Inflammation and oxidant-stress in β-thalassemia patients treated with iron chelators deferasirox (ICL670) or deferoxamine: an ancillary study of the Novartis CICL670A0107 trial

Patrick B. Walter,[1] Eric A. Macklin,[2] John Porter,[3] Patricia Evans,[3] Janet L. Kwiatkowski,[4] Ellis J. Neufeld,[5] Thomas Coates,[6] Patricia J. Giardina,[7] Elliott Vichinsky,[1] Nancy Olivieri,[8] Daniele Alberti,[9] Jaymes Holland,[9] and Paul Harmatz,[1] for the Thalassemia Clinical Research Network

[1]Children's Hospital & Research Center Oakland, Oakland, CA,USA; [2]New England Research Institutes, Watertown, MA, USA (*current address: Massachusetts General Hospital, Boston, MA*); [3]University College London, London, UK; [4]Children's Hospital of Philadelphia, Philadelphia, PA; [5]Children's Hospital Boston, Boston, MA, USA; [6]Children's Hospital Los Angeles, Los Angeles, CA, USA; [7]New York-Presbyterian Hospital, New York, NY, USA; [8]University Health Network, Toronto, Canada and [9]Novartis Pharmaceuticals Corp., East Hanover, NJ, USA

This is publication number four of the Thalassemia Clinical Research Network (TCRN). A list of TCRN member institutions and staff is given in the Appendix.

Funding: this work was supported by the following NIH-NHLBI cooperative agreements: U01-HL65232 to the Children's Hospital of Philadelphia (The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center of Research Resources or the National Institutes of Health), U01-HL65233 to the University Health Network Toronto General Hospital, U01-HL65239 to the Children's Hospital & Research Center Oakland, U01-HL65244 to Weill Medical College of Cornell University, U01-HL65260 to the Children's Hospital Boston and U01-HL65238 to New England Research Institutes. The study was also supported, in part, at the Children's Hospital & Research Center Oakland, by NIH-NIDDK grant R01-DK57778 and NIH-NCRR grant M01-RR01271, at the Children's Hospital of Philadelphia by NIH-NCRR grant UL1-RR024134 and at the Children's Hospital Boston by NIH-NCRR grant M01-RR02172. Furthermore, we gratefully acknowledge the expert technical assistance of Esther Roitman in the GC-MS experiments.

Manuscript received May 22, 2007. Revised version arrived on December 21, 2007. Manuscript accepted January 21, 2008.

Correspondence:
Paul Harmatz, Children's Hospital & Research Center Oakland, 747 52nd Street, Oakland, Ca, 94609, USA. E-mail: pharmatz@mail.cho.org

ABSTRACT

Background
We assessed whether oxidant-stress and inflammation in β-thalassemia could be controlled by the novel oral iron chelator deferasirox as effectively as by deferoxamine.

Design and Methods
Forty-nine subjects were enrolled from seven sites and studied at baseline, and after 1, 6, and 12 months of therapy. Malondialdehyde, protein carbonyls, vitamins E and C, total non-transferrin bound iron, transferrin saturation, C-reactive protein, cytokines, serum ferritin concentration and liver iron concentration were measured.

Results
Liver iron concentration and ferritin declined significantly in both treatment groups during the study. This paralleled a significant decline in the oxidative-stress marker malondialdehyde (deferasirox -22%/year, deferoxamine -28%/year, average decline p=0.006). The rates of decline did not differ between treatment groups. Malondialdehyde was higher in both treatment groups than in a group of 30 non-thalassemic controls (p<0.001). The inflammatory marker high-sensitivity C-reactive protein decreased significantly only in the group receiving deferasirox (deferasirox -51%/year, deferoxamine +8.5%/year, p=0.02). This result was confounded by a chance difference in the level of high-sensitivity C-reactive protein between the two groups at baseline, but analyses controlling for this difference suggested an equally large treatment effect.

Conclusions
Iron chelation therapy with deferoxamine or with deferasirox was equally effective in decreasing iron burden and malondialdehyde. The possible differential effect of the two chelators on inflammation warrants further investigation.

Key words: iron overload, thalassemia, oxidative stress, inflammation, hsCRP, C-reactive protein, malondialdehyde, lipid peroxidation, vitamin E, vitamin C.

Citation: Walter PB, Macklin EA, Porter J, Evans P, Kwiatkowski JL, Neufeld EJ, Coates T, Giardina PJ, Vichinsky E, Olivieri N, Alberti D, Holland J, and Harmatz P for the Thalassemia Clinical Research Network. Inflammation and oxidant-stress in β-thalassemia patients treated with iron chelators deferasirox (ICL670) or deferoxamine: an ancillary study of the Novartis CICL670A0107 trial. Haematologica 2008 June; 93(6):817-825.
doi: 10.3324/haematol.11755

©2008 Ferrata Storti Foundation. This is an open-access paper.

Introduction

The outlook for patients with thalassemia has improved in recent decades with the use of modern transfusion practice and iron chelation, but patients continue to be at high risk for iron overload and its toxicities.[1,3] Iron-related cardiac disease remains the most common cause of death in patients with thalassemia.[1] More than 70% of adult patients suffer from hypogonadism, osteoporosis, and other endocrine disorders.[4] Long-term chelation therapy with deferoxamine lowers iron burden and reduces or prevents end-organ toxicity in compliant patients.[3] However, deferoxamine is administered parenterally, over 8-12 hours or more per day - a difficult regimen which limits patients' compliance.[5] Furthermore, the short half-life of deferoxamine in the body after stopping an infusion results in a prolonged period each day when non-transferrin bound iron (NTBI) can be detected at elevated levels in the circulation.[6,7] NTBI has been suggested to be toxic to the heart and other iron-susceptible tissues.[8,9] Deferasirox (ICL670, Exjade®, Novartis) is an orally administered tridentate iron chelator with a relatively long circulating half-life of 8 to 16 hours[10,11] and documented cellular permeability.[12,13] Deferasirox was approved by the Food and Drug Administration, in late 2005, for treatment of transfusional iron overload in chronic anemias (this approval can be found at: *http://www.fda.gov/bbs/topics/news/2005/NEW01258.html*). During the pivotal Novartis-sponsored phase III randomized, open-label comparison of deferasirox *vs.* deferoxamine,[11] the National Institutes of Health (NIH)-sponsored Thalassemia Clinical Research Network (TCRN) initiated an ancillary study to determine whether iron-induced oxidant-stress and inflammation in β-thalassemia, as precursors to cell and organ injury, could be controlled by deferasirox as effectively as or better than by deferoxamine through analysis of body iron burden and biomarkers of oxidative stress and inflammation. Biomarkers of oxidant stress included plasma malondialdehyde (a marker of lipid peroxidation[14-17]) and plasma protein carbonyls, a marker of oxidation to circulating proteins. Inflammatory biomarkers were cytokines (including interleukin-6) and high-sensitivity C-reactive protein (hsCRP), markers previously found useful in thalassemia[15,18,19] and other disease states including heart disease and diabetes.[20-22]

Design and Methods

Study design

The study was ancillary to the pivotal phase III Novartis CICL670A0107 trial, a randomized, open-label comparison of deferasirox, an oral iron chelator, *vs.* deferoxamine.[11] Participants in the Novartis CICL670A0107 trial weighing at least 12 kg and co-enrolled in the TCRN, an NIH-sponsored consortium of North American and UK thalassemia clinical research centers, were eligible. Of 586 participants from 65 sites in 12 countries in the Novartis CICL670A0107 trial, 66 thalassemia patients from seven sites in three countries were eligible for the ancillary study of whom 49 (28 male, 22.4±10.7 years [mean age±SD], range 3 to 42 years) were enrolled. An additional 30 healthy controls (15 male, 24.5±9.0 years) frequency matched for age (in 5-year intervals), sex, race and use of vitamin supplements were enrolled for a single visit. The study was approved by the institutional review board at each site and conducted under the supervision of a Data and Safety Monitoring Board convened by the National Heart, Lung, and Blood Institute of the NIH. All participants provided written, informed consent.

Fasting blood samples were collected at the following times: (i) after a 5-day washout of deferoxamine prior to commencing treatment with either deferasirox (average dose 18.6±7.6 mg/kg/day) or deferoxamine (average dose 46.8±8.8 mg/kg/day), and (ii) 24 hours post-chelator and antioxidant supplementation at 1, 6, and 12 months on study. Because the Novartis CICL670A0107 trial initiated enrollment prior to approval of the ancillary study at several sites, baseline blood samples were obtained from only 30 participants (61%). Six participants (12%) had their first blood sample drawn after 1 month of treatment and 13 participants (27%) after 6 months of treatment.

Laboratory analyses

Forty-five milliliters of blood were collected in the morning from fasting subjects who were told to take no medications including chelators and nutritional supplements in the preceding 24 hours. Blood for NTBI assays was collected in trace element-free tubes containing $AlCl_3$ at a final concentration of 200 μM. Blood samples were collected at trough chelator levels. After centrifugation, aliquots of serum and plasma (plasma for measurement of vitamin C was acidified with 2% (w/v) oxalic acid) were stored at -80°C.

Malondialdehyde

Malondialdehyde was assayed in duplicate 250 μL plasma samples using gas chromatography-mass spectrometry (GC-MS), as previously described.[15,23,24] Diethylenetriaminepentaacetate (200 μM) and butylated hydroxytoluene (2.5 mM) were added to prevent oxidation. Samples were spiked with 1 μM $^2H_2$- malondialdehyde as an internal standard. To hydrolyze protein-bound malondialdehyde, 10 μL of 6.6N H_2SO_4 were added for 10 min at room temperature. Malondialdehyde was derivatized to pentafluorophenylhydrazine at room temperature for 1 hour. Derivatized malondialdehyde was extracted with isooctane, and 70 μL were injected into a GC-MS (Hewlett-Packard model 5888, Agilent Technologies, San Jose, CA, USA).

Protein carbonyls

Carbonyl groups on plasma proteins were measured by reaction with 2,4-dinitrophenylhydrazine to form a spectrophotometrically detectable hydrazone.[25,26]

Vitamins E and C

Plasma α-tocopherol levels were determined using high performance liquid chromatography with fluorescent detection by the method of Hansen and Warwick.[27] Plasma vitamin C was measured using a spectrophotometric method. Assays were performed at ARUP Laboratories (Salt Lake City, UT, USA).

Total non-transferrin bound iron

Total NTBI (referred to as NTBI) was determined by nitrilotriacetic acid capture of NTBI and high performance liquid chromatographic detection[28] with modification to account for either iron-free or iron-bound study chelator in the sample. $AlCl_3$ was added to blood samples to prevent NTBI shuttling onto unbound chelator and thus being underestimated. Although iron-bound deferoxamine has no effect on the assay, the deferasirox-iron complex is disrupted by the high concentrations of nitrilotriacetic acid. Blood samples were taken at trough chelator levels to limit over-estimation of NTBI in the presence of deferasirox-iron complex. Concentrations of the deferasirox-iron complex were also measured in separate samples by Novartis. To determine the proportion of NTBI attributable to disruption of deferasirox-iron complex, increasing concentrations of deferasirox-iron complex were spiked into normal and thalassemic sera (both n=6) and the observed NTBI levels measured. Both types of sera gave consistent graphs with the same slope (0.1264 μM NTBI/μM deferasirox-iron complex). The estimated excess NTBI attributable to the deferasirox-iron complex was subtracted from the crude NTBI measurement.

Transferrin saturation

Transferrin saturation was determined on serum samples as described elsewhere.[29] Twenty-five microliters of serum were treated with an excess of rivanol to remove most of the serum proteins excluding transferrin. After centrifugation, samples of the supernatants were run on polyacrylamide gels containing 6 M urea. Under these partially denaturing conditions, the four different species of transferrin (apo-, diferric-, C- and N-terminal monoferric) denature to differing extents and separate on the gel, allowing densitometric quantitation, from which saturation was calculated.

C-reactive protein

Plasma hsCRP was determined by a nephelometric method utilizing latex particles coated with C-reactive protein monoclonal antibodies by Quest Diagnostics (San Bernardino, CA, USA).

Cytokines

Plasma levels of ten cytokines (interleukin [IL]-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, granulocyte-monocyte colony-stimulating factor [GM-CSF], interferon [IFN]-γ and tumor necrosis factor [TNF]-α) were determined in samples by a multiplex antibody bead assay from BioSource International, Camarillo CA, USA.

Liver iron

Liver iron concentration (LIC) was estimated either by atomic absorption spectrometry of tissue extraction from paraffin blocks and digested at Clinique des Maladies du Foie, Centre Hospitalier Universitaire, Rennes, France[11] or by bio-magnetic susceptibility (BLS) (n=7) using SQUID biosusceptometers at Oakland (Ferritometer® Model 5700, Tristan Technologies, San Diego, CA, USA) and Hamburg (Hamburg biosusceptometer, Biomagnetic Technologies Inc., San Diego, USA).[30,31] SQUID estimates of LIC per unit wet weight were converted to dry weight units according to Fischer *et al.*,[32] using a conversion factor of 5.8.

Ferritin

Serum ferritin concentrations were measured by immunoassay at B.A.R.C. Laboratories, Gent, Belgium.[11]

Statistics

Demographic and anthropometric data, baseline medical characteristics, and trial follow-up parameters were compared by *t*-tests and Fisher's exact test. Malondialdehyde, α- and γ-tocopherol, cytokine and hsCRP values were log-transformed prior to analysis. Back-transformed means are reported. Longitudinal changes in biomarker levels among thalassemia patients were analyzed using linear mixed models of treatment group x time controlling for cumulative chelator dose nested within treatment group, self-reported illness in the previous 2 weeks, and baseline LIC with random subject-specific intercepts and slopes. Partial correlation coefficients were estimated from residuals of the longitudinal mixed model. To adjust for confounding of treatment group and baseline hsCRP levels, change in hsCRP was analyzed in a model controlling for baseline hsCRP. Because many participants enrolled after their baseline visit, baseline hsCRP data were estimated by multiple imputation[33] using a regression-based imputation model based on low-sensitivity C-reactive protein levels, absolute neutrophil counts, alanine transaminase levels, and terms in the final longitudinal model.

Results

Demographics

The demographic and anthropometric data of the study populations are shown in Table 1. The groups were well-matched with no significant statistical differences among them, including the transfusion volume during the study (0.32±0.10 mL red blood cells/kg/day for deferasirox, and 0.34±0.11 mL for deferoxamine, p=0.57).

Iron burden

There were no significant differences in baseline LIC, ferritin or duration of transfusion between the two treatment groups (p=0.46, 0.23 and 0.66, respectively, Table 1). The mean LIC of both treatment groups declined significantly from baseline during treatment (deferasirox -2.3 mg/g dry weight/year, deferoxamine -1.9 mg/g dry weight/year, average decline p=0.02, Figure 1A), with no difference between chelator groups for the mean rate of decline (p=0.81) and no difference between the groups at the end of treatment (p=0.54). The mean serum ferritin of both treatment groups at baseline also declined significantly during treatment (deferasirox -148 μg/L/year, deferoxamine -499 μg/L/year, average decline p=0.004; Figure 1B) with no significant difference between treatment groups in the rate of change of ferritin by longitudinal analysis (p=0.11). When controlling for age, gender and race, there was no significant difference in ferritin levels between chelator groups at baseline (p=0.26), but there was at the end of follow-up with ferritin levels being lower in the deferoxamine group (deferoxamine

1711 μg/L, deferasirox 2983 μg/L, p=0.01).

NTBI was significantly higher in both the deferasirox and deferoxamine groups than in the control group at baseline (p<0.001, Table 1) and at the end of the study (deferasirox 4.31 μmol/L, deferoxamine 4.43 μmol/L, p=0.001). However, there was no significant change in NTBI during the study period (deferasirox 0.91 μmol/L/year, deferoxamine -0.26 μmol/L/year, average rate p=0.49, treatment difference p=0.21) and no difference between treatment groups at the end of the study (p=0.85). NTBI was positively associated with transferrin saturation (r=0.70, p<0.001), but, interestingly, was inversely correlated with hsCRP (r=-0.21, p<0.01, Table 3) which declined during treatment with deferasirox.

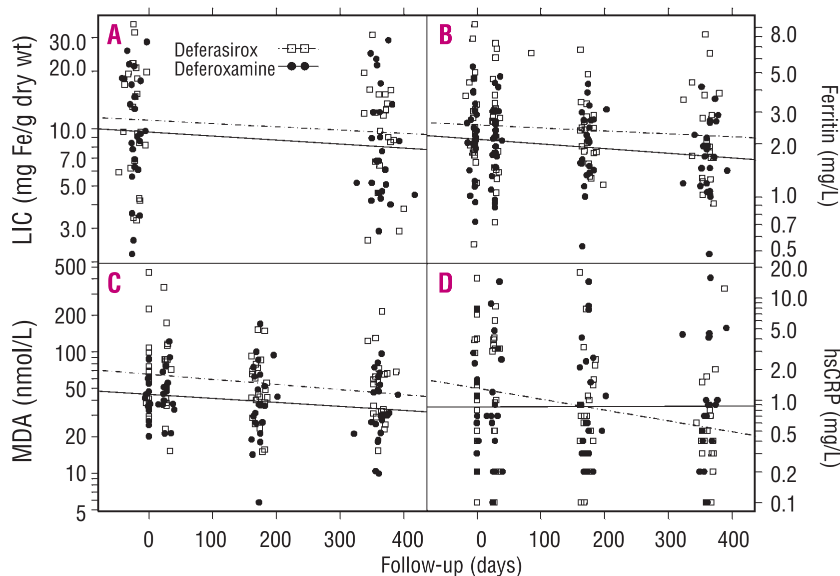

Figure 1. Distributions of LIC (panel A), ferritin (panel B), malondialdehyde (MDA) (panel C), and hsCRP (panel D) vs. time stratified by treatment group (deferasirox □; deferoxamine ●). Day zero (0) is the first day of treatment. Regression lines of the observed data are overlaid (deferasirox · – · – ·; deferoxamine ——).

Table 1. Baseline demographic data, iron status, oxidant stress and inflammation of the thalassemic patients and control subjects.

Parameter	*Deferasirox (n=24)*	*Deferoxamine (n=25)*	*Control (n=30)*	*Thalassemia vs. control p value*	*Deferasirox vs. deferoxamine p value*
Demographics					
Age, years, mean±SD	22.6±10.9	22.2±10.8	24.5±9.0	>0.25	>0.25
Sex, male/female	16/8	12/13	15/15	>0.25	>0.25
Race, Asian/Caucasian/Other	11/11/2	12/13/0	12/16/2	>0.25	>0.25
Body mass index, kg/m^2, mean±SD	20.9±4.1	20.9±4.1	21.8±4.1	>0.25	>0.25
Splenectomy, n (%)	13 (54%)	15 (60%)		–	>0.25
Alanine transferase (U/L)	42.3±41.9	38.3±40.8	–	–	>0.25
HCV Ab positive, n (%)*	4 (17%)	5 (20%)	–	–	>0.25
Iron status					
Liver iron (mg Fe/g dw)	13.7±8.7	12.0±7.4	–	–	>0.25
Ferritin (μg/L)	2985±2058	2398±1163	–	–	0.23
Transfusion (years)	15.6±10.4	17.3±10.9	–	–	>0.25
NTBI (μmol/L)	3.19±2.21	4.81±1.94	-1.48±0.48	<0.001	0.04
Transferrin saturation (%)	89.1±19.8	95.3±11.0	38.1±11.5	<0.001	>0.25
[1]Oxidant stress					
Malondialdehyde (nmol/L)	68.2 (28.1, 451)	42.6 (20.1, 88.3)	19.2 (1.9, 75.8)	<0.001	0.06
Vitamin C (mg/dL)	0.70±0.52	0.82±0.40	1.40±0.33	<0.001	>0.25
α-tocopherol (mg/L)	5.67 (2.80, 11.2)	6.12 (3.20, 9.2)	8.55 (5.60, 12.1)	<0.001	>0.25
Inflammation					
hsCRP (mg/L)	1.46 (0.10, 15.6)	0.78 (0.20, 7.7)	0.48 (0.10, 5.1)	0.012	0.18
IL-10 (pg/mL)	3.09 (1.17, 8.32)	2.67 (1.91, 4.90)	1.27 (0.39, 2.86)	<0.001	>0.25

*Values are arithmetic means±SD unless otherwise noted. [1]Ranges are given for log transformed variables. *No patients showed current hepatitis C virus (HCV) active disease by either inflammation noted on study liver biopsy or detection of HCV RNA by polymerase chain reaction analysis for subjects not undergoing liver biopsy.*

Table 2. Regression coefficients and 95% confidence intervals (CI) from a mixed model analysis of biomarker change over time.

Parameter	Malondialdehyde (log nmol/L), 95% CI		hsCRP (log mg/L), 95% CI	
Sick within previous 2 weeks (yes *vs.* no)	0.224 (0.034 to 0.415)	*p*=0.02	0.784 (0.431 to 1.137)	*p*<0.001
Baseline LIC (mg Fe/g dry wt)	0.025 (0.001 to 0.049)	*p*=0.04	-0.015 (-0.066 to 0.036)	*p*=0.56
Time (years)	-0.29 (-0.49 to -0.09)	*p*=0.006	-0.32 (-0.65 to 0.02)	*p*=0.06
Treatment by time (years)	0.08 (-0.31 to 0.48)	*p*=0.68	-0.80 (-1.47 to -0.13)	*p*=0.02

Table 3. Correlations among biomarkers of iron stores, inflammation, and oxidative injury.

Variables	Patients (observations)	Partial correlation	p value
LIC (mg Fe/g dw) *vs.* MDA (log nmol/L)	47 (72)	0.38	0.001
NTBI (μmol/L) *vs.* MDA (log nmol/L)	48 (135)	0.10	0.25
Transferrin saturation (%) *vs.* NTBI (μmol/L).	47 (134)	0.70	< 0.001
Transferrin saturation (%) *vs.* hsCRP (log mg/L)	48 (144)	-0.29	< 0.001
hsCRP (log mg/L) *vs.* NTBI (μmol/L).	47 (134)	-0.21	0.01
hsCRP (log mg/L) *vs.* IL-6 (pg/mL)	49 (143)	0.44	< 0.001

Oxidative injury

At baseline the mean plasma malondialdehyde concentration of the two treatment groups (56 nmol/L) was significantly higher than that of the control group (19.5 nmol/L; see Table 1). The value in the control group was in the range found for healthy controls in other studies.[15,23] The mean plasma malondialdehyde concentration of the two treatment groups declined significantly during treatment (Figure 1C, deferasirox –21%/year, deferoxamine –25%/year, *p*=0.006 for average decline, controlling for baseline LIC and self-reported recent illness). However, there was no difference between the treatment groups in the mean rate of decline of malondialdehyde (*p*=0.68, Table 2). Malondialdehyde concentration was positively correlated with baseline LIC (Table 3) and remained elevated above the level in controls in both chelator groups at the end of follow-up (deferasirox 49.5 nmol/L, deferoxamine 33.9 nmol/L, both *p*=0.001 compared to controls). Plasma protein carbonyls, a marker of protein oxidative damage, did not differ between controls and thalassemia patients at baseline (0.52 and 0.54 nmol/mg respectively) and did not change significantly over the course of treatment (*p*=0.63).

Further evidence of oxidative stress in the thalassemia patients was apparent from the lower levels of antioxidant vitamins C and E (α-tocopherol) at baseline (Table 1) and at 1 year, compared to the levels in normal controls (*p*<0.001, *data not shown*). However, there were no significant differences in the levels of either vitamin C or α-tocopherol between the treatment groups (Table 1), nor were there any significant changes in the concentrations of these vitamins during the follow-up. These vitamins were also correlated amongst themselves (vitamin C *vs.* α-tocopherol r=0.23, *p*=0.007; and α-tocopherol *vs.* γ-tocopherol r=–0.20, *p*=0.01). The negative relationship between γ- and α-tocopherol is not surprising since we have previously shown this in thalassemia patients[15] and the opposing rise in γ-tocopherol has been shown in other inflammatory conditions.[34]

Inflammation

Markers of inflammation including hsCRP and ten cytokines were compared in each of the groups (see Figure 1D for hsCRP). At baseline, mean plasma hsCRP was elevated only among individuals randomized to deferasirox (deferasirox 1.30 mg/L, deferoxamine 0.74 mg/L, controls 0.43 mg/L, *p*=0.001 and *p*=0.10 compared to controls, respectively; see Table 1). Similarly, IL-6 was only elevated at baseline among individuals randomized to deferasirox (deferasirox 1.40 pg/mL, deferoxamine 1.06 pg/mL, controls 0.87 pg/mL, *p*=0.05 and *p*=0.47 compared to controls, respectively). IL-10 was higher in both treatment groups at baseline (*p*<0.001, Table 1) and during the follow-up (deferasirox 2.32 pg/mL, deferoxamine 2.49 pg/mL, *p*<0.001 compared to controls). In longitudinal analysis, there was a significant difference between treatment groups in the mean rate of decline of hsCRP (deferasirox –51% year, deferoxamine +8.6% year, *p*=0.02, Figure 1D and Table 2). The confounding effect of treatment groups and baseline hsCRP levels and the lack of baseline hsCRP levels for 40% of participants made inferences on the relationship between treatment and changes in hsCRP difficult. In a model controlling for baseline hsCRP levels using data obtained by multiple imputation, the treatment difference in the rate of change in hsCRP was equally large (deferasirox –27%/year, deferoxamine +34%/year, *p*=0.02).

Inflammatory markers also correlated with other biomarkers in samples from thalassemia patients. hsCRP concentration was weakly associated with LIC (r=0.23, *p*=0.06), but inversely associated with transferrin saturation (r=-0.29, *p*<0.001, Table 3) and, as with pointed out above, NTBI. IL-6 was positively correlated with hsCRP (r=0.43, *p*<0.001) and serum ferritin (r=0.18, *p*=0.03) but inversely correlated with transferrin saturation (r=0.20, *p*=0.02). Participant-reported sickness in the 2 weeks prior to samples being taken was associated with higher levels of hsCRP (+113%, *p*<0.001, Table 2), IL-2 (+75%), IL-6 (+45%), IL-10 (+36%) and TNF-α (+30%, *p*<0.05 for all).

Discussion

We examined the relationship between decrease in total body iron burden induced by deferoxamine or deferasirox and direct measures of oxidant damage and inflammation in transfused thalassemia patients. In general, decreased iron burden was associated with decreased oxidant damage and inflammation. Iron chelation therapy with deferasirox or deferoxamine was equally effective in decreasing LIC and malondialdehyde by the end of the study period.

Iron burden and oxidative stress

Although we are interested in the specific role of iron overload in mediating injury, the unique pathophysiology of thalassemia may also play a role in promoting changes in the observed oxidative and inflammatory biomarkers. Specifically, the surplus of α-globin chains and intramedullary ineffective erythropiesis are important factors.[35] Furthermore, while each class of biomarker is traditionally thought to represent oxidative or inflammatory processes, it is important to note that these are not distinct entities and have considerable interaction, i.e., iron-induced oxidative stress can initiate tissue injury and/or inflammation.

We followed three parameters of iron burden: LIC, serum ferritin and total NTBI. Both LIC and ferritin declined during the study period in both treatment groups, confirming previous findings.[10,11] While total NTBI levels were significantly higher in thalassemia patients than in controls, we did not find a change in total NTBI with either deferoxamine or deferasixon treatment, although the assay was modified to account for iron present in chelator complexes (see methods). The present study measured total NTBI, which represents the total plasma iron not bound to transferrin. However, because less than 10% of the total plasma NTBI is in a rapidly chelatable form[7] our small sample size would be unlikely to identify a modest difference between the chelators. Our analysis could have had greater sensitivity if we had collected samples at earlier time points when deferasirox would have been present at higher concentrations. A recent report suggests that *labile plasma iron*, a redox active rapidly chelatable subfraction of total NTBI,[36] is decreased by the use of deferasirox.[12] In the present study we chose to measure total NTBI because NTBI tissue uptake is unlikely to be limited to the redox active *labile* fraction. Further studies are needed to clarify the relationship between total NTBI and labile plasma iron.

We found a positive association between NTBI and transferrin saturation but noted interesting inverse associations of NTBI and transferrin saturation with hsCRP. Parallel with this there was also an inverse correlation between transferrin saturation and IL-6. These findings may be related to the high levels of IL-10 found in our thalassemia patients since there are previous reports that IL-10 promotes retention of iron within the reticuloendothelial system thus lowering blood circulating free iron.[37,38] It is also tempting to speculate that this retention could be promoted by hepcidin[39,40] and a study of the relationship of hepcidin levels to NTBI, transferrin saturation and inflammatory markers is indicated in these patients. We and others also suggest that this response enables the reticuloendothelial system to retain damaging free iron during inflammation.[15,37,41] The falling hsCRP levels during the study, together with their inverse correlation with NTBI could mean that any fall in NTBI resulting from chelation is being countered by the opposing effects from decreased inflammation.

Some plasma malondialdehyde assays have previously been limited by lack of specificity and demonstrate a very wide range of normal values probably related to artifacts generated during the process.[42] In the present study, we used a GC-MS assay which is specific for malondialdehyde[14,15] and avoids the oxidation during processing seen in thiobarbituric acid reactive substances (TBARS) analysis.[42] Longitudinal analysis in our study showed that malondialdehyde levels were controlled by both deferoxamine and deferasirox, and to our knowledge this is the first study to show that plasma malondialdehyde levels can be reduced by chelation, although it was previously shown that deferiprone could lower TBARS in a 1 year study of eight patients.[43] In agreement with our previous work, LIC from thalassemia patients correlated with plasma malondialdehyde levels.[15] In experimental animal models, high liver iron levels were shown to induce elevation of lipid peroxides and oxidants[44,45] presumably through iron initiated Fenton chemistry. A similar mechanism is likely to be contributing in our present study. Increased plasma lipid peroxidation markers such as malondialdehyde have previously been observed in patients with thalassemia.[14,15,46,47]

Whereas NTBI and liver iron overload are both reasonable candidates for initiating malondialdehyde formation, only LIC, not NTBI, correlated with malondialdehyde. This might seem surprising because of the potential for free iron in NTBI to initiate the Fenton chemistry *in vivo* that could induce lipid peroxidation leading to increased malondialdehyde. However, this was not the case. Of the recent published studies that investigated the relationship between NTBI and malondialdehyde (excluding our previous paper),[14,48] one found no correlation between NTBI and malondialdehyde;[48] while another did find a correlation.[14] The weakness in the relationship between NTBI and malondialdehyde in the present work and others may be explained by variation in circulating NTBI related to the specific protocol design - such as the variation of NTBI at any specific time point or consistency of the time point in the transfusion cycle selected for blood sampling. The largest variation of NTBI levels between patients has been shown to occur at the midpoint of the transfusion cycle.[49] In contrast to Cighetti *et al*.[14] who completed their blood sampling just prior to transfusion, we did not standardize the blood sampling to the transfusion date. Discrepancies between results could also occur if the timing of the blood sampling in relationship to chelation is not similar between studies. Cighetti *et al*.[14] took their blood samples 48 hours after stopping chelation while we took ours 24 hours after stopping chelation.

In a previous study,[15] we suggested that the increased levels of plasma malondialdehyde in thalassemia might

be explained by three mechanisms: (i) the excess α-chains in β-thalassemic erythrocytes and erythroblasts being unstable and prone to denaturation and oxidation;[35] (ii) peroxidation of tissues that leak malondialdehyde into the blood; and (iii) depleted antioxidant capacity (described below) lowering defense to oxidants. While all three may contribute, the parallel decline of LIC and malondialdehyde during our study is more consistent with the mechanism of malondialdehyde leaking from the iron overloaded liver.

Antioxidant capacity is also a determinant of sensitivity to oxidant-stress and resulting tissue injury, especially in patients with increased iron burden such as thalassemics. We found significantly lower antioxidant capacity (vitamins E and C) and elevated malondialdehyde in the thalassemia patients compared to controls at baseline and during the follow-up. This increase in oxidative stress has been found in other studies on thalassemia[15,50-52] and previously only antioxidant supplementation was successful in decreasing oxidant stress.[53]

Inflammation

In contrast to the results found for markers of iron burden and oxidative stress noted above, for which both chelators were similar, only the group treated with deferasirox had a significant decline in the inflammatory marker hsCRP. The interpretation of this finding is hampered by the chance difference in baseline hsCRP levels. Nevertheless, the difference in hsCRP change was equally large after controlling for baseline hsCRP levels using data obtained by multiple imputation. The reason for this difference is not, however, certain. One recognized difference between the chelators is that deferasirox has a longer plasma half-life (8–16 hours) than deferoxamine (18 min),[11] enabling deferasirox to exert its effects for a much longer time after dosing. Although we did not see any differences in total NTBI between the treatment groups at chelator trough levels, it is possible that the longer chelator half-life may lower inflammation by better controlling levels of NTBI earlier in the 24-hour period after chelator administration. Deferasirox may also better control the levels of labile plasma iron in our patients.[12] Lower levels of either NTBI or labile plasma iron might be expected to diminish iron-induced oxidative injury and possible stress to circulating monocytes and cells of the reticuloendothelial system. Reduced oxidant-stress has been shown to lower monocyte IL-6 release in other inflammatory disease models.[54,55] In addition, the present work shows that IL-6, which can be produced by monocytes and macrophages,[56-59] is well correlated with hsCRP levels. Given the established relationship of IL-6 to hepatocyte production of C-relative protein[60-62] it is possible that this is a relevant mechanism. Clearly, more studies are needed to further investigate these interesting findings.

In conclusion, given the central role of iron-dependent redox reactions and attendant inflammatory responses in the complications of thalassemia, further studies of the general and specific effects of iron chelators on oxidation and inflammation are needed.

Appendix

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, Gladys Warr and Annie Lui, Laboratory Technicians; 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.

Authorship and Disclosures

PBW designed and performed the research, analyzed the data and wrote the paper; EAM designed the research, analyzed 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; EJN: 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; 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; DA designed the research and wrote the paper; JH designed the research and wrote the paper; PH designed the research, recruited and administered procedures to patients, analyzed the data and wrote the paper.

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; DA and JH are employees of Novartis Pharmaceuticals Corp. All authors gave final approval for the manuscript to be published.

References

1. Zurlo MG, De Stefano P, Borgna-Pignatti C, Di Palma A, Piga A, Melevendi C, et al. Survival and causes of death in thalassaemia major. Lancet 1989;2:27-30.
2. Aldouri MA, Wonke B, Hoffbrand AV, Flynn DM, Ward SE, Agnew JE, et al. High incidence of cardiomyopathy in b-thalassaemia patients receiving regular transfusion and iron chelation: reversal by intensified chelation. Acta Haematol 1990;84: 113-7.
3. Borgna-Pignatti C, Rugolotto S, De Stefano P, Zhao H, Cappellini MD, Del Vecchio GC, et al. Survival and complications in patients with thalassemia major treated with transfusion and deferoxamine. Haematologica 2004;89:1187-93.
4. Vichinsky E, Butensky E, Fung E, Hudes M, Theil E, Ferrell L, et al. Comparison of organ dysfunction in transfused patients with SCD or beta thalassemia. Am J Hematol 2005;80: 70-4.
5. Cappellini MD. Overcoming the challenge of patient compliance with iron chelation therapy. Semin Hematol 2005;42 [Suppl 1]:S19-21.
6. Gosriwatana I, Loreal O, Lu S, Brissot P, Porter J, Hider RC. Quantification of non-transferrin-bound iron in the presence of unsaturated transferrin. Anal Biochem 1999;273:212-20.
7. Porter JB, Abeysinghe RD, Marshall L, Hider RC, Singh S. Kinetics of removal and reappearance of non-transferrin-bound plasma iron with deferoxamine therapy. Blood 1996; 88:705-13.
8. Kontoghiorghes GJ. Iron mobilization from transferrin and non-transferrin-bound-iron by deferiprone. Implications in the treatment of thalassemia, anemia of chronic disease, cancer and other conditions. Hemoglobin 2006;30:183-200.
9. Porter JB, Rafique R, Srichairatanakool S, Davis BA, Shah FT, Hair T, et al. Recent insights into interactions of deferoxamine with cellular and plasma iron pools: Implications for clinical use. Ann Y Acad Sci 2005;1054:155-68.
10. Piga A, Galanello R, Forni GL, Cappellini MD, Origa R, Zappu A, et al. Randomized phase II trial of deferasirox (Exjade, ICL670), a once-daily, orally-administered iron chelator, in comparison to deferoxamine in thalassemia patients with transfusional iron overload. Haematologica 2006;91:873-80.
11. Cappellini MD, Cohen A, Piga A, Bejaoui M, Perrotta S, Agaoglu L, et al. A phase 3 study of deferasirox (ICL670), a once-daily oral iron chelator, in patients with b-thalassemia. Blood 2006;107:3455-62.
12. Glickstein H, Ben El R, Link G, Breuer W, Konijn AM, Hershko C, et al. Action of chelators in iron-loaded cardiac cells: accessibility to intracellular labile iron and functional consequences. Blood 2006;108:3195-203.
13. Glickstein H, El RB, Shvartsman M, Cabantchik ZI. Intracellular labile iron pools as direct targets of iron chelators: a fluorescence study of chelator action in living cells. Blood 2005;106:3242-50.
14. Cighetti G, Duca L, Bortone L, Sala S, Nava I, Fiorelli G, et al. Oxidative status and malondialdehyde in b-thalassaemia patients. Eur J Clin Invest 2002;32[Suppl 1]:55-60.
15. Walter PB, Fung E, Killilea DW, Jiang Q, Hudes M, Madden J, et al. Oxidative stress and inflammation in iron-overloaded Patients with b-thalassemia or sickle cell disease. Br J Haematol 2006;135:254-63.
16. Kadiiska MB, Gladen BC, Baird DD, Germolec D, Graham LB, Parker CE, et al. Biomarkers of oxidative stress study II: are oxidation products of lipids, proteins, and DNA markers of CCl4 poisoning? Free Radic Biol Med 2005;38:698-710.
17. Kadiiska MB, Gladen BC, Baird DD, Graham LB, Parker CE, Ames BN, et al. Biomarkers of oxidative stress study: III. Effects of the nonsteroidal anti-inflammatory agents indomethacin and meclofenamic acid on measurements of oxidative products of lipids in CCl4 poisoning. Free Radic Biol Med 2005;15:25-40.
18. Tong PC, Ng MC, Ho CS, So WY, Li JK, Lam CW, et al. C-reactive protein and insulin resistance in subjects with thalassemia minor and a family history of diabetes. Diabet Care 2002;25:1480-1.
19. Archararit N, Chuncharunee S, Pornvoranunt A, Atamasirikul K, Rachakom B, Atichartakarn V. Serum C-reactive protein level in postsplenectomized thalassemic patients. J Med Assoc Thai 2000;83 [Suppl 1]:S63-9.
20. Ridker PM, Morrow DA. C-reactive protein, inflammation, and coronary risk. Cardiol Clin 2003;21:315-25.
21. Pradhan AD, Cook NR, Buring JE, Manson JE, Ridker PM. C-reactive protein is independently associated with fasting insulin in nondiabetic women. Arterioscler Thromb Vasc Biol 2003;23:650-5.
22. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. JAMA 2001;286:327-34.
23. Yeo HC, Helbock HJ, Chyu DW, Ames BN. Assay of malondialdehyde in biological fluids by gas chromatography-mass spectrometry. Anal Biochem 1994;220:391-6.
24. Yeo HC, Liu J, Helbock HJ, Ames BN. Assay of malondialdehyde and other alkanals in biological fluids by gas chromatography-mass spectrometry. Methods Enzymol 1999; 300:70-8.
25. Houglum K, Filip M, Witztum JL, Chojkier M. Malondialdehyde and 4-hydroxynonenal protein adducts in plasma and liver of rats with iron overload. J Clin Invest 1990;86: 1991-8.
26. Houglum K, Ramm GA, Crawford DH, Witztum JL, Powell LW, Chojkier M. Excess iron induces hepatic oxidative stress and transforming growth factor b1 in genetic hemochromatosis.Hepatology 1997; 26:605-10.
27. Hansen LG, Warwick WJ. An improved assay method for serum vitamins A and E using fluorometry. Am J Clin Pathol 1978;70:922-3.
28. Singh S, Hider RC, Porter JB. A direct method for quantification of non-transferrin-bound iron. Anal Biochem 1990;186:320-3.
29. Evans RW, Williams J. Studies of the binding of different iron donors to human serum transferrin and isolation of iron-binding fragments from the N- and C-terminal regions of the protein. Biochem J 1978;173:543-52.
30. Fischer R. Liver iron susceptometry. In: Andrae W, Nowak H, editors. Magnetism in medicine. A Handbook. Berlin: Wiley-VCH, 1998. p. 286-301.
31. Fung E, Fischer R, Pakbaz Z. The New SQUID biosusceptometer at Oakland: first year of experience. Neurol Clin Neurophysiol 2004;5.
32. Fischer R, Harmatz P, Nielsen P. Does liver biopsy overestimate liver iron concentration? Blood 2006;108: 1775-6.
33. Little RJA, Rubin DB. Statistical Analysis with Missing Data. New York: John Wiley & Sons, Inc., 2002.
34. Himmelfarb J, Kane J, McMonagle E, Zaltas E, Bobzin S, Boddupalli S, et al. α and γ tocopherol metabolism in healthy subjects and patients with end-stage renal disease. Kidney Int 2003;64:978-91.
35. Scott MD, van den Berg JJ, Repka T, Rouyer-Fessard P, Hebbel RP, Beuzard Y, et al. Effect of excess alpha-hemoglobin chains on cellular and membrane oxidation in model beta-thalassemic erythrocytes. J Clin Invest 1993;91:1706-12.
36. Pootrakul P, Breuer W, Sametband M, Sirankapracha P, Hershko C, Cabantchik ZI. Labile plasma iron (LPI) as an indicator of chelatable plasma redox activity in iron-overloaded β-thalassemia/HbE patients treated with an oral chelator. Blood 2004;104:1504-10.

37. Ludwiczek S, Aigner E, Theurl I, Weiss G. Cytokine-mediated regulation of iron transport in human monocytic cells. Blood 2003;101:4148-54.
38. Fillet G, Beguin Y, Baldelli L. Model of reticuloendothelial iron metabolism in humans: abnormal behavior in idiopathic hemochromatosis and in inflammation. Blood 1989;74:844-51.
39. Ludwiczek S, Theurl I, Artner-Dworzak E, Chorney M, Weiss G. Duodenal HFE expression and hepcidin levels determine body iron homeostasis: modulation by genetic diversity and dietary iron availability. J Mol Med 2004;82:373-82.
40. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 2004;306:2090-3.
41. Pippard MJ. Secondary iron overload. In: Brock JH, Halliday JW, Pippard MJ, eds. Iron metabolism in health and disease. London: W.B. Saunders Ltd.; 1994. p. 272-300.
42. Liu J, Yeo HC, Doniger SJ, Ames BN. Assay of aldehydes from lipid peroxidation: gas chromatography-mass spectrometry compared to thiobarbituric acid. Anal Biochem 1997; 245:161-6.
43. Pootrakul P, Sirankapracha P, Sankote J, Kachintorn U, Maungsub W, Sriphen K, et al. Clinical trial of deferiprone iron chelation therapy in β-thalassaemia/haemoglobin E patients in Thailand. Br J Haematol 2003;122:305-10.
44. Knutson MD, Walter PB, Ames BN, Viteri FE. Both iron deficiency and daily iron supplements increase lipid peroxidation in rats. J Nutr 2000; 130:621-8.
45. Walter PB, Knutson MD, Paler-Martinez A, Lee S, Xu Y, Viteri FE, et al. Iron deficiency and iron excess damage mitochondria and mitochondrial DNA in rats. Proc Natl Acad Sci USA 2002;99:2264-9.
46. Laksmitawati DR, Handayani S, Udyaningsih-Freisleben SK, Kurniati V, Adhiyanto C, Hidayat J, et al. Iron status and oxidative stress in β-thalassemia patients in Jakarta. Biofactors 2003;19:53-62.
47. Livrea MA, Tesoriere L, Maggio A, D'Arpa D, Pintaudi AM, Pedone E. Oxidative modification of low-density lipoprotein and atherogenetic risk in β-thalassemia. Blood 1998; 92:3936-42.
48. Livrea MA, Tesoriere L, Pintaudi AM, Calabrese A, Maggio A, Freisleben HJ, et al. Oxidative stress and antioxidant status in β-thalassemia major: iron overload and depletion of lipid-soluble antioxidants. Blood 1996;88:3608-14.
49. Grosse R, Lund U, Caruso V, Fischer R, Janka GE, Magnano C, et al. Non-transferrin-bound iron during blood transfusion cycles in β-thalassemia major. Ann N Y Acad Sci 2005;1054:429-32.
50. Rachmilewitz EA. The role of intracellular hemoglobin precipitation, low MCHC, and iron overload on red blood cell membrane peroxidation in thalassemia. Birth Defects Orig Artic Ser 1976;12:123-33.
51. Rachmilewitz EA, Shohet SB, Lubin BH. Lipid membrane peroxidation in β-thalassemia major. Blood 1976; 47:495-505.
52. Rachmilewitz EA, Weizer-Stern O, Adamsky K, Amariglio N, Rechavi G, Breda L, et al. Role of iron in inducing oxidative stress in thalassemia: Can it be prevented by inhibition of absorption and by antioxidants? Ann NY Acad Sci 2005; 1054:118-23.
53. Tesoriere L, D'Arpa D, Butera D, Allegra M, Renda D, Maggio A, et al. Oral supplements of vitamin E improve measures of oxidative stress in plasma and reduce oxidative damage to LDL and erythrocytes in beta-thalassemia intermedia patients. Free Radic Res 2001;34:529-40.
54. Jain SK, Rains JL, Croad JL. High glucose and ketosis (acetoacetate) increases, and chromium niacinate decreases, IL-6, IL-8, and MCP-1 secretion and oxidative stress in U937 monocytes. Antioxid Redox Signal 2007;9:1581-90.
55. Jialal I, Devaraj S, Venugopal SK. Oxidative stress, inflammation, and diabetic vasculopathies: the role of alpha tocopherol therapy. Free Radic Res 2002;36:1331-6.
56. Fuller GM, Bunzel RJ, Woloski BM, Nham SU. Isolation of hepatocyte stimulating factor from human monocytes. Biochemical and biophysical research communications. 1987;144:1003-9.
57. Bauer J, Ganter U, Geiger T, Jacobshagen U, Hirano T, Matsuda T, et al. Regulation of interleukin-6 expression in cultured human blood monocytes and monocyte-derived macrophages. Blood 1988;72:1134-40.
58. Woloski BM, Fuller GM. Identification and partial characterization of hepatocyte-stimulating factor from leukemia cell lines: comparison with interleukin 1. Proc Natl Acad Sci USA 1985;82:1443-7.
59. Gauldie J, Richards C, Harnish D, Lansdorp P, Baumann H. Interferon beta 2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. Proc Natl Acad Sci USA 1987;84:7251-5.
60. Kushner I, Rzewnicki DL. The acute phase response: general aspects. Bailliere's Clin Rheumatol 1994;8:513-30.
61. Li SP, Goldman ND. Regulation of human C-reactive protein gene expression by two synergistic IL-6 responsive elements. Biochemistry 1996;35:9060-8.
62. Majello B, Arcone R, Toniatti C, Ciliberto G. Constitutive and IL-6-induced nuclear factors that interact with the human C-reactive protein promoter. EMBO J 1990;9:457-65.