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CD34+ Viable Cells in Thawed Apheresis Samples May Be More Than Expected

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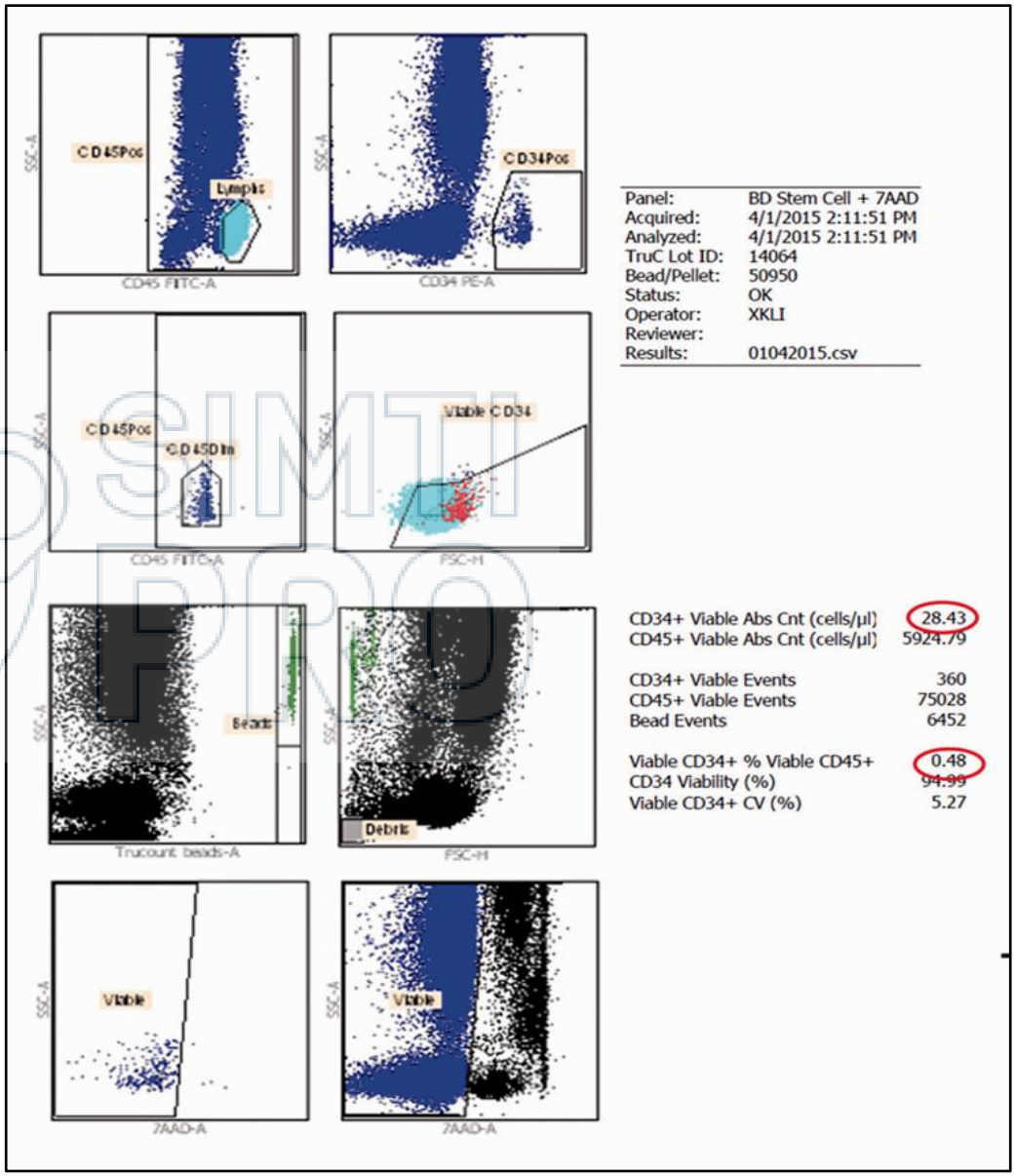
Il sottoscritto, in qualità di Relatore
dichiara che

nell'esercizio della Sua funzione e per l'evento in oggetto, NON È in alcun modo portatore di interessi commerciali propri o di terzi; e che gli eventuali rapporti avuti negli ultimi due anni con soggetti portatori di interessi commerciali non sono tali da permettere a tali soggetti di influenzare le sue funzioni al fine di trarne vantaggio.

INTRODUCTION

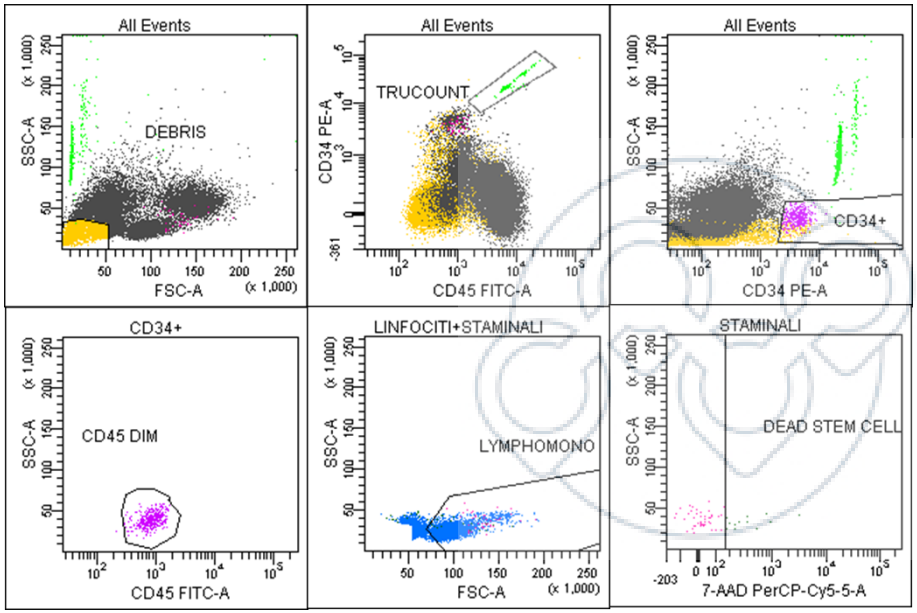
- The ISHAGE (International Society of Hematotherapy and Graft Engineering) assay, combined with the 7-aminoactinomicin D (7-AAD) viability stain is considered the flow cytometric (FC) standard technique for quantifying CD34+ viable cells in fresh apheresis units.

- The recovery of viable CD34+ cells assessed in the thawed products is frequently lower than 70-80%, but it is difficult to estimate it precisely, due to a number of technical issues.



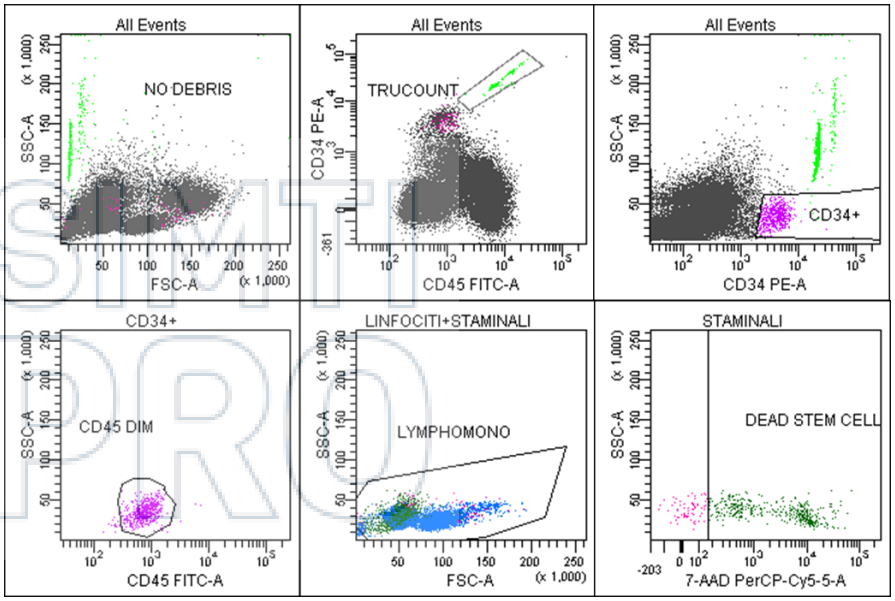
Modifications to the ISHAGE methodology (MI) were also proposed by several authors with the aim of improving the assessment of CD34+ cells viability in thawed samples.

ISHAGE METHOD



CD34+ cells: 260/ µl
Percentage of viable CD34 + cells: 72%

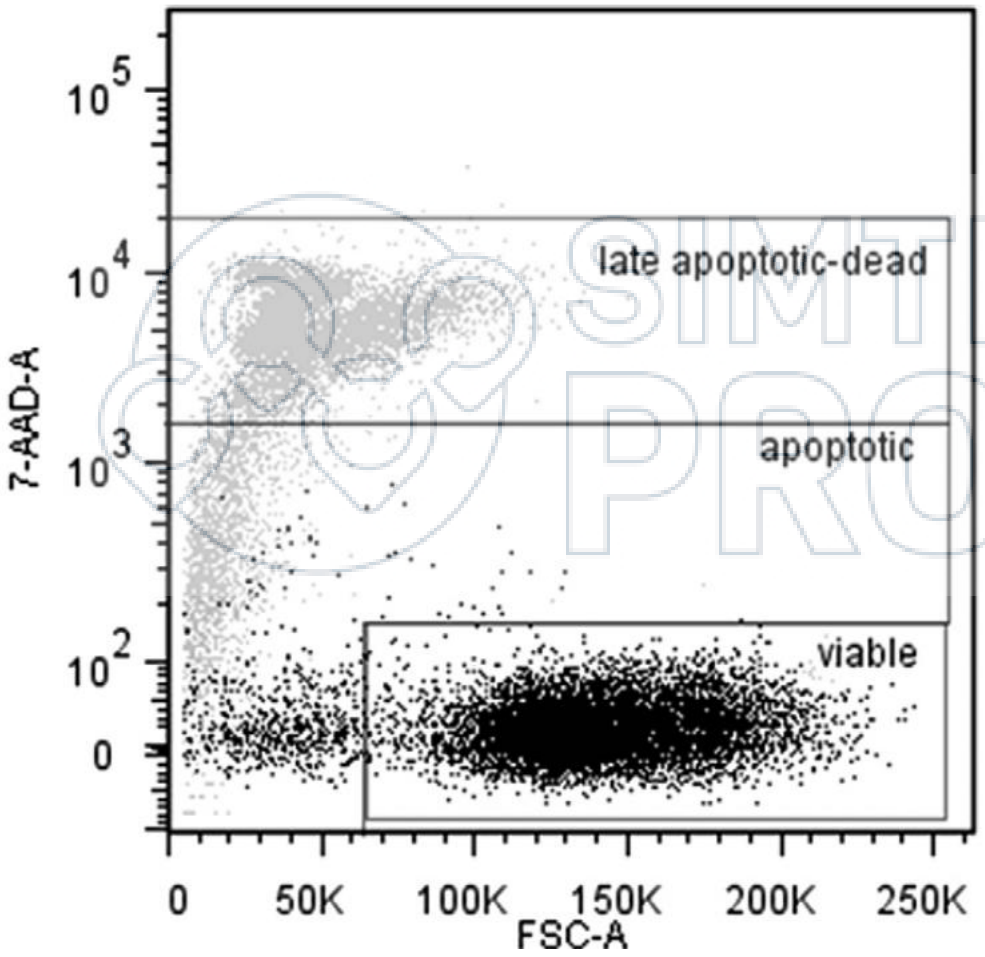
ISHAGE: modified METHOD (ISHAGE MOD)



CD34+ cells: 2316/ µl
Percentage of viable CD34 + cells: 15%

Lanza F., Saccardi R. Seghatchian J. New horizons on stem cells cryopreservation through the artificial eyes of CD34+ using modern flow cytometry tool. Transfusion and Apheresis Science 59 (2020): 102785

7-AAD differently stains viable, apoptotic, and late apoptotic/dead cells. It is known that pre-apoptotic cells can recover their full viability after removing the harmful stimuli by a short incubation in a favorable media.



C.L Nadine et al. Anal. Biochem. 429 (2012) 79–81

Key Strategies to Minimize Apoptosis: Incubation of cells in supplemented RPMI medium

- To prevent apoptosis in cell suspensions, **RPMI medium** is crucial to optimize the environmental conditions and nutrient supply.
- Careful handling techniques must be also applied to minimize cellular stress *(See the forthcoming CLSI H63).*
- The use of RPMI- medium supplemented with 10% Fetal Bovine Serum (FBS) provides essential growth factors that can prevent apoptosis.
- Maintain CO_2 levels between 5-10% to ensure correct pH (7.2-7.5)
- Ensure the incubator remains consistently at 37°C

Procedure Particolari per l'Analisi della Vitalità nei Preparati di Aferesi Scongelati

- Tutti i materiali criopreservati (Sacchette, Criotubi/Segmenti Satelliti) vanno rapidamente scongelati a +37°C (a secco o in bagnetto) con gentile agitazione.
- Qualche residuo pezzetto di ghiaccio può ancora essere visibile al termine della procedura: il materiale deve risultare **freddo**, non caldo al termine dello scongelamento.
- Il materiale scongelato va immediatamente posto a **+4°C**. Non sussiste il rischio di rigelo per il DMSO al 10%.
- Prelevare un'aliquota di campione scongelato (ad es. 100 µL) e aggiungere 20 µL alla volta PBS+BSA preraffreddato con pipettamento di precisione, con brevi pause, raggiungendo gradualmente la diluizione finale di 1:10 del campione originario, sempre a +4°C.
- Massima cura fino all'aggiunta di 200-300 µL. Poi si può aumentare la quantità di ogni step aggiunto a 50-100 µL. Non diluire comunque **MAI tutto in una volta**, per prevenire lo shock osmotico che aumenta molto la mortalità.
- È difficile e un po' laborioso, ma mantenere sempre il pipettamento di precisione per risalire alle conte assolute.
- Un'aliquota di 50 µL di scongelato diluito viene quindi marcata con CD45, CD34 e 7-AAD e analizzata in citometria non appena possibile. Può essere **omesso il lisante** (le emazie si lisano da sole nel ciclo congelamento/scongelamento).
- Applicare la strategia di gating ottimizzata per i campioni con elevata mortalità e riportare i conteggi assoluti di CSE CD34+ vitali (opzionali i valori percentuali) (ricordando di correggere per il fattore di diluizione applicato).

Excerpt from **CLSI H-63-1 Guideline** on CD34+ Cell Enumeration (to be published in 2027)

AIM OF THE STUDY

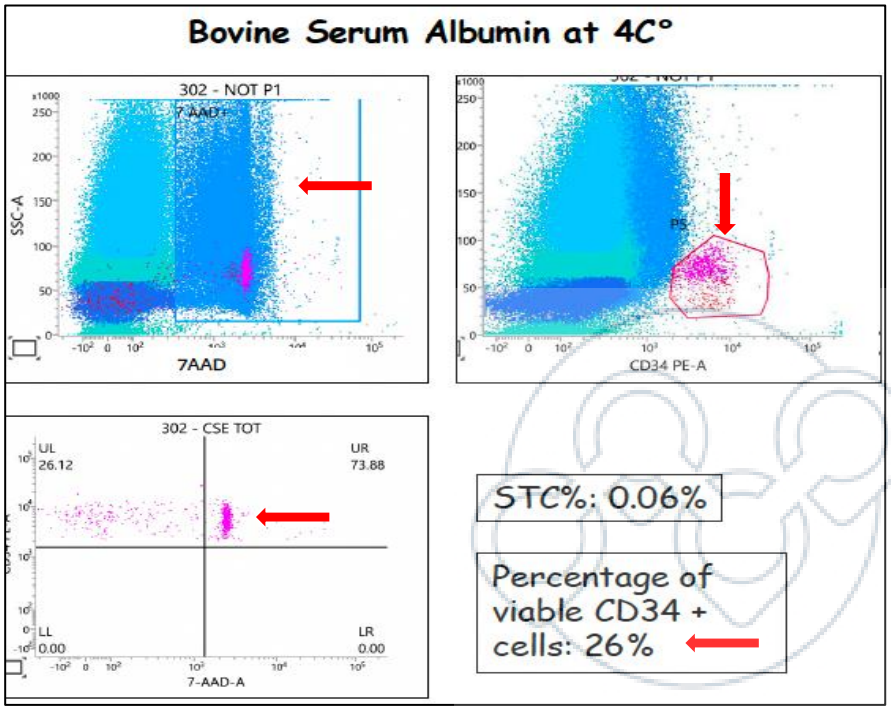
- The aim of the present study was to analyze and compare the percentage of viable CD34+ cells in thawed apheresis units, using a ISHAGE methodology approach, with and without incubation in complete RPMI medium.

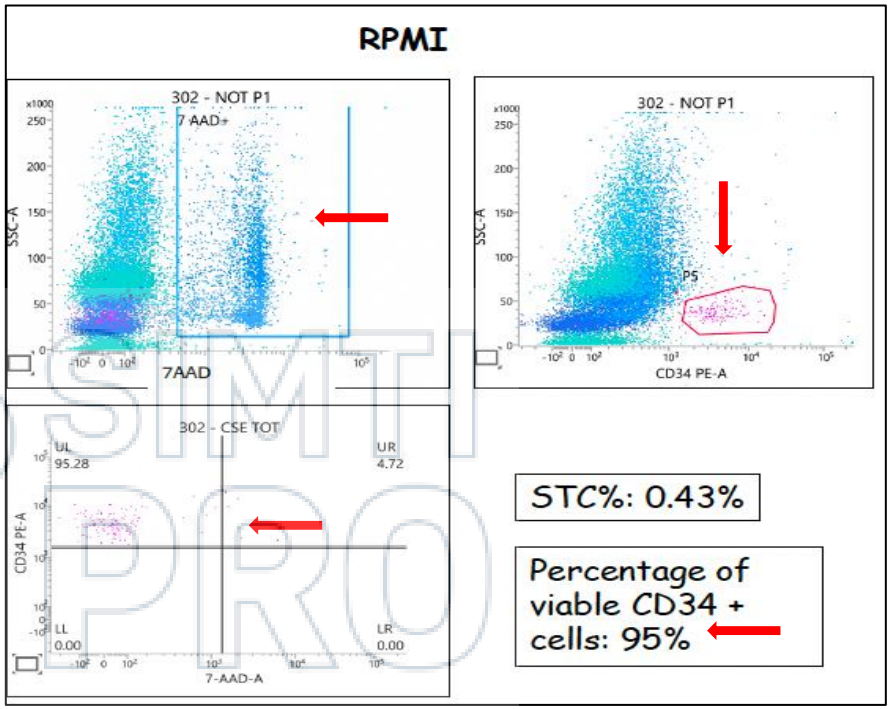
- The results were compared to those obtained in fresh apheresis and to the viability data on the respective satellite cryotubes, as evaluated by a conventional trypan blue exclusion assay.

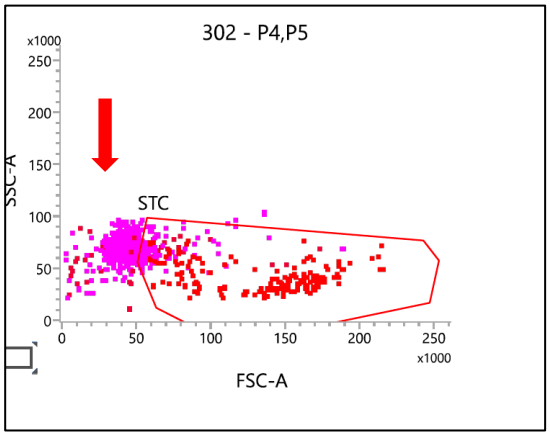
Method

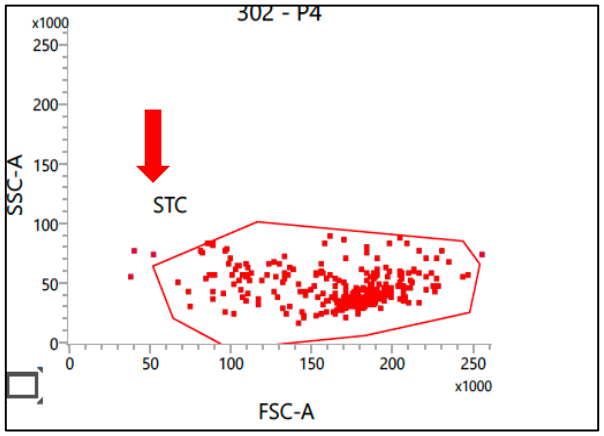
- 26 post-thawed apheresis samples from 12 patients were studied.
- Thawed apheresis samples were immediately processed without pre-dilution. Briefly, 25µl of thawed samples were diluted in 75µl of RPMI Medium and incubated for 20 min at 37°C in 5% CO_2.
- After incubation, the cells were stained with 10µl of anti-CD45-FITC, 10µl CD34-PE and 5µl of 7-AAD and analyzed with an ISHAGE FC template.
- Coupled control samples were left at +4°C for 20 min without RPMI preincubation and analyzed the same way.
- The respective satellite cryovials were independently evaluated for viability with a trypan blue exclusion test.

ISHAGE: modified METHOD (ISHAGE MOD) with RPMI (growth medium) versus Bovine Serum Albumin collection









RESULTS

- All reinfused aphereses allowed a prompt neutrophil (10 days) and platelet (20 days) engraftment in all patients.

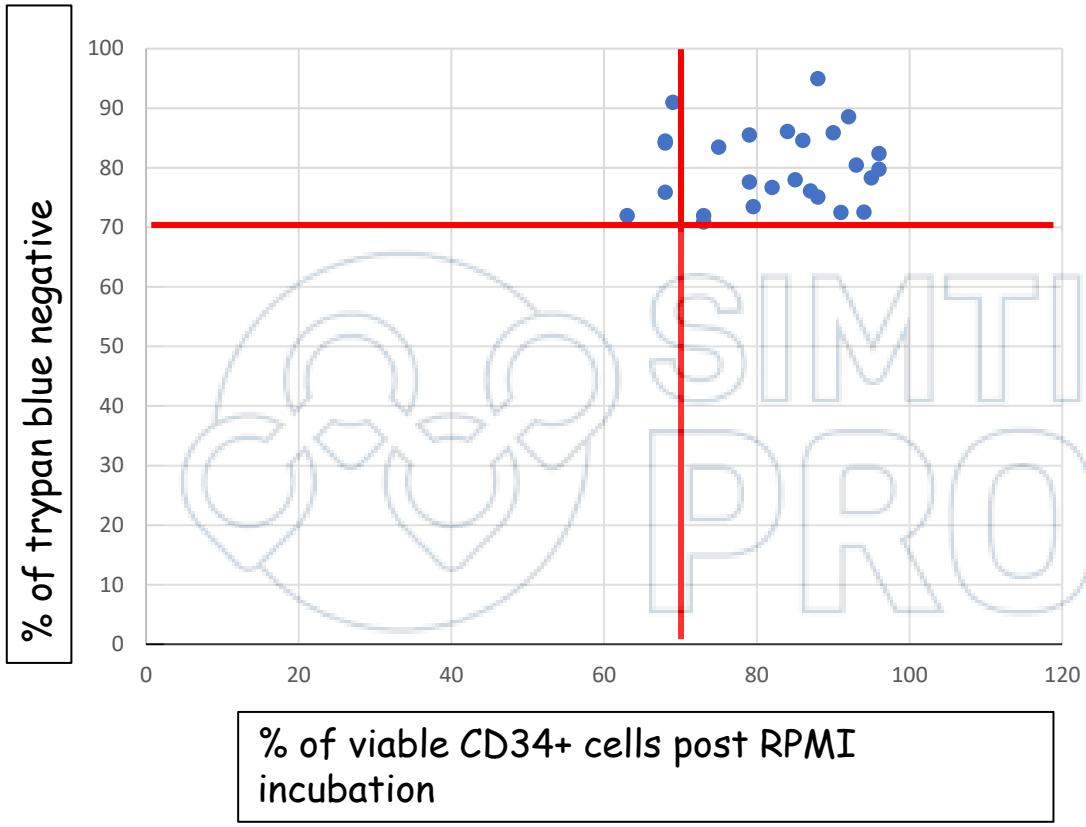
- In fresh aphereses the median CD34+ cell percentage was 0.48% (ranging 0.3-2.83%) with a 99.6% viability.

- In the thawed samples preincubated in RPMI the median global CD34+ cell percentage was 0.55% (ranging 0.2-2.86%), in agreement with the respective fresh apheresis samples.

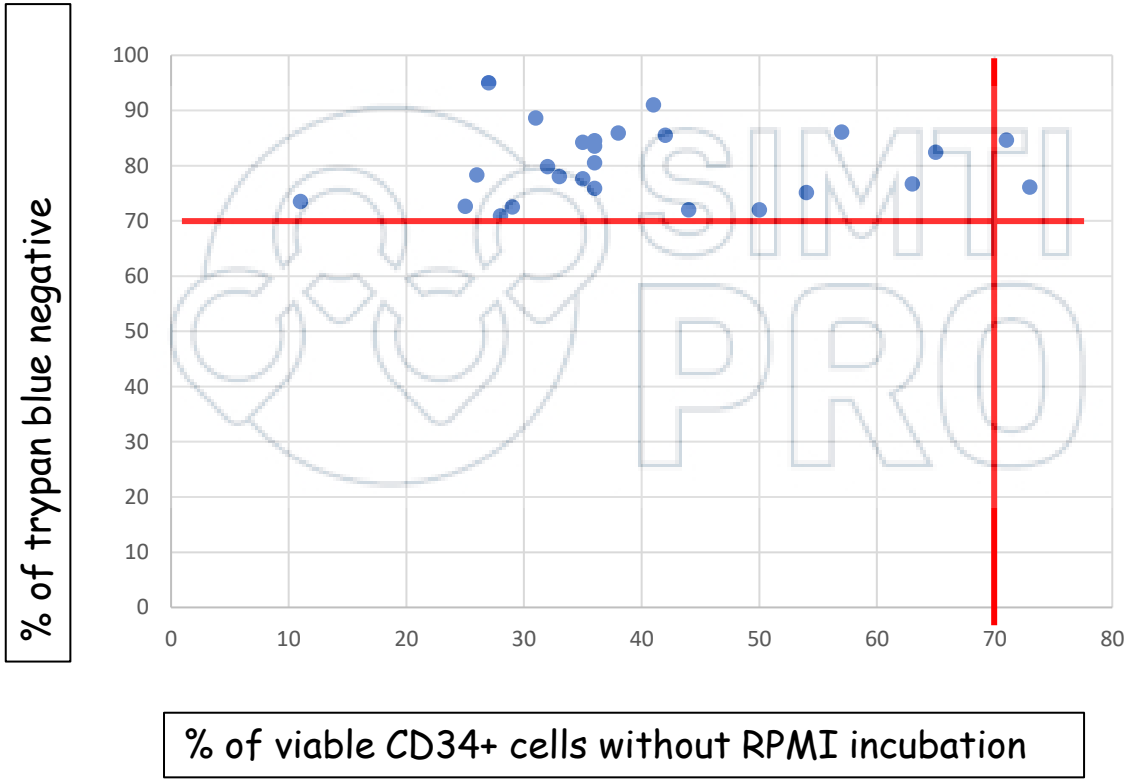
- In thawed cryopreserved vials, the percentage of trypan blue negative cells was 80% (ranging 70.9-95%) in agreement with the percentage of viable CD34+ cells post RPMI incubation evaluated by FC (median 79%, ranging 63%-96%).

- In thawed control samples not incubated in RPMI the CD34+ cell percentage and viability were significantly lower than those in RPMI-preincubated ones (median CD34+ cells 0.13% and median 7-AAD viability 36%, $p<0.01$).

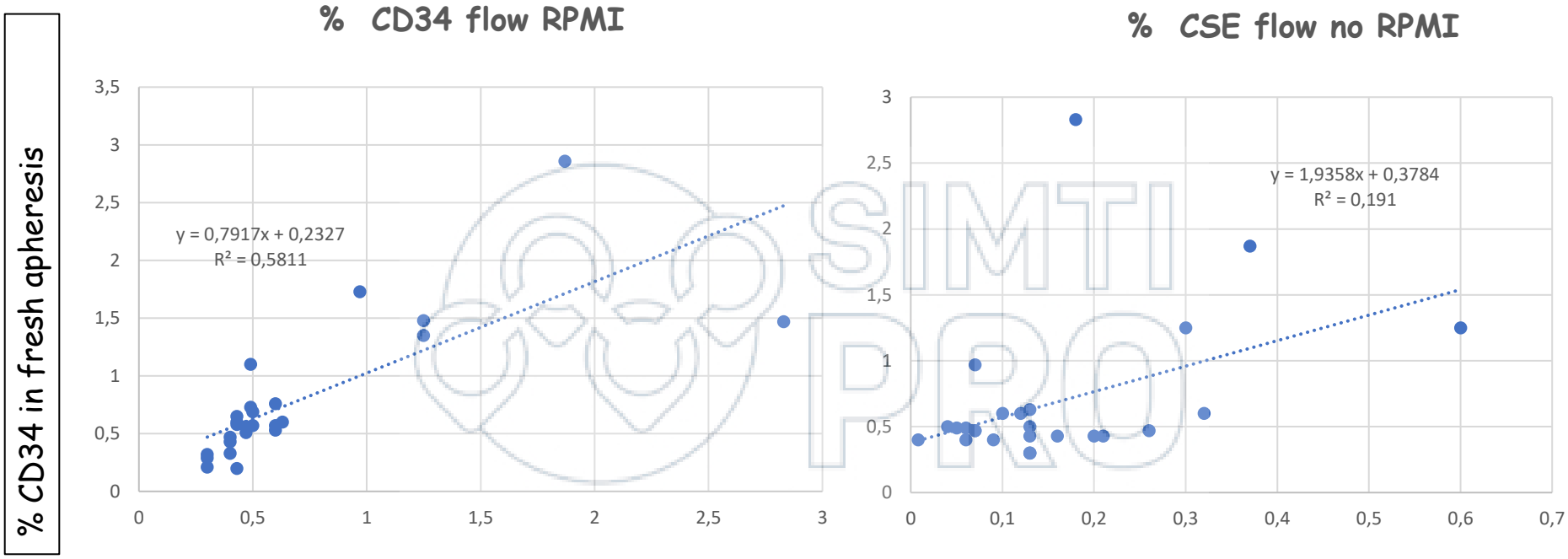
The percentage of trypan blue negative vs the percentage of viable CD34+ cells post RPMI incubation



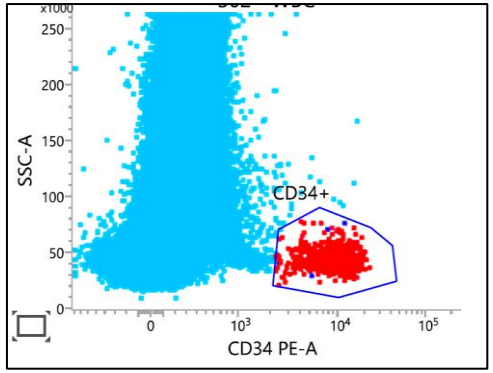
The percentage of trypan blue negative vs the percentage of viable CD34+ cells without RPMI incubation (Bovine Serum Albumin collection)

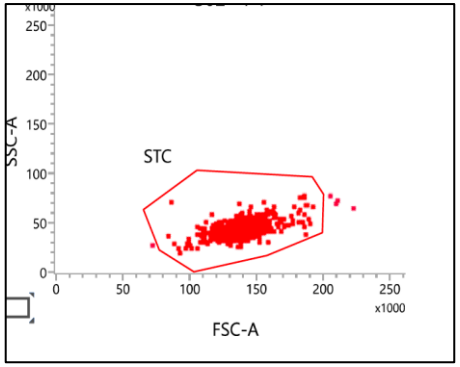


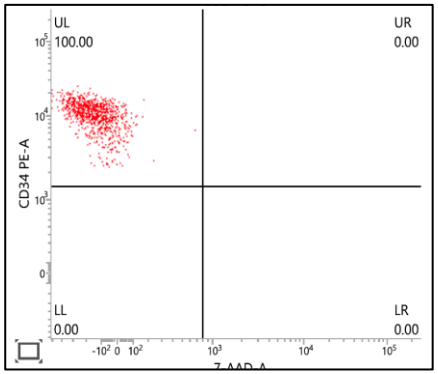
The percentage of CD34 in fresh apheresis vs the percentage of viable CD34+ cells post RPMI incubation and without RPMI incubation



CD34 cells in fresh apheresis

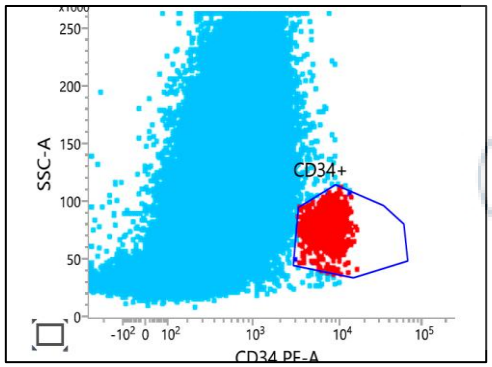


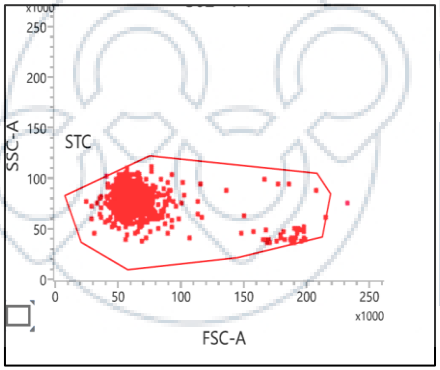


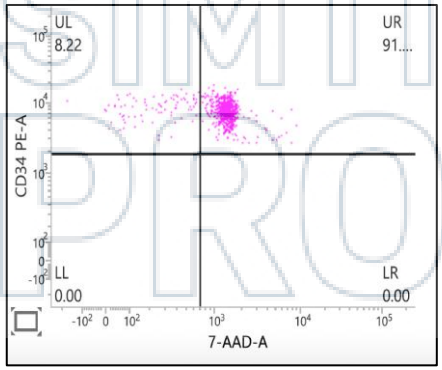


CD34/μL: 940
CD34%:0.77
viable CD34+ cells: 100%

CD34 cells in Bovine Serum Albumin

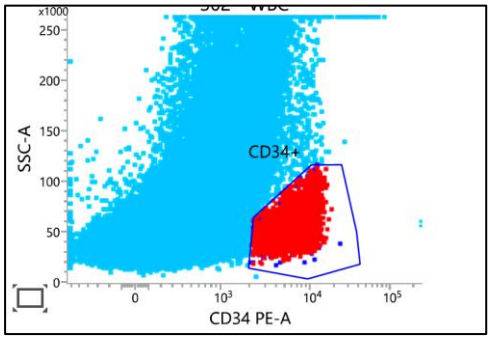


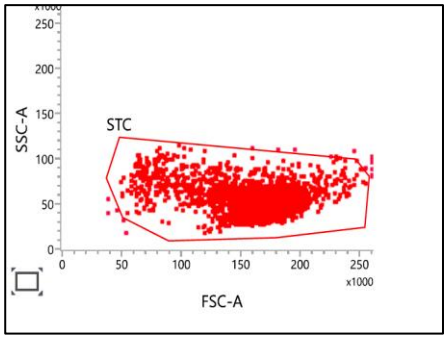


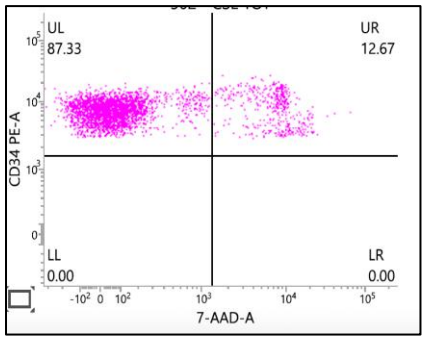


CD34/μL: 1004 (live & dead cells)
CD34%: 0.05%(live)-0.43% (live & dead)
viable CD34+ cells:10%

CD34+ cells post RPMI incubation







CD34%: 1%
viable CD34+ cells:87%

Conclusion

- The number of post-thaw viable CD34+ cells is a good predictor of the clinical outcome.
- Some modifications of the Flow Cytometry ISHAGE protocol have been implemented in order to detect more precisely viable and nonviable CD34+ cells in the thawed material.
- Early apoptotic CD34+ cells in thawed apheresis samples seem more resilient than expected, as evaluated by our in-vitro assay, and may regain full viability after a short incubation in favorable conditions.
- This finding, if confirmed, may at least partly envisage similar events taking place in-vivo after reinfusion, and may modify our way of using the traditional methods to evaluate CD34+ viable cells in thawed products.