predictor for transfusion response. It can be done on available apheresis

platelets, making a compatible transfusion available in few hours rather than the several days it takes to schedule, draw, and test an HLA-typed donor **[9]**. Potential donors, not identified by HLA matching, may be selected by cross-matching. This may be because such recipients have platelet antibodies rather than HLA alloantibodies and therefore would not respond to HLA-matched platelets **[23]**. This indicates the importance of transfusing of flow cytometric cross-matched platelets and that cross-matched platelets can be given to patients with acute leukemia from the start to predict the transfusion response. In children it may provide the most useful way for selecting donors even in presence of clinical factors and alloimmunization.

REFERENCES

1- Stroncek DF, Rebulla P. Platelet transfusions. Lancet. 2007, 370 (9585): 427-438.

2- Hod E, Schwartz J. Platelet transfusion refractoriness. Br J Hematol. 2008, 142 (3): 348-60.

3- Slichter SJ. Evidence-based platelet transfusion guidelines. Hematology. 2007, 172-8.

4- Balduini CL, Salvaneschi L, Klersy C, Noris P, Mazzucco M, Rizzuto F, Giorgiani G, Perotti C, Stroppa P, Pumpo MD, Nobili B, Locatelli F. Factors influencing post-transfusional platelet increment in pediatric patients given hematopoietic stem cell transplantation. Leukemia. 2001, 15: 1885-1891.

5- Slichter SJ, Davis K, Enright H, Braine H, Gernsheimer T, Kao KJ, Kickler T, Lee E, McFarland J, McCullough J, Rodey G, Schiffer CA, Woodson R. Factors affecting post-transfusion platelet increments, platelet refractoriness, and platelet transfusion intervals in thrombocytopenic patients. Blood. 2005, 105 (10): 4106-4114.

6- Laundy GJ, Bradley BA, Rees BM, Younie M, Hows JM. Incidence and specificity of HLA antibodies in multitransfused patients with acquired aplastic anemia. Transfusion. 2004, 44: 814-825.

7- Novotny VM, van Doorn R, Witvliet MD, Claas FH, Brand A. Occurrence of allogeneic HLA and non-HLA antibodies after transfusion of pre-storage filtered platelets and red blood cells: a prospective study. Blood. 1995, 85: 1736-1741.

8- Sanz C, Freire C, Alcorta I, Ordinas A, Pereira A. Platelet specific antibodies in HLA-immunized patients receiving chronic platelet support. Transfusion. 2001, 41: 762-765.

9- Sacher RA, Kickler TS, Schiffer CA, Sherman LA, Bracey AW, Shulman IA. Management of patient's refractory to platelet transfusion. Archives of Pathology and Laboratory Medicine. 2003, 12: 409-414.

10- Rebulla P, Morelati F, Revelli N, Villa MA, Paccapelo C, Nocco A, Greppi N, Marconi M, Cortelezzi A, Fracchiolla N, Martinelli G, Deliliers GL. Outcomes of an automated procedure for the selection of effective platelets for patients' refractory to random donors based on cross- matching locally available platelet products. Br J Haematol. 2004, 125: 83-89.

11- Rebulla P. A mini-review on platelet refractoriness. Haematologica. 2005, 90: 247-253.

12- Secord A, Goldfinger D. Refractoriness to platelet transfusion therapy. www.uptodate.com. 2005, (800) 998-6374 (781) 237-4788.

13- Gates K, MacPherson BR. Retrospective evaluation of flow cytometry as a platelet crossmatching procedure. Cytometry. 1994, 18 (3): 123-128.

14- Sintnicolaas K, Lownberg B. A flow cytometric platelet immunofluorescence crossmatch for predicting successful HLA matched platelet transfusions. Br J Hematol. 1996, 92: 1005-1010.

15- Pamphilon HD, Murphy FM, Navarrete C, Ouwehana HW. Guidelines for the use of platelet transfusion. Br J Haematol. 2003, 122: 10-23.

16- Quality handbook, Department of immunology and transfusion medicine, University hospital of Northern Norway HF.

17- Julmy F, Ammann RA, Taleghani BM, Fontana S, Hirt A, Leibundgut K. Transfusion efficacy of ABO major-mismatched platelets (PLTs) in children is inferior to that of ABO-identical PLTs. Transfusion. 2009, 49(1):21-33.

18- Sensebé L. Factors affecting post-transfusion platelet efficiency "close relationship between patient and product".Transfus Clin Biol. 2007, 14 (1): 90-3.

19- Slichter SJ, Schiffer CA, Davis KA, et al. The trial to reduce alloimmunization to platelets study group. Leukocyte reduction and ultraviolet _ irradiation of platelets to prevent alloimmunization and refractoriness to platelet transfusions. N Engl J Med. 1997, 337: 1861-1869.

20- Moroff G, Garratty G, Heal JM, MacPherson BR, Stroncek D, Huang ST, Ho W, Petz LD, Leach MF, Lennon SS, et al. Selection of platelets for refractory patients by HLA matching and prospective crossmatching. Transfusion. 1992, 32: 633-640.

21- Gelb AB, Leavitt AD. Crossmatch compatible platelets improve corrected count increments in patients who are refractory to randomly selected platelets. Transfusion. 1997, 37: 624-630.

22- Petz LD, Garratty G, Calhoun L, Clark BD, Terasaki PI, Gresens C, Gornbein JA, Landaw EM, Smith R, Cecka JM. Selecting donors of platelets for refractory patients on the basis of HLA antibody specificity. Transfusion. 2000, 40: 1446-1456.

23- Schiffer CA, Anderson KC, Bennett CL, Bernstein S, Elting LS, Goldsmith M, Goldstein M, Hume H, McCullough JJ, McIntyre RE, Powell BL, Rainey JM, Rowley SD, Rebulla P, Troner MB, Wagnon AH. American Society of Clinical Oncology. Platelet transfusion for patients with cancer: clinical practice guidelines of the American Society of Oncology. J Clin Oncol. 2001, 19: 1519-1538.