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### Permalink

<https://escholarship.org/uc/item/6460j1q8>

### Journal

British Journal of Haematology, 160(3)

### ISSN

0007-1048

### Authors

Walter, Patrick B  
Porter, John  
Evans, Patricia  
[et al.](#)

### Publication Date

2013-02-01

### DOI

10.1111/bjh.12076

Peer reviewed



# HHS Public Access

Author manuscript

*Br J Haematol.* Author manuscript; available in PMC 2015 October 08.

Published in final edited form as:

*Br J Haematol.* 2013 February ; 160(3): 399–403. doi:10.1111/bjh.12076.

## Increased leucocyte apoptosis in transfused $\beta$ -thalassaemia patients

Patrick B. Walter<sup>1,2</sup>, John Porter<sup>3</sup>, Patricia Evans<sup>3</sup>, Janet L. Kwiatkowski<sup>4</sup>, Ellis J. Neufeld<sup>5</sup>, Thomas Coates<sup>6</sup>, Patricia J. Giardina<sup>7</sup>, Robert W. Grady<sup>7</sup>, Elliott Vichinsky<sup>1</sup>, Nancy Olivieri<sup>8</sup>, Felicia Trachtenberg<sup>9</sup>, Daniele Alberti<sup>10</sup>, Ellen Fung<sup>1</sup>, Bruce Ames<sup>1</sup>, Annie Higa<sup>1</sup>, Paul Hartz<sup>1,\*</sup>, and Thalassemia Clinical Research Network

<sup>1</sup>Children's Hospital & Research Center Oakland, Oakland, CA, USA

<sup>2</sup>Department of Biology, University of Victoria, Victoria, Canada

<sup>3</sup>University College London, London, UK

<sup>4</sup>Children's Hospital of Philadelphia, Philadelphia, PA

<sup>5</sup>Children's Hospital Boston, Boston, MA

<sup>6</sup>Children's Hospital Los Angeles, Los Angeles, CA

<sup>7</sup>New York-Presbyterian Hospital, New York, NY, USA

<sup>8</sup>University Health Network, Toronto, ON, Canada

<sup>9</sup>New England Research Institutes, Watertown, MA

<sup>10</sup>Novartis Pharmaceuticals Corporation, East Hanover, NJ, USA

### Summary

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Correspondence: Paul Hartz, Children's Hospital & Research Center Oakland, 747 52nd Street, Oakland, CA 94609, USA. [phartz@mail.cho.org](mailto:phartz@mail.cho.org).

This is publication number eleven of the Thalassemia Clinical Research Network (TCRN).

A list of TCRN member institutions and staff appears in Appendix I.

#### Author contributions

PBW: designed and performed the research, analysed the data and wrote the paper; JP: designed the research, recruited and administered procedures to patients and wrote the paper; PE: designed and performed the research and wrote the paper; JLK: designed the research, recruited and administered procedures to patients and wrote the paper; EJV: designed the research, recruited and administered procedures to patients and wrote the paper; TC: designed the research, recruited and administered procedures to patients and wrote the paper; PJG: designed the research, recruited and administered procedures to patients and wrote the paper; RWG: designed and performed the research, analysed and the data; EV: designed the research, recruited and administered procedures to patients and wrote the paper; NO: designed the research, recruited and administered procedures to patients and wrote the paper; FT: designed the statistics, analysed the data and wrote the paper; DA: designed the research and wrote the paper; EF: analysed the data and wrote the paper; AH: performed the research and analysed the data; BA: helped with data acquisition, analysis and interpretation of data and wrote the paper; PH: designed the research, recruited and administered procedures to patients, analysed the data and wrote the paper.

#### Disclosures

PBW has received research support from Novartis Pharmaceuticals Corp. PH has received an educational grant and research support from Novartis Pharmaceuticals Corp.; EV, JP and PJG received research funding from Novartis Pharmaceuticals Corp.; TC has received funding and honoraria from Novartis Pharmaceuticals Corp.; DA is an employee of Novartis Pharmaceuticals Corp. All authors gave final approval for the manuscript to be published.

This exploratory study assessed apoptosis in peripheral blood leucocytes (PBL) from  $\beta$ -thalassaemia patients receiving chronic transfusions and chelation therapy (deferasirox or deferoxamine) at baseline, 1, 6, and 12 months. At baseline, thalassaemic PBLs presented 50% greater levels of Bax (BAX), 75% higher caspase-3/7, 48% higher caspase-8 and 88% higher caspase-9 activities and 428% more nucleosomal DNA fragmentation than control subjects. Only neutrophils correlated significantly with apoptotic markers. Previously, we showed that over the treatment year, hepatic iron declined; we now show that the ratio of Bax/Bcl-2 (BCL2), ( $-27.3\%$ /year), and caspase-9 activity ( $-13.3\%$ /year) declined in both treatment groups, suggesting that chelation decreases body iron and indicators of PBL apoptosis.

## Keywords

apoptosis; Bax; caspase; oxidant-stress; inflammation

Frequent transfusions in  $\beta$ -thalassaemia major patients cause cyclic changes in haemoglobin and increased non-transferrin-bound iron (NTBI). Despite improved chelation therapies there is risk for iron overload, organ failure, infection, oxidative stress and chronic inflammation, resulting in elevated levels of pro- and anti-inflammatory proteins such as tumour necrosis factor  $\alpha$ , interleukin (IL)2, 10 and high sensitivity C-reactive protein (hsCRP) (Del Vecchio *et al*, 2002; Walter *et al*, 2008). These proteins participate in response and resolution to infection and tissue injury by balancing leukocyte proliferation and apoptotic cell death. Thalassaemia patients are chronically immuno-stimulated by transfusions, NTBI, chelation and organ injury and without resolution of inflammation, there is chronic inflammatory protein production. Some of these proteins are proliferative and some are resolving (apoptotic) in nature. Persistence or lack of immune cells at inflammatory sites or the development of chronic inflammation (Scheel-Toellner *et al*, 2004) observed in  $\beta$ -thalassaemia patients may result from dysregulation of the apoptotic cell death pathway. Furthermore, increased apoptosis reduces neutrophil function (Whyte *et al*, 1993) and could contribute to increased infection risk in thalassaemia patients.

Apoptosis initiated extrinsically by death receptor ligation eventually activates procaspase-8 (Scheel-Toellner *et al*, 2004), while increases in Bax (BAX) and/or decreases in Bcl-2 (BCL2) intrinsically activates the mitochondrial pathway (Brenner & Mak, 2009) with eventual activation of caspase-9. The balance between Bax and Bcl-2 forms an apoptotic rheostat that is regulated by oxidants (Korsmeyer *et al*, 1993). Executioner caspases 3 and 7 subsequently activate endonucleases cleaving DNA into nucleosomal fragments. Other pro-apoptotic stimuli include reactive oxygen species (ROS), DNA damage, and anti-inflammatory cytokines, such as IL10 (Turina *et al*, 2007).

Thalassaemia patients have increased markers for ROS (such as malondialdehyde, MDA), and inflammation (such as CRP and IL10) and these levels of MDA and CRP can be controlled by chelation (Walter *et al*, 2008). Iron overload increases leucocyte mitochondrial dysfunction (Walter *et al*, 2002) and may also be implicated in increased apoptotic mechanisms (Ichii *et al*, 2012). Young paediatric thalassaemia patients (<5 years, not chronically transfused), showed no increases in neutrophil apoptosis (Ören *et al*, 2003),

however the impact of chronic iron burden and chelation on the expression of leucocyte apoptotic markers in older, chronically transfused thalassaemia patients has not been determined.

This study evaluated six markers of peripheral blood leucocyte (PBL) apoptosis in thalassaemia compared with control subjects, to assess whether improved control of iron overload with deferasirox or deferoxamine was associated with changes in the level of apoptotic markers.

## Materials and methods

### Patients and study design

Details of this ancillary study of the phase III deferasirox trial, (CICL670A0107) and trial subjects have been described (Walter *et al*, 2008). The study was approved by the institutional review board at each site and conducted under supervision of a Data and Safety Monitoring Board convened by the National Heart, Lung, and Blood Institute. Forty-nine  $\beta$ -thalassaemia subjects (from seven Thalassaemia Clinical Research Network sites) (28 male, age =  $22.4 \pm 10.7$ , range 3–42 years), weighing  $72 \pm 12$  kg, with chronic iron overload [liver iron concentration (LIC)  $2.5 \pm 1.2$  mg Fe/g dw] from blood transfusions ( $8$  l/year) were randomized to receive deferasirox ( $n = 24$ ) or deferoxamine ( $n = 25$ ). Thirty controls (15 male,  $24.5 \pm 9.0$  years) frequency-matched for age, sex, race and vitamin supplement intake were enrolled for a single visit. All participants provided written informed consent.

### Laboratory analyses

Fasting blood samples were collected from subjects that had abstained from medications, chelators and nutritional supplements for the previous 24 h, at 1, 6, and 12 months. Peripheral blood leucocytes were isolated from Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA) gradients and the levels of Bax and Bcl-2 determined by enzyme immunometric assay (Assay Designs, Ann Arbor, MI, USA). Activity of caspase-3/7, -8 and -9, determined by luminescent enzyme activity assay (Caspase – Glo™ assay, Promega, Madison, WI, USA), is reported as average relative light units (RLU)/ $\mu$ g PBL protein, or RLU was converted to ng by comparison to a standard curve of recombinant caspase (BioVision, Mountain view, CA, USA). Nucleosomal DNA fragmentation was determined by enzyme-linked immunosorbent assay (Roche, Indianapolis, IN, USA). Vitamin E, iron measures, CRP, cytokines, LIC, NTBI and ferritin were measured as described (Walter *et al*, 2008).

### Statistics

Analysis of covariance (ANCOVA) and repeated measures ANCOVA evaluated associations controlling for age, gender, race, treatment and dose, illness (previous 2 weeks), and baseline LIC.  $P < 0.05$  was considered statistically significant. Outcomes were log-transformed in models, but back-transformed for figures. Outliers were removed (visual inspection) and associations were modelled by repeated measures regression with subject-specific intercepts and slopes. Fisher's exact test and  $t$ -test compared demographics.

## Results and discussion

Thalassaemic PBLs had increased levels of Bax ( $P = 0.006$ ), no change in Bcl-2 ( $P > 0.05$ ), but greater caspase-3/7 ( $P = 0.01$ ), -8 ( $P = 0.01$ ), and -9 ( $P = 0.001$ ) activities compared to controls (Fig 1A–C). Both neutrophils and lymphocytes are known to increase the translocation of pro-apoptotic Bax to mitochondria during intrinsic apoptosis (Scheel-Toellner *et al*, 2004; Brenner & Mak, 2009), but in our study only neutrophils correlated with Bax and other tested apoptotic markers (Table I). Neutrophils are sensitive to redox changes and upregulate Bax on exposure to increased oxidants (Geering & Simon, 2011). In parallel with our finding of increased Bax expression, others have reported elevated Bax and apoptosis in neutrophils during infection (Perskvist *et al*, 2002), which could reflect high levels of neutrophil turnover during inflammation or exposure to oxidants. Enhanced exposure of thalassaemic PBLs to NTBI could increase intracellular iron and cause nuclear oxidative DNA damage. This could upregulate p53 (TP53) leading to the observed increase in Bax expression. A high ratio of Bax/Bcl-2 indicates decreased stability of the mitochondrial outer membrane and increased potential for mitochondrial dysfunction and increased oxidant production. In the one year follow-up, the Bax/Bcl-2 ratio decreased 27.3%/year, (from 6.16 to 5.28,  $P = 0.033$ , Fig 1D) during treatment with either deferoxamine or deferasirox in parallel with decreased body iron burden. Given that we previously found enhanced markers for circulating levels of lipid peroxidation (MDA) (Walter *et al*, 2008) and now find enhanced expression of Bax and increased caspase-9 activity, our data also supports the work of others who showed that the Bax/Bcl2 ratio may constitute an ‘apoptotic rheostat’, which determines susceptibility to oxidant-induced cell death (Korsmeyer *et al*, 1993; Perskvist *et al*, 2002). Thus, the shift in balance of the Bax-Bcl-2 ratio may determine neutrophil fate, and although some doubt Bcl2 expression in neutrophils, recent reports have confirmed the expression of Bcl2 in neutrophils (Sarkar *et al*, 2012). What may be relevant to thalassaemic neutrophils is the increased expression of Bax, possibly because of their exposure to NTBI and oxidants. Also suggestive of the role of iron and oxidants in thalassaemic PBL apoptosis are the correlations of caspase-8 with transferrin saturation,  $\gamma$ -tocopherol with caspase-3/7 and  $\alpha$ -tocopherol and hsCRP with nucleosomal DNA fragmentation (Table I).

Caspase-9 activity also declined during treatment (13.3%/year,  $P = 0.049$ , Fig 1E), from 19.2 to 17.9 ng/ $\mu$ g protein for both chelators (control levels = 14.9 ng/ $\mu$ g). Bax (and Bax/Bcl-2) and caspase-9 (and -8) were amongst our strongest correlations (Table I), which has also been found in other studies in both healthy neutrophils and lymphocytes during inflammation (Scheel-Toellner *et al*, 2004). Caspase-9 activity has also been found to be closely associated with changes in the expression of Bax (Scheel-Toellner *et al*, 2004). Another possible mechanism for enhanced apoptosis in thalassaemic PBLs concerns the high levels of IL10 that we have previously shown in the same study population (Walter *et al*, 2008). IL10 is an important regulatory anti-inflammatory cytokine involved in the resolution of acute inflammatory and oxidative stress responses (Haddad & Fahlman, 2002). It also promotes neutrophil apoptosis during resolution of inflammation perhaps by enhancing caspase-8 activity (Turina *et al*, 2007).

In support of this possibility, we found enhanced caspase-8 activity in thalassaemic PBLs. Caspase-8 participates in extrinsic, receptor-mediated apoptosis. However, procaspase-8 can become activated not only by this extrinsically mediated pathway, but also by ROS (Scheel-Toellner *et al*, 2004) and/or possibly by IL10 (Turina *et al*, 2007). Thus, in thalassaemia patients, enhanced caspase-8 activity may be due to enhanced levels of ROS and/or anti-inflammatory cytokines. Thalassaemic PBLs also had increased caspase-3 activity relative to the controls, providing the final common pathway to both death receptor and mitochondria-dependent apoptotic mechanisms. Because we observed both the enhanced activity of caspase-9, and -8, our results suggest that both apoptotic pathways are stimulated during apoptosis of thalassaemic PBLs although we cannot rule out caspase-8 activated bid may be initiating the mitochondrial pathway. The increase in caspase-3 activity invokes the activity of downstream nucleases, as shown by the 5.3-fold elevation of nucleosomal fragmentation in patients compared to controls ( $P < 0.001$ , Fig 1C). Nucleosomal DNA fragmentation did not change during the one-year follow-up, however, Bax and the Bax/Bcl-2 ratio correlated with nucleosomal DNA fragmentation (Table I). This high level of nucleosomal DNA fragmentation confirms that circulating PBLs of thalassaemia patients had high levels of apoptotic proteins. This unusually high level of fragmentation is suggestive of reactive oxygen- or inflammatory-induced apoptosis occurring in thalassaemic PBLs.

We also explored the possible contribution of splenectomy and chelation to the enhanced levels of thalassaemic PBL apoptosis. 57% of the thalassaemia patients were splenectomized; however, analysis controlling for splenectomy showed no effect. Besides controlling iron load, it is also possible that the chelators themselves may affect PBL apoptosis; for example deferoxamine and deferiprone were found to reduce leucocyte cytokine production (Del Vecchio *et al*, 2002), although other iron chelators, such as lactoferrin, have recently been found to delay apoptosis (Francis *et al*, 2011). While we have seen an increase in apoptotic proteins in thalassaemic PBLs, we have not seen a decrease in any one type of leukocyte that is similar to that seen with infection. This may seem paradoxical; however the increase in apoptotic proteins without a decrease in cell number could be due in part to the unresolved inflammation present in the disease and may become another marker of chronic inflammation in thalassaemia.

In conclusion, this ancillary study found high levels of apoptotic markers in thalassaemic PBLs, including increased Bax expression, caspase activity and nucleosomal DNA fragmentation. Neutrophils were the only PBL type that consistently correlated with the measured apoptotic markers. In the one-year follow-up, iron chelation therapy with deferasirox or deferoxamine was equally effective in decreasing LIC (Walter *et al*, 2008), the ratio of Bax/Bcl-2 and caspase-9 activity. Thus, with effective chelation, it may be possible to decrease the PBL mitochondrial Bax/Bcl-2 ratio and the level of some pro-apoptotic proteins.

## Acknowledgments

This work was supported by the following NIH-NHLBI cooperative agreements: U01-HL65232 to Children's Hospital of Philadelphia, U01-HL65233 to University Health Network Toronto General Hospital, U01-HL65239 to Children's Hospital & Research Center Oakland, U01-HL65244 to Weill Medical College of Cornell University, U01-HL65260 to Children's Hospital Boston and U01-HL65238 to New England Research Institutes. The study

was also supported, in part, at Children's Hospital & Research Center Oakland by NIH-NIDDK grant R01-DK57778 and NIH-NCRR grant M01-RR01271, at Children's Hospital of Philadelphia by NIH-NCRR grant UL1-RR024134 and at Children's Hospital Boston by NIH-NCRR grant M01-RR02172.

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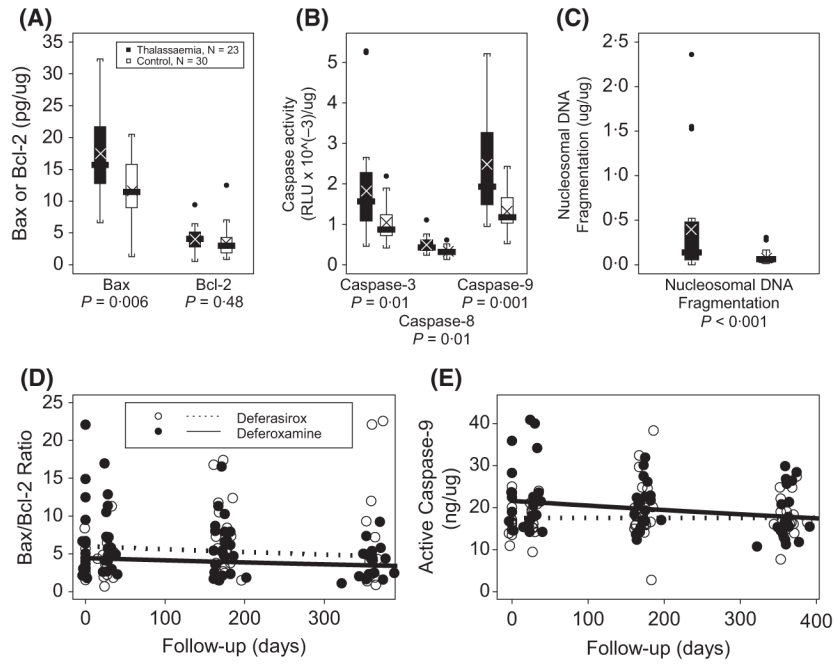
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## Appendix I

The following institutions and researchers contributed to the Thalassemia Clinical Research Network Mitochondrial Ancillary Study reported in this paper (listed in alphabetical order). Children's Hospital Boston: Ellis Neufeld, MD, PhD, Principal Investigator, Melody Cunningham, MD, Co-Principal Investigator, Jennifer Braunstein, RN, Study Coordinator, Joanna Hedstrom Study Coordinator. Children's Hospital of Philadelphia: Alan R. Cohen, MD, Principal Investigator, Janet L. Kwiatkowski, MD, Co-Principal Investigator, Catherine S. Manno, MD, Coinvestigator, Debra Hillman, Study Coordinator, Marie Martin, RN, Nurse Coordinator. Children's Hospital & Research Center Oakland: Elliott Vichinsky, MD, Principal Investigator, Sylvia Singer MD, Co-Principal Investigator, Paul Harmatz, MD, Co-Investigator, Patrick Walter, PhD, Co-Investigator, Nancy Sweeters RN PNP and Eun-Ha Pang, Study Coordinators, Dru Foote, RN PNP, Thalassemia Nurse, Annie Higa, Laboratory Technician; Satellite, Children's Hospital of Los Angeles: Thomas Coates, MD, Principal Investigator, Robert Weihing, MD, Ph.D. Research Associate, Kerry Wymbs, Study Coordinator. Toronto General Hospital: Nancy Olivieri, MD, Principal Investigator, Laura Merson, Giulia Muraca, Clinical Research Managers, Melissa Stamplecoski, Study Coordinator. University College London: John Porter, MD, Principal Investigator, Michelle Cummins, Study Coordinator, Patricia Evans, Research Associate. Weill Medical College of Cornell: Patricia J. Giardina, MD, Principal Investigator, Robert W. Grady, PhD, Co-Investigator, Dorothy Kleinert, NP, MPH, MA, Thalassemia Nurse, Jeffrey E. Mait, Study Coordinator. Network Steering Committee Chair: David Nathan, MD. National Heart, Lung, and Blood Institute: Charles Peterson, MD, Project Officer. Data Coordinating Center: New England Research Institutes, Inc., Libby Wright, PhD, and Sonja McKinlay, PhD, Principal Investigators, Eric Macklin, PhD, Co-Principal Investigator, Ellen McCarthy, Project Director.





**Fig. 1.** A–C (top) Baseline peripheral blood leucocyte levels of Bax (A); caspase activities (B) and nucleosomal DNA fragmentation (C). The values for thalassaemia patients are means of both chelator groups with the *P* values indicated for each comparison. The boxplots show the median value with box around the interquartile range with whiskers for range and dotted outliers. The symbol ‘x’ marks the mean value. D,E (bottom) Distributions of the Bax/Bcl-2 ratio (D) and the amount of active Caspase 9 (E) *versus* time stratified by treatment group (deferiasirox, open symbols; deferoxamine, filled symbols). Day zero (0) is the first day of treatment. Regression lines of the observed data are overlaid (deferiasirox ● – ● – ●; deferoxamine —), with no significant interaction between time and chelator (*P* = 0.98 for Bax/Bcl-2; *P* = 0.14 for Caspase-9).

**Table I**

Significant correlations between Biomarkers and: A – PBL Apoptotic biomarkers, B – Neutrophils, C – Inflammatory Indicator, D – Tocopherols and E – Transferrin saturation.

	<b>Biomarker</b>	<b>Correlation</b>	<b>P-value</b>
A. PBL Apoptotic biomarkers			
Caspase-9	Bax	0.45	<0.001
Caspase-8	Bax	0.47	<0.001
Nucleosomal DNA fragmentation	Bax	-0.37	<0.001
Caspase-3/7	Bcl-2	0.075	0.010
Caspase-9	Bax/Bcl-2 ratio	0.35	0.001
Caspase-8	Bax/Bcl-2 ratio	0.38	<0.001
Nucleosomal DNA fragmentation	Bax/Bcl-2 ratio	-0.18	0.007
B. Neutrophils			
ANC	Bax/Bcl-2 ratio	0.29	0.004
ANC	Bcl-2	-0.27	0.0035
ANC	Bax	0.21	0.045
ANC	Nucleosomal DNA fragmentation	0.19	0.04
ANC	Caspase-9	0.16	0.048
C. Inflammatory Indicator hsCRP			
	Nucleosomal DNA fragmentation	0.25	0.01
D. Tocopherols			
$\alpha$ -tocopherol	Nucleosomal DNA fragmentation	0.28	0.002
$\gamma$ -tocopherol	Caspase-3/7	-0.23	0.02
E. Transferrin saturation			
	Caspase-8	-0.24	0.010

PBL, peripheral blood leucocyte; ANC, absolute neutrophil concentration; hsCRP, high sensitivity C-reactive protein; Note: alanine transaminase, aspartate transaminase, malondialdehyde, Vitamin C, non-transferrin bound iron, liver iron concentration, ferritin,  $\gamma$ -interferon, tumour necrosis factor- $\alpha$ , interleukin (IL)5 and IL8 did not correlate with PBL apoptotic markers.