# Mitapivat Treatment Increases β-Thalassemic Erythroblasts Energy Production and Responsiveness to Oxidative Stress

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## BACKGROUND

- In thalassemic syndromes, oxidation plays a key role in both ineffective erythropoiesis and reduced survival of circulating red cells. [1]
- In a mouse model of β-thal (Hbb3th/+), we showed that mitapivat, a Pyruvate Kinase (PK) activator, improves anemia by targeting both ineffective erythropoiesis and chronic hemolysis.[2,3]
- We corroborated this finding by the observation that *in vitro* mitapivat ameliorates the human β-thal erythroid cell maturation index and reduces the amount of Annexin-V$^+$ cells. [2]
- **The results of the phase 2 proof-of-concept study (NCT03692052) in patients with non-transfusion-dependent thalassemic (NTDT) support mitapivat as a new potential therapeutic strategy in thalassemic syndromes. [4]**

## METHODS

**In vitro cell cultures of human erythoid precursors from CD34$^+$ cells:** We studied CD34+ derived erythroblasts from either healthy controls or β-thal (cod $\beta^{039}$) patients (n=5). Light-density mononuclear cells were obtained by centrifugation on Lymphoprep (STEMCELL Technologies) density gradient, as previously described.[2] The CD34$^+$ cells were positively selected by anti-CD34-tagged magnetic beads (MiniMACS columns; Miltenyi Biotech) according to the manufacturer's protocol. The recovery was more than 90% CD34$^+$ cells, as determined by flow cytometry. CD34$^+$ cells were grown at a density of $10^5$ cells/mL in α-MEM supplemented with 100 U/mL penicillin-streptomycin, 2 mmol/L L-glutamine, $10^{-6}$ mol/L hydrocortisone, $10^{-3}$ g/L nucleotide, 25 × $10^{-3}$ mg/L gentamicin, $10^{-4}$ mol/L 2-mercaptoethanol, 1% deionized BSA, 1 μg/mL cyclosporine A (all from Sigma Aldrich), and 30% fetal bovine serum (GIBCO) using a two step based procedure. The following recombinant cytokines were added to the media: 3 U/mL recombinant human (rH) EPO (Janssen-Cilag), 20 ng/mL rH stem cell factor, and 10 ng/mL rH interleukin-3 (both PeproTech). Whenever indicated mitapivat was added to the culture medium at 5, 7, 10, 13 days of culture (final concentration: 2 μM). Cell samples were collected for cell counting and determination of cell viability.[2]

**Analysis PKM2 and PKLR activity:** Recombinant PKM2 and PKLR (R&D systems) were diluted in 1X Buffer: (100 mM KCl, 50 mM Tris 7.5, 5 mM MgCl2, 1 mM DTT, 0.03% BSA). 1 uL compound was added into wells first, and then 40 uL reaction mix was added. Reactions were assembled except for ADP, and plates were stored for 60 minutes at RT. 10 uL ADP was added to start reaction and read OD340 at Multiskan. [5]

**qRT-PCR on erythroblasts: :** 0.5 million of cells collected at day 11$^{th}$ and 14$^{th}$ of culture were used to isolate mRNA and reverse transcribed into high-purity complementary DNA (cDNA) using μMACS One-step cDNA Kit according to the manufacturer's instructions (Miltenyi Biotec). qRT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) using Applied Biosystems Model 7900HT Sequence Detection System. All PCR reactions were performed in triplicate. Relative gene expression was calculated using the 2-ΔCt method. HbA1 (hemoglobin subunit alpha 1) was used as internal control gene. [6]

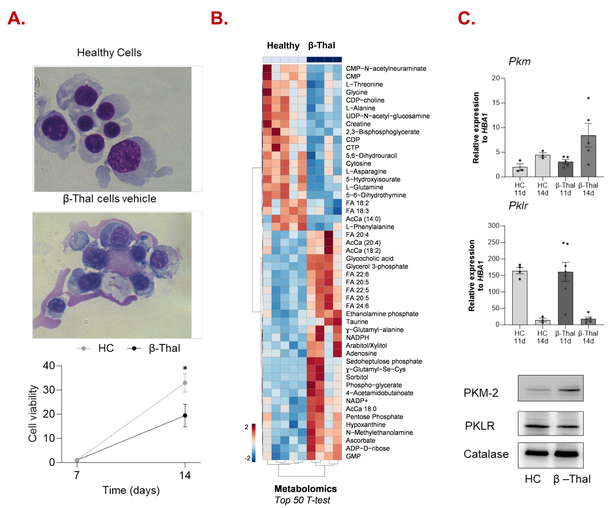
**Western blot analysis:** 1.5 million of cells collected at day 11$^{th}$ and 14$^{th}$ of culture were solubilized as previously described [2] and analysed by SDS-PAGE followed by western blot analysis using specific antibodies anti-Pklr (Santa Cruz Biotechnology, USA), anti-Pkm2 (Cell Signaling Technologies), anti Prx-2 (kindly gifted by Ho Zoo Chae, School of Biological Science and Technology, Chonnam National University, Gwangju, Korea). Catalase was used as protein loading control. Images were acquired using the Alliance Q9 Advanced Chemiluminescence Imager (Uvitec, Cambridge, UK).

**Metabolomic analysis:** 1.5 million of cells collected at day 14$^{th}$ of culture were resuspended in ice-cold methanol:acetonitrile: water (5:3:2, v/v/v) and vortexed at 4°C for 30min prior to a 96-well plate-compatible positive pressure-assisted filtration of the extracts. Filtered extracts were analysed using a Vanquish UHPLC coupled online to a Q Exactive mass spectrometer (Thermo Fisher, Bremen, Germany). Samples were analyzed using a high-throughput 1min gradient, as generally described.[7] Solvents were supplemented with 0.1% formic acid for positive mode runs and 10mM ammonium acetate +0.1% ammonium hydroxide for negative mode runs. MS acquisition, data analysis and elaboration was performed as previously described (Nemkov et al., Frontiers in Physiology, 2022). [8]

**Immunofluorescence analysis:** 0.25 million of cells were cytospun onto a slide, fixed with buffered paraformaldehyde 4%, permeabilized with Triton 0.1% and immunostained using specific anti Prx2 (kindly gifted by Ho Zoo Chae) followed by the secondary anti Rabbit Alexa Fluor 633 conjugated (Thermo Fisher Scientific, USA). DAPI was used to stain nuclei. Cells were imaged using the Inverted confocal microscope Leica TCS SP5 AOBS, magnification 63x (1.4). [9]

## RESULTS

### Erythroblasts from β-Thal show altered metabolomic profile and compensatory upregulation of PKM2.

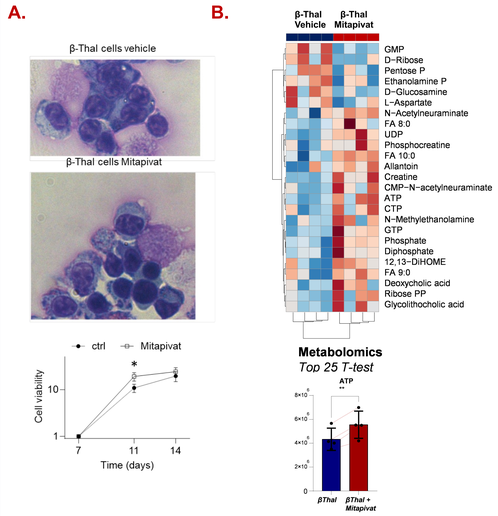



**A. The morphologic analysis of β-Thal erythroid precursors shows irregular nuclear shape and chromatin condensation when compared to healthy controls.** McGrawald-Giemse staining for erythroblast morphology (upper panel) of CD34+ derived erythroid precursors, at 14 days of culture, from healthy controls (HC) and β-thal patients (cod β039). One representative image from 5 with similar results is shown. Original magnification 100×. Cell growth (lower panel) of the erythroid precursors as in A. Data are mean ± SEM ($n$ = 5). * p<0.05 compared to HC cells.

**B. Metabolomic profile of β-Thal erythroid precursors is characterized by an altered metabolite profile suggestive of high oxidative stress.** Heat map of the top 50 significant metabolites of erythroid precursors, at 14 days of culture, from healthy controls (HC) and β-thal patients. (n= 5-4).

**C. β-Thal erythroblasts show persistent expression of PKM2 in the late phases of erythropoiesis.** qRT-PCR (upper panel) of erythroid precursors, at 11 and 14 days of culture, from healthy control (HC) and β-thal patients. HBA1 served as constitutive gene. Data are mean±SEM (n=3-5). **Lower panel.** Western-blot analysis with specific antibodies against PKM2 and PKLR of erythroid precursors as in A at 14 days of culture. Catalase was used as loading control. One representative immunoblots of 3 others with similar results.

### Mitapivat, targeting both PKLR and PKM2 isoforms, boosts glycolysis with increased ATP cell content and modulates the PPP pathway, reducing oxidation and improving *in vitro* human β-Thal erythropoiesis.




Kung et al have previously shown that mitapivat activates both PKLR and PKM.[5] We in vitro lidated the ability of mitapivat to target both PK isoforms, using commercially available Pk oform PKM2 and PKLR (R&D systems). We then evaluated the impact of mitapivat on etabolome of β-thal erythroblasts.

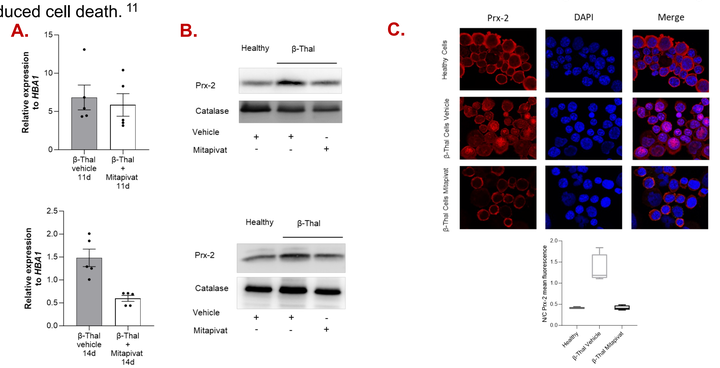
**Mitapivat treated** β-thal cells show less irregular nuclei and more condensed chromatin mpared to vehicle treated β-thal cells **(upper panel).** One representative micropicture at days of culture from β-thal patients (cod $\beta^{039}$) *in vitro* treated with either vehicle or tapivat (2 μM). Data are mean ± SEM ($n$ = 5). * p<0.05 compared to Vehicle (lower panel).

**Metabolomic analysis shows that mitapivat stimulates glycolysis and energy oduction in β-Thal erythroid precursors promoting cell response to oxidative stress.** eat map of the top 25 significant metabolites of CD34+ derived erythroid precursors, at 14 ys of culture, from β-thal patients (cod β039) *in vitro* treated with either vehicle or mitapivat (2 μM). Data are single samples run in 3 technical replicates ($n$ = 4).

**Lower panel.** A bar plot focusing on ATP content in β-Thal erythroblasts treated with either vehicle or Mitapivat as in the upper panel.

### Mitapivat treated human β-thal erythroblasts display down-regulation of the Prx2 oxidative sensor and normalization of Prx2 cellular distribution.

Previous studies have shown that Peroxiredoxin-2 (Prx2) acts as anti-oxidant and atypical chaperone, with a distribution between cytoplasm and nucleus in presence of intense oxidation. In β-thal erythropoiesis, we demonstrated a key role of Prx2 as cytoprotector to ensure erythroid maturation and survival. [9,10] In the nucleus, Prx2 has been show to protect against DNA damage induced cell death. [11]




**A. Down regulation of Prx2 characterized β-Thal erythroblasts treated with mitapivat** qRT-PCR and **B.** Western-blot analsysis (Wb) of erythroid precursors, at 11 and 14 days of culture, from β-thal patients (cod β039) *in vitro* treated with either vehicle or mitapivat (2 μM). HBA1 (for qPCR) and catalase (for Wb) served as constitutive controls. Data are mean±SEM (n=3-5) * p<0.05 compared to vehicle treated cells. One representative western blot of other 3 with similar results is shown.

**C. In β-Thal erythroblasts, Prx2 translocates into the nucleus to counteract oxidative stress. Mitapivat (2 μM) normalizes Prx2 localization, which display mainly a cytoplasmic localization.** Prx2 ratio of nuclear/cytoplasmic staining of 4 different square and at least 150 cells. Data are presented as box plots, whiskers representing min to max.

## CONCLUSIONS

- ***In vitro* human β-Thal erythroblasts show alteration of metabolomic profile with a shift towards PPP, due to intense cell oxidation.**
- **In human β-Thal erythroblasts, mitapivat boostes glycolysis, favoring energy production and responsiveness to oxidative stress.**
- **Mitapivat treated β-Thal erythroblasts displayed downregulation of Prx2 with normalization of its cellular distribution, supporting a reduction in DNA damaged induced block in cell maturation.**

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