Darbepoietin-alfa has comparable erythropoietic stimulatory effects to recombinant erythropoietin whilst preserving the bone marrow microenvironment

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ABSTRACT

Erythropoiesis stimulating agents are widely used for the treatment of anemia. Recently, we reported erythroid expansion with impaired B lymphopoiesis and loss of trabecular bone in C57BL/6 mice following ten days of treatment with low-dose short acting recombinant human erythropoietin. We have assessed erythropoietin against longer-acting darbepoietin-alfa at a comparable erythroid stimulatory dosage regime. Darbepoietin-alfa and erythropoietin induced similar *in vivo* erythropoietic expansion. Both agents induced an expansion of the colony-forming unit-erythroid populations. However, unlike erythropoietin, darbepoietin-alfa did not impair bone marrow B lymphopoiesis. Strikingly the bone loss observed with erythropoietin was not apparent following darbepoietin-alfa treatment. This analysis demonstrates that whilst darbepoietin-alfa has similar *in vivo* erythropoietic potency to erythropoietin, it preserves the bone marrow microenvironment. Thus erythropoietin and darbepoietin-alfa manifest different action showing that erythropoiesis stimulating agents have differential non-erythroid effects dependent on their duration of action.

Introduction

Erythropoietin (Epo) is a 34kDa glycoprotein essential for erythropoiesis.[1] Since 1986, a variety of recombinant forms of erythropoietic stimulating agents (ESAs) have been used widely for the treatment of anemia.[1,2] The most widely used form is recombinant human erythropoietin (rhEpo), a full-length unmodified Epo polypeptide identical to endogenous Epo with a short half-life requiring frequent administration.[3] Long-acting preparations have been introduced allowing for less frequent administration. Darbepoietin-alfa (darbepoietin-α, Darbo) was the first long-acting preparation approved for clinical use.[4,5] It was prepared with specific modifications including 5 substitutions (Asn-57, Thr-59, Val-114, Asn-115 and Thr-117) creating 2 new glycosylation sites conferring a 3-4 times longer half-life than rhEpo.[6] No major differential effects have been reported between the preparations to date. Other ESAs including the long-acting pegylated preparation and newer forms linked to polymers (e.g. hematide) are also in clinical trials.[3,7]

While its essential role in erythropoiesis has been widely described, several non-hematologic effects of Epo have also been recently reported.[1,8] Within the bone marrow microenvironment, elevated levels of Epo caused changes including non-erythroid hematologic effects and an alteration of bone homeostasis.[9-11] Recently, we reported erythropoietic expansion with impaired bone marrow B lymphopoiesis and a rapid loss of trabecular bone in C57BL/6 mice following ten days of alternate day treatment with low-dose rhEpo (~300U/kg).[10] Here we report that administration of darbepoietin-alfa in a once per week regimen consistent with its use in humans, compared to once a week rhEpo treatment, does not impact on B lymphopoiesis or bone homeostasis. Therefore, different ESAs can exert significant differential non-erythroid effects that may be attributed to their specific modifications or the duration of their action *in vivo*.

Design and Methods

Experimental mice

Nine-week old male C57Bl/6 mice (A.R.C., WA, Australia) were injected intra-peritoneally (ip) with PBS control, 200 μg/kg (2400 U/kg) of recombinant human Epo (Janssen Cilag, epoitin alfa) or 6.25 μg/kg darbepoietin alfa (Amgen, Aranesp) once a week.[12] Peripheral blood (PB), bone marrow (BM), spleen and bone analysis was carried out at ten days post treatment. All experiments were performed with the approval of St Vincent's Health Melbourne institutional ethics committee.

Cell preparations and flow cytometry analysis

PB was analyzed on a blood cell analyzer (Sysmex KX-21N, Roche Diagnostics, Australia). Bones were flushed, spleens crushed and strained through a 40-μm cell strainer (BD Biosciences, NSW, Australia). Flow cytometry antibodies and color conjugates were as previously described.[10] Cells were analyzed on an LSRFortessa Cell Analyzer (BD). Results were analyzed with FlowJo software version 9 (Tree Star, Ashland, OR, USA).

CFU-E and BFU-E analysis

For CFU-E and BFU-E assays, 50,000 cells/mL for bone marrow and 100,000 cells/mL for spleen were plated in MethoCult M3334 methylcellulose based media (Stem Cell Technologies, Canada) as

 doi:10.3324/haematol.2012.078709
Manuscript received on September 30, 2012. Manuscript accepted on December 10, 2012.
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described by the manufacturer. CFU-E were counted on Day 2 and BFU-E at Day 6.

Bone analysis

Micro-computed tomography (μCT) analysis was performed according to standard procedures in the secondary spongiosa of the proximal tibia using Skyscan1076 (X-ray potential 50KVp, Kontich, Belgium)[13] as previously described.[10]

Statistical analysis

Data were analyzed using the paired two-tailed Student's t-test; $P<0.05$ was considered significant. All data are presented as mean±S.E.M.

Results and Discussion

Darbepoietin-alfa has similar in vivo erythropoietic potency and induces lower extramedullary erythropoiesis than rhEpo

To examine whether the effects on the bone marrow microenvironment previously observed with frequently administered rhEpo treatment[10] also occurred with long-acting preparations of Epo, we injected 9-week old male C57BL/6 mice with PBS (control), rhEpo or darbepoietin-alfa at a previously defined comparable once a week treatment schedule (Figure 1A).[12] As expected, analysis after ten days showed that both rhEpo and darbepoietin-alfa treatment induced a significant increase in peripheral blood (PB) red blood cells with an accompanying elevation in hemoglobin and hematocrit (Figure 1B and C). Consistent with previous reports,[4,15] the level of increase in PB parameters with rhEpo or darbepoietin-alfa using this once a week dosage regime was equivalent indicating a similar *in vivo* potency of both preparations for inducing erythropoiesis.

Total BM cellularity remained unchanged with either treatment (*data not shown*). As assessed by Ter119/CD71 surface markers (Figure 1D) both preparations induced a reduction in $Ter119^{+}/CD71^{med}$ expressing erythroblasts and an expansion of the $Ter119^{+}/CD71^{hi}$ population (Figure 1E). Analysis of bone marrow erythroid progenitors by FACS[14,16] (Figure 1F) indicated that the major effect of rhEpo and darbepoietin-alfa was an over 50% expansion of the pre-CFU-E phenotypic fraction (Figure 1G). The darbepoietin-alfa treated cohort also had a significant increase in the preMegE phenotypic fraction. Colony forming assays confirmed the increase in CFU-E in both the rhEpo-treated and darbepoietin-alfa treated bone marrow, with no significant increase in the more mature BFU-E population in either group (Figure 1H). The differences in the erythroid progenitor response may be due to duration of stimulation or differential requirements for erythroid progenitors stimulated by each agent.

Consistent with our previous report administering low-dose rhEpo,[10] weekly high-dose rhEpo was accompanied by extensive extra-medullary erythropoiesis in the spleen. Spleen weight and cellularity were increased (Figure 1I) with expansions in all erythroid populations (Figure 1J). However, there was a trend toward lower extra-medullary erythropoiesis as assessed by splenic weight, splenic cellularity and erythroid populations in the spleen with darbepoietin-alfa compared to rhEpo. CFU-E and BFU-E numbers were, as anticipated, elevated in both rhEpo-treated and darbepoietin-alfa treated groups, but to a significantly greater extent in rhEpo-treated animals (Figure 1K), further supporting the concept that darbepoietin-alfa induces a lesser increase in extramedullary erythropoiesis than rhEpo. Together, the data suggested that erythroid expansion stimulated by darbepoietin-alfa treatment was accommodated more completely in the BM compared to extensive splenic erythropoiesis following rhEpo treatment.

B lymphopoiesis is largely unaffected by darbepoietin-alfa treatment

As seen with low-dose rhEpo treatment, high-dose rhEpo impaired BM B lymphopoiesis. Detailed fractionation of B-cell progenitors (Figure 2A and B) revealed major reductions in the immature $B220^{+}IgM^{-}$ fraction. Further fractionation of B lymphopoiesis revealed that there was an impairment of differentiation at the pre-B stage of maturation (Figure 2C and D). Unexpectedly, bone marrow B lymphopoiesis was unaffected by darbepoietin-alfa treatment. There were no changes in pre-B cell populations or the total numbers of immature $B220^{+}IgM^{-}$ cells. However, we did observe a significant decrease in the circulating mature $B220^{+}IgM^{+}$ population compared to control (Figure 1B).

Darbepoietin-alfa does not induce bone remodeling

We have reported that low-dose rhEpo resulted in a rapid loss of trabecular bone.[10] Similarly, high-dose once weekly rhEpo induced a rapid loss of trabecular bone as assessed by microCT with reductions in both bone volume and trabecular number evident and a commensurate increase in trabecular separation (Figure 3A-F). These changes were absent following darbepoietin-alfa treatment with levels of bone comparable to that of control-treated mice (Figure 3A-F).

Taken together, our data indicate that darbepoietin has similar *in vivo* potency to rhEpo as an erythroid-expanding agent. However, unlike rhEpo, darbepoietin-alfa does not cause changes in bone marrow B lymphopoiesis or bone homeostasis. The exact mechanism of how these differential effects occur remains to be clarified. It has been shown that Epo-receptor (Epo-R)-mediated endocytosis is possibly the main form of metabolism for both rhEpo and darbepoietin-alfa.[17] As darbepoietin-alfa has a low affinity of the Epo-receptor (Epo-R), it would be internalized and degraded more slowly than Epo resulting in sustained increase in serum levels.[18] In contrast, higher affinity with rapid uptake and degradation of rhEpo would lead to pulsatile increments. Therefore, it is possible that the non-erythroid effects are caused by pulsatile increments of Epo in contrast to sustained actions of darbepoietin-alfa. A precedent for the differential effects of a pulsatile compared to sustained activation model can be seen in the actions of parathyroid hormone on bone homeostasis. Pulsatile PTH is anabolic whereas continuous infusion (or hyperparathyroidism) results in net catabolism and low bone mass.[19]

Becker *et al*. have shown that the Epo-R levels are not ligand-dependent and are responsive to wide variations in ligand availability.[20] Thus, we do not expect any differences in Epo-R levels to be a contributory factor for the differential effects. Recently, it was reported that long-acting pegylated Epo causes the same intracellular signaling events to occur as rhEpo.[21] Therefore, it would be antici-

pated that darbepoietin-alfa has a similar Epo-R regulated transcriptome to that of rhEpo[22] and consider the duration of stimulation to be more important for the differential effects. We have previously demonstrated that osteoblasts do not respond to Epo treatment and do not express Epo-R.[10] However, Rankin *et al.* has proposed that *de novo* production of Epo by osteoblasts through HIF signaling pathway could directly modulate erythropoiesis.[23] Thus, it

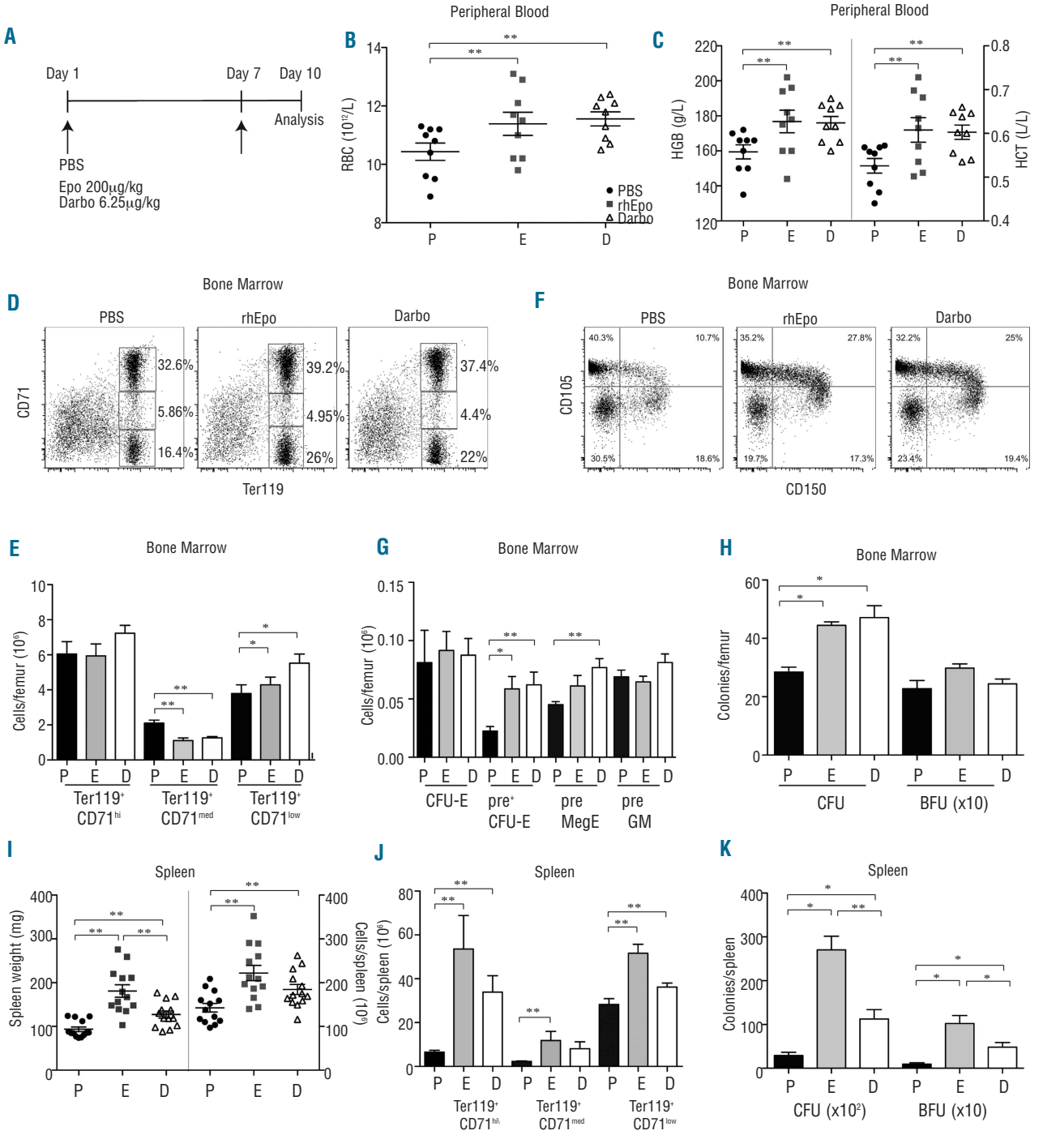

Figure 1. Darbepoietin-alfa has similar *in vivo* potency with unaffected B lymphopoiesis and lower extra-medullary stress erythropoiesis than recombinant human erythropoietin. (A) 9-week old C57Bl/6 mice were treated with once per week high-dose rhEpo or Darbepoietin-α and analyzed 10 days after the first injection (n=9 per treatment; 3 independent experiments of 3 per group). (B) Red blood cell count, (C) hemoglobin and hematocrit in PB 10 days post treatment are shown. (D) Representative FACS plots of erythroid fractions using CD71/Ter-119; (E) quantitation of erythroid differentiation in the bone marrow using CD71 and Ter119; (F-G) Analysis of myeloerythroid progenitor fractions based on CD105/CD150 staining as described by Pronk *et al.*[14] (H) CFU-E and BFU-E in the bone marrow (n=4 per group). (I) Spleen weight and cellularity (n=13 per group). (J) Erythroid populations in spleen based on CD71/Ter-119 staining (n=9 per group). (K) and CFU-E and BFU-E numbers in the spleen (n=4 per group). Data are represented as mean±SEM. **P*<0.05, ***P*<0.01.

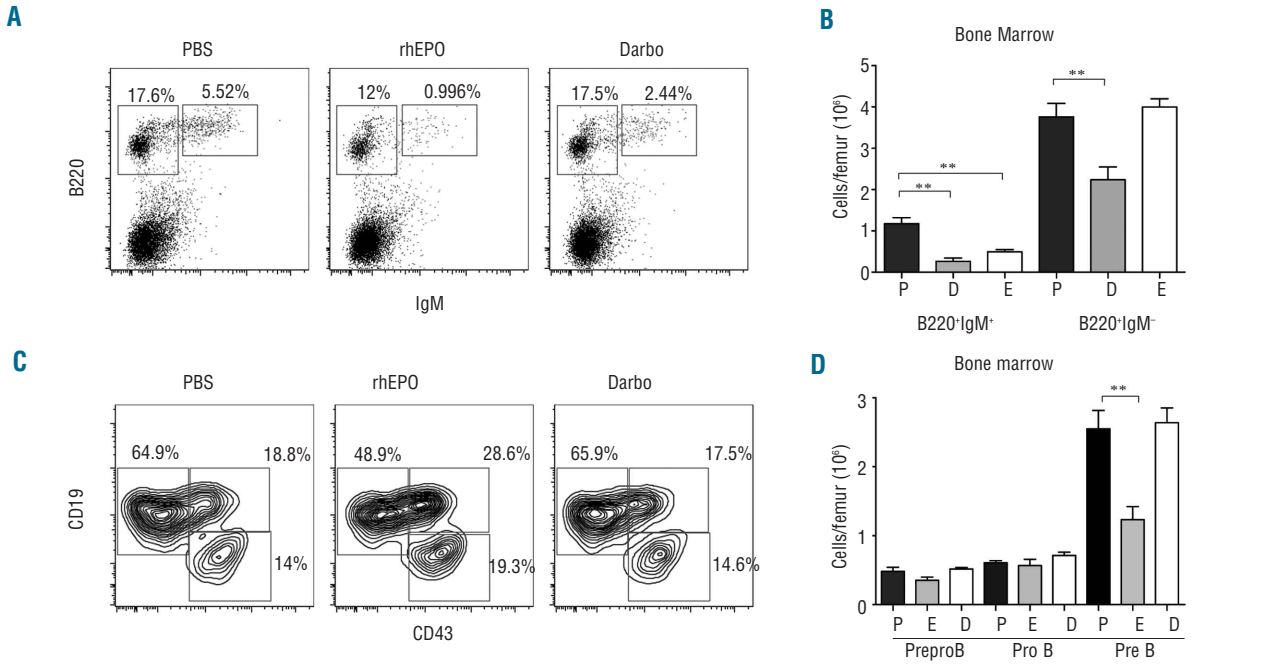

Figure 2. Largely unaffected B lymphopoiesis with Darbepoietin-α treatment. (A-B) B lymphopoiesis after rhEpo and Darbo treatment analyzed by B220 and IgM expression; (n=9 per group). (C-D) B-cell progenitor populations analyzed by FACS using CD43 and CD19 expression. Representative FACS plots and quantitation of Pre-ProB, Pro-B and Pre-B fractions are indicated. FACS data representative from 3 independent experiments. Data are represented as mean±SEM; (n=9 per group). *P<0.05, **P<0.01.

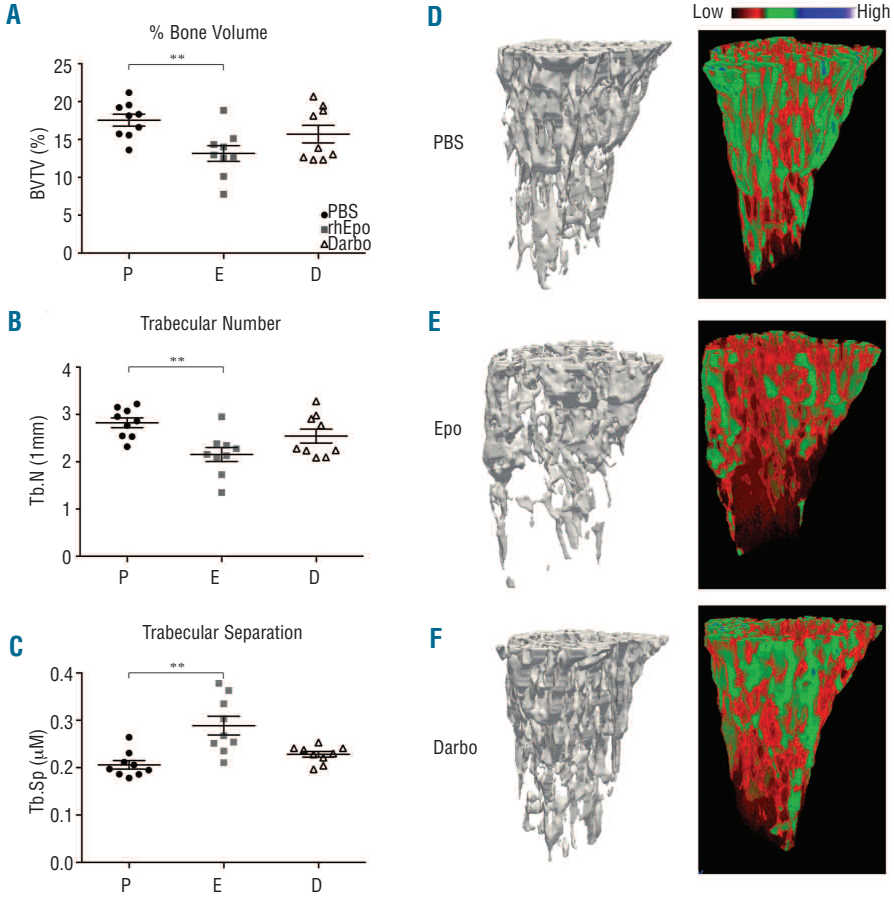

Figure 3. Darbepoietin-alfa treatment does not cause bone loss compared to rhEpo. Three-dimensional μCT analysis of the secondary spongiosa of proximal tibia. (A) Quantitation of total bone volume (Bone Volume/Total Volume; %), (B) trabecular number (number of trabecular per mm) and (C) the distance between the trabeculae (trabecular separation; μm) from PBS – Epo- and Darbo-treated mice respectively (n=9 per group; 3 independent experiments of 3 per group). Data represented as mean±SEM. (D-F) Representative images (CTvol) of the trabecular bone within the secondary spongiosa and color-coded quantitative mineralization paraview images of trabecular bone in each treatment category. Red indicates areas of lowest mineral density and blue represents regions of highest mineral density. *P<0.05, **P<0.01.

would be of interest to find whether pulsatile compared to sustained increase in serum Epo levels would have differential effects on activation of these pathways that couple osteogenesis and erythropoiesis. Regardless of the exact mechanism, our data are of direct relevance to the application of ESAs and further indicate significant involvement of regulatory mechanisms coupling erythropoiesis, B lymphopoiesis and osteogenesis within the bone marrow microenvironment.

Acknowledgments

The authors would like to thank L. Purton, M. Wall, N. Walsh, V. Sankaran and J. Martin for comments and discussion, SVH BioResources Centre, T. Jovic and E. DeLuca for technical assistance.

Funding

This work was supported by grants from the Leukaemia Foundation, National Health and Medical Research Council of Australia (NHMRC to CW) NHMRC Career Development Award (CW); in part by the Victorian State Government Operational Infrastructure Support Program (to St. Vincent's Institute). CW is the Philip Desbrow Senior Research Fellow of the Leukaemia Foundation.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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