# Aldehyde Dehydrogenase Activity may Replace Colony Forming Units as A Quality Marker in Hematopoietic Peripheral Stem Cells Collections

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## Abstract

### Background and Objectives

In daily routine stem cell harvests for stem cell transplantation are measured by CD34 antigen and the colony-forming ability (CFU) and checked for viability by 7-amino actinomycin D (7-AAD). We tested whether the stem cell marker ALDH could be useful for characterizing PBSC graft quality

### Materials and Methods

In a prospective analysis we investigated the ALDH activity in freshly collected PBSC and in frozen products (n = 42) of 25 patients (15 males/10 females, median age 52 yrs., range 21-67). The results were correlated with CD34, viability and engraftment data in already transplanted patients (n = 7).

### Results

In general we found a significant correlation between ALDH activity, CD34 yield and CFU-GM/unit in fresh and thawed products with a p value of < 0.05. A low ALDH activity was predictive for poor stem cell function, expressed as colony forming capacity of collected stem cells.

### Conclusion

The ALDH assay is readily available and provides information about the quality of the majority of leukocyte-apheresis products instantly which may be of special interest for scheduled transplants with long-term stored PBSC.

Keywords: Aldehyde dehydrogenase; Hematopoietic stem cell- mobilization; CD34; Colony forming unit; Quality

## Introduction

Transplantation of autologous hematopoietic stem cells (HSC) has gained widespread application for the treatment of hematological malignancies, solid tumours, and autoimmune diseases [1-3]. In the autologous setting the HSC harvests are cryopreserved sometimes up to several years before transplantation. Although it was shown previously that after autologous transplantation of long-term cryopreserved HSC (up to 5 years) hematological reconstitution was not impaired [4], extended storage time bears the risk of deterioration of the stem cells [5]. Thus some functional and viability testing prior to transplantation are recommended [5]. The number of HSC is usually measured by the surface marker CD34 and checked for viability by 7-amino actinomycin D (7-AAD) [6]. The viability of these cells alone gives no information about colony forming capacity of stem cells which is important for engraftment. Therefore, to verify sufficient functional capacity of harvested HSC colony forming units are also performed (CFU) [7-8]. But results of CFU's cannot be seen until after an average of 14 days and may vary depending on culture conditions. At this time the patient may have already been transplanted and exposed to several risks of complications like bleeding, infection, and toxicity. Recently, a new marker aldehyde dehydrogenase, has been described as stem cell marker that correlates well with stem cell functions [9-14]. Aldehyde dehydrogenase is a polymorphic enzyme responsible for the oxidation of aldehydes to carboxylic acids, which leave the liver and are metabolized

by the body's muscle and heart [15]. An association with stem cells has been described [9,16-20]. This enzyme has been described to be involved in the metabolization of several cytostatic drugs including cyclophosphamide and thereby may be associated with chemoresistance [11,21]. In the setting of allogeneic peripheral blood stem cell transplantation there is already evidence that aldehyde dehydrogenase activity serves as a marker for the quality of hematopoietic stem cell transplants. ALDH correlated with CFU-GM and full hematopoietic donor cell chimerism [14,22].

Measuring the ALDH content of the harvested cells is done by FACS analysis which is performed concomitantly to the CD34 and viability determination. The results may be helpful in the pre and post transplant management of the patients. To this end we prospectively analysed ALDH activity in freshly collected PBSC and in frozen products from autologous patients. The results were correlated with CD34, viability and engraftment data in already transplanted patients. Here we attempted to show that measuring of ALDH activity in stem cell collections may replace the extensive and time consuming CFU assay especially in frozen products.

## Patients and Methods

The study was approved by the local ethics committee and all patients signed written informed consent.

## Patients

Between January and August 2009 42 stem cell collections from 25 patients (15 males/10 females, median age 52 yrs., range 21-67) were performed. In 34 harvests COBE®/Spectra blood cell separator (TerumoBCT, Lakewood, CO, USA) and in 8 the AMICUS™ (Fresenius/Fenwal, Europe, Zaventem, Belgium) blood cell separator was used. The leading diagnosis was NHL (n=12) followed by MM (n=7), Morbus Hodgkin (n=4), acute lymphoblastic leukemia (n=1), and germ cell tumour (n=1). The percentage of CD34+ cells in the peripheral blood at start was in median 0.14% (0.02 – 5.77). Patient characteristics are given in Table 1.

In the autologous setting, patients underwent a conditioning regimen consisting of chemotherapy and G-CSF. The specific chemotherapy regimen was based on the diagnosis. The following regimens were used, B-COPP, mini-BEAM and R-ICE. From day 4 after G-CSF administration the peripheral blood count and the CD34+ percentage were determined daily. Apheresis was started from a median leukocyte count of 30.000/µL (range 4000-80.000) and a median CD34+ count of 36 cells/µL (range 5-751) (Table 1).

Table 1: Patient characteristics

| Mobilization groups | Poor mobilizers n = 7 | adequate mobilizer n = 18 | All patients n = 25 |
|---|---|---|---|
| Male/female | 3/4 | 12/6 | 15/10 |
| Age (yrs) | 55 (21 - 66) | 50 (21 – 67) | 52 (21-67) |
| **Underlying disease** | | | |
| MM/M.Hodgkin/NHL/ALL/n.testis | 2/2/2/1/0 | 5/2/10/0/1 | 7/4/12/1/1 |
| **Prevalues** | | | |
| WBC x 10^9/L | 28 (15 – 45) | 31 (4 – 80) | 30 (4-80) |
| CD34% | 0.05 (0.02-0.09) | 0.20 (0.05 – 5.77) | 0.14 (0.02-5.77) |
| CD34a/µL | 15 (5 – 18) | 76 (20 – 751) | 36 (5-751) |

MM = multiple Myeloma, M. Hodgkin = Morbus Hodgkin, NHL = non Hodgkin lymphoma, ALL = acute lymphatic leukemia, n.testis = neoplasma testis
WBC = white blood cell count, CD34a/µL = absolute count of CD34+ cells/µL

All values are given in median and (range)

## Stem Cell Collection

In majority of patients (n=12) only one harvest procedure was necessary to collect the required amount of stem cells for transplantation. In 9 patients 2 and in 4 patients 3 procedures were needed. The mean duration of a stem cell collection session was 273 (±36) minutes with a mean use of 1141ml (±301) ACD-A (Fresenius, Eugendorf, Austria) processing a mean of 14448ml (± 4696) blood. In 14 patients the apheresis procedure was performed via peripheral venous access and in 11 patients a central venous catheter (CVC) had to be placed. Except in MM patients, where a mixture of ACD-A and Heparin was used for anticoagulation, all other procedures were anticoagulated with ACD-A solely. All patients received calcium gluconate 10% (B. Braun, Melsungen AG, Germany) continuously (3.92 g absolute) to avoid citrate reactions. The vital parameters of all patients were monitored during the procedure. The collection and product characteristics in freshly collected and thawed products are detailed in Table 2.

Of all 25 patients 7 underwent high-dose chemotherapy followed by autologous peripheral blood stem cell transplantation. Grafts were cryopreserved and thawed immediately before transplantation. Aliquots were stored under the same conditions and measured shortly before transplantation. Autologous transplantations took place after a median of 139 days after the stem cell harvest. The outcome of the patients is displayed in the results section.

Table 2: collection and product characteristics in freshly collected and thawed products

| Collection groups | Group 1 n = 14 low mobilize | Group 2 n = 17 adaquate mobilize | Group 3 n = 11 super mobilize |
|---|---|---|---|
| **Cell separator** | | | |
| Amicus/Comtec/CobeSpectra | 2/1/11 | 4/1/12 | 2/0/9 |
| **Collection** | | | |
| PBV (L) | 16 (11 – 20) | 14 (7.4 – 20) | 14.3 (5.6 – 23) |
| times of TBV | 3.0 (1.9 -3.7) | 3.1 (2.0 – 4.2) | 2.7 (1.3 – 3.4) |
| **Products fresh** | | | |
| Vol / mL | 121 (87 – 324) | 215 (81 – 328) | 189 (63 – 333) |
| CD34% | 0.41 (0.11 – 0.78) | 0.57 (0.19 – 2.0) | 2.9 (0.8 – 7.63) |
| CD34a | 99 (3 – 251) | 261 (135 – 386) | 812 (483 – 2003) |
| CD34 x 10^6/kg BW | 1.19 (0.03 – 1.97) | 3.41 (2.09 – 4.49) | 10.9 (5.42 – 18.2) |
| ALDH activity % | 0.21 (0.15 – 0.76) | 0.59 (0.15- 1.33) | 3.07 (0.3 – 8.89) |
| CFU (10^5 / mL) | 0.9 (0.19 – 5.9) | 1.2 (0.0 – 7.02) | 2.3 (0.56 – 8.9) |
| CFUa (10^5) | 157 (18 – 516) | 245 (0.0 – 828) | 300 (185 – 1273) |
| **Products thawed** | | | |
| Vol / mL | 170 (100-300) | 200 (120-800) | 255 (110 – 300) |
| CD34a | 89.7 (16.1-163) | 157 (24-263) | 836 (51 - 1460) |
| CD34 x 10^6/kg BW | 0.99 (0.15-2.33) | 2.2 (0.24-3.06) | 10.08 (0.36 – 15.5) |
| ALDH activity % | 0.33 (0.12-0.82) | 0.58 (0.08-2.23) | 2.45 (0.16 – 4.8) |
| CFU (10^5 / mL) | 0.13 (0.07 – 0.43) | 0.74 (0.01 – 4.42) | 1.15 (0.89 – 5.79) |
| CFUa (10^5) | 28 (12.4-77.3) | 103 (5.23 – 662) | 330 (203 – 1129) |

PBV = processed blood volume, TBV = total blood volume, vol = volume, CD34a = abslute CD3 count, BW = body weight, ALDH = aldeydlactatdehydrogenase, CFU = colony forming units, CFUa = absolute count of CFU in the products  0.0 = below detection limit
All values are given in median and (range)

## Laboratory Analysis

Samples were taken before stem cell collection from the peripheral blood for the measurement of WBC counts and CD34 pre-values and from the whole grafts for determination of cell counts, CD34 percentage, viability, ALDH expression and colony forming units (CFU-GM) on the day of harvest (day 0), 1 week before the scheduled transplantation and after a maximum of 15 months in the not transplanted cases.

For this purpose aliquots were frozen under the same conditions as the grafts and thawed for measurements according to standard procedures.

Peripheral blood and differential counts as well as cell counts of the harvests were assessed by a Cell Dyn 3500® (Abbott Diagnostics, South Pasadena, CA). WBC differential counts from the products were done from blood smears stained according to a Wright-Giemsa stain (Sigma, St. Louis, MO, U.S.A.). The total number of collected MNCs was calculated from WBC, differential counts, and product volume.

## Expression of CD34 and Viability

CD34+ cells in the peripheral blood and in the products were measured according to the ISHAGE protocol with a dual laser FACSCalibur (Becton Dickinson, San Jose, CA, USA) using the CellQuest software [7,23-24]. In brief, WBC were stained after a lyse and wash procedure by double colour staining (anti-CD45 (FITC)/anti-CD34 (PE), Becton Dickinson, San Jose, CA, USA), were identified with forward and side scatter and the CD34 population was determined. CD34+ cell frequencies were converted into counts/L by multiplying the percent of stained CD34/CD45+ cells by the peripheral blood leukocyte count/L and into absolute counts by multiplying the CD34/CD45+ percentage by the absolute WBC count of the products.

In the same analysis cell viability with 7-amino actinomycin D (7-AAD) dye (BD Pharmigen™, BD Biosciences, Europe), which binds to DNA of apoptotic or necrotic cells, was determined [25].

## ALDH Expression

ALDH expression was measured using Aldeflour® Kit from Aldagen® (Aldagen Inc., USA). The processing of the probes was done according to the manufacturers recommendations. FACS analysis was performed with a dual laser FACSCalibur (Becton Dickinson, San Jose, CA, USA) using the CellQuest software [7,9]. In brief, Aldefluor substrate (0.625 μg/mL) was added to 1 to $5 \times 10^6$ cells/mL suspended in Aldefluor assay buffer and incubated for 20 to 30 minutes at 37°C to allow the conversion of Aldefluor substrate, a green fluorescent product retained within the cell due to its negative charge. For each experiment, an aliquot of Aldefluor-stained cells was immediately quenched with 5 μL 1.5-mM diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor, to serve as a negative control.

## Granulocyte–Macrophage Colony-Forming Units (CFU-GM)

In brief, $1 \times 10^5$ cells were seeded in 1 ml methyl cellulose (MC) supplemented with growth factors (IL3, EPO, GM-CSF) to define numbers of CFU-GM in the analyzed samples. CFU-GM were counted after an incubation of 14 days in a 5% $CO_2$ incubator at 37°C.

## Statistics

Results are expressed as mean ± standard deviation (SD) or as median and range for descriptive purposes in the text. Statistical analyses of matched pairs (freshly collected and frozen products) were performed by Wilcoxon signed - rank test. For correlation testing the Spearman's rank correlation coefficient was calculated.

Patients were classified as poor (<20 CD34+/L) and adequate mobilizers (≥20 CD34+/L) according their pre-values [26]. For comparison between these two groups the Man-Whitney U Test was used.

The products were assigned to three groups according the collection yield (< 2, 2 -5 and > 5 x106 CD34+/kgBW) [27] .

For comparisons between the 3 groups the Kruskal Wallis Test and one way analysis of variance was applied. A p-value < 0.05 was considered significant.
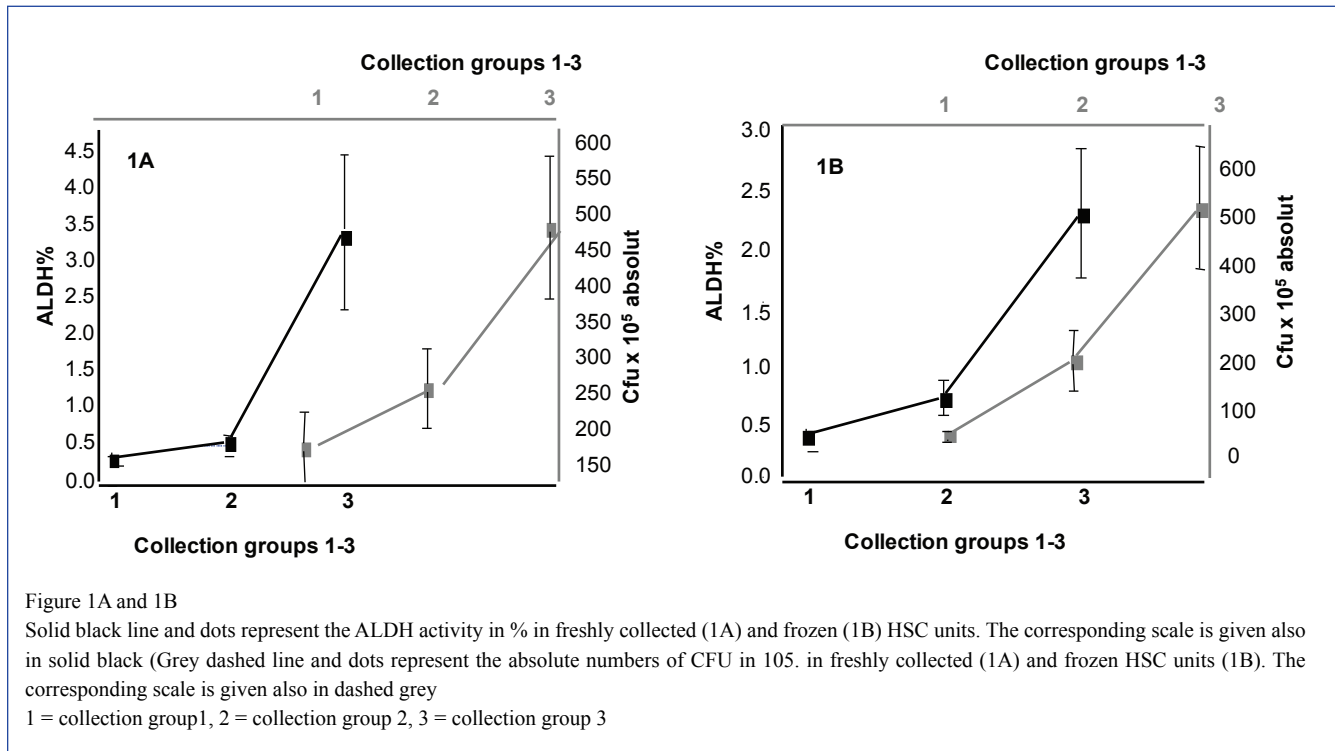
## Results

### Freshly Collected HSCs

The overall ALDH activity in the products showed a significant correlation with CD34 pre-values (R 0.65 p < 0.05), CD34 yield (R 0.86, p < 0.05) and CFUs in the harvests (R 0.47, p = 0.005) (Figure 1A). In freshly collected products overall viability of CD34+ cells was in median 99% (range, 76-100). No correlation between ALDH activity and viability was seen (R 0.2, p = 0.14). In a second step patients were assigned to two groups according to their CD34 pre-values. Patients whose circulating CD34+ cells were < 20 x 106/L after an adequate mobilization regimen were defined as "poor mobilizers" (7/25 pts) [26]. Interestingly in this cohort of patients no correlation between CD34 pre-values and ALDH activity in the products was measurable, but both, ALDH activity and the amount of CFUs were significantly lower than in the products of adequate mobilizers (p = 0.02 and 0.004).

### Cryopreserved and Thawed HSCs

In thawed products a CD34% recovery of in median 84% (range, 6.5-132) and a CFU recovery of in median 40% (range, 3-91) was measured. Fifty-four% (range 5-86) of baseline ALDH activity was detectable in thawed products. Viability after thawing ranged between 35% and 100% (median 77%). Again a positive correlation was found between ALDH activity in the thawed products and the number of CFUs (R 0.73, p < 0.05) and CD34+ cells (R 0.89, 0 < 0.05) as well as between baseline ALDH activity and CFU- and CD34 recovery (R 0.61, p = 0.007, R 0.43, p = 0.04) (Figure 1B). In one collection of one patient CFUs from the freshly collected product was lower than expected with regard to the CD34 yield and ALDH activity. The patient was diagnosed with Hodgkin's lymphoma and assigned to "adequate mobilizer" and "collection group 2". CFU capacity was below the detection limit in the fresh product and about 6 x 105 absolute after thawing.

To enable a more detailed description of the harvest results the collections were assigned to three groups according their CD34 yield/run as described by Oszan in 2011: < 2.0 x10^6/kg BW = poor mobilizer, 2.0 - 5x10^6/kg BW = adequate mobilizer and > 5x10^6/kg BW = super mobilizer [27]. (Table 2)

Figure 1A and 1B

Solid black line and dots represent the ALDH activity in % in freshly collected (1A) and frozen (1B) HSC units. The corresponding scale is given also in solid black (Grey dashed line and dots represent the absolute numbers of CFU in 105. in freshly collected (1A) and frozen HSC units (1B). The corresponding scale is given also in dashed grey

1 = collection group1, 2 = collection group 2, 3 = collection group 3

## Collection Group 1 (< 2x10^6/kg BW), Poor Mobilizer

In general CD34 pre-values predicted the collection success (R² 0.83). In 14 collections of 7 pts the minimum of 2x10^6/kg BW was not reached. CD34% in these products was in median 0.41% (range, 0.11-0.78) As expected for this group, in the freshly collected leukocyte-apheresis products the lowest ALDH activity with a median of 0.32% (range, 0.15 - 0.76%) was measured. Also the CFU counts were the lowest in this group with a median of 0.9 x $10^5$/ml (range, 0.19 – 5.93 x 105/ml). The median recoveries after thawing for CD34, CFU, and ALDH were 84% (53-132), 31% (8-41) und 53% (44-54) of baseline values, respectively.

## Collection Group 2 (2 - 5x10^6/kg BW), Adequate Mobilizer

This group covers 17 products of 12 patients. CD34% in products was in median 0.57% (range, 0.19 - 2%), ALDH% was in median 0.59% (range, 0.15-1.33) and CFU counts were in median of 1.2 x $10^5$/ml (range, 0.3 - 7.0 x $10^5$/ml). The median recoveries for CD34, CFU, and ALDH after thawing were 73% (6.5-97), 45% (3-91) and 55% (5-86) of baseline values, respectively.

## Collection Group 3 (> 5x10^6/kg BW), Super Mobilizer

CD34% in these products was in median 2.9% (range, 0.8 – 7.6%) and correlated with ALDH% (median 3.07%, range 0.3 - 8.8) CFU counts were in median 2.3 x 105/ml (range, 0.56 - 8.9 x 105/ml). The median recoveries for CD34, CFU, and ALDH after thawing were 86% (6.7 - 126), 76% (33 - 100) and 82% (78-86) of baseline values, respectively. In group 3 the recoveries of CFU and CD34+ cells were significant higher than in groups 1 and 2.

## Outcome of Transplantation

Meanwhile 7 out of the 25 donors have been transplanted with their autologous harvests. The harvests have been cryopreserved between 32 and 264 days and were thawed right before transplantation. Seven patients received in median 5.34 CD34+ cells (range, 4.05 – 16.56) harvested in 9 collections (2 pts had 2 collections), with an ALDH activity of median 1.06% (range 0.18 – 4.8) and a median of 3.06 x105/kg (range 0.46 - 9.46) CFU. The average leukocyte engraftment occurred on day +11 (± 1 day), the platelet engraftment was day +10 on average (± 1 day). During a median hospital stay of 22 days (17-30) 2 RBC units (range, 0 -14) and 4 single donor platelet units (range, 0 -28) were administered. According to their pre-values all 7 patients were adequate mobilizers. Their harvests were classified as poor- (1 graft), adequate- (4 grafts) and super mobilizers (4 grafts). The transplanted cell dose was in median 5.34 x 106 CD34+ cells/kg BW (range 4.05-9.74), 4.17x105 CFU / kg BW (range 2.5–8) with a median percentage of ALDH 0.76% (range 0.64–4.88).

## Discussion

Hematological malignancies are often life threatening and fast progressing diseases. Therefore, sometimes high-dose chemotherapy with subsequent autologous peripheral blood stem cell transplantation is needed to control the disease. If transplantation is planned autologous peripheral blood stem cells have to be collected. Stem cell mobilization consists of conventional chemotherapy that is effective against the underlying disease combined with filgrastim. The stem-collection is done by leukocyte-apheresis. Mostly, only one stem-cell collection is needed to obtain a sufficient number of HSCs to allow engraftment. But the number of collections needed varies depending on age, gender, underlying disease, former therapies, and mobilization regimen ranging from 1-3 times.

Since the number of collected HSCs is critical for the success of transplantation every improvement of quality control is welcome. Currently, the number of collected HSCs is determined by measuring the CD34 surface antigen with a threshold level of at least 2x106/kg body weight and by assessing the CD34 viability. In addition, CFU assays serve as functional assay, but they are time consuming. Therefore this assay is done in the frequency of a quality control and not every graft is tested.

This study is aimed at evaluating aldehyde dehydrogenase as an additional marker in the setting of autologous peripheral blood stem cell transplantation. We looked out especially for a correlation of ALDH with the clinical outcome of transplantation and/or the CD34- and CFU assays. Our patient population as shown in Table 1 showed a big variation range of CD34+ cells in the blood at the beginning of the harvest procedure. This variation between patients is also reflected by all three parameters (CD34, CFU, aldehyde dehydrogenase) in the products. This is best explained by the disparity of the multiple factors listed above that influence the harvest of HSC in each patient. Consequently, ALDH results mirrored CD34 and CFU results in all fresh collections, except in one where ALDH activity did not predict the lack of CFUs. According to the patients harvest results this collection was assigned to collection group 2. After thawing the frozen product a low amount of CFUs (6 x 105 absolute) were detectable, which was within the range of group 2 (range 5.23 – 662 x 105, Table 2). An initial failure of the CFU assay cannot be excluded. Due to the variation of parameters the patients could be assigned to 3 collection groups according to their CD34 yield (see results, Table 2). Hereby, we found that a low ALDH expression correlated significantly with a low CFU and CD34 yield. Thus, being in group 1 was predictive for a low CFU content. Likewise the absolute CD34 yield and the recovery of all tested parameters in this group were the lowest of all groups. But given the low CFU content and recovery in group 1 a low ALDH expression may translate in poor engraftment. The other groups showed higher CD34 and CFU values as well as a better recovery of all tested parameters. Since we did not observe a significant difference of CFUs of fresh collections between group 2 and 3 our findings suggest the existence of a threshold value for ALDH expression for successful transplantation. The fact that all transplanted patients (group 2 and 3) showed an engraftment after a median of 14 days supports this observation. Unfortunately up to now no patient receiving grafts of mobilization group 1 solely was transplanted.

Regarding quality control of the leukocyte-apheresis products ALDH expression offers two advantages. First, the prediction of CFU and the recovery is provided instantly by the ALDH expression. This is an advantage over the CFU assay because it allows a better planning of a successful stem cell harvest. Second, in contrast to the outcome of the CFU assay measurement of ALDH by FACS is not depending on varying culture conditions. Third, in contrast to CD34 assays an additional viability assay becomes dispensable because ALDH can only be measured in living cells.

In summary, our study showed that in general a low ALDH value is predictive for poor stem cell function as confirmed by low CFUs and poor recovery of all parameters (CD34, ALDH, and CFU) after thawing of the stored stem cell product. The fact that the outcome data after transplantation do not differ between groups 2 and 3 supports the existence of a minimal ALDH threshold level. Measuring ALDH expression is a quick, cost effective, and easy method to get instantly information about the quality of the stem cell product. Our study provides evidence for the inclusion of ALDH assay in the quality control of autologous blood stem cell harvest either as additional assay or even as a substitute for CFU. The big advantage of this assay is the availability within one hour which may be of importance in the validation of thawed products pre-transplant and the viability testing can be omitted as ALDH activity is solely present in viable cells.

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