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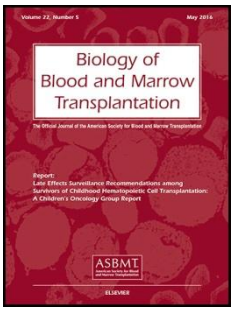

Title: A Modified Post-Transplant Cyclophosphamide (PT-CY) Regimen, for Unmanipulated Haploidentical Marrow Transplantation, in Acute Myeloid Leukemia: a Multicenter Study

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A MODIFIED POST-TRANSPLANT CYCLOPHOSPHAMIDE (PT-CY) REGIMEN, FOR UNMANIPULATED HAPLOIDENTICAL MARROW TRANSPLANTATION, IN ACUTE MYELOID LEUKEMIA: A MULTICENTER STUDY.

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HIGHLIGHTS

- A favourable use of Baltimore protocol for Haplo bone marrow transplant is described.
- It is associated to a very low rate of relapse in remission patients.
- We have seen a low incidence of GvHD, despite the use of cyclosporine before PT-CY.
- These results may not translate automatically to unmanipulated peripheral blood.

ABSTRACT

We are reporting a modified post-transplant cyclophosphamide (PT-CY) regimen, for unmanipulated haploidentical marrow transplants (HAPLO), in 150 patients with acute myeloid leukemia (AML).

All patients received a myeloablative regimen, cyclosporine (CsA) on day 0, mycophenolate on day +1, and PT-CY 50 mg/kg, on days +3 and +5. The median age was 51 years (17-74), 51 patients (34%) had active disease at transplant, and the median follow up of surviving patients 903 days (150-1955).

The cumulative incidence (CI) of engraftment, acute graft versus host disease (GVHD) grade II-IV and moderate/severe chronic GvHD was respectively 92% , 17% and 15%. The 4 year CI of transplant related mortality (TRM) and relapse was respectively 20% and 24%. Four year survival for remission patients was 72% (74% vs 67% for age </$\underline{\geq}$ 60 years) and 26% for advanced patients (17% vs 41% for age </$\underline{\geq}$ 60 years). In a multivariate analysis, active disease at transplant was the only negative predictor of survival, TRM and relapse.

The original PT-CY regimen can be modified with CsA on day zero, still providing protection against GvHD, low toxicity, and encouraging low relapse incidence in AML patients, also over the age of sixty years.

Keywords: Acute myeloid leukemia,haploidentical stem cells transplant, PT-CY

INTRODUCTION

The introduction of high dose post transplantation cyclophosphamide (PT-CY) for unmanipulated HLA haploidentical transplants (**1)** has been a major breakthrough of the past decade, perhaps the most significant one, and is largely responsible for the worldwide increase in unmanipulated HAPLO transplants **(2).** It is based on pre-clinical studies **(3)** showing that donor allo-reactive T cells will proliferate in the first 72 hours after transplantation, and will thus be killed when PT-CY is administered on day+3 and +4. For this reason the original Baltimore protocol calls for tacrolimus and mycophenolate (MMF) to start on day +5, following the administration of PT-CY **(4):** regulatory T-cells and hematopoietic stem cells express aldehyde dehydrogenase (ALDH) that makes them resistant to PTCY, and would be spared the effect of PT-CY **(4, 5, 6)**. One issue with the original Baltimore PT-CY protocol, based on a non myeloablative conditioning regimen, has been the risk of relapse in patients with acute leukemia **(1),** although further studies have shown a strong correlation of relapse with disease risk **(7)**.

We reasoned that patients with AML required a fully myeloablative conditioning regimen, and were concerned of adding, on top of this, high dose cyclophosphamide on two consecutive days. We therefore administered PT-CY on days +3 and +5 and started cyclosporine (CsA) on day zero and mycophenolate on day +1 **(8).** We also reasoned that some alloreactive T cell exposed to CsA, would not proliferate and would be spared the purging effect of PT-CY, possibly enhancing the graft versus leukemia (GvL) effect, in our initial advanced patients. The same reasoning leads to an increased risk of graft versus host disease (GvHD), which was however disproved by the observation of very little acute and chronic GvHD, very little toxicity and TRM, and encouraging survival in our initial reports **(8,9).** These results were achieved in a single Center (Genova) **(8),** and the question was whether they would be reproduced also in a multicentre setting. We therefore retrospectively analysed 150 AML patients, grafted in seven different Units, from a HAPLO donor, all receiving the same modified PT-CY protocol, with CsA on day 0 and PT-CY on days +3+5, following a myeloablative conditioning regimen.

PATIENTS AND METHODS

Patients' characteristics

This retrospective study was approved by the IRB of the Istituto di Ematologia, Fondazione Policlinico Universitario Gemelli, Rome, Italy, and included 150 AML patients. Eligible for this study were AML patients, receiving umanipulated HAPLO bone marrow, between 2010 and 2016, with a uniform prophylaxis for GvHD, as detailed below. Patients were treated at different institutions as follow: 93 patients in Genova, 25 patients in Rome, 10 patients in Frankfurt, 7 patients both in Ancona and Pavia and 5 patients in Cuneo. Clinical characteristics are shown in **Table 1.** High resolution HLA typing was performed both on patients' and donors' DNA to confirm allele matching, and haplotypes were determined on family studies when possible. The donor had a major ABO mismatch in 17 pairs (12%), a minor ABO mismatch in 38 pairs (27%), and in 7 pairs (5%) there was a double ABO mismatch.

Conditioning regimens and GVHD prophylaxis

All patients received a myeloablative regimen: younger patients were eligible for full dose total body irradiation (TBI), whereas older patients received a combination of thiotepa, busulfan, fludarabine (TBF). The TBF regimen consisted of thiotepa (5 mg/kg) on day -6 and -5 (total dose 10 mg/kg), busulfan (BU) (3.2 mg/kg) on day -4,-3,-2 (total dose 9.6 mg/kg), fludarabine (FLU) (50 mg/m^2) on day -4,-3,-2 (total dose 150 mg/kg). The TBI regimen consisted of FLU 120 mg/m^2, followed by 9-12 Gy total body irradiation (FLU-TBI). The median age of patients receiving TBF (n=114) was 55 years (17-74); the median age of patients receiving FLU-TBI (n=28) was 38 years (range 20-58) ($p<0.0001$). Busulfan was capped at 2 days in patients over 60 years of age **(Table 1)**. GVHD prophylaxis was uniform and consisted of intravenous cyclosporine A (CsA), from day 0 to day +20, 3 mg/kg, adjusting for blood levels (200–400 ng/ml), and then orally until day +180; mycophenolate (MMF) (15 mg/kg every 12 hours) from day +1, to day +28 and cyclophosphamide 50 mg/kg on day +3 and +5. G-CSF was started on day+6 until neutrophil recovery.

The stem cell source was unmanipulated bone marrow for all patients, and the median dose of cells collected and infused on day 0, was $3.1x10^8$/kg (range 0.8-6.7). Donor specific antibodies (DSA) were not routinely assessed pre-transplant.

Supportive care

Antimicrobial prophylaxis was given as per Institutional standard of care. Twice weekly monitoring for cytomegalovirus (CMV), by PCR or antigenemia, was started on day –7 until day+100 and thereafter once a week, or at each outpatient examination; weekly Epstein Barr Virus (EBV) monitoring by PCR was started on day +15 up to day+100.

Diagnosis and treatment of GVHD

The clinical diagnosis of acute GVHD was made according to standard criteria, and confirmed histologically by skin and/or rectal/colon biopsies. First and second line therapy of GVHD was provided according to Institutional protocols.

Statistical analysis

The NCSS 11 Data for Windows (Kaysville, UT, USA), was used for contingency tables, rank sum test, cumulative incidence (CI) rates, and actuarial survival. When calculating the CI of transplant-related mortality (TRM), the competing risk was relapse, and viceversa; when calculating the CI of engraftment or GVDH, the competing risk was death due to any cause. The log rank test was used for differences between survival curves; the Grays' test was used to assess differences between cumulative incidence curves. Multivariate Cox analyses on engraftment, GvHD, TRM, relapse, and survival were run with the following variables: nucleated cell dose, disease phase, donor, and patient age, TBI or busulfan based conditioning, number of nucleated cells infused, interval between diagnosis and transplant, and transplant Center (Genova vs others). The number of patients who died of leukemia relapse in this study, was compared to the number of leukemic deaths in a recent analysis of the Center for International Blood and Marrow Transplant Registry (CIBMTR) **(10)**: contingency table analyses were used to test for statistical significance**.**

RESULTS

Engraftment

The median number of nucleated bone marrow cells infused was 3.1 × 10^8/kg (range 0.8-6.7); the 25 and 75 percentile were 2.3 and 4.1x10^8/kg. Eight patients died before day +20 and could not be evaluated; two were in CR1, two in CR2 and 4 in advanced disease at transplant. Six patients failed to achieve durable engraftment, one of whom could be rescued with a second transplant from the same donor: the overall CI of neutrophil engraftment at 80 days was 92% **(Table 2)**: it was 90%, 92%, and 95% for patients receiving <2.3 -4.1 and >4.1x10^8/kg nucleated marrow cells (p=0.01) **(Fig.1)**. The median day of neutrophil engraftment was day +21 (range 15-36), vs day +18 (range 13-56) (p=0.004), vs day +17 (range 13-31) (p=0.0007), for the 3 groups respectively. In a multivariate Cox analysis, factors predicting neutrophil engraftment were the nucleated cell dose infused, entered as a continuous variable, with a RR of 1.37 (p=0.001) and advanced disease, with a lower probability of neutrophil engraftment (RR 0.6, p=0.03) **(Table 3).** There was no effect of ABO matching. The CI of platelet engraftment was 87%: it was 85%, 88%, 89% for patients receiving <2.3 -4.1 and >4.1x10^8/kg nucleated marrow cells (p=0.03) **(Fig.1)**. The median day of platelet engraftment was day +27 (range 13-180), for patients receiving $\leq$ 2.3x10^8 cells/kg vs day +24 (range 9-90) for patients receiving 2.4-4.0x10^8 cells/kg (p=0.057), vs day +21 (range 13-31) (p=0.01), for patients receiving $\geq$4.1x10^8 cells/kg **(Fig.1)**.

Graft versus host disease

Acute GvHD grade I, II, III, IV was diagnosed respectively in 56, 19, 5, and 1 patient: the CI of acute GVHD grade II-IV was 17%**,** and 5% for grades III-IV **(Table 2)**. In a multivariate analysis, patients with advanced disease had a lower probability of grade II-IV acute GvHD (p=0.01), with no other significant predictor, including donor and patients age. There was no effect of cell dose on acute GvHD: patient receiving the highest cell dose had 0% rate of grade III-IV GvHD. Chronic GVHD was classified as minimal, moderate or severe, respectively in 47, 14 and 6 patients: The CI of moderate-severe chronic GVHD was 15% **(Table 2)**, and there were no significant predictors in multivariate analysis, including cell dose: older donors (> 36 years) were associated with a higher risk of cGvHD (RR 1.79), but this did not reach

statistical significance (p=0.19). Sixty five patients were available for examination at 1 year: 41 (63%) were off immunosuppressive treatment.

Transplant related mortality and relapse

At a median follow up of 903 days (range 120-1955), 89 patients are alive, 29 died of TRM and 32 died of recurrent disease. The overall cumulative incidence of transplant related mortality (TRM) at 100 days was 9%, at 1 year 15% and at 4 years it was 20% **(Table 2)**; it was 14% and 18% respectively in patients in CR1 and CR2 and 30% in patients with active disease (p=0.05) **(Fig.2)**. In multivariate analysis advanced disease (RR 3.2, p=0.03) was the only negative predictor of TRM.

Thirty five patients relapsed, and 32 patients died of recurrent leukemia: the 4 year CI of relapse was 24% **(Table 2)**; it was 13% for remission patients (14% and 10% for CR1 and CR2) and 47% for patients with active disease at transplant (p= 0.00006) **(Fig. 3):** in multivariate analysis advanced disease was the strongest negative predictor of relapse (RR=11.3, p<0.0001)**(Table 3)**; there was a trend for a lower relapse in patients with a longer interval diagnosis to transplant. Sixty two patients had WT1 levels before transplant: relapse was seen in 3/52 with WT1 copies <100/10^4 ABL vs 2 relapses out of 10 patients with WT1 levels >100 copies/10^4 ABL (p=0.1). The number of events is very small and limits the potency of the test.

Survival

The overall actuarial 4 year survival is 57% **(Table 2):** it is 74%, 70% and 26% for patients grafted in CR1, CR2 or with active disease **(Fig.4)** (p<0.0001). When comparing patients grafted in Genova (n=93) or in other Centers (n=57), 4 year survival for remission patients was 73% vs 72%, and for patients with active leukemia it was 23% vs 30%, respectively. **Figure 5** outlines actuarial survival of patients stratified by age (≤/> 60 years) and remission status: 74% and 67% for younger and older remission patients; 41% and 17% for older and younger advanced patients. The 4yr probability of survival free of GvHD and relapse (GRFS), for remission patients was 55% **(Table 2)**. In a multivariate analysis, advanced disease was the only significant negative predictor of survival (**Table 3).** There was no effect of the conditioning regimen (TBI based vs busulfan based), despite a significant difference in age (37 vs 55 years respectively), with comparable survival, both for remission patients (69% vs 75%, p=0.7) and relapsed patients (25% vs 27%, p=0.5).

The causes of death were as follows: relapse (n=32), infections (n=14), graft failure (n=5), multiorgan failure (n=2), acute GvHD (n=1), chronic GvHD (n=2), second tumour (n=1), and interstitial pneumonia (n=4). The proportion of different causes of death was not different (p=0·8) in patients receiving TBI or TBF, although graft failure was reported in 0/27 and 5/123 patients respectively (0% vs 4%).

DISCUSSION

We have shown in the present study that the Baltimore protocol of high dose PT-CY for HAPLO bone marrow transplants can be modified and still retain a favourable profile, in terms of GvHD protection and effective GvL. The results can be summarized as follows: high rate of engraftment, low incidence of GvHD and transplant mortality, very low relapse in remission patients, encouraging survival, including patients over the age of 60, no Center effect.

As to the first point, all patients received unmanipulated marrow, and the cumulative incidence of engraftment was 92%: in 5 patients (3.3%) rejection was reported as the primary cause of death, and donor specific antibodies (DSA) were detected in 3 of them. We found a strong effect of cell dose on time to engraftment, but not on the risk of rejection: the median cell dose, for 6 patients rejecting, was 3.4x10^8/kg, compared to 3.0x10^8/kg for patients engrafting. In keeping with this observation, a recent study on unmanipulated HAPLO transplants shows a comparable rate of engrafment for patients receiving marrow (92%) or peripheral blood grafts (95%) **(11)**. The issue of graft rejection in haploidentical stem cell transplantation has been recently reviewed **(12)**, and does not seem to be linked to the stem cell source, but rather to the presence of DSA. In order to prevent graft rejection it is important to test for DSA, in order to select for the best possible donor.

As to the second point we have seen a low incidence of GvHD, despite the use of cyclosporine before PT-CY. Perhaps the use of marrow for all patients, may have played a role, since higher rates of GvHD grades III-IV, have been reported with the use of peripheral blood **(11)**. The standard PT-CY regimen (day +3+4), following a non myeloablative regimen produces a risk of GVHD grade II-IV and III-IV of 34% and 6% **(1)**, which is quite similar to what we have seen in our series of patients with PT-Cy given on days +3+5, but following a myeloablative conditioning regimen. Our low GvHD rates were associated with relatively low rates of TRM, both in young patents, as well as in patients over the age of 60 years: the overall TRM was 18% and 23% in patients less than or over 60 years (p=0·3); for remission patients these figures are 13% and 18%, suggesting that also older AML patients can be scheduled for a HAPLO graft with a relatively low toxicity profile. Overall TRM is reported to be low, also in other studies, both with MA or NMA regimens, with

figures ranging from 3% **(13)** to 21% **(14)**. We not have data on the use of peripheral blood as a stem cell source with this platform: it may be that the early use of CsA, before PT-CY will expose patients to a high risk of acute and chronic GvHD: therefore these results cannot be extrapolated to unmanipulated HAPLO peripheral blood.

Relapse remains one of the key problems of allogeneic transplantation for leukemia, and has remained unchanged over the past decades **(15)**: in this study we have seen a very low rate of relapse in remission patients (13%), perhaps lower when compared to other series of patients, and the use of PT-CY following cyclosporine administration, may have played a role. In a study of the Center for International Blood and Marrow Transplantation Research (CIBMTR), on 104 myeloablative HAPLO grafts in AML with PT-CY on days+3 and +4 **(10)**, the proportion of patients in first remission was 46%, 20% were in second remission, and the median follow up was 900 days: 41 patients (39%) died of leukemia relapse. In the present study on 150 AML patients, 45% were in first and 21% in second remission, and the median follow up was identical (900 days): 32 patients died of leukemia relapse (21%), which is significantly lower when compared to the CIBMTR study (Fisher- p=0.002). There may be a role for the myeloablative conditioning regimen, given in our study, since the CIBMTR included also RIC regimens. Nevertheless, relapse remains high for patients with advanced disease, 47% in our series, and new strategies are needed to prevent leukemia relapse. A pre-emptive approach with cellular therapy **(16),** or with azacytidine **(17),** is one option, but requires close monitoring of a marker for minimal residual disease (MRD)**.** Post-transplant prophylaxis of relapse is another possibility, as shown in a German cooperative study with panobinostat **(18),** or again with azacytidine **(19-22).** Salvage strategies may need to be personalized in HAPLO graft recipients, since relapse can occur with loss of the HLA mismatched haplotype **(23).**

Older age has long been a contraindication for allogeneic transplantation, especially from alternative donors. A recent report has shown encouraging outcome for patient above the age of 55 years, with both acute and chronic hematologic malignancies **(24),** receiving HAPLO grafts, mainly with peripheral blood as a stem cell source, and the original PT-CY protocol day+3+4. We were thus interested in looking at the outcome of our AML patients over the age of 60: for remission AML, survival in older patients was comparable to younger patients (67% vs 74%

respectively). For advanced AML, there was a trend for a reduced risk of relapse in the older group, leading to improved survival (41% vs 17%), despite an equal distribution of high ELN risk: among 20 advanced patients over the age of 60, there were 5 relapses (25%) compared to 17/31 in younger patients (51%) (p=0.03).
Finally all major outcomes were comparable for patients grafted in Genova or elsewhere, and there was no Center effect in a multivariate Cox model.

In conclusion, the original Baltimore PT-CY regimen can be modified with CsA administered before PT-CY, in AML patients undergoing an unmanipulated HAPLO marrow graft, following a myeloablative conditioning regimen. In a multicenter setting we have seen a low incidence of GvHD and TRM, and relapse has been lower than in other series. These results were obtained with marrow as a stem cell source , and may not translate automatically to unmanipulated peripheral blood. It could be speculated that CsA given before PT-CY has prevented some allo-reactive T cells from PT-CY purging, thus increasing the graft versus leukemia effect. This hypothesis is being tested in an EBMT registry based study comparing CsA following PT-CY, given on days +3+4 with CsA preceeding PT-CY, given on days +3+5.

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Legend for Figures

Fig.1. Cumulative incidence of neutrophil and platelet engraftment, stratified according to the nucleated BM cells at transplant (<2.3 (A), 2.3-4.1 (B) , >4.1 (C) x10^8/kg). A strong effect of the cell dose on time to engraftment is shown .

Fig.2. Cumulative incidence of transplant related mortality, in patients stratified according to disease phase: first remission (CR1), second remission (CR2), or active disease.

Fig.3. Cumulative incidence of leukemia relapse, in patients stratified according to disease phase.

Fig.4. Overall survival in patients stratified according to disease phase.

Fig.5. Overall survival in patients stratified according to remission status and age: a strong effect of disease phase can be seen, for both young and older patients (>60 years). Of note is the comparable survival of remission AML patients aged </> 60 years.

Table 1. Clinical characteristics of patients

Number		150
Patients' age in years: median(range)		51 (17-74)
Patients over 60 years: n(%)		42 (28%)
Patients' gender: males/females		76/74
Donors' age in years :median(range)		36 (17-67)
ABO matched		80 (56%)
Disease phase		
CR1		68 (45%)
CR2		31 (21%)
Active disease at transplant		51 (34%)
ELN risk group	low	4 (3%)
	Intermediate	51 (34%)
	High	95 (63%)
Flt3 positive		34 (22%)
Interval diagnosis Transplant		201 (48-3004)
Conditioning regimen		
FLU-TBI		27 (19%)
TBF (BU3)		80 (53%)
TBF (BU2)		43 (28%)
Median nucl.cellsx10^8/kg		3.1 (0.8- 6.7)
Median Follow up in days :median(range)		903 (120-1955)

Abbreviations: CsA =cyclosporine; MMF= mycophenolate; PT-CY post-transplant cyclophosphamide; TBF= thiotepa busulfan, fludarabine ;BU3= busulfan 3.2 mg/kg/dayx3; BU2= busulfan 3.2 mg/kg/dayx2; FLU= fludarabine; TBI= total body irradiation; ELN= European Leukemia Network; CR= complete remission; Flt3= fms like tyrosine kinase3.

Table 2 Main outcomes

			95% Confidence Interval
Proportion engrafted	CI	92%*	88% - 98%
Acute GvHD grade II-IV	CI	17%*	12% - 24%
Acute GvHD grade III-IV	CI	5%*	2% -10%
Chronic GvHD moderate/ severe	CI	15%**	10% - 22%
Transplant related mortality	CI	20%**	14% - 28%
Relapse	CI	24%**	18% - 33%
Actuarial survival	KM	57%**	49% - 66%
Actuarial survival for remission patients	KM	73%**	63% - 82%
Disease free survival	KM	52%**	43% - 52%
Disease free survival for remission patients	KM	67%**	55% - 79%
Survival free of GvHD and relapse (GRFS)	KM	45%**	36% - 55%
GRFS for remission patients	KM	55%**	42% - 68%

Abbreviations= CI: cumulative incidence; KM: Kaplan Meier survival curves

*at 100 days; **at 4 years

Table 3. Multivariate Cox analysis on outcome

Variable	Baseline value	Compared value	PMN RR	PMN P	TRM RR	TRM P	Relapse RR	Relapse P	OS RR	OS P
Phase	CR1	CR2	0.8	0.5	1.9	0.3	1.5	0.5	1.8	0.2
		Advanced	**0.6**	**0.03**	**3.2**	**0.03**	**11.3**	**0.000**	**9.0**	**0.000**
Don Age	< 35	≥35 years	0.8	0.4	0.6	0.3	0.6	0.2	0.6	0.1
Rec Age	< 60	≥60 years	0.7	0.5	2.0	0.1	0.4	0.23	0.6	0.3
Condit.	TBI	TBF-BU3	0.7	0.3	2.4	0.2	0.5	0.3	0.8	0.8
		TBF-BU2	0.8	0.5	1.2	0.8	0.8	0.7	0.7	0.7
Cell dose		**continuous**	**1.37**	**0.001**	0.8	0.2	0.9	0.6	0.8	0.1
In DxTx		continuous	1.0	0.3	0.9	0.4	0.9	0.08	0.9	0.07
Center	Genova	other	0.8	0.5	0.9	0.8	0.5	0.1	0.7	0.3

Abbreviation: RR = risk ratio; PMN= neutrophils; TRM= transplant related mortality; OS= overall survival; DxTX= interval diagnosis transplant; other= see table 2.

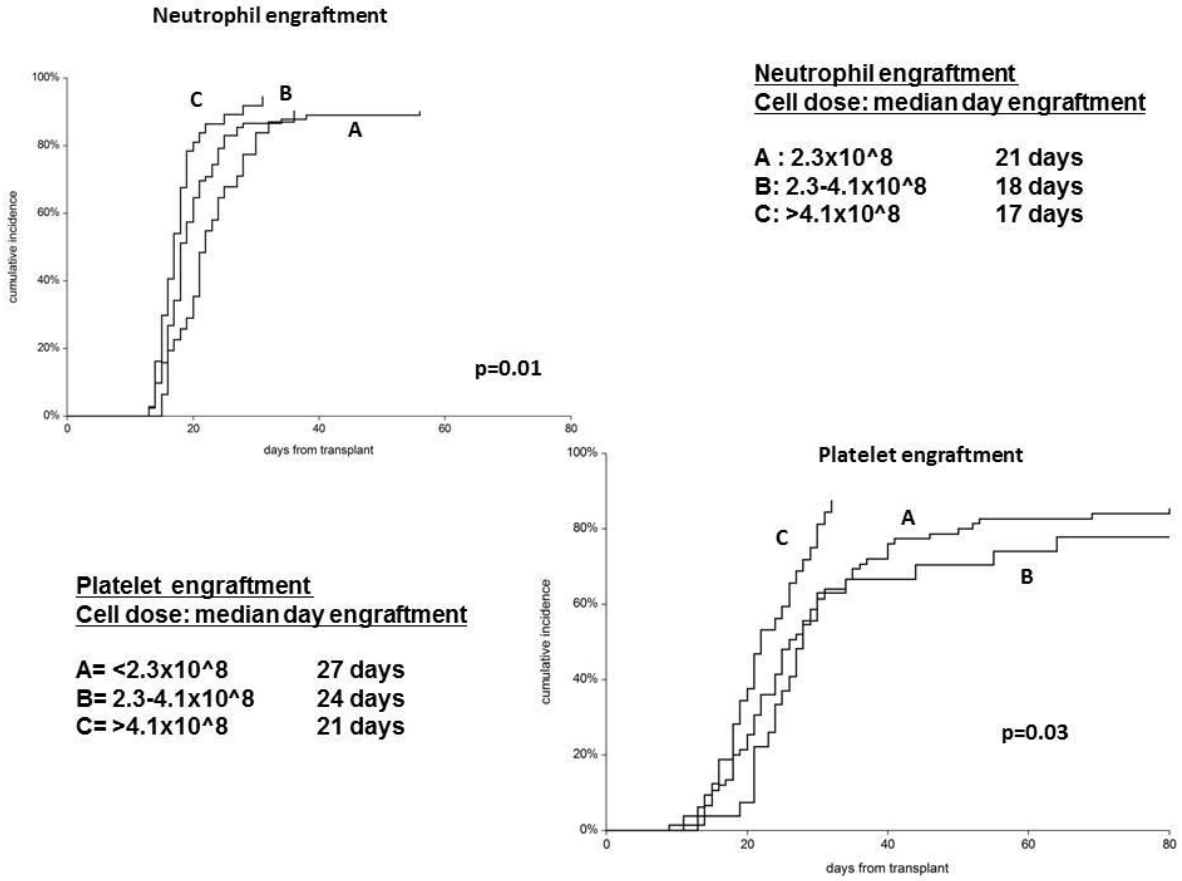

Figure 1.jpg

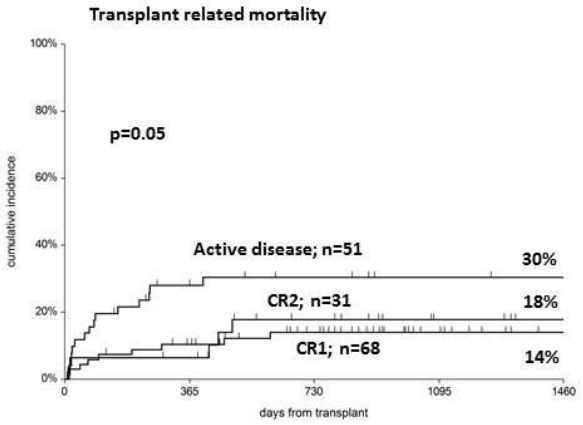

Figure 2.jpg

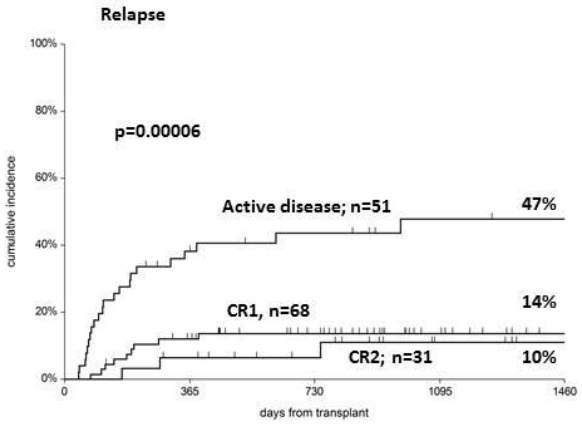

Figure 3.jpg

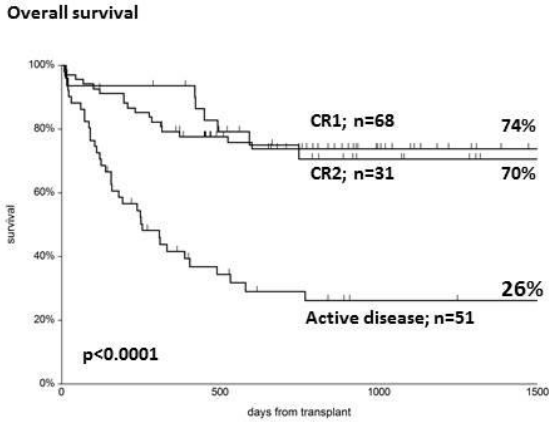

Figure 4.jpg

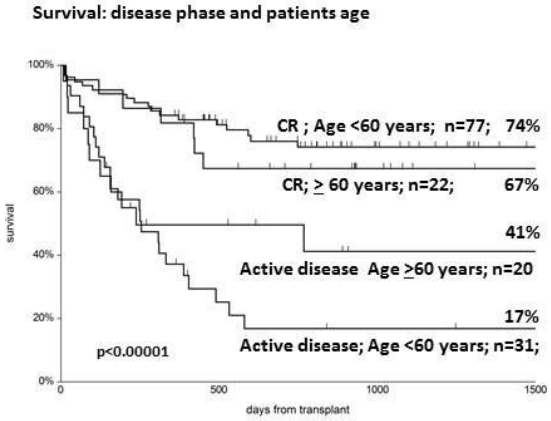

Figure 5.jpg