British Journal of Haematology, 2000, **108**, 784–792

Reduction of intra- and interlaboratory variation in CD34$^+$ stem cell enumeration using stable test material, standard protocols and targeted training

D. Barnett,[1] V. Granger,[1] J. Kraan,[2] L. Whitby,[1] J. T. Reilly,[1] S. Papa[3] and J. W. Gratama[2] for and on behalf of the CD34 Task Force of the European Working Group of Clinical Cell Analysis (EWGCCA)*
[1]*UK NEQAS for Leucocyte Immunophenotyping, Department of Haematology, Royal Hallamshire Hospital, Sheffield, UK,* [2]*Department of Clinical and Tumour Immunology, Academisch Ziekenhuis-Daniel den Hoed Kliniek, Rotterdam, The Netherlands, and* [3]*Istituto di Scienze Morfologiche, Università di Urbino, Urbino, Italy*

Received 28 September 1999; accepted for publication 29 November 1999

Summary. The European Working Group on Clinical Cell Analysis (EWGCCA) has, in preparation for a multicentre peripheral blood stem cell clinical trial, developed a single-platform flow cytometric protocol for the enumeration of CD34$^+$ stem cells. Using this protocol, stabilized blood and targeted training, the EWGCCA have attempted to standardize CD34$^+$ stem cell enumeration across 24 clinical sites. Results were directly compared with participants in the UK National External Quality Assessment Scheme (NEQAS) for CD34$^+$ Stem Cell Quantification that analysed the same specimens using non-standardized methods. Two bead-counting systems, Flow-Count and TruCount, were also evaluated by the EWGCCA participants during trials 2 and 3. Using Flow-Count, the intralaboratory coefficient of variation (CV) was $\leq 5\%$ in 39% of the laboratories (trial 1), increasing to 65% by trial 3. Interlaboratory variation was reduced from 23·3% (trial 1) to 10·8% in trial 3. In trial 2, 70% of laboratories achieved an intralaboratory CV $\leq 5\%$ using TruCount, increasing to 74% for trial 3; the interlaboratory CV was reduced from 23·4% to 9·5%. Comparative analysis of the EWGCCA and the UK NEQAS cohorts revealed that EWGCCA laboratories, using the standardized approach, had lower interlaboratory variation. Thus, the use of a common standardized protocol and targeted training significantly reduced intra- and interlaboratory CD34$^+$ cell count variation.

Keywords: CD34$^+$ stem cell enumeration, flow cytometry, standardized protocols, clinical cell analysis.

The use of specific treatment and mobilization regimes that can significantly increase the numbers of circulating CD34$^+$ stem cells in both patients and healthy donors is now the preferred option for autografting (Henon, 1993). As a result, peripheral blood stem cells (PBSCs) have now virtually replaced bone marrow as the primary source of stem cells for autologous transplantation (Gratwohl *et al*, 1996, 1998). Monitoring the rise in circulating CD34$^+$ PBSCs using flow cytometry enables the optimum time point to harvest such cells by leucapheresis to be determined. However, a variety of different flow cytometric approaches have been developed (Bender *et al*, 1992; Sutherland *et al*, 1996; Gratama *et al*, 1997; Verwer & Ward, 1997; Keeney *et al*, 1998). This has consequently resulted in a lack of standardization and unacceptably high interlaboratory variation, with respect to reagents, gating strategies and techniques used to derive absolute CD34$^+$ PBSC values (Brecher *et al*, 1996; Chang & Ma, 1996; Johnsen & Knudsen, 1996; Lowdell & Bainbridge, 1996; Lumley *et al*, 1996; Chin-Yee *et al*, 1997; Gratama *et al*, 1997; Dzik *et al*, 2000) Thus, the minimum threshold dose of PBSCs required to obtain adequate engraftment can be affected by this lack of flow cytometric standardization

*Participating centres in this EWGCCA CD34 clinical task force study are listed in the Appendix.

Correspondence: Dr D. Barnett, Department of Haematology, Royal Hallamshire Hospital, Glossop Road, Sheffield S10 2JF, UK. E-mail: d.barnett@sheffield.ac.uk.

Members of the EWGCCA CD34 Task Force: S. Papa (Urbino, Italy), Chairman; D. Barnett (Sheffield, UK); B. Brando (Milan, Italy); J. Gratama (Rotterdam, The Netherlands); A. Huber (Aarau, Switzerland); A. Orfao (Salamanca, Spain) and G. Rothe (Regensburg, Germany). These individuals also acted as the (inter)national co-ordinators.

© 2000 Blackwell Science Ltd

and also by other factors, such as: (i) bone marrow function at the time of stem cell collection (i.e. effects of prior cytoreductive therapy); (ii) the preparative regimen before reinfusion (i.e. myeloablative vs. non-myeloablative); and (iii) the composition of the stem cell graft, i.e. the balance of subpopulations of $CD34^+$ haematopoietic stem cell (HSC) (Perey *et al*, 1998).

The CD34 Task Force of the European Working Group of Clinical Cell Analysis (EWGCCA) wishes to address, in a multicentre study, the effect of absolute number of $CD34^+$ cells infused as well as subset composition of the stem cell graft on short- and long-term engraftment. Therefore, it is essential that the flow cytometric determination of $CD34^+$ stem cells be standardized. Thus, the 24 centres enrolled into this study agreed to do so using a single-platform flow cytometric technique and standardized reagents to assess absolute $CD34^+$ cell counts and $CD34^+$ subsets (Gratama *et al*, 1999). Importantly, the use of single-platform approaches have recently been shown to result in interlaboratory variations of < 20% when compared with the so-called 'dual-platform' approach (Barnett *et al*, 1999). Therefore, we initiated an educational external quality assessment (EQA) programme for absolute $CD34^+$ cell counting and $CD34^+$ subset enumeration in order to minimize the variation within and between laboratories. This educational programme differs from previous approaches (Johnsen *et al*, 1999) in that 'on-site' laboratory standardization was undertaken rather than a workshop on a single site and that stabilized blood products, validated for use in EQA programmes for $CD34^+$ PBSC enumeration (Barnett *et al*, 1998), were used. In this way, the influence of specimen deterioration, occurring with fresh or cryopreserved material on the EQA results was eliminated (Barnett *et al*, 1998).

The first part of this programme set out to standardize absolute $CD34^+$ cell counts at a level representative for apheresis products (i.e. 150–200 $CD34^+$ cells/mm^3). The goals were: (i) to reduce the interlaboratory coefficient of variation (CV) to < 10% and (ii) to obtain, in > 75% of the participants, an intralaboratory CV < 5%, but not exceeding 10% for any participant. Here, we report the results of three send-outs and show, in comparison to the results of simultaneous send-outs by the UK National External Quality Assessment Scheme (NEQAS) for $CD34^+$ Stem Cell Quantification Scheme using the same test samples, the beneficial effects of such an educational programme on the interlaboratory variation in $CD34^+$ stem cell enumeration.

METHODS

Study design. This study was designed in order to standardize, monitor and improve the assessment of CD34 absolute counts in a group of 24 different laboratories located in seven European countries. For that purpose, several items were considered in advance: (i) sample to be used; (ii) reagents; (iii) sample preparation protocols; (iv) data acquisition, analysis and reporting; and (v) monitoring of potential problems occurring during the trial.

The specimen used in each instance was a well-characterized stabilized sample, similar in composition to mobilized peripheral blood (PB) (Barnett *et al*, 1998). To standardize sample staining/labelling, reagents from the same source and clones were used in each trial in all participating centres. Moreover, any technical issues that may have an impact on the quality of the results obtained, i.e. type of lysing solution to be used, single- vs. dual-platform assay, were resolved before the trials (Menendez *et al*, 1998; Barnett *et al*, 1999). The definition of the single-platform and dual-platform approaches have been described elsewhere (Barnett *et al*, 1999). Briefly, a single-platform approach facilitates the absolute count determination of leucocyte subsets directly from the flow cytometer by either adding a known number of microbeads to the sample or by calculating the number of cells in a known volume. In the dual-platform approach, the absolute leucocyte subset count is a product of the absolute total leucocyte count derived from an haematology analyser and the percentage of the leucocyte subset derived immunologically from the flow cytometer. Furthermore, to ensure standardized sample preparation procedures, a written protocol was distributed to all participants and, before commencement, a 'wet workshop' was conducted for all technical and clinical staff responsible for the study in each laboratory.

A common reporting form was distributed with samples to each participating centre, with all data generated being analysed centrally. To resolve any technical problems that occurred during the trial, a co-ordinating centre existed in each country that was responsible for direct contact with each laboratory within their territory.

Specimen collection and distribution. For each trial, 200 ml of peripheral blood was obtained, after informed consent, from a normal individual and was spiked with a known number of $CD34^+$ cells, typically to a final concentration of between 150 and 210 cells/μl. An acute myeloid leukaemia that presented with a 100% $CD34^+$ blast cell count was used as the source of $CD34^+$ cells, the blasts having characteristics identical to the normal human PB HSC regarding their light scatter characteristics [forward light scatter $(FSC)^{\text{low to intermediate}}$, side light scatter $(SSC)^{(\text{low})}$], and the expression of the CD34 and CD45 antigens ($CD34^+$ and $CD45^{\text{dim}}$). The material was stabilized using a procedure described in detail previously (Barnett & Granger, 1998). Longitudinal studies have shown that flow cytometric profiles and the expression of CD34 and CD45 are stable for over 600 d by this protocol (Barnett *et al*, 1996, 1998).

Three trials involving a total of 24 laboratories in seven European countries (four laboratories in The Netherlands, two in Germany, one in France, one in Switzerland, four in the UK, two in Spain and 10 in Italy) were conducted over a 6-month period. Of the 24 participating laboratories, 21 used a Becton–Dickinson flow cytometer, two used a Coulter flow cytometer and one used an Ortho Cytoron*Absolute*. Before commencement of the study, each centre was randomly assigned a laboratory code number (1–24) that maintained between-site confidentiality, this code number was retained throughout. Only each national co-ordinator and the relevant site knew the code numbers assigned. One laboratory (site 16) stopped routine $CD34^+$ cell enumeration after the second trial and did not participate further in the study. Another site (site 24) joined the project from trial 2

onwards. The first trial determined the absolute $CD34^{+}$ stem cell count using Flow-Count beads, and Flow-Count and TruCount beads were evaluated in parallel in trials 2 and 3. Each participant was requested to perform six replicate analyses using Flow-Count on three occasions for trial 1 and three replicate analyses for each type of counting beads on three occasions for trials 2 and 3. Before commencement of the study, it was agreed that after each trial the participating laboratories were debriefed and advice given where appropriate. It was also agreed that to consider the planned clinical multicentre trial feasible: (i) $> 75\%$ of laboratories should have attained an intralaboratory CV $< 5\%$ but not exceeding 10% for any participant and (ii) an interlaboratory variation of $\leq 10\%$ should be reached at the level of 150–200 $CD34^{+}$ cells/μl.

For each trial, the participating sites were provided with 1·5 ml of stabilized peripheral blood and sufficient reagents to perform single-platform $CD34^{+}$ stem cell enumeration, and an English language standard operating procedure (see below). The reagents issued for trial 1 were CD45 FITC (clone 2D1), CD34 PE (clone 8G12) both from Becton-Dickinson BioSciences (San Jose, USA), Ortho-Mune lysing reagent (Ortho Diagnostic Systems, Raritan, New Jersey, USA) and Flow-Count beads (Coulter-Immunotech, Miami, Florida, USA). For trials 2 and 3, the same set of reagents were issued along with TruCount tubes (Becton-Dickinson BioSciences, San Jose, USA).

The samples used for trials 2 and 3 were simultaneously issued to 110 laboratories enrolled in the UK NEQAS for $CD34^{+}$ Stem Cell Quantification, of which 98 and 97 participants, respectively, returned results for the trials. This group of laboratories used either non-standardized techniques (86 and 80 laboratories for trials 2 and 3 respectively) or commercial kits – Stem-Kit (Coulter-Immunotech) or ProCount (Becton-Dickinson) (12 laboratories in trial 2 and 17 laboratories in trial 3).

Standard protocol for CD34+ cell enumeration. Sample preparation using Flow-Count beads, flow cytometric data acquisition and data analyses were performed as described by Gratama *et al* (1999). Briefly, 10 μl of mAb (dilution) were pipetted in duplicate to the bottom of 12 × 75 mm polystyrene tubes, followed by 100 μl of sample. After a 15-min incubation of the samples with mAb mixture at room temperature (RT), 2 ml of each lysing reagent was added to the respective tubes for a further 10 min incubation at RT. To each tube, 100 μl of the Flow-Count bead suspension was added immediately before data acquisition. All samples were kept on melting ice until data acquisition, which was performed within 1 h after completion of sample preparation. Samples and counting beads were aliquoted using an electronic pipette using reverse pipetting (Barnett *et al*, 1999). For data acquisition of samples evaluated using Flow-Count, the forward light scatter (FSC) threshold was set at a sufficiently low level so as not to exclude the counting beads.

Samples issued for trials 2 and 3 were also analysed using TruCount tubes. These are 12 × 75 mm polystyrene tubes containing a predefined number of TruCount beads. The same staining procedure was adopted as described above except that the Flow-Count beads were omitted. During acquisition, the very low FSC signals of TruCount beads precluded the use of the FSC threshold as described for the Flow-Count beads and, thus, FL1 was used as the threshold to exclude debris, i.e. $CD45^{-}$ events (Fig 1A and B). Sufficient events were collected so as to contain at least 100 $CD34^{+}$ cells ($FSC^{low\ to\ intermediate}$,$SSC^{low}$,$CD34^{+}$,$CD45^{dim}$).

An overview of the sequential Boolean gating strategy used for list mode data analysis for Flow-Count beads has been published in Gratama *et al* (1999) and is shown for TruCount beads in Fig 1. In the first step, $CD45^{neg}$ events (i.e. most debris, platelets and unlysed erythrocytes) were excluded (R1 in Fig 1A). The $CD45^{dim}$ border of the CD45 window (R1) was verified on a CD45 vs. CD34 plot of ungated data (Fig 1E) to prevent exclusion of any $CD34^{+}$ events at this stage. The selected leucocytes (R1) were then displayed in a CD34 vs. SSC dot plot (Fig 1B). The $CD34^{+}$ events were selected (R2) and displayed on a CD45 vs. SSC dot plot (Fig 1C). The cluster of SSC^{low},$CD45^{dim}$ events was selected (R3) and displayed on a FSC vs. SSC dot plot (Fig 1D). Any events that did not meet the FSC,SSC criteria of viable leucocytes were excluded by setting a fourth window (R4), of which the FSC^{low} left border was verified using the FSC and SSC signals of lymphocytes (selected by R5 and displayed in Fig 1F). 'True' $CD34^{+}$ HPC must fulfil the criteria of R1, R2, R3 and R4. The counting beads were analysed by drawing a small rectangular gate (R6) to include the brightest events that fall in the highest FL1 and FL2 fluorescence channels (Fig 1E). The events from R6 were displayed in a time vs. FSC dot plot (Fig 1G) in order to exclude any 'non-bead' events (i.e. falling outside R7). The absolute number of $CD34^{+}$ cells (per μl) was calculated by multiplying the number of 'true' $CD34^{+}$ events by bead concentration and dilution factor, followed by division by the number of beads counted (i.e. number of events fulfilling R6 and R7).

Statistical analyses. Statistical analyses were performed using SAS (Statistical Analysis Systems, Cary, NC, USA) and Stata (Stata Corporation, College Station, TX, USA) software. For each trial and type of counting bead, the median result and CV of the number of $CD34^{+}$ cells for each laboratory were calculated. The median result of each laboratory was used to calculate the group median, 10th and 90th percentiles (indicated in each panel of Fig 2), as well as the interlaboratory CV for each trial and type of counting bead. A laboratory exceeding the 10th and 90th percentile was considered as an outlier.

RESULTS

Intralaboratory variation

Figure 2 shows the results of the $CD34^{+}$ stem cell enumerations performed six times (trial 1) or in triplicate (trials 2 and 3) stratified by trial and type of counting bead. Histograms of the intralaboratory CVs, plotted by trial and type of counting bead, are shown in Fig 3.

Flow-Count beads. For trial 1, the median intralaboratory CV was 6·1%. Nine (39%) of the 23 participants had an intralaboratory CV $< 5\%$. Laboratories 4 and 8 had the highest intralaboratory CVs of 10·5% and 14·1% respectively. For trial 2, the median intralaboratory CV improved to

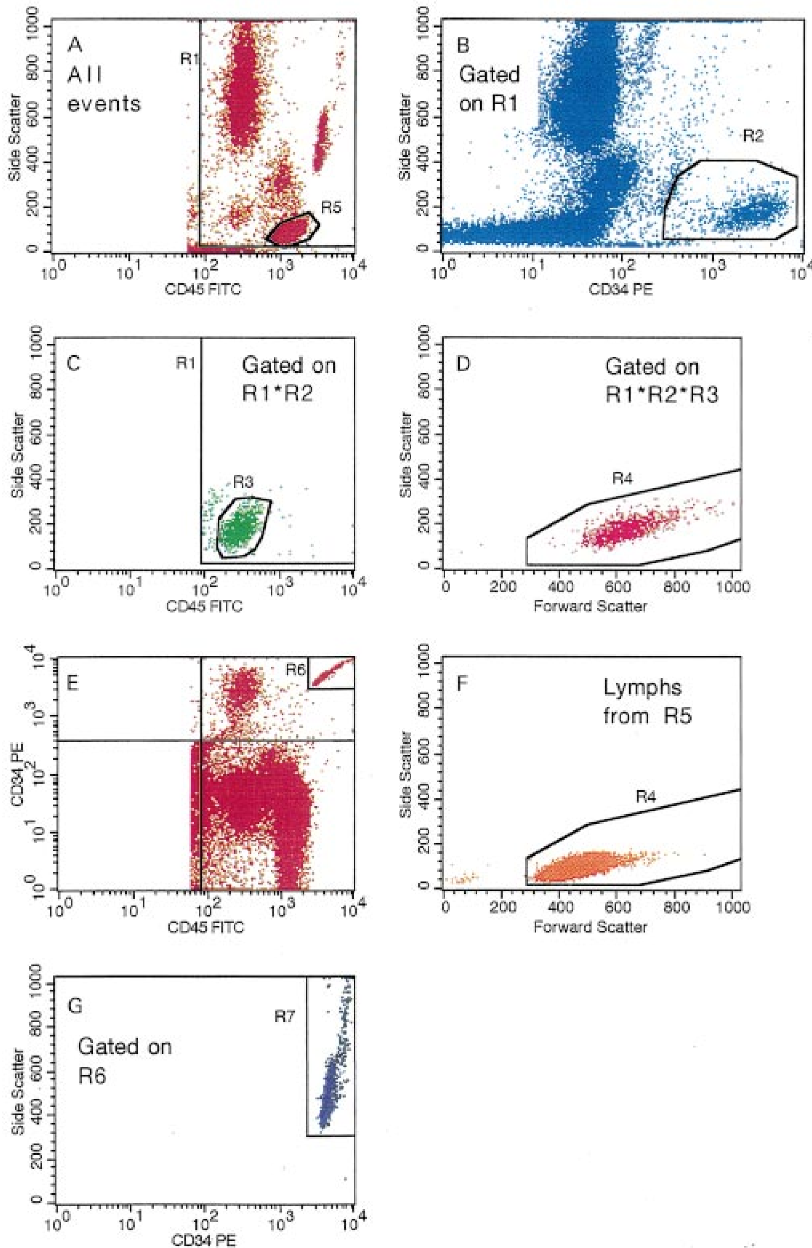

Fig 1. Analysis of sample 3 prepared with TruCount beads then acquired and analysed according to the standard protocol on a Becton Dickinson FACSCan. See further information in Materials and methods.

2·7% with 16 (70%) of the 23 participants having an intralaboratory CV < 5%. Two laboratories, 9 and 23, were outliers, having intralaboratory CVs of 13·4% and 25·1% respectively. Laboratories 4 and 8 had improved their performance with intralaboratory CVs of < 1% in both cases. For trial 3, the median intralaboratory CV was 3·5%; 15 of the 23 participants (65%) now attained an intralaboratory CV < 5%. Laboratory 23 had improved its intralaboratory CV to 2·6%, whereas laboratory 9 kept underperforming, with an intralaboratory CV of 15·0%. Interestingly, both Coulter users achieved intralaboratory CVs of 2·9% and 2·4% respectively.

TruCount beads. The number of laboratories attaining the target intralaboratory CV of < 5% in trials 2 and 3 was 15 and 19 respectively. For trial 2, the median intralaboratory CV was 3·8%, with 15 (65%) of the 23 participants having an intralaboratory CV < 5%. Laboratories 6 and 10 performed relatively poorly with intralaboratory CVs of 10·8% and 15·0% respectively. For trial 3, the median intralaboratory CV was reduced further to 2·1%, with 19 (83%) of the 23 participants having an intralaboratory CV < 5%. In this round, laboratory 18 was an outlier, with an intralaboratory CV of 14·3%, whereas laboratories 6 and 10 had improved their intralaboratory CVs to 4·9% and 1·8% respectively. Indeed, using TruCount, 13 laboratories showed a decrease in their intralaboratory CVs between trials 2 and 3, with 11 of these having an intralaboratory CV of between 0·98% and 3·57%.

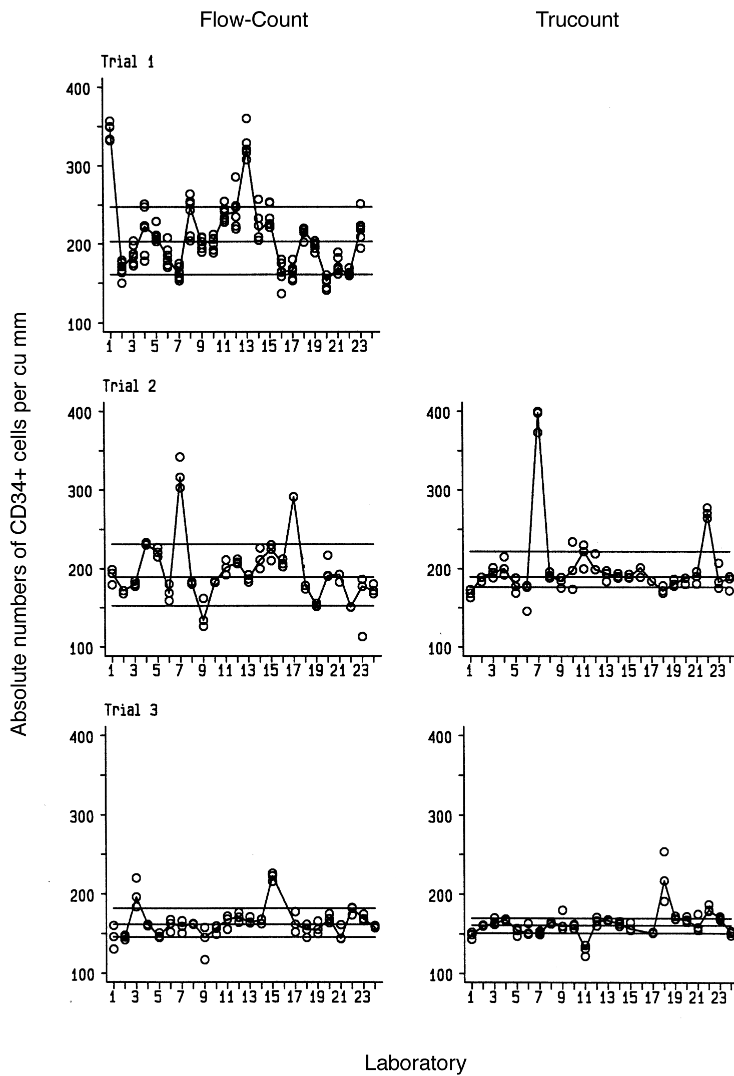

Fig 2. Intra- and interlaboratory variation in results of assessments of absolute numbers of $CD34^+$ cells. The median values of assessments carried out six times (trial 1) or in triplicate for each type of counting bead (trials 2 and 3) are connected with a line. The horizontal lines indicate, for each trial and type of counting bead, the 10th, 50th (median) and 90th percentiles (for computation, see Materials and methods).

Differences in intralaboratory CVs using Flow-Count and TruCount beads did not reach statistical significance either for trial 2 ($P = 0{\cdot}23$) or for trial 3 ($P = 0{\cdot}06$ using the Wilcoxon test).

Interlaboratory variation

The variation between the laboratories is shown in Fig 4 and is summarized in Table I.

Flow-Count beads. During trial 1, laboratories 1 and 13 were clear outliers, with median results > 300 $CD34^+$ cells/mm^3, whereas the 90th percentile of the group was 248 $CD34^+$ cells/mm^3 (Fig 2). The interquartile range (Fig 4) was 61 $CD34^+$ cells/mm^3 and the interlaboratory CV was 23·3%. During trial 2, the interquartile range decreased to 35 $CD34^+$/mm^3 and interlaboratory CV decreased slightly to 18·7%; this time, laboratories 7 and 17 (the latter performing only a single analysis because of instrument problems) obtained exceedingly high $CD34^+$ cell counts. The interquartile range decreased further to 14 $CD34^+$ cells/mm^3 in trial 3. Only moderate outliers were observed (laboratories 3 and 15), hence the interlaboratory CV fell to 10·8%, i.e. almost at the target level set out at the beginning of the project.

TruCount beads. The interquartile range in trial 2 was only 15 $CD34^+$ cells/mm^3. However, laboratories 7 and 22 significantly contributed with their high outliers to the

Table I. Interlaboratory CV of absolute $CD34^+$ cell numbers in the EWGCCA trials and the UK NEQAS send-outs.

	EWGCCA		UK NEQAS	
Trial	Flow-Count	TruCount	Dual platform	Single platform
1	23·3	ND		
2	18·7	23·4	17·6	17·2
3	10·8	9·5	34·4	17·5

ND, not done.

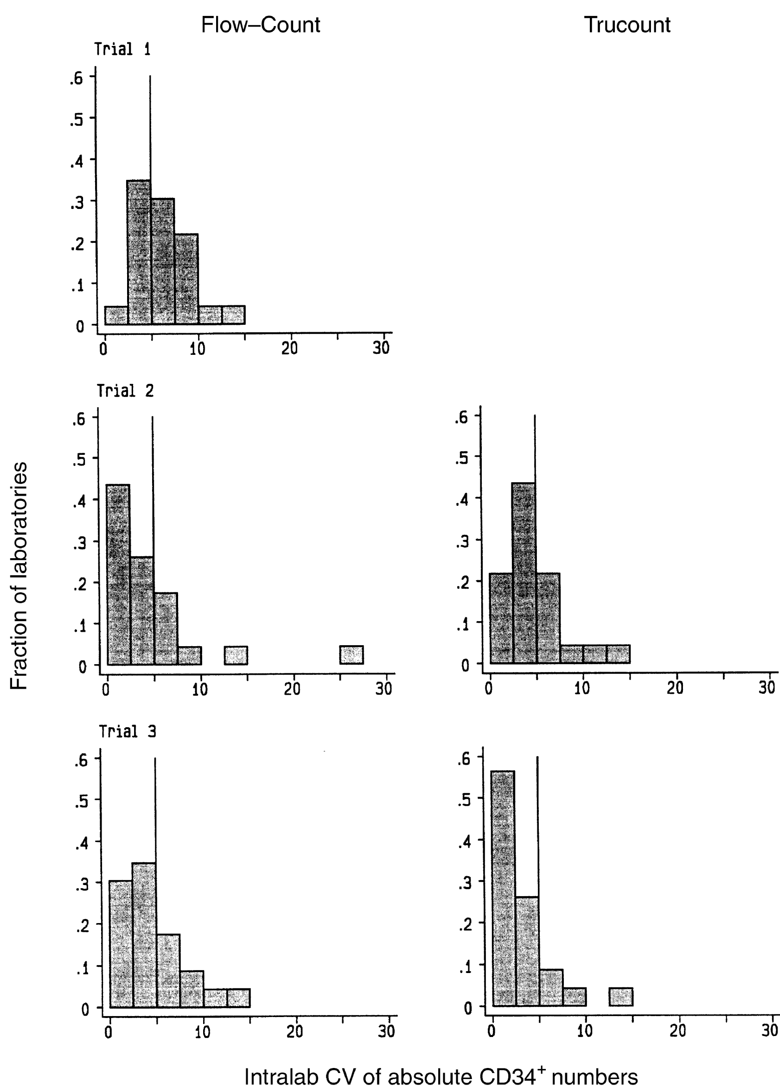

Fig 3. Histograms showing the distribution of intralaboratory CVs per trial and type of counting bead. The lines at CV = 5% indicate the target level set for the multicentre clinical trial and are shown as a reference.

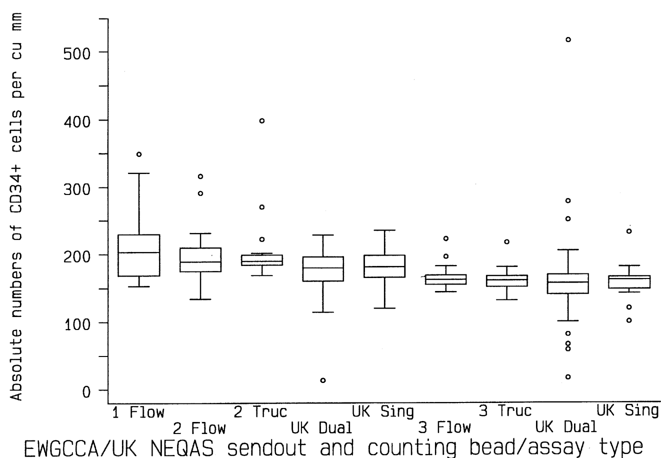

Fig 4. Variation in absolute numbers of $CD34^{+}$ cells/mm^3 between laboratories participating in the three EWGCCA trials and the two UK NEQAS $CD34^{+}$ Stem Cell Enumeration Scheme send-outs. The boxes extend from the 25th (p25) to the 75th (p75) percentile; the line in the middle represents the median. The whiskers extend to the upper and lower adjacent values, which are defined as 1·5 × (p75–p25), rolled back to where there are data. Outliers more extreme than the adjacent values have been individually plotted. Numbers 1–3 denote EWGCCA trial numbers. The UK NEQAS results on the same samples as used for trials 2 and 3 are shown adjacent to the corresponding EWGCCA results. Flow, Flow-Count; Truc, TruCount; Dual, dual-platform assays; Sing, single platform assays.

© 2000 Blackwell Science Ltd,

interlaboratory CV of 23·4% (Fig 2). The interquartile range remained at 16 $CD34^+$ cells/mm^3 in trial 3. This time, only moderate outliers were seen (Fig 4) by laboratories 11 (low), 18 (high) and, again, 22 (high); as a result, the interlaboratory CV fell to 9·5%, i.e. to target level.

Comparison between laboratories participating in EWGCCA trials and UK NEQAS send-outs

For trials 2 and 3, the same samples were also analysed and reported by 98 and 97 laboratories participating in the UK NEQAS for $CD34^+$ Stem Cell Quantification programme respectively. The results of both groups are shown in Fig 4, with results stratified for type of counting bead (i.e. Flow-Count vs. TruCount), for the EWGCCA participants and for assay type (i.e. dual platform vs. single platform) for the UK NEQAS participants.

For trial 2, the UK NEQAS laboratories (using dual platform or single platform) obtained slightly, but significantly, lower absolute $CD34^+$ cell counts (median 180 and 181 $CD34^+$ cells/mm^3 respectively) than the EWGCCA labs (Flow-Count, 189 $CD34^+$ cells/mm^3; TruCount, 190 $CD34^+$ cells/mm^3; $P = 0{\cdot}03$, Kruskal–Wallis test). The smallest interquartile range was observed for the EWGCCA participants using TruCount beads (15 $CD34^+$ cells/mm^3 vs. 35, 36 and 32 $CD34^+$ cells/mm^3 for EWGCCA participants using Flow-Count beads and UK NEQAS participants using dual- and single-platform assays respectively). However, because of the presence of five outliers in the EWGCCA results compared with only a single outlier in the UK NEQAS results, the interlaboratory CVs of the UK NEQAS dual- and single-platform groups (17·6% and 17·2% respectively) were lower than those of the EWGCCA groups (18·7% and 23·4% respectively; (Table I).

For trial 3, the EWGCCA and UK NEQAS participants obtained similar absolute numbers of $CD34^+$ cells. The medians were: EWGCCA (Flow-Count), 162 $CD34^+$ cells/mm^3; EWGCCA (TruCount) 161 $CD34^+$ cells/mm^3; UK NEQAS (dual platform), 157 $CD34^+$ cells/mm^3; and UK NEQAS (single platform), 162 $CD34^+$ cells/mm^3. The smallest interquartile range was observed for EWGCCA (Flow-Count), 14 $CD34^+$ cells/mm^3, followed by EWGCCA (TruCount), 16 $CD34^+$ cells/mm^3, UK NEQAS (single platform), 18 $CD34^+$ cells/mm^3, and UK NEQAS (dual platform) 28 $CD34^+$ cells/mm^3. Because of the large number of outliers ($n = 8$), the UK NEQAS double-platform group had increased from 17·6% in trial 2 to 34·4% in trial 3, whereas the CV of the UK NEQAS single-platform group remained at the same level (17·2% in trial 2 and 17·5% in trial 3). These CVs were clearly higher than those obtained by the EWGCCA group (Flow-Count, 10·8%; TruCount, 9·5%).

Problems identified during the course of the study

During the first trial, several problems became apparent, such as that the templates supplied on disc were not compatible with all software used by participants, thus two laboratories had to define gating criteria that were slightly different to the remaining participants. This problem was rectified before the next trial. During trial 1, several laboratories reported increased doublet, triplet and quadruplet bead formation with the Flow-Count beads. The cause of this problem was thought to arise from the aliquoting of Flow-Count beads by the co-ordinating laboratory from manufacturer vials into smaller secondary vials before distribution among participants. Variation in how these populations were included within the calculation of PBSCs would therefore be one of the factors affecting the intra- and interlaboratory CVs. Therefore, all participants were provided with factory-sealed Flow-Count vials before commencing trials 2 and 3.

During the course of the study, several sites were identified as having specific problems. The staff performing trial 2 at laboratory 7 were insufficiently proficient in English and made errors using both counting bead systems (Fig 1). After instructions and trouble-shooting had been provided in their native language, their performance in trial 3 improved. Laboratory 17 returned only one result for trial 2 as a consequence of instrumentation failure. Laboratory 9 had results out of consensus for trials 2 and 3. Further investigation found the causes to be (i) an increased number of doublet, triplet and quadruplet beads using Flow-Count for trial 2 and (ii) the automatic pipette used during trial 3 was dispensing incorrect volumes as a result of low battery power. When the batteries were replaced, the problem was rectified and the intra- and interlaboratory CV improved to achieve the target CVs required. Laboratory 18, with results in trial 3 higher than the 90th percentile, had problems owing to faulty TruCount tubes. Their performance improved significantly when new reagents, tubes and samples were issued.

After trials 2 and 3, several sites received additional training in order to address problems that had occurred. The problems identified were either a high intralaboratory CV and/or outlying results (> 90th or < 10th percentiles). After training, all but one site showed improved performance when they tested the same material again, i.e. intralaboratory CVs were reduced to < 5% and/or the median of the triplicate results fell within the 10th and 90th percentiles.

DISCUSSION

Using stabilized samples prepared by UK NEQAS for leucocyte immunophenotyping, and having PBSC concentrations representative of leucapheresis samples (typically > 150 cells/μl), three separate EWGCCA CD34 task force (TF) trials were conducted. It is important to stress that the stabilized whole blood material used in this study had minimal, if any, matrix effects. Such an effect is defined as having interassay properties similar to those of a patient specimen and does not contain, within the specimen, a component that will influence the parameter(s) to be analysed. Material stabilized in a manner previously described (Barnett & Granger, 1998) has been previously demonstrated to satisfy these criteria and to be compatible with all flow cytometers (Barnett *et al*, 1996, 1998, 1999).

It is well recognized that single-platform flow cytometric analysis of cellular subsets results in lower interlaboratory variation and is the preferred approach (Margolick *et al*, 1998; Barnett *et al*, 1999; Reimann *et al*, 2000; Schnizlein-Bick *et al*, 2000). Thus, using this state-of-the-art approach, the EWGCCA CD34 TF have evaluated two single-platform

approaches using beads as reference values, one using Flow-Count beads and the other TruCount beads (Keeney *et al*, 1998; Gratama *et al*, 1999). Both of the protocols utilized the previously described International Society for Haematotherapy and Graft Engineering (ISHAGE) gating strategy, but have been modified to enable single-platform analysis. After each trial, participants were debriefed and assistance was provided where required. This study had a primary objective of reducing interlaboratory variation to < 10% while achieving an intralaboratory CV of < 5% in at least 75% of participating centres, but not exceeding 10% in all centres, using a standardized single-platform flow cytometric technique. The EWGCCA CD34 TF arbitrarily set these goals to enable generation of reliable data in a multicentre clinical study aimed at determining the precise numbers of $CD34^+$ HSC (and subsets) required that obtained adequate engraftment.

The high intralaboratory CV observed in the first trial using Flow-Count and the fact that the majority of laboratories failed to achieve an intralaboratory CV of < 5% could be the result of two factors: (i) unfamiliarity of the reverse pipetting technique, a prerequisite for single-platform flow cytometric analysis (Connelly *et al*, 1995; Mercolino *et al*, 1995; Strauss *et al*, 1996) and (ii) problems encountered when the Flow-Count beads were subaliquoted centrally. However, when trial 2 was conducted, the majority of laboratories attained a CV of 5% (or less), suggesting that laboratories had become familiar with the technique. This hypothesis was supported by the fact that for trial 3 using Flow-Count the improvement was sustained, with all except three laboratories attaining an intralaboratory CV of < 10% and the interlaboratory CV was very close to the target CV of 10%. However, by trial 3 when using TruCount tubes, all laboratories, except one, had an intralaboratory CV of < 10% and the interlaboratory CV reached the target value of < 10%.

Trials 2 and 3 were also performed in parallel with the UK NEQAS for $CD34^+$ Stem Enumeration scheme. The EWGCCA sites were compared with the UK NEQAS sites using the single-platform approaches Stem-Kit and ProCount. The trial 2 median value for the UK NEQAS cohort was slightly, but significantly, lower than the median value obtained by the EWGCCA cohort, but this was not reproduced in trial 3. No obvious cause for this difference was determined. It has previously been demonstrated by the UK NEQAS scheme that use of single-platform methods significantly reduces interlaboratory variance compared with the use of dual-platform methods (Barnett *et al*, 1999). However, it should be noted that the UK NEQAS cohort did not use standardized methodology. Furthermore, Bergeron *et al* (1998) demonstrated that when using a standardized approach for $CD4^+$ T-lymphocyte enumeration a significant improvement in interlaboratory variance is observed. This current EWGCCA study has extended these findings to $CD34^+$ stem cell enumeration and also supports the UK NEQAS observations that a single-platform flow cytometric approach does result in reduced interlaboratory variation. However, this EWGCCA study has extended these findings and has shown that, with appropriate training, the use of TruCount tubes and a standardized single-platform approach, interlaboratory CVs lower than those previously reported by UK NEQAS can be obtained and the target CV defined before study commencement can be attained.

Thus, in conclusion, we have demonstrated that, using a standardized 'state-of-the art' single-platform $CD34^+$ stem cell flow cytometric method intralaboratory, CVs can be reduced to < 5%. Continuous education and targeted training of individual laboratories form a critical component of this improvement. Furthermore, using such an approach, i.e. EQA with stable whole-blood material free from any matrix effect, interlaboratory variation can be reduced to below 10% in the majority of laboratories. This is a prerequisite to achieving meaningful multicentre clinical study data. Further trials are now planned to monitor these parameters and also to undertake the monitoring of $CD34^+$ subsets.

REFERENCES

Barnett, D. & Granger, V. (1998) Stabilisation of Cell Suspensions. European patent no. 0754301, pp. 1–31.

Barnett, D., Granger, V., Mayr, P., Storie, I., Wilson, G.A. & Reilly, J.T. (1996) Evaluation of a novel stable whole blood quality control material for lymphocyte subset analysis: results from the UK NEQAS Immune Monitoring Scheme. *Cytometry*, **26**, 216–222.

Barnett, D., Granger, V., Storie, I., Peel, J., Pollitt, R., Smart, T. & Reilly, J.T. (1998) Quality assessment of CD34+ stem cell enumeration: experience of the United Kingdom National External Quality Assessment Scheme (UK NEQAS) using a unique stable whole blood preparation. *British Journal of Haematology*, **102**, 553–565.

Barnett, D., Granger, V., Whitby, L., Storie, I. & Reilly, J.T. (1999) Absolute CD4+ T-lymphocyte and CD34+ stem cell counts by single platform flow cytometry: the way forward. *British Journal of Haematology*, **106**, 1059–1062.

Bender, J.G., To, L.B., Williams, S. & Schwartzberg, L.S. (1992) Defining a therapeutic dose of peripheral blood stem cells. *Journal of Hematotherapy*, **1**, 329–341.

Bergeron, M., Faucher, S., Minkus, T., Lacroix, F., Ding, T., Phaneuf, S., Somorjai, R., Summers, R., Mandy, F. & Participating Flow Cytometry LabsCana (1998) Impact of unified procedures as implemented in the Canadian quality assurance program for T lymphocyte subset enumeration. *Cytometry*, **33**, 146–155.

Brecher, M.E., Sims, L., Schmitz, J., Shea, T. & Bentley, S.A. (1996) North American multi-center study on flow cytometric enumeration of $CD34^+$ hematopoietic stem cells. *Journal of Hematotherapy*, **5**, 227–236.

Chang, A. & Ma, D. (1996) The influence of flow cytometric gating strategy on the standardisation of CD34+ cell quantitation: an Australian multi-centre study. *Journal of Hematotherapy*, **5**, 605–616.

Chin-Yee, I., Anderson, L., Keeney, M., Sutherland, D.R. & Canadian, Q.E.S. (1997) Quality assurance of stem cell enumeration by flow cytometry. *Cytometry*, **30**, 296–303.

Connelly, M.C., Knight, M., Giorgi, J.V., Kagan, J., Landay, A.L., Parker, J.W., Page, E., Spino, C., Wilkening, C. & Mercolino, T. (1995) Standardisation of absolute CD4+ lymphocyte counts across laboratories: an evaluation of the Ortho Cytoron Absolute flow cytometry system on normal donors. *Cytometry*, **22**, 200–210.

Dzik, W.H., Sniecinski, I., Fisher, J. & the Biomedical Excellence for Safer Transfusion Working Party (2000) Towards standardisation of CD34+ cell enumeration: an international study. *Transfusion*, in press.

Gratama, J.W., Kraan, J., Levering, W., van Bockstaele, D.R., Rijkers, G.T. & van der Schoot, C.E. (1997) Analysis of variation in results of CD34$^+$ hematopoietic progenitor cell enumeration in a multicenter study. *Cytometry,* **30,** 109–117.

Gratama, J.W., Keeney, M. & Sutherland, D.R. (1999) Enumeration of CD34$^+$ hematopoietic stem and progenitor cells. *Current Protocols in Cytometry* (ed. by J.P. Robinson, Z. Darzynkiewicz, P.N. Dean, L.G. Dressler, P.S. Rabinovitch, C.C. Stewart, H.J. Tanke & L.L. Wheeless), pp. 6.4.1–6.4.22. John Wiley & Sons, New York.

Gratwohl, A., Baldomero, H. & Hermans, J. (1996) Haemopoietic precursor transplant activity in Europe, 1994. *Bone Marrow Transplantation,* **17,** 137–148.

Gratwohl, A., Passweg, J., Baldomero, H., Hermans, J. & E.B.M.T. (1998) Blood and marrow transplantation activity in Europe 1996. *Bone Marrow Transplantation,* **22,** 227–240.

Henon, P.R. (1993) Peripheral blood stem cell transplantations: past, present and future. *Stem Cells,* **11,** 154–172.

Johnsen, H.E. & Knudsen, L.M. (1996) Nordic flow cytometry standards for CD34$^+$ cell enumeration in blood and leukapheresis products: report from the second Nordic workshop. *Journal of Hematotherapy,* **5,** 237–245.

Johnsen, H.E., Baech, J. & Nikolajsen, K. (1999) Validation of the Nordic flow cytometry standard for CD34+ cell enumeration in blood and autografts: report from the third workshop. Nordic Stem Cell Laboratory Group. *Journal of Hematotherapy,* **8,** 15–28.

Keeney, M., Chin-Yee, I., Weir, K., Popma, J., Nayar, R. & Sutherland, D.R. (1998) Single platform flow cytometric absolute CD34+ cell counts based on the ISHAGE guidelines. *Cytometry,* **34,** 61–70.

Lowdell, M.A. & Bainbridge, D.R. (1996) External quality assurance for CD34 cell enumeration – results of a preliminary national trial. *Bone Marrow Transplantation,* **17,** 849–853.

Lumley, M.A., McDonald, D.F., Czarnecka, H.M., Billingham, L.J. & Milligan, D.W. (1996) Quality assurance of CD34$^+$ cell estimation in leucapheresis products. *Bone Marrow Transplantation,* **18,** 791–796.

Margolick, J.B., Donnenberg, A.D., Chu, C., O'Gorman, M.R., Giorgi, J.V. & Muñoz, A. (1998) Decline in total T cell count is associated with onset of AIDS, independent of CD4$^+$ lymphocyte count: implications for AIDS pathogenesis. *Clinical Immunology and Immunopathology,* **88,** 256–263.

Menendez, P., Redondo, O., Rodriguez, A., Lopez-Berges, M.C., Ercilla, G., Lopez, A., Duran, A., Almeida, J., Perez-Simon, J.A., San Miguel, J.F., Gratama, J.W. & Orfao, A. (1998) Comparison between a lyse-and-then-wash method and a lyse-non-wash technique for the enumeration of CD34+ hematopoietic progenitor cells. *Cytometry,* **34,** 264–271.

Mercolino, T.J., Connelly, M.C., Meyer, E.J., Knight, M.D., Parker, J.W., Stelzer, G.T. & DeChirico, G. (1995) Immunologic differentiation of absolute count with an integrated flow cytometric system: a new concept for absolute T cell subset determinations. *Cytometry,* **22,** 48–59.

Perey, L., Peters, R., Pampallona, S., Schneider, P., Gross, N. & Leyvraz, S. (1998) Extensive phenotypic analysis of CD34 subsets in successive collections of mobilized peripheral blood progenitors. *British Journal of Haematology,* **103,** 618–629.

Reimann, K.A., O'Gorman, M.R., Spritzler, J., Wilkening, C., Sabath, D.E., Campbell, D.E. & the DAIDS/NIAID, New Technology Evaluation Group (2000) A multisite comparison of CD4 and CD8 T lymphocyte counting by single vs. multiple platform methodologies: evaluation of Beckman Coulter Flow Count Fluorospheres and TetraONE System. *Diagnostic Laboratory Immunology,* in press.

Schnizlein-Bick, C.T., Spritzler, J., Wilkening, C., Nicholson, J.K.A., O'Gorman, M.R. & the DAIDS/NIAID, New Technology Evaluation Group (2000) Evaluation of TruCount Absolute Count Tubes for determining CD4 and CD8 cell numbers and their comparison to conventional immunophenotyping. *Diagnostic Laboratory Immunology,* in press.

Strauss, K., Hannet, I., Engel, S., Shiba, A., Ward, D., Ullery, S., Jinguji, M.G., Valinsky, J., Barnett, D., Orfao, A. & Kestens, L. (1996) Performance evaluation of Facscount: a dedicated system for clinical cellular analysis. *Cytometry,* **26,** 52–59.

Sutherland, D.R., Anderson, L., Keeney, M., Nayar, R. & Chin-Yee, I. (1996) The ISHAGE guidelines for CD34+ cell determination by flow cytometry. *Journal of Hematotherapy,* **5,** 213–226.

Verwer, B.J.H. & Ward, D.M. (1997) An automated classification algorithm for ProCount flow cytometric acquisition and analysis. (Abstract). *Journal of Hematotherapy,* **6,** 169.

APPENDIX

The following centres participated in this study: France, E. Wunder and H. Sovalat (Hôpital Hasenrain, Mulhouse); Germany, M. Bornhäuser and U. Oelschlägel (Universitätsklinikum, Dresden), G. Rothe and S. Barlage (Klinikum der Universität Regensburg, Regensburg); Italy, R. Lemoli and D. Rondelli (Istituto Di Ematologia 'Seragnoli', Bologna), F. Lanza and S. Moretti (Ospedale S. Anna, Ferrara), R. Saccardi and R. Caporale (Azienda Ospedaliera Careggi, Firenze), R. Cairoli and B. Brando (Ospedale Niguarda-Ca' Granda, Milan), M. Bregni and S. Rossini (H. San Raffaele, Milan), M. Danova and G. Comolli (Oncologia Medica e Biotecnologie, IRCCS Policlinico S. Matteo, Pavia), G. Pagnucco and L. Vanelli (Ematologia, IRCCS Policlinico S. Matteo, Pavia), A. Tabilio and D. Alfonsi (Policlinico Monteluce, Perugia), F. Benedetti and D. De Sabata (Ospedale Policlinico, Verona); The Netherlands, G. J. Schuurhuis and G. Westra (Academisch Ziekenhuis Vrije Universiteit), F. Preijers and M. Leenders (Academisch Ziekenhuis St. Radboud, Nijmegen), M. B. van 't Veer and J. Kraan (Academisch Ziekenhuis-Daniel den Hoed Kliniek, Rotterdam), E. Braakman and P. van Geel (Academisch Ziekenhuis-Erasmus Universiteit, Rotterdam); Spain, J. J. Lahuerta and M. A. Montalban Pallares (Hospital Universitario 12 de Octubre, Madrid), A. Orfao and P. Menéndez (Hospital Universitario de Salamanca, Salamanca); Switzerland, A. Huber and L. Stempora (Kantonsspital Aarau, Aarau); UK, S. Maclennan and A. Lubenko (Leeds Blood Centre, Leeds), S. Richards and A. Rawstron (Leeds General Infirmary, Leeds), N. H. Russell and C. Stainer (Nottingham City Hospital, Nottingham), J. T. Reilly and I. Storie (Royal Hallamshire Hospital, Sheffield).