

ORIGINAL RESEARCH

Deferiprone therapy improves the oxidative status of LDL in patients with β -thalassaemia/HbE

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Abstract

Background: Oxidative modifications of low-density lipoproteins (LDL) have been reported in patients with β -thalassaemia/haemoglobin E (HbE) and are related to cardiovascular complications. Deferiprone (L1) is an iron chelator that decreases iron overload and, consequently, reduces oxidative stress. This study assesses the protective effect of L1 on the oxidative status of LDL in patients with β -thalassaemia/HbE.

Methods: Twenty-nine patients with β -thalassaemia/HbE treated with L1 were recruited. The study included a 4-week washout period followed by 4 and 12 weeks of L1 treatment. Non-transferrin-bound iron (NTBI) levels and oxidative stress markers, including thiobarbituric acid reactive substances and α -tocopherol, were monitored at each visit. The rate and content of lipid radical formation following Cu^{2+} -induced LDL oxidation in vitro were detected by NBD-Pen, a specific fluorescence probe.

Results: L1 was shown to prevent the depletion of α -tocopherol, decrease thiobarbituric acid reactive substances and preserve the levels of lipid compo-

nents in LDL. A negative correlation between serum NTBI and LDL α -tocopherol indicated that the circulating non-redox-active NTBI can lead to the depletion of α -tocopherol. LDL from the washout period showed the highest oxidative susceptibility when evaluated by NBD-Pen.

Conclusion: Iron chelation therapy with L1 improves the oxidative status of LDL in patients with β -thalassaemia/HbE.

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Keywords: β -Thalassaemia/HbE, cholesteryl esters, deferiprone, iron overload, LDL oxidation.

Citation

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Introduction

β -Thalassaemia is an inherited haemolytic anaemia disease. Amongst several variants, β -thalassaemia/haemoglobin E (HbE) is commonly found in Southeast Asia.¹ Iron overload has been implicated in oxidative stress, which contributes to complications in patients with

β -thalassaemia/HbE. Oxidative modification of lipoproteins, particularly low-density lipoproteins (LDL), induced by iron overload, is associated with the development of lipid-related diseases such as atherosclerosis, which may present as complications of β -thalassaemia.²⁻⁴

Although the underlying mechanisms of LDL oxidation in β -thalassaemia have not been fully elucidated,

lipid peroxidation induced by iron overload through non-enzymatic pathways is proposed to play a significant role.⁵ The hydrophobic region of LDL is particularly susceptible to oxidation, as significant changes in lipid fluidity⁶ and alterations in major lipid components in the core region have been observed.⁷ These components include cholesteryl linoleate (CL), cholesteryl arachidonate (CA) and cholesteryl oleate (CO). The CL-to-CO ratio has been proposed as a biomarker for LDL oxidative damage and disease severity in β -thalassaemia/HbE. Additionally, different oxidized products are generated at different stages of LDL oxidation, ranging from minimally to fully oxidized LDL.⁸ Therefore, the oxidative status of LDL might be a critical parameter indicating oxidative damage and the severity of disease.

Deferiprone (LI), an iron chelator, is effective in reducing iron levels in patients. Beyond iron chelation, LI has shown benefits in addressing complications associated with heart failure, renal disease, Parkinson's disease and cancer.^{9–11} However, its potential role in mitigating LDL oxidation remains to be fully explored.

This study aims to investigate whether LI, beyond its iron-chelating capabilities, can improve the oxidative status of LDL. Parameters such as iron levels, depletion of major lipid components, endogenous antioxidants in LDL, and the formation of lipid peroxidation products will be evaluated in patients with β -thalassaemia/HbE. The findings may confirm the protective effect of LI on the prevention of LDL oxidation caused by iron overload in β -thalassaemia/HbE. Furthermore, assessing the oxidative status of LDL could serve as a valuable measure for the evaluation of LI efficacy in managing the progression of the disease.

Methods

Patient recruitment and study design

Twenty-nine patients with β -thalassaemia/HbE (18 men and 11 women, aged 18–55 years) who regularly received blood transfusions and were treated with LI (GPO-L-ONE®, Government Pharmaceutical Organization, Thailand) were included in the study following informed consent. According to the study design, 20 mL of blood was collected from each patient at four time points (visits 1–4). The first time point (visit 1) was used as the baseline. All patients were asked to discontinue their regular dose of LI to allow for a 4-week washout period before collecting blood at the second time point (visit 2). Subsequently, LI was represcribed to patients for 4 and 12 weeks, and blood samples were collected prior to LI administration at visits 3 and 4, respectively. Blood samples from each visit were analyzed for clinical

blood chemistry, and serum and LDL were separated for further analysis.

Institutional Review Board statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Thailand (COA No. 751/2019).

Informed consent statement

Informed consent was obtained from all participants involved in the study. Written informed consent was obtained from the patients for the publication of this article.

Serum and LDL separation

Blood samples were centrifuged at 2,454 *g* for 15 min at 4°C to separate serum fractions, which were stored at –80°C until LDL separation. Serum fractions were further processed using Hitachi CP100 NX ultracentrifuge with P100AT2 fixed-angle rotor (Himac, Tokyo, Japan) and a modified sequential density gradient method,¹² as described by Havel et al.¹³ The LDL fractions were collected at a density of 1.019–1.063 g/mL.

Determination of NTBI in serum

Non-transferrin-bound iron (NTBI) levels were determined by using the colorimetric method described by Jittangprasert et al.¹⁴ Bathophenanthroline-disulfonic acid (BPT) was used to form a complex with ferrous iron (Fe^{2+}) of NTBI, and formation of the $[\text{Fe}^{2+}\text{-BPT}]$ complex was measured at a wavelength of 537 nm using a microplate reader (CLARIO Star, BMG Labtech, Ortenberg, Germany).

Lipid extraction and determination of lipid composition in LDL

Lipid extraction and determination of lipid components were modified from Luechapudiporn et al.⁷ Lipid samples were analyzed using an HPLC system with a Hypersil BDS C-18 stainless-steel column (5 μm ; 4.6×250 mm) (Thermo Scientific, CA, USA), a 2690 Separation Module and a 2487 Dual Wavelength Detector (Waters). The interesting lipid compositions of LDL, including α -tocopherol, CA, CL and its oxidized product, 13-hydroxy-octadecadienoic acid cholesteryl ester (13-HODE-CE), were separated. All chromatograms were analyzed using Empower software version 3.0 (Waters).

Determination of TBARs in LDL

Levels of thiobarbituric acid reactive substances (TBARs), a marker of lipid peroxidation, were measured using a spectrofluorometric method adapted from Asakawa et al.¹⁵ The fluorescence was quantified using a CLARIOstar microplate reader (BMG Labtech, Germany).

Determination of FRAP in LDL

The antioxidant capacity of LDL was measured using a ferric reducing antioxidant power (FRAP) assay, which quantifies the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). The assay was performed using a CLARIOstar microplate reader (BMG Labtech, Germany) and the method was as described by Benzie et al.¹⁶

Determination of oxidative susceptibility of LDL

The oxidative susceptibility of LDL was assessed by monitoring lipid radical production in Cu^{2+} -induced LDL oxidation. A solution of LDL (44 μg protein) was added to 20 μL of 2,2,6-trimethyl-4-(4-nitrobenzol[1,2,5]oxadiazol-7-ylamino)-6-pentylpiperidine-1-oxyl (NBD-Pen) at a final concentration of 10 μM and the oxidation reaction was initiated with 20 μL CuSO_4 at a final concentration of 5 μM . The lipid radicals generated in the system were trapped by NBD-Pen to form NBD-Pen-lipid radical adducts (NBD-Pen-L). Fluorescence intensity of NBD-Pen-L was monitored at an excitation wavelength of 470 nm and an emission wavelength of 530 nm at 37°C for 3 h. The maximum fluorescence intensity was recorded and the slope of fluorescence intensity over 3 h was calculated.

Statistical analysis

All data were analyzed using IBM SPSS Statistics software version 25.0 (Chicago, IL, USA). The Wilcoxon signed-rank test was employed for comparison between visits. Spearman's rank correlation was used for correlation analysis amongst parameters.

Results

Characteristics of patients with β -thalassaemia/HbE

Twenty-nine patients with β -thalassaemia/HbE (18 men and 11 women, 13 splenectomized and 16 non-splenectomized, age 18–53 years; duration of L1 treatment ranging from 1 to 17 years) were recruited. No significant differences were observed in haematological parameters or serum ferritin levels between the washout period (visit 2) and after 12 weeks of L1 retreatment (visit 4). The average haemoglobin levels were 7.7 g/dL (range 6.1–9.2 g/dL) at visit 2 and 7.7 g/dL (range 6.4–9.2 g/dL) at visit 4. Serum ferritin levels averaged 1,987 ng/mL (range 826–11,191 ng/mL) at visit 2 and 2,030 ng/mL (range 628–11,104 ng/mL) at visit 4. L1 levels have been previously measured using a reverse-phase high-performance liquid chromatography (HPLC) system adapted from Limenta et al.¹⁷ and reported in detail.¹⁸ In summary, trough levels gradually increased over the treatment period (7.0 $\pm$ 5.0, 3.4 $\pm$ 1.0, 5.9 $\pm$ 2.7 and 7.4 $\pm$ 3.9 μM at visits 1, 2, 3 and 4, respectively), whilst peak levels also rose, with no significant differences observed between patient groups.¹⁸

Levels of α -tocopherol and TBARs in LDL patients with β -thalassaemia/HbE

The levels of α -tocopherol and TBARs in LDL at each visit were recorded (Figure 1a,b). At baseline (visit 1), the mean α -tocopherol level was 4.10 $\pm$ 2.31 $\mu\text{g}/\text{mg}$. After the 4-week L1 washout period (visit 2), α -tocopherol levels significantly dropped to 3.42 $\pm$ 1.96 $\mu\text{g}/\text{mg}$ ($p<0.05$) compared to baseline. After 12 weeks of L1 retreatment (visit 4), α -tocopherol levels significantly increased to 4.23 $\pm$ 2.24 $\mu\text{g}/\text{mg}$ ($p<0.01$) compared to visit 2. Conversely, TBAR levels significantly increased to 0.25 $\pm$ 0.09 μM at visit 2 compared to baseline (0.21 $\pm$ 0.10 μM ; $p<0.001$). After 4 and 12 weeks of L1 retreatment, TBAR levels significantly decreased to 0.22 $\pm$ 0.10 μM and 0.22 $\pm$ 0.09 μM , respectively ($p<0.001$) compared to visit 2. A significant negative correlation was observed between TBAR level and FRAP of LDL ($r=-0.546$, $p<0.001$; Figure 1c).

The lipid components of LDL, including CA and CL, as well as the ratio of 13-HODE-CE to CL in patient samples, are presented in Table 1. No significant changes in CA, CL or the 13-HODE-CE-to-CL ratio were observed across visits, suggesting that lipid composition remained stable despite variations in oxidative stress markers.

These findings indicate that the L1 washout increased lipid peroxidation in LDL, as evidenced by a significant reduction of α -tocopherol and a concurrent increase in TBARs. Retreatment with L1 restored α -tocopherol levels and reduced TBARs, reflecting a reduction in oxidative stress, whilst the lipid composition in LDL was preserved.

Levels of NTBI in serum of patients with β -thalassaemia/HbE

Levels of NTBI were measured in the serum of patients at each visit (Figure 2a). At baseline (visit 1), NTBI levels were 2.43 $\pm$ 1.36 μM . NTBI levels significantly decreased to 1.53 $\pm$ 0.74 μM during the L1 washout period (visit 2; $p<0.001$). After 4 and 12 weeks of L1 retreatment (visits 3 and 4), NTBI levels significantly increased to 2.66 $\pm$ 1.33 μM and 2.85 $\pm$ 1.99 μM , respectively ($p<0.01$ compared to visit 2). This indicated that the treatment of L1 led to the elevation of NTBI being detected in the circulation. A negative correlation between NTBI levels and LDL α -tocopherol ($r=-0.202$, $p<0.05$) was observed (Figure 2b).

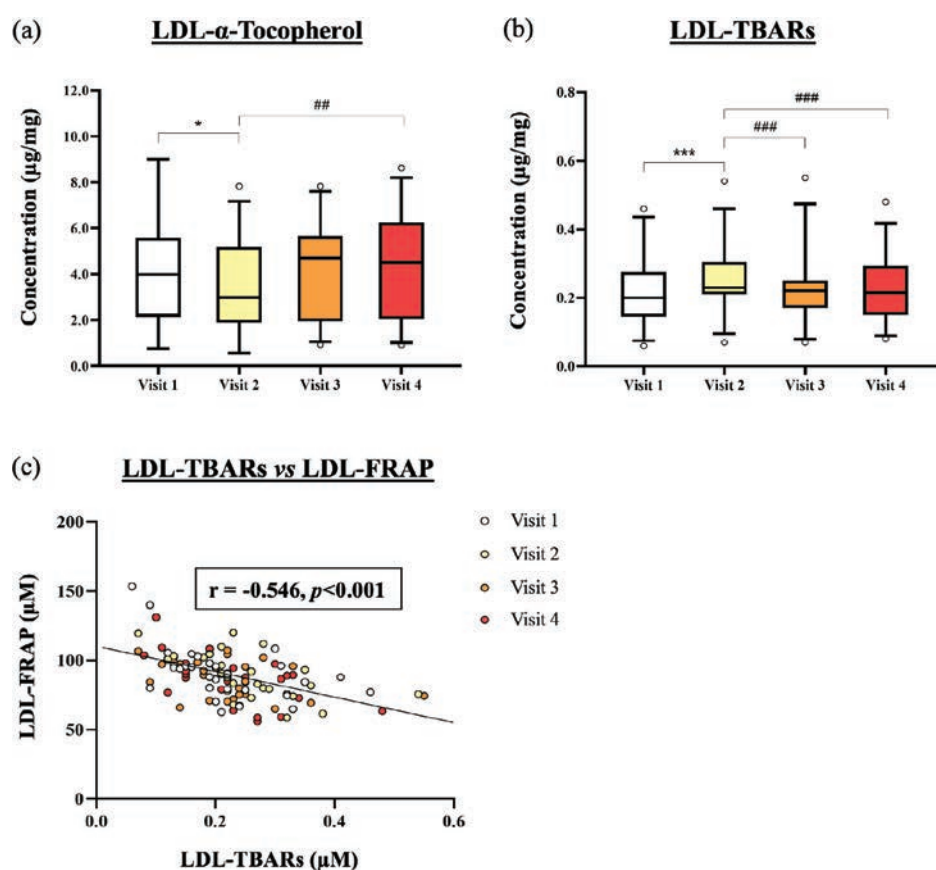
These results demonstrated that lower serum L1 levels during the washout period led to reduced NTBI levels. Retreatment with L1 significantly increased NTBI levels in circulation at visits 3 and 4. However, the observed correlation between NTBI and LDL α -tocopherol indicates that circulating NTBI contributes to α -tocopherol depletion in LDL.

Table 1. Levels of CA, CL and the ratio of 13-HODE-CE to CL in LDL of patients with β -thalassaemia/HbE at each visit.

Lipids	Visit 1	Visit 2	Visit 3	Visit 4
CA ($\mu\text{g/mL}$)	101.71 $\pm$ 40.85	110.28 $\pm$ 43.90	101.94 $\pm$ 39.84	113.33 $\pm$ 50.16
CL ($\mu\text{g/mL}$)	225.96 $\pm$ 76.66	227.04 $\pm$ 68.29	210.38 $\pm$ 68.99	222.91 $\pm$ 75.30
13-HODE-CE:CL ratio	0.12 $\pm$ 0.11	0.12 $\pm$ 0.10	0.16 $\pm$ 0.16	0.14 $\pm$ 0.15

Data are presented as mean $\pm$ SD ($n=29$). 13-HODE-CE, 13-hydroxy-octadecadienoic acid cholesteryl ester; CA, cholesteryl arachidonate; CL, cholesteryl linoleate; HbE, haemoglobin E; LDL, low-density lipoproteins.

Figure 1. The levels of α -tocopherol (a) and thiobarbituric acid reactive substance (TBARs) formation, (b) in low-density lipoproteins (LDL) of patients with β -thalassaemia/haemoglobin E ($n=29$) at each visit and the correlation curve between TBARs and ferric reducing antioxidant power (FRAP) and (c) in LDL of the patients across all visits. Visits include baseline (visit 1), after 4 weeks of L1 washout (visit 2), and after 4 weeks (visit 3) and 12 weeks (visit 4) of L1 retreatment.



*** $p < 0.001$; * $p < 0.05$ compared to baseline at visit 1; ### $p < 0.001$; ## $p < 0.01$ compared to the washout period (visit 2) (Wilcoxon signed-rank test).

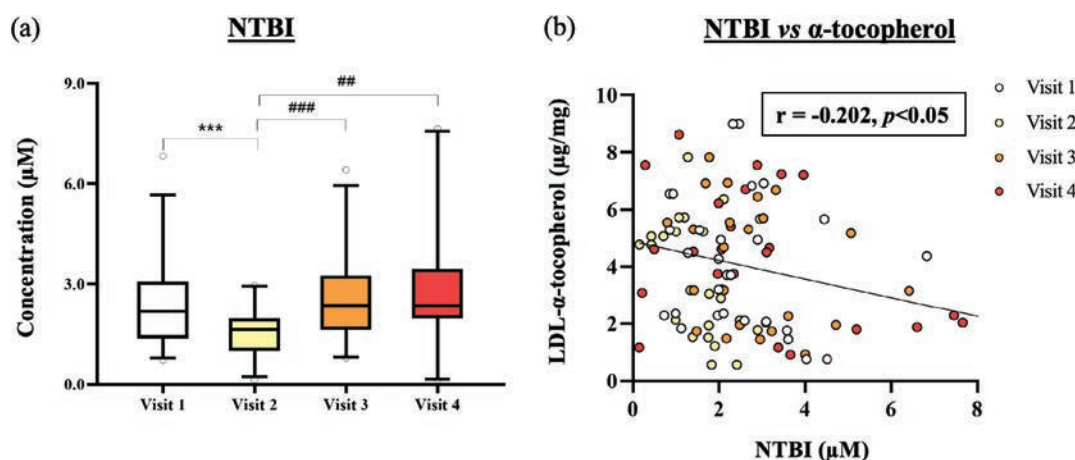
Correlations amongst lipid compositions and oxidative stress parameters in LDL of patients with β -thalassaemia/HbE

Correlations between LDL lipid compositions and oxidative stress parameters were assessed (Figure 3). A significant positive correlation was observed between α -tocopherol

and CA in LDL ($r=0.355, p < 0.001$; Figure 3a) as well as between α -tocopherol and CL ($r=0.290, p < 0.01$; Figure 3b). Additionally, CA was positively correlated with TBAR levels in LDL ($r=0.244, p < 0.01$; Figure 3c).

These findings suggest that α -tocopherol plays a protective role in preserving lipid components (CA and CL)

Figure 2. The levels of non-transferrin-bound iron (NTBI) (a) in the serum of patients with β -thalassaemia/haemoglobin E ($n=29$) at each visit and the correlation curve between serum NTBI and low-density lipoprotein α -tocopherol and (b) across all visits (Spearman's rank correlation). Visits include baseline (visit 1), after 4 weeks of L1 washout (visit 2), and after 4 weeks (visit 3) and 12 weeks (visit 4) of L1 retreatment.



Significant differences are indicated as follows: *** $p < 0.001$ compared to baseline (visit 1); ### $p < 0.001$; ## $p < 0.01$ compared to the washout period (visit 2) (Wilcoxon signed-rank test).

from peroxidation. The positive correlation between CA and TBAR levels highlights that CA may be a major target of lipid peroxidation under oxidative stress.

Oxidative susceptibility of LDL

The oxidative susceptibility of LDL was assessed by evaluating both the rate (slope) and the total extent (maximum fluorescence intensity) of NBD-Pen-L formation during Cu^{2+} -induced oxidation (Figure 4). The slope and maximum intensity of fluorescence during Cu^{2+} -induced LDL oxidation revealed the rate and extent of lipid radical formation (Figure 4a,b). Both parameters significantly increased during the L1 washout period (visit 2), indicating increased oxidative susceptibility of LDL to Cu^{2+} .

Correlations between the slope of lipid radical formation (NBD-Pen-L formation rate) and CA ($r=0.489, p<0.001$) as well as CL ($r=0.220, p<0.05$) were observed (Figure 5a,b). The stronger positive correlation between the slope and CA suggests that CA is more rapidly oxidized than CL under oxidative conditions.

Oxidative susceptibility of LDL, as indicated by NBD-Pen-L content, was positively correlated with lipid peroxidation markers, including TBARs ($r=0.593, p<0.001$; Figure 5c) and 13-HODE-CE ($r=0.319, p<0.01$; Figure 5d). A negative correlation was observed between NBD-Pen-L content and FRAP ($r=-0.458, p<0.001$; Figure 5e), reflecting an inverse relationship between lipid radical formation and

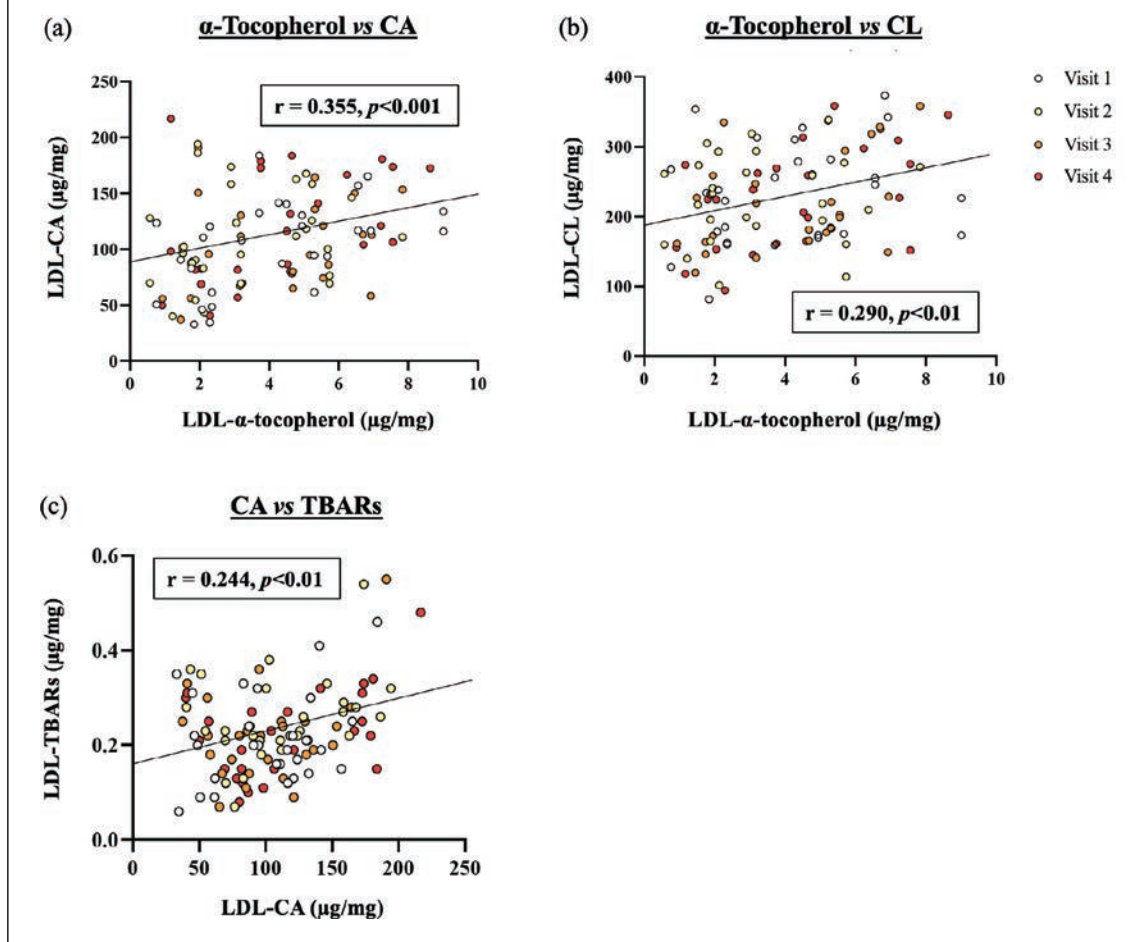
antioxidant capacity in LDL. These findings indicate that the oxidative susceptibility of LDL significantly increased during the L1 washout period, driven by lipid peroxidation processes primarily affecting CA. Retreatment with L1 effectively reduced oxidative susceptibility and restored antioxidant defences in LDL, highlighting its protective effects against oxidative damage.

Discussion

Oxidative modification of lipoproteins, particularly LDL, underlies various diseases. The evaluation of oxidative status of LDL may serve as an indicator of disease severity. For instance, Miljkovic et al.¹⁹ reported that the oxidative status of several lipoproteins, including LDL, is associated with renal disease. Similarly, Luechapudiporn et al.⁷ demonstrated that the reduction of CL in LDL could be used as a severity index for β -thalassaemia/HbE.

In this study, we focused on TBAR and α -tocopherol levels as markers of LDL oxidative status. Serum TBAR level, a classical marker for lipid peroxidation, has been linked to the incidence of atherosclerosis in patients with β -thalassaemia.^{4,7} α -Tocopherol, a key antioxidant in LDL, plays a critical role in maintaining redox balance and determining oxidative susceptibility of LDL.²⁰ Our findings suggest that the levels of TBARs and α -tocopherol in LDL of patients with β -thalassaemia/HbE reflect the oxidative status of LDL in the circulation and could serve as useful markers to monitor disease

Figure 3. The correlation curves between (a) low-density lipoprotein (LDL)- α -tocopherol and LDL cholesteryl arachidonate (CA) and (b) LDL- α -tocopherol and LDL cholesteryl linoleate (CL) and (c) CA and thiobarbituric acid reactive substances (TBARs) in LDL of patients across all visits (Spearman's rank correlation). Visits include baseline (visit 1), after 4 weeks of L1 washout (visit 2), and after 4 weeks (visit 3) and 12 weeks (visit 4) of L1 retreatment.



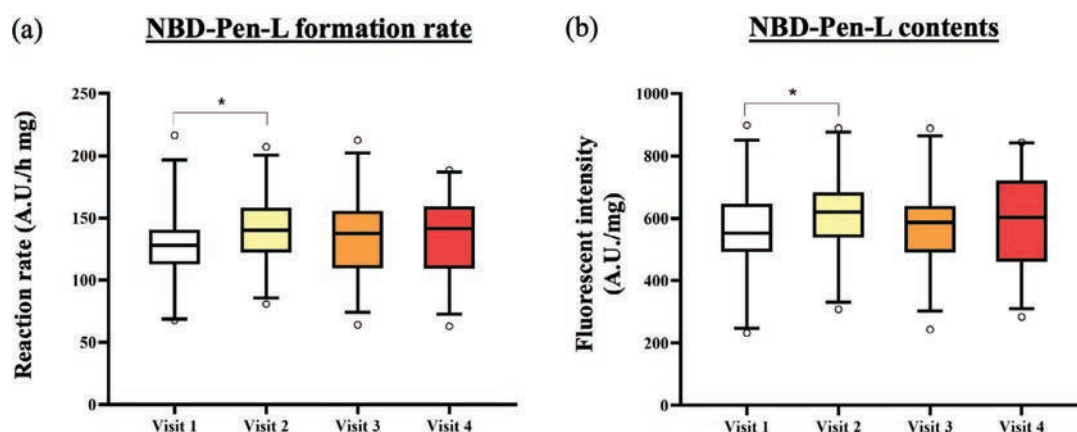
progression and evaluate the effectiveness of iron chelation therapy.

Our results showed that, during the L1 washout period, the reduced concentration of L1 in circulation led to a significant drop in α -tocopherol levels and a simultaneous increase in TBAR formation, indicating heightened lipid peroxidation. After retreatment with L1, α -tocopherol levels increased, and TBAR levels decreased, demonstrating the protective effect of L1 on the oxidative status of LDL, including the levels of antioxidant and lipid aldehyde product. Furthermore, the strong negative correlation between TBAR levels and FRAP of LDL (Figure 1c) supports the idea that the greater the production of oxidized lipid, the greater the reduction in antioxidant capacity of LDL.

Regarding iron overload, NTBI is considered a redox-active form of iron present in both tissue and the circulation of patients with β -thalassaemia, where it

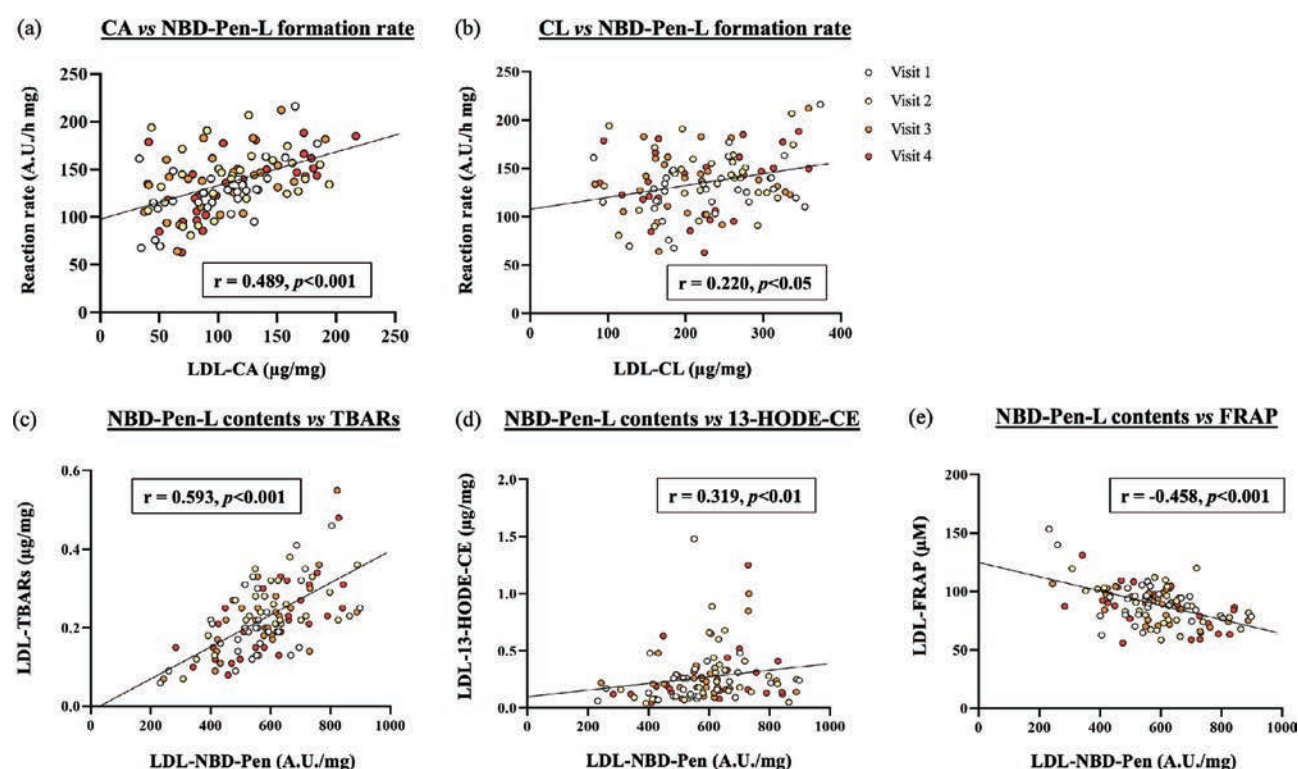
induces oxidative damage to nearby biological components.²¹ Here, we observed a significant reduction in NTBI levels during the L1 washout period, followed by a significant increase after 4 and 12 weeks of L1 retreatment. Although this paradoxical rise in NTBI might initially appear unfavourable, it aligns with previous findings that L1 can efficiently penetrate tissues, chelate intracellular NTBI and deliver it into the circulation for subsequent clearance.²² From this perspective, elevated NTBI during therapy may reflect mobilization of labile iron pools rather than enhanced redox toxicity, particularly when the L1-to-NTBI ratio exceeds 3:1 and the redox activity of NTBI is effectively suppressed.^{23,24} Clinically, this suggests that NTBI levels should be interpreted with caution as transient increases may indicate ongoing clearance processes. Supporting this view, our data demonstrated that, despite elevated NTBI during L1 treatment, oxidative markers improved: α -tocopherol levels increased, TBARs decreased, and a negative correlation was found between serum NTBI and

Figure 4. The reaction rate of NBD-Pen-L adduct formation during 3 h of CuSO_4 -induced oxidation (a) and the final contents of NBD-Pen-L adducts and (b) in low-density lipoprotein (LDL) of patients with β -thalassaemia/HbE ($n=29$) at each visit. Visits include baseline (visit 1), after 4 weeks of L1 washout (visit 2), and after 4 weeks (visit 3) and 12 weeks (visit 4) of L1 retreatment.



Significant differences are indicated as $*p<0.05$ compared to baseline (visit 1) (Wilcoxon signed-rank test).

Figure 5. The correlation curves between (a) cholesteryl arachidonate (CA) and NBD-Pen-L formation rate, (b) cholesteryl linoleate (CL) and NBD-Pen-L formation rate, (c) final NBD-Pen-L contents and thiobarbituric acid reactive substances (TBARs), (d) final NBD-Pen-L contents and 13-hydroxy-octadecadienoic acid cholesteryl ester (13-HODE-CE) and (e) final NBD-Pen-L contents and ferric reducing antioxidant power (FRAP) in LDL of patients across all visits (Spearman's rank correlation). The visits include baseline (visit 1), after 4 weeks of L1 washout (visit 2), and after 4 weeks (visit 3) and 12 weeks (visit 4) of L1 retreatment.



LDL- α -tocopherol. These findings suggest that LI exerts an independent protective effect on NTBI and α -tocopherol. Despite the non-redox-active nature of NTBI when chelated by LI, circulating NTBI still appears capable of depleting α -tocopherol in LDL. Our study thus highlights that even non-redox-active NTBI may contribute to the oxidative stress observed in patients with β -thalassaemia by diminishing the antioxidant defences of LDL. Thus, monitoring both NTBI and oxidative markers in parallel may provide a more accurate assessment of chelation efficacy in patients with β -thalassaemia.

In terms of lipid composition, it has been established that major lipid compositions in the core region of LDL can be modified by oxidative stress induced by iron overload. Our previous study demonstrated that the ratio of CL to CO is correlated with the severity of β -thalassaemia/HbE and could serve as a biomarker for the disease.⁷ However, the present study found no significant differences in lipid composition across visits. This finding suggests a compensatory adaptation mechanism that maintains the levels of lipid core components in LDL, making them less susceptible to changes in serum NTBI or LI concentration at different visits. This contrasts with the observed variations in α -tocopherol or lipid radical levels. Significant positive correlations were observed amongst α -tocopherol, CA and CL (Figure 3), suggesting a protective role of α -tocopherol in preserving other lipid components of LDL. α -Tocopherol can trap the generated lipid-derived radicals and break the chain reaction of lipid peroxidation.²⁵ Consequently, higher levels of α -tocopherol may reduce the consumption of lipid components, such as CA and CL, during lipid peroxidation. Interestingly, only CA was significantly correlated with the production of TBARs and FRAP in LDL, suggesting that CA is more actively involved in LDL oxidation compared to CL. This preference for CA in LDL oxidation warrants further investigation to better understand its role in oxidative stress and lipid peroxidation in patients with β -thalassaemia/HbE.

NBD-Pen is a fluorescence probe developed for the selective and specific detection of lipid radicals.²⁶ Recently, NBD-Pen was utilized to study the kinetics of lipid radical production in hemin-induced and AAPH-induced lipoprotein oxidation.²⁵ The results showed that the lag phase and propagation rate of oxidation were associated with the content of α -tocopherol and cholesteryl esters, respectively. Consistent with these findings, our data demonstrated that the slope of lipid radical production was significantly correlated with cholesteryl esters, particularly CA. In the LDL oxidation process, the core region, which is composed of cholesteryl

esters, is more sensitive to oxidation than the surface region.⁶ CL and CA are the most abundant lipid components in the core of lipoproteins,⁷ and several oxidation products of CL and CA have been implicated in atherosclerosis.²⁷ This explains why the rate of lipid radical formation correlated primarily with CA and CL, whilst the lipid radical contents correlated with markers of later lipid peroxidation such as TBARs and FRAP. With its ability to detect correlations with cholesteryl esters, NBD-Pen may be particularly useful for identifying the early phase of lipid oxidation in patients with thalassaemia. The significant increase in slope and content of lipid radicals during the LI intermission period further supports the protective role of LI in preventing LDL oxidation. In addition to reducing reactive iron, LI may help preserve antioxidants in LDL. Consequently, the higher susceptibility of LDL to oxidation observed during LI intermission highlights the importance of continuous treatment. The slope and content of lipid radical production during LDL oxidation were strongly correlated with lipid peroxidation markers, reinforcing the value of these parameters in evaluating oxidative susceptibility and damage.

The antioxidant properties of LI identified in our study may also provide mechanistic insight into the efficacy of combination chelation regimens. James and Prakash²⁸ demonstrated that the combination of deferasirox and LI significantly reduced serum ferritin levels in children with β -thalassaemia major who were inadequately controlled on deferasirox monotherapy. Whilst their study primarily assessed iron burden, our findings suggest that the protective effect of LI on LDL oxidative status — through preservation of α -tocopherol and reduction of lipid peroxidation — may represent an additional pathway by which chelation therapy provides clinical benefit. Thus, the combined use of two chelators may not only enhance iron removal but could also provide complementary antioxidant protection, thereby mitigating oxidative damage in patients with β -thalassaemia. Future studies incorporating oxidative LDL markers, such as TBARs, α -tocopherol and lipid radicals, into combination chelation regimens may further clarify their protective impact in patients with β -thalassaemia.

Despite these encouraging findings, certain limitations should be acknowledged. The relatively small sample size ($n=29$) and the limited follow-up period of 12 weeks restrict the ability to generalize our results to long-term cardiovascular outcomes in patients with β -thalassaemia. Larger studies with extended follow-up will be necessary to confirm the durability of the observed antioxidant effects of LI and to establish their clinical relevance in reducing cardiovascular risk.

Conclusion

The parameters derived from NBD-Pen serve as reliable indicators of LDL oxidative susceptibility and lipid per-oxidation processes. Our findings demonstrate that LI

treatment not only reduces reactive iron levels but also preserves LDL antioxidant levels, providing protection against oxidative stress. These results highlight the importance of continuous iron chelation therapy in mitigating LDL oxidation and preventing oxidative complications in patients with β -thalassaemia/HbE.

Contributions: Conceptualization: NPM and RL. Methodology: PL, NTT, NPM and RL. Validation: PL, NTT and NPM. Formal analysis: NTT, PL, NPM and RL. Investigation: NTT, PL and PS. Resources: PS, PR, KY, NPM and RL. Data curation: NTT, PL, PS, NPM and RL. Writing — original draft preparation: PL, NTT, NPM and RL. Writing — review and editing: NTT, NPM, KY, PR and RL. Visualization: PL, NTT, NPM. Supervision: NPM and RL. Project administration: NPM and RL. Funding acquisition: RL. NTT and PL contributed equally to this work. All authors have read and agreed to the published version of the manuscript. All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this article, take responsibility for the integrity of the work as a whole and have given their approval for this version to be published.

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