Received: 5 July 2021 | Accepted: 8 December 2021
DOI: 10.1111/bjh.18012

RESEARCH PAPER

Eltrombopag in paediatric immune thrombocytopenia: Iron metabolism modulation in mesenchymal stromal cells

Alessandra Di Paola[1] | Giuseppe Palumbo[2] | Chiara Tortora[3] | Maura Argenziano[3] | Marialuigia Catanoso[2] | Caterina Di Leva[3] | Giulia Ceglie[2] | Silverio Perrotta[3] | Franco Locatelli[2] | Francesca Rossi[3]

[1]Department of Experimental Medicine, University of Campania "Luigi Vanvitelli", Naples, Italy

[2]Department of Haematology, Bambino Gesù Hospital, Rome, Italy

[3]Department of Woman, Child and General and Specialist Surgery, University of Campania "Luigi Vanvitelli", Naples, Italy

Correspondence
Francesca Rossi, Department of Woman, Child and General and Specialist Surgery of University of Campania "Luigi Vanvitelli", Via Luigi De Crecchio, 4, Naples, Italy.
E-mail: francesca.rossi@unicampania.it

Funding information
This work was financially supported by VALERE 2019 "In-CHICAS".

Summary

Immune thrombocytopenia (ITP) is an autoimmune disease caused by platelet destruction mediated by auto-antibody production. It is characterized by a compromised immune system and alteration of the inflammatory response. Mesenchymal stromal cells (MSCs) play an important role in modulating immune and inflammatory processes, exerting immune-suppressing and anti-inflammatory properties. In ITP-MSCs the activity and survival are strongly impaired. Eltrombopag (ELT) is a thrombopoietin receptor agonist approved in chronic ITP for stimulating platelet production. It has immunomodulating properties by stimulating T and B regulatory cell activity and by promoting a macrophage switch from the pro-inflammatory to the anti-inflammatory phenotype. ELT also exhibits iron-chelating properties. Iron is a crucial element involved in several physiologic processes, but its intracellular accumulation determines cell damages. Therefore, for the first time we analysed the effect of ELT on ITP-MSCs demonstrating its ability to restore survival and activity of MSCs directly and to promote their survival and proliferation indirectly, by iron metabolism modulation.

KEYWORDS
eltrombopag, immune thrombocytopenia, iron metabolism, mesenchymal stromal cells.

INTRODUCTION

Immune thrombocytopenia (ITP) is an autoimmune disorder caused by the production of autoantibodies against platelets, determining their disruption.[1–4] This leads to thrombocytopenia and, in only 5% of patients, to a severe risk of bleeding.[1,3,4] In ITP an alteration of both inflammatory and immune profiles has been reported.[5,6] In particular, the increase of T-helper 1/T-helper 2 (Th1/Th2) and of classically activated/alternatively activated macrophage (M1/M2) ratios is responsible for an impairment of immune and inflammatory responses,[5,6] with a consequent alteration of cytokine release.[5–7] In ITP, the function and activity of mesenchymal stromal cells (MSCs) are also compromised.[6–8] MSCs are multipotent non-haematopoietic cells which exert anti-inflammatory and immunomodulatory properties.[6–8] In particular, they inhibit proliferation of T, B and natural killer (NK) cells,[9–12] thus influencing both innate and adaptive immunity and also the release of inflammatory cytokines.[13,14] In patients with ITP, MSCs show a reduced proliferation capacity and also a high grade of apoptosis and senescence,[15,16] and consequently their immunosuppressive

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and anti-inflammatory activities are compromised.[6,8,16,17] Rossi et al. demonstrated that the stimulation of cannabinoid receptor 2 (CB2) together with dexamethasone (Dexa) administration improves MSCs' activities and survival, restoring their anti-inflammatory and immunosuppressive properties.[6]

Iron plays a crucial role in cell survival, participating in several physiological processes.[18] Its metabolism is finely regulated by several proteins, such as transferrin (TF), transferrin receptor 1 (TFR-1), ferritin, ferroportin 1 (FPN-1) and hepcidin.[19–21] TF binds circulating iron and triggers it to target cells expressing TFR1, which is responsible for iron internalization. The intracellular iron could be stored in ferritin or exported by FPN-1, the only iron exporter.[20] FPN-1 activity is regulated by hepcidin, a peptide hormone highly expressed in several inflammatory conditions, which inhibits iron release by binding FPN-1 and inducing its degradation.[20,22] In particular, high levels of pro-inflammatory cytokines, such as interleukin-6 (IL-6), cause an increased expression of hepcidin, resulting in iron retention in cells.[20,22]

Nevertheless, deregulation of iron metabolism is responsible for the onset of different disorders.[18,21] Iron excess determines production of reactive oxygen species (ROS) that are toxic for cells, causing DNA, protein and lipid damages.[18,21] Iron overload-mediated ROS production induces apoptosis of MSCs and decreases their viability.[18] It has been reported that administration of iron chelators protects MSCs from iron overload effects.[18] In particular, deferasirox restores MSCs' viability and proliferation by chelating intracellular iron.[18] Moreover, Khoshlahni *et al.* demonstrated that the administration of desferal inhibits MSCs apoptosis induced by increased levels of iron-mediated ROS.[23]

Eltrombopag (ELT) is a thrombopoietin receptor agonist used in paediatric and adult patients with chronic ITP to stimulate platelet production.[2,6,24] It also exerts immunomodulating properties both by promoting T and B regulatory cells' activity[25,26] and by inducing the switch of macrophages from the pro-inflammatory phenotype to the anti-inflammatory one in ITP patients.[5] It has been reported that ELT also exerts iron-chelating properties by binding intracellular iron.[5,27]

Considering that in ITP impairment of the inflammatory state could lead to an increase of the intracellular iron concentration and consequently compromised survival and activities of MSCs, we evaluated the capability of ELT to restore MSCs' viability, survival and immune-modulating properties in ITP patients.

MATERIALS AND METHODS

Source of MSCs

Mesenchymal stromal cells (MSCs) were obtained from the bone marrow of 10 newly diagnosed ITP children (median age 6 ± 2 years) and 10 healthy donors (median age 6 ± 2 years). ITP patients and healthy subjects were enrolled in the Department of Women, Child and General and Specialized Surgery of the University of Campania Luigi Vanvitelli. Healthy subjects were bone marrow donors for siblings affected by malignant or non-malignant haematological pathologies that require haematopoietic stem cell transplantation. ITP children enrolled in this study were those with atypical presentation for which there is indication for bone marrow aspiration.[28] All procedures performed in this study were in accordance with the Helsinki Declaration of Principles, the Italian National Legislation, and the Ethics Committee of the University of Campania Luigi Vanvitelli, which formally approved the study (Identification code 0005511/i del 24/02/2021). Written informed consent was obtained from parents, and assent was acquired from children before any procedures.

Cultures of MSCs

Mesenchymal stromal cells were obtained from mononuclear cells, isolated from bone marrow by density gradient centrifugation (Ficoll 1.077 g/ml; Lympholyte, Cedarlane Laboratories Ltd., Uden, The Netherlands). Mononuclear cells were diluted in complete culture medium consisting of a low-glucose Dulbecco's Modified Eagle Medium (LG-DMEM) (Euroclone, Milan, Italy) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies Ltd, Paisley, UK), 50 U/ml penicillin, 50 mg/ml streptomycin, and 2 mM L-glutamine (Euroclone, Pero, Italy) and plated in non-coated T25 polystyrene culture flasks (Corning Costar, Celbio, Milan, Italy). Non-adherent cells were discarded after 48 h, while adherent cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was replaced twice a week until 80% confluence was reached; then MSCs were split, re-plated for expansion and harvested until the sixth passage (P0–P6). MSCs were characterized by flow cytometry using monoclonal antibodies, conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE), specific for these antigens: HLA-DR, HLA-ABC, CD45, CD34, CD13, CD14, CD31, CD80, CD90 (BD PharMingen, San Diego, CA, USA), CD73, and CD105 (Serotec, Kidlington, Oxford, UK). Labelled cells were acquired using a FACSCanto flow cytometer (BD PharMingen, San Diego, CA, USA), and data were elaborated using FACSDiva software (TreeStar Inc., Ashland, OR, USA).

Supernatants and proteins of MSCs were isolated after each passage. In particular, supernatants derived from CTR-MSCs and ITP-MSCs at P6 were collected to analyse IL-4, IL-10, interferon (IFN)-γ and IL-6 release with an enzyme-linked immunosorbent assay (ELISA) and to perform iron assay. Furthermore, ITP-MSCs were treated with ELT [6 μM] at P6. After 48 h of ELT incubation, cells were harvested for protein extraction, and cell culture supernatants were collected to analyse IL-4, IL-10, IFN-γ and IL-6 release with ELISA and to perform iron assay.

Drugs and treatments

Eltrombopag (ELT; Novartis S.p.a., Origgio, Italy) was dissolved in sterile water at a concentration of 10 mM. Cells were treated with ELT at the final concentration of 6 μM. This concentration was determined following a viability assay. Non-treated cultured cells were maintained in incubation media during the relative treatment time with and without vehicle (sterile water).

Count and viability assay kit

After 48h of ELT [2 μM], [6 μM] and [10 μM] incubation a count and viability assay was performed by using the Muse cell analyser machine. Briefly, 450 μl of the Muse Count & Viability reagent (Luminex Corporation, Austin, TX, USA) were added to 50 μl of MSC suspension and incubated for 5 min at room temperature. This reagent differentially stains viable and non-viable cells based on their permeability to the two DNA-binding dyes present in the reagent. The results were analysed with Muse 1.4 analysis software for data acquisition and analysis.

Cell dead and annexin V Assay

Apoptosis in MSCs treated was evaluated by a fluorometric assay on the Muse cell analyser machine with the Cell Dead and Annexin V Assay Kit. The Muse Annexin V & Dead Cell Assay utilizes annexin V to detect phosphatidylserine (PS) on the external membrane of apoptotic cells. A dead-cell marker, 7- amino-actinomycin D (7-AAD), is also used as an indicator of cell membrane structural integrity. Briefly, 100 μl of a cell suspension (1×10^5 cells/ml) was mixed with 100 μl of Muse Annexin V & Dead Cell Reagent and incubated for 20 min at room temperature in the dark. The results, automatically displayed, were analysed with Muse 1.4 Analysis software for data acquisition and analysis.

Total RNA extraction and reverse transcription quantitative polymerase chain reaction

Total RNA from MSCs cultures was extracted using Quiazol® (Quiagen, Hilden, Germania) following the manufacturer's instructions. EasyScript™ cDNA Synthesis Kit (abm, Richmond, Canada) was used to synthesize the first-strand cDNA from approximately 1 000 ng mRNA. The transcript levels of TFR-1 and FPN-1 were detected by reverse transcription quantitative polymerase chain reaction (RT-qPCR) using a CFX96 Real-Time PCR system (Bio-Rad, Hercules, CA, USA) using I-Taq Universal SYBR® Green Master Mix (Bio-Rad, Hercules, CA, USA). The cycling conditions were 10 min at 95°C (initial denaturation) followed by 40 cycles of 15 s at 94°C (denaturation) and 1 min at 68°C (annealing/extension/data collection). The β-actin gene served as the reference gene for normalization of the real-time PCR products. The PCR primers used to detect each gene were designed using the Primer 3 programme and synthesized by Sigma Aldrich (St. Louis, MO, USA). The linearity and efficiency of the assays were tested over dilutions of input cDNA spanning five orders of magnitude. Assays were performed in technical triplicate for each subject. Dissociation curve analysis of the amplification products was performed at the end of each PCR reaction to confirm the specificity of the amplification. The $2^{-\Delta\Delta Ct}$ method was used to analyse the data and obtain the relative gene expression levels.

Protein isolation and western blot

Proteins were extracted from treated and non-treated MSCs using RIPA Lysis Buffer (Millipore, Burlington, MA, USA), following the manufacturer's instructions. TFR-1, FPN-1, Bcl-2 and pERK proteins were detected in total lysates from MSCs cultures by western blotting. Membranes were incubated overnight at 4°C with these antibodies: rabbit monoclonal anti-TFR-1 (1:1000 dilution; abcam, Cambridge, UK), rabbit polyclonal anti-FPN-1 (1:1000 dilution; Novus Biologicals, Centennial, CO, USA), mouse monoclonal anti-Bcl-2 antibody (1:50 dilution; Santa Cruz Biotechnology, Dallas, TX, USA), and rabbit polyclonal anti-pERK antibody (1:1000 dilution; Bethyl Laboratories, Montgomery, TX, USA). Reactive bands were detected by chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate, Millipore, Burlington, MA, USA) on a C-DiGit® Blot Scanner (LI-COR Biotechnology®, Lincoln, NE, USA). A mouse monoclonal anti-β-actin antibody (1:100, Santa Cruz Biotechnology) was used as housekeeping protein. Images were captured, stored and analysed using Image studio Digits ver. 5.0 software.

ELISA

Interleukin-4, IL-10, IFN-γ, IL-6 and hepcidin levels were measured in cell culture supernatants by ELISA Assays (Invitrogen by Thermo Fisher, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, microplate wells were coated with monoclonal antibodies specific to the compounds of interest. All the standards and supernatants were pipetted into the wells and were run in duplicate. After the plate was washed, enzyme-linked polyclonal antibodies specific for IL-4, IL-10, IFN-γ, IL-6 and hepcidin were added to the wells. Then substrate solution was added, and the optical density was measured at a wavelength of 450 nm by using the Tecan Infinite M200 (Tecan Group Ltd, Männedorf, Switzerland) spectrophotometer. Cytokine concentrations (pg/ml) were determined against a standard concentration curve.

Iron assay

Cell culture supernatants were also used to measure iron (III) by using the Iron Assay Kit (Abcam, Cambridge,

UK) according to the manufacturer's instructions. Briefly, all the standards and MSCs supernatants were pipetted into microplate wells and were incubated with an acidic buffer to allow iron release. Then, an iron probe was incubated at 25°C for 60 min in the dark. Released iron reacted with the chromogen resulting in a colorimetric product (593 nm), proportional to the amount of iron. The optical density was measured at a wavelength of 593 nm by using the Tecan Infinite M200 spectrophotometer. Iron (II) and total iron (II + III) contents of the test samples (nmol/µl) were determined against a standard concentration curve. Iron (III) content can be calculated as: iron (III) = total iron (II + III) − iron (II).

Statistical analysis

Results are expressed as means ± standard deviation (SD). The experiments were conducted in triplicate. Statistical analyses on data derived from western blot and real-time PCR were performed using the Student's *t*-test (XLSTAT by Addinsoft 2020, Boston, MA, USA) to evaluate differences between quantitative variables. Statistical analyses on data derived from ELISA and cellular assays were performed using one-way ANOVA or ANOVA followed by a Bonferroni test. A *P* value ≤0·05 (*^) was considered statistically significant.

RESULTS

Characterization and morphology of MSCs

Immunophenotypic assay revealed no differences in immunophenotypes between healthy donors and ITP patients. Both CTR and MSC cells express surface CD105, CD73, CD90, HLA-ABC markers at high levels (100%) but are negative for CD45, CD14, CD34, CD13, CD31, CD80 and HLA-DR markers (Table 1). Morphologically, we observed that ITP-MSCs *in vitro* expanded slower, appeared larger and more flattened than CTR-MSCs.

TABLE 1 Immunophenotypic characterization of control mesenchymal stromal cells (CTR-MSCs) and immune thrombocytopenia (ITP)-MSCs. The table shows the antigens revealed using a FACSCanto flow cytometer (BD Biosciences, Milan, Italy) and the relative percentage of positivity

	Positivity (%)	
	CTR-MSCs	ITP-MSCs
CD105	100	100
CD73	100	100
CD90	100	100
HLA-ABC	96.35	97.34
CD45	0.04	0.15
CD14	0.21	0.33
CD34	0.43	0.55
CD13	0.12	0.18
CD31	0.02	0.09
CD80	0.43	0.31
HLA-DR	0.13	0.21

Effect of ELT on viability of ITP-MSCs

We performed a cytofluorimetric assay to evaluate the effects of ELT on the viability of ITP-MSCs. This treatment at concentration of 6 µM did not alter the percentage viability of ITP-MSCs compared to that of the untreated cells (NT) (Table 2). This result suggests that ELT [6 µM] is not cytotoxic for ITP-MSCs.

Effect of ELT on release of cytokines

To investigate the effect of ELT [6 µM] on cytokine release, we performed several ELISAs (Figure 1A–D).

We evaluated the release of the anti-inflammatory cytokines IL-4 and IL-10 by ELISA. We observed a statistically significant reduction of IL-10 levels in ITP-MSCs with respect to CTR-MSCs. We also found a trend towards a reduction in IL-4 levels, but this was not statistically significant. The administration of ELT [6 µM] did not induce variation in IL-4 and IL-10 levels in the supernatant of ITP-MSCs (Figure 1A, B). Moreover, we analysed the release of the pro-inflammatory cytokine interferon-γ (IFN-γ) and we showed a statistically significant increase of its levels in ITP-MSCs compared to CTR. ELT [6 µM] administration induced a strong reduction of IFN-γ with respect to non-treated ITP-MSCs (ITP NT). We also evaluated IL-6 levels in supernatants of ITP-MSCs. We confirmed that IL-6 levels are increased in ITP-MSCs compared to CTR. After treatment with ELT [6 µM], we observed a statistically significant reduction of IL-6 release (Figure 1C, D).

Effect of ELT on iron metabolism

Firstly, we performed an ELISA to determine hepcidin levels (Figure 2A). We observed a trend toward an increase of hepcidin in ITP-MSCs compared to CTR. Although not significant, this result suggested that high levels of IL-6 could led to hepcidin up-regulation. Then, we measured intracellular iron concentration [Fe^{3+}] and analysed mRNA and protein expression levels of transferrin receptor-1 (TFR-1) and ferroportin (FPN-1), two important compounds in the regulation of iron metabolism. TFR-1 is responsible for iron intake, while FPN-1 is the only iron exporter. We revealed an increase in intracellular iron concentration (Figure 2B) and a reduction of FPN-1 protein expression level (Figure S1D) in ITP-MSCs compared to CTR, confirming hepcidin's capability to induce iron accumulation in cells. We also observed

higher levels of TFR-1 protein in ITP-MSCs than in CTR-MSCs, further increasing intracellular iron concentration (Figure S1C). We demonstrated that ELT [6 μM] administration led not only to a strong reduction of the iron concentration, confirming its iron-chelating properties, but it also induced a reduction of *TFR-1* gene and protein expression levels and an increase of *FPN-1* gene and protein levels, causing a further decrease in intracellular iron levels (Figure 2B–D). No change in TFR-1 and FPN1 expression was induced by ELT administration in the CTR cells (Figure S2C, D).

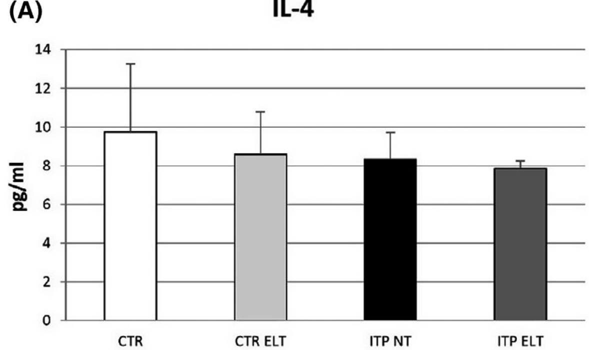
TABLE 2 Viability in immune thrombocytopenia mesenchymal stromal cells (ITP-MSCs) after eltrombopag (ELT) treatment

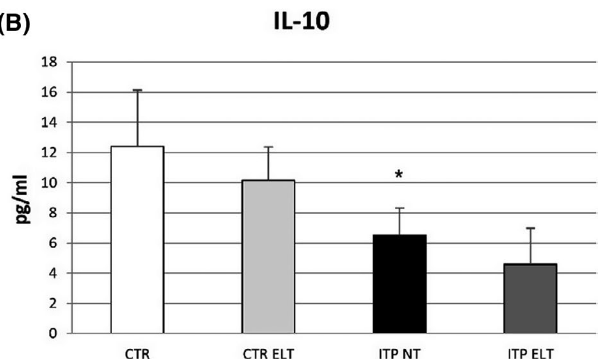
ITP- MSC	% Viable cells
NT	70.66 ± 3.3
ELT [2 μM]	75.4 ± 4.4
ELT [6 μM]	78.07 ± 6.4
ELT [10 μM]	57.82 ± 1.2*

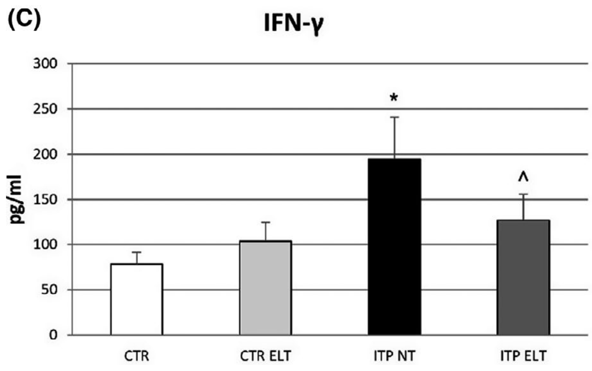
Percentage of viable cells in MSCs derived from ITP patients after 48 h of treatment with ELT at different concentrations (2 μM, 6 μM, 10 μM). The results are presented as the mean percentage ± standard deviation (SD) of independent experiments on each individual sample. A *t*-test was used for statistical analysis. $P < 0{\cdot}05$ was considered statistically significant compared to the untreated control (NT).

Effect of ELT on ITP-MSCs survival and proliferation

We firstly evaluated protein expression levels of Bcl-2 and pERK in ITP-MSCs compared to CTR-MSCs. We revealed lower levels of both proteins in ITP than in CTR, confirming that survival and proliferation of ITP-MSCs were impaired (Figure S1A, B). To strengthen our results, we also performed a cell count assay, which revealed a reduction of number of ITP-MSCs, supporting once again the evidence that ITP-MSCs' proliferative capacity is impaired (Table 3). To evaluate if ELT [6 μM] could restore survival and proliferation capability of ITP-MSCs, we performed western blotting to analyse Bcl-2 (Figure 3A) and pERK (Figure 3B) protein expression level after treatment and also a cell count assay (Table 3). Bcl-2 is an anti-apoptotic protein, while pERK is the activated form of mitogen-activated protein kinase 1 involved in cell proliferation. ELT [6 μM] administration increased expression levels of both proteins and of cell number. No change in Bcl-2 and pERK expression was induced by ELT administration on the CTR cells (Figure S2A, B). We also performed an apoptosis assay (Table 4) that, as expected, showed an increase of the percentage of total apoptotic cells in ITP-MSCs respect to CTR-MSCs. ELT administration did not induce any significant change in percentage of total







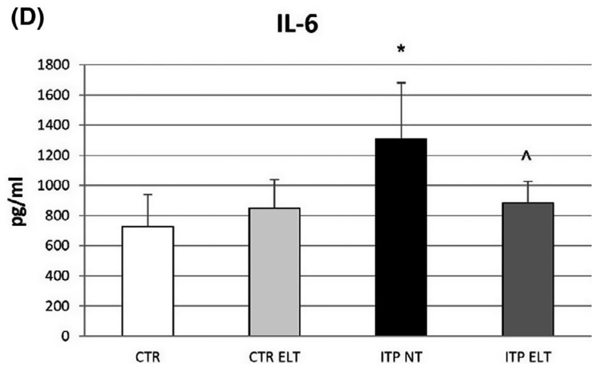


FIGURE 1 Quantification of pro-inflammatory and anti-inflammatory cytokines levels by enzyme-linked immunosorbent assay (ELISA). Interleukin (IL)-4 (A), IL-10 (B), INF-g (C) and IL-6 (D) levels in mesenchymal stromal cells (MSCs) from five control (CTR) and five immune thrombocytopenia (ITP) patients before and after 48 h of treatment with eltrombopag (ELT) [6 μM] determined through ELISA. Histograms show cytokine concentration as the mean ± standard deviation (SD) of independent experiments on each individual sample. One-way ANOVA has been used for statistical analysis. *, $P \leq 0{\cdot}05$ compared to CTR; ^, $P \leq 0{\cdot}05$ compared to the untreated control (NT).

FIGURE 2 Evaluation of iron metabolism. Hepcidin (A) from five control mesenchymal stromal cells (CTR-MSCs) and five immune thrombocytopenia (ITP)-MSCs before and after 48 h of treatment with eltrombopag (ELT) [6 μM] investigated through enzyme-linked immunosorbent assay (ELISA). The experiment was performed for each patient, and the graphs show hepcidin levels [pg/ml] as mean ± standard deviation (SD). One-way ANOVA followed by Bonferroni test has been used for statistical analysis. (B) Fe^{3+} intracellular concentrations (nmol/μl) in five CTR-MSCs and five ITP-MSCs before and after 48 h of treatment with ELT [6 μM] determined by iron assay. Histogram shows Fe^{3+} concentration as the mean ± SD of independent experiments on each individual sample. One-way ANOVA followed by Bonferroni test has been used for statistical analysis. *, $P \leq 0{\cdot}05$ compared to CTR; ^, $P \leq 0{\cdot}05$ compared to the untreated control (NT). Transferrin receptor (TFR1) (C) and ferroportin (FPN-1) (D) mRNA expression levels in MSCs from five ITP patients determined by quantitative polymerase chain reaction (qPCR). For the RT reaction, 1000 ng of total mRNA was used. Results normalized for the housekeeping gene β-actin are shown as mean ± SD of independent experiments on each individual sample. A *t*-test has been used for statistical analysis. *, $P \leq 0{\cdot}05$ compared to NT. Transferrin receptor (TFR1) (E) and ferroportin (FPN1) (F) protein expression levels in MSCs from five ITP patients determined by western blotting, starting from 15 μg of total lysates, before and after 48-h exposure to ELT [6 μM]. The most representative images are displayed. The protein bands were detected through Image Studio Digits software (LI-COR Biotechnology®, Lincoln, NE, USA) and the intensity ratios of immunoblots compared to NT, taken as 1, were quantified after normalizing with respective controls. The relative quantification for TFR1 and FPN1 expression, normalized for the housekeeping protein β-actin, is represented in the histogram as mean ± SD of independent experiments on each individual sample. A *t*-test has been used for statistical analysis. *, $P \leq 0{\cdot}05$ compared to NT.

TABLE 3 Cell count viability in mesenchymal stromal cells (MSCs) from five control (CTR) and five immune thrombocytopenia (ITP) patients before and after 48 h of exposure to eltrombopag (ELT) [6 μM].

	Total cells/ml
CTR-MSC	
NT	585 000.1
ELT	576 000.4
ITP-MSC	
NT	224 949.8*
ELT	280 200.2^

The results are presented as the mean percentage ± standard deviation (SD) of independent experiment on each individual sample. A *t*-test was used for statistical analysis. $P < 0{\cdot}05$ was considered statistically significant.

*Compared to CTR-MSCs for untreated patients (NT).

^Compared to ITP-MSCs NT.

TABLE 4 Annexin-V and propidium iodide (PI) double-stained apoptosis assay in mesenchymal stromal cells (MSCs) from five control (CTR) and five immune thrombocytopenia (ITP) patients before and after 48 h of exposure to eltrombopag (ELT) [6 μM]. The results are presented as the mean percentage ± standard deviation (SD) of independent experiments on each individual sample

	% Early apoptosis	% Late apoptosis	% Total apoptotic cells
CTR-MSC			
NT	18.23 ± 2.15	4.33 ± 2.50	22.56 ± 2.33
ELT	19.34 ± 2.30	4.98 ± 2.18	24.32 ± 3.55
ITP-MSC			
NT	33.58 ± 3.30*	12.16 ± 2.90*	45.74 ± 4.21*
ELT	35.90 ± 2.80	7.97 ± 3.30	43.87 ± 2.13

One-way ANOVA has been used for statistical analysis

*, $P \leq 0{\cdot}05$ compared to CTR-MSCs for untreated patients (NT).

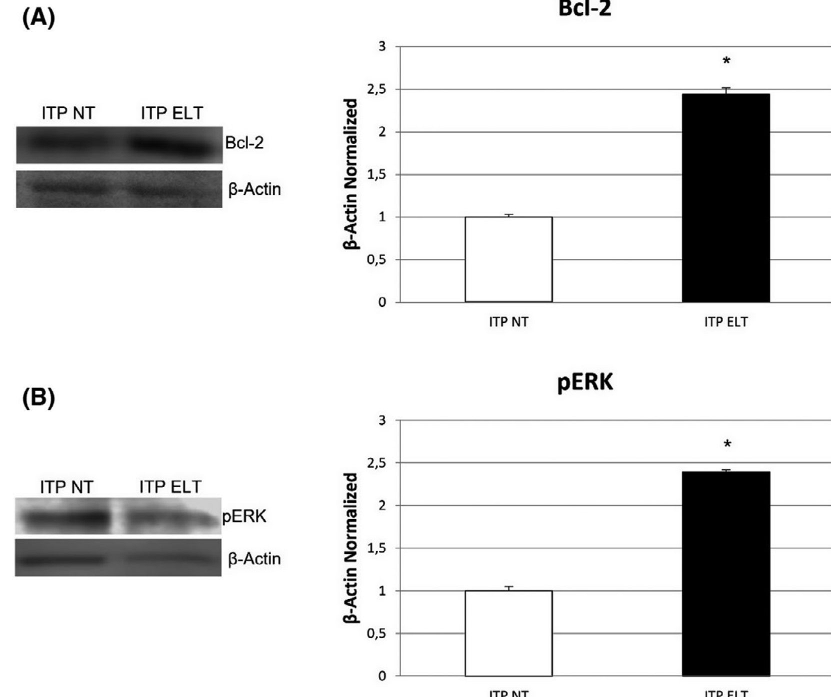


FIGURE 3 Evaluation of survival and proliferation. Bcl-2 (A) and pERK (B) protein expression levels in mesenchymal stromal cells (MSCs)_ from five immune thrombocytopenia (ITP) patients was determined by western blotting, starting from 15 μg of total lysates, before and after 48-h exposure to eltrombopag (ELT) [6 μM]. The most representative image is displayed. The proteins were detected using Image Studio Digits software (LI-COR Biotechnology®, Lincoln, NE, USA) and the intensity ratios of immunoblots compared to the untreated control (NT), taken as 1 (arbitrary unit), were quantified after normalizing with respective loading controls for the housekeeping protein β-actin. The graph represents the relative quantification for Bcl-2 and pERK protein expression as mean ± standard deviation (SD) of independent experiments on each individual sample. A *t*-test has been used to evaluate the statistical differences. *, $P \leq 0{\cdot}05$ compared to NT.

apoptotic cells both in CTR and ITP-MSCs, but it prevented other cells from going into apoptosis, blocking them in the early phase of apoptosis.

DISCUSSION

Immune thrombocytopenia is an autoimmune disease caused by platelet destruction mediated by production of auto-antibodies.[1,4] It is characterized by immune system compromise and alteration of the inflammatory response, with an excess release of pro-inflammatory cytokines.[5–7] MSCs play an important role in modulating immune and inflammatory processes, exerting immune-suppressing and anti-inflammatory properties.[6,8] In fact, MSCs inhibit the proliferation and activities of T, B and NK cells,[9–12] regulating innate and adaptive immunity and inflammatory response.[13,14] It has been demonstrated that MSCs from

ITP patients displayed enhanced senescence and apoptosis and showed a lower capacity in inhibiting the proliferation of activated T cells.[6,8,16,17] ITP-MSCs showed an increased secretion of the pro-inflammatory cytokines IFN-γ and IL-6, which may contribute to their reduced function. Eltrombopag (ELT) is a thrombopoietin receptor agonist, commonly approved in chronic ITP, responsible for stimulation of platelet production.[6] ELT also exerts immunomodulating properties by stimulating T and B regulatory cells' activity and by promoting macrophages' switch from phenotype M1 to M2.[5,25,26] Considering its immunomodulating properties,[27] in this study we evaluated ELT's capability to restore viability, survival and immune-modulating properties of MSCs in newly diagnosed ITP children. As expected, ELT administration considerably reduced levels of pro-inflammatory cytokines. It is known that inflammation could induce iron accumulation in cells, which causes ROS production and consequently cell damage.[20,22] In particular, high levels of IL-6 induce an increase in hepcidin production and activation.[20,22] Hepcidin plays a key role in modulating iron metabolism: it mediates ferroportin (FPN-1) degradation, thus preventing iron release by cells with a consequent increase of intracellular iron concentration.[18,20] Growing evidence suggests that iron overload could damage the bone marrow microenvironment.[18] In particular, Pittenger *et al*. in 2019 suggested a role of iron in creating an important bone marrow niche for haematopoiesis and that MSCs support for haematopoiesis could be dependent on iron regulation.[29] Moreover, Jiaxin Hu *et al*. demonstrated that iron overload regulates the release of cytokines by MSCs.[30] Considering the well-known correlation between IL-6 levels and hepcidin activation,[20,22] we hypothesize that the increased levels of IL-6 detected in ITP patients could induce hepcidin activation and consequently an intracellular iron increase, thus contributing to the function and viability impairment of MSCs. In effect, we observed a trend toward an increase for hepcidin and accumulation of Fe^{3+} in ITP-MSCs. MSCs damaged by iron could be unable to support their important functions. Therefore, new possible therapeutic strategies for ITP could aim to restore function and activity of MSCs, which are strongly impaired by iron accumulation. Recently, ELT's iron-chelating properties are emerging.[31] ELT mobilizes iron from the intracellular compartment, thus reducing availability of the metal to cells. We have widely proved its chelating properties in osteoclasts from beta thalassemia major patients,[27] in osteosarcoma[32] and interestingly in macrophages obtained from ITP patients.[5] In particular, we suggested for the first time ELT as a mediator of the switch of macrophage phenotype from the M1 pro-inflammatory type to the M2 anti-inflammatory one in paediatric patients with ITP.[5]

According to our previous data, also in ITP-MSCs ELT administration resulted in a reduction of intracellular iron concentration, confirming once again its iron-chelating properties. Moreover, in order to identify a new possible ELT mechanism of action on the regulation of intracellular iron concentration, we evaluated ELT's effect on two important key regulators of iron metabolism: TFR-1 and FPN-1. TFR-1 is responsible for iron intake, while FPN-1 is the only known iron exporter.[19–21] We revealed increased levels of TFR-1 and reduced levels of FPN-1 in ITP-MSCs compared to CTR-MSCs. These interesting results not only confirmed our hypothesis about iron accumulation determined by hepcidin-induced FPN-1 degradation but suggested an overexpression of TFR-1 in ITP-MSCs which led to increased iron uptake. For the first time in our knowledge, we demonstrated that ELT is able to modulate these iron transport proteins levels. In particular, ELT reduced TFR-1 expression levels, thus inhibiting iron uptake; at the same time, it increased FPN-1 levels, promoting iron release. Considering these results, ELT seems to have an important role in modulating iron efflux, both inhibiting its inflow into cells and allowing its release by cells, with the important consequence of reducing intracellular iron concentration. This dual function of ELT together with its iron-chelating properties could be crucial for restoring the proliferative capacity and the survival of MSCs damaged by iron excess.[18] We previously demonstrated that MSCs from ITP show an impaired proliferative capacity and a high percentage of cells in apoptosis, observing an important reduction of Bcl-2, a key protein of the apoptosis pathway.[6] Accordingly, we observed a reduction of Bcl-2 protein expression level and an increase of total apoptotic cells in ITP-MSCs compared to CTR-MSCs. Interestingly, we also revealed a decrease of pERK protein expression levels in ITP-MSCs and a reduction of their count, confirming the impairment of their proliferative capacity. Moreover, we demonstrated that ELT induced an increase of both Bcl-2 and pERK protein expression levels and cell count, highlighting its beneficial effects on MSC survival and its capability to restore MSC proliferation. Furthermore, although ELT did not reduce the total of apoptotic cells, interestingly, we observed that its administration prevented other cells from going into apoptosis, establishing a blockade of cells in the early phase. This result indicates the possible effect of ELT in counteracting apoptotic processes, even though additional data should be useful for a deeper evaluation. In future it could be interesting to investigate how ELT influences apoptosis and identify its molecular targets.

In conclusion, our study adds new evidence on the immunomodulating properties of ELT, demonstrating its capability to reduce the inflammatory condition also in ITP-MSCs. Moreover, we have demonstrated for the first time that ELT directly acts on the survival and activity of MSCs, restoring their survival and proliferation, and indirectly, by influencing iron efflux. These data suggest the possibility of using ELT in newly diagnosed ITP not only to stimulate the production of platelets but also to restore the well-known anti-inflammatory and immune-suppressing properties of MSCs[6,33] derived from ITP patients.

ACKNOWLEDGEMENTS

The authors would like to thank the subjects enrolled in the study and their parents.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

ADP: supervision, data curation, validation, methodology and writing — original draft preparation; GP: investigation and resources; CT: data curation, formal analysis, methodology and validation; MA: data curation, formal analysis, methodology and validation; MC: investigation and resources; CDL: methodology and data analysis; GC: investigation and resources; SP: contribution to revisions; FL: investigation and resources; FR: conceptualization, project administration, funding acquisition, supervision, and writing — original draft preparation.

ORCID

Giulia Ceglie https://orcid.org/0000-0002-4127-6914
Franco Locatelli https://orcid.org/0000-0002-7976-3654
Francesca Rossi https://orcid.org/0000-0003-2879-6277

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How to cite this article: Di Paola A, Palumbo G, Tortora C, Argenziano M, Catanoso M, Di Leva C, et al. Eltrombopag in paediatric immune thrombocytopenia: Iron metabolism modulation in mesenchymal stromal cells. Br J Haematol. 2022;197:110–119. https://doi.org/10.1111/bjh.18012