Communication

The α-lipoic acid improves hepatic metabolic dysfunctions in Acute Intermittent Porphyria: a proof-of-concept study

Miriam Longo[1,2]; Erika Paolini[1,3];Marica Meroni[1]; Lorena Duca[4], Irene Motta[1,2]; Anna L. Fracanzani[1,5]; Elena Di Pierro[4*]; Paola Dongiovanni[1*]

1 General Medicine and Metabolic Diseases; Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Milan, Italy.

2 Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milan, Italy.

3Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milan, Italy.

4Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, U.O.C Medicina Generale, Milan, Italy.

5Department of Pathophysiology and Transplantation, Università degli Studi di Milano, Italy.

*Equal contributors

Corresponding Author

Paola Dongiovanni

General Medicine and Metabolic Diseases, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Milan, Italy,

Via Francesco Sforza 35, 20122 Milano.

Phone: +39-02-55033467. Fax +39-02-50320296.

e-mail: paola.dongiovanni@policlinico.mi.it

Abstract

Background: Acute intermittent porphyria (AIP) is caused by haploinsufficiency of porphobilinogen deaminase (PBGD) enzymatic activity. Acute attacks occur in response to fasting and alterations in glucose metabolism, insulin resistance and mitochondrial turnover may be involved in AIP pathophysiology. Therefore, we investigated the metabolic pathways in PBGD-silenced hepatocytes and assessed the efficacy of an insulin-mimic, the α-lipoic acid (α-LA) as a potential therapeutic strategy.

Methods: HepG2 cells were transfected with a siRNA targeting PBGD (siPBGD). Cells were cultured with low glucose concentration to mimic fasting and exposed to α-LA alone or with glucose.

Results: At baseline, siPBGD cells showed lower expression of genes involved in glycolysis and mitochondrial dynamics along with reduced total ATP levels. Fasting further unbalanced glycolysis by inducing ATP shortage in siPBGD cells and activated DRP1, which mediates mitochondrial separation. Consistently, siPBGD cells in fasted state showed the lowest protein levels of Complex IV which belong to the oxidative phosphorylation (OXPHOS) machinery. α-LA upregulated glycolysis and prompted ATP synthesis and triglyceride secretion, thus possibly providing energy fuels to siPBGD cells by improving glucose utilization. Finally, siPBGD exposed to α-LA plus glucose raised mitochondrial dynamics, OXPHOS activity and energy production.

Conclusions: α-LA-based therapy may ameliorate glucose metabolism and mitochondrial dysfunctions in siPBGD hepatocytes. Keywords: AIP, PBGD, glucose metabolism, mitobiogenesis, α-lipoic acid

Keywords: AIP, PBGD, glucose metabolism, mitochondrial dynamics, alpha-lipoic acid

1. Introduction

Acute Intermittent Porphyria (AIP) is the most common and severe form of acute hepatic porphyria (AHP), a heterogeneous group of hereditary metabolic disorders characterized by defects of heme biosynthesis. Clinical manifestations include acute neurovisceral attacks (abdominal pain, nausea, constipation, vomiting) occurring in

response to precipitating factors such as fasting, reproductive hormones, infections, drugs, alcohol, and physical stress, which have in common the increased demand of hepatic heme production. Heme exerts a negative feedback on the first rate-limiting enzyme of biosynthetic pathway, the 5-aminolaevulinic acid (ALA) synthase 1 (ALAS1) [1-4]. Loss of regulatory heme pool causes the upregulation of ALAS1 together with the overproduction of heme precursors (porphyrins) as ALA and porphobilinogen (PBG), which play a key role in neurotoxicity [5-7]. Indeed, some AIP patients displayed elevated levels of ALA and PBG outside of attacks, which reached the peak during the crisis, and they were associated with debilitating symptoms, and long-term complications as sensorimotor neuropathy [8].

Causative mutations of AIP affect the *Hydroxymethylbilane Synthase* (*HMBS*) gene, encoding the porphobilinogen deaminase (PBGD) enzyme, and their prevalence is relatively high in the general population (~1/1700 subjects). Notwithstanding, the clinical penetrance hovers just around 0.5-1% of AIP carriers [9]. More than 400 genetic mutations identified in the *HMBS* gene may predispose to the effects of precipitating factors, but they are not sufficient to account for the phenotypic variability observed in AIP individuals. Indeed, over 90% of AIP subjects, some of whom are highly excreters of both ALA and PBG, remain asymptomatic throughout their life, suggesting that pathophysiology of acute events may not be directly triggered by the accumulation of the neurotoxic byproducts.

Alterations of glucose metabolism and mitochondrial bioenergetics have been described in AIP experimental models, but their role in precipitating the AIP symptomatology has not been demonstrated yet. Impaired glucose tolerance and low glucose availability were observed in AIP murine models exposed to porphyrinogen drugs inducing the acute attack [10, 11]. Collantes *et al*. revealed that AIP mice aberrantly responded to caloric restriction by stimulating gluconeogenesis and ketogenesis rather than glycogenolysis in the liver [11]. *PBGD*-deficient rodents showed NADH, FADH2 and succinyl-CoA shortage, the primary sources for the electron flux of the respiratory chain and heme synthesis, respectively [11, 12]. Furthermore, hepatic transcriptome of AIP mice revealed that the majority of differentially expressed genes during a stress-induced attack were involved in circadian rhythm, mitochondrial biogenesis and oxidative phosphorylation (OXPHOS), whose regulation closely depends by peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) [13].

Intravenous hemin administration represents the first-line therapy in AIP patients to manage severe episodes even if its chronic infusion was associated with persistent ALAS1 activation thus contributing to the occurrence of debilitating attacks [14]. Recently, ALAS1-directed small interfering RNA (Givorisan) was introduced for AIP treatment in subjects with recurrent attacks, although its efficacy was paralleled by increased hepatic and renal adverse complications [15]. In sum, the current standards of care are mostly addressed to reduce hepatic ALAS1 activity although they may present several shortcomings [16].

Foregoing studies have highlighted that AIP is featured by an impaired glucose metabolism in experimental models to the extent that carbohydrate loading has been proposed as alternative strategy in patients with mild attacks. Moreover, AIP patients showed a higher prevalence of hyperinsulinemia and insulin resistance (IR) compared to control volunteers including family members without *HMBS* mutations and porphyrin accumulation [17, 18], thereby opening new perspectives for the development of novel medical care aiming to improve insulin sensitivity. Consistently, a liver-targeted insulin therapy in AIP mice promoted ALAS1 downregulation and improved glucose metabolism [17]. Therefore, this study aimed to reproduce *in vitro* a condition which parallels human AIP by silencing PBGD mRNA in human HepG2 hepatoma cells (siPBGD) and to investigate metabolic alterations occurring at baseline and during a stressful factor (fasting), pointing to an in-depth characterization of glucose metabolism and mitochondrial turnover. Furtherly, in the attempt to offer a novel therapeutic option for the AIP prophylaxis, we assessed the potential efficacy of a nutraceutical supplement,

the insulin-mimic alpha-lipoic acid (α-LA), which has been already tested for the treatment of metabolic disorders as type 2 diabetes, turning out to be effective and safe at improving glucose handling, insulin sensitivity and hepatic inflammation [19-21].

2. Materials and Methods

RNA interference

HepG2 human hepatoma cells, which represent the most used *in vitro* model to study liver metabolism and related disorders, were transiently transfected for 48 hours by pooling 3 different target-specific siRNA oligo duplexes (MyBioSource, Inc., USA) of human *HMBS* gene (siPBGD) at final concentration of 10 μM. Cyclophilin B (10 μM) was used as scramble negative control (Horizon Discovery, Italy). To mimic fasting, both scramble and siPBGD cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with low glucose concentration (1.125 mg/L) for 6 hours. Cultured medium of siPBGD cells was supplemented with 0.5 mM α-LA alone (siPBGD+ α-LA) or in combination with 0.33 mM glucose (α-LA+Gluc) for 24 hours before inducing fasting. Treatments were freshly prepared and administered when appropriate. The potential efficacy of α-LA alone and α-LA+Gluc were compared to 0.33 mM glucose (siPBGD+Gluc) [17].

Evaluation of PBGD enzymatic activity

siPBGD and scramble cells were lysed in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.2% Triton-X 100. 50 μl cell lysates were incubated with 200 μl 0.1 M Tris-HCl and 25μl of 1 mM PBG for 1 hour at 37°C, in order to evaluate whether PBGD enzyme could convert PBG substrate into uroporphyrin according to Hsiao et al. procedure [22]. After incubation, the enzymatic reaction was stopped through 10% trichloroacetic acid and centrifuged at 13.000 rpm for 10 minutes. The uroporphyrin fluorescence emission (Ex/Em = 405/655nm) was measured by a spectrofluorometer (Shimadzu Corporation, Japan) and uroporphyrin concentration was determined using uroporphyrin I standard calibrator (Sigma). The PBGD activity (pmol Uro/h) was normalized to the total amount of proteins (mg) and expressed as percentage of residual activity for each condition.

Gene expression analysis

RNA was extracted from cell cultures using Trizol reagent (Life Technologies-ThermoFisher Scientific, Carlsbad, U.S.A). 1μg of total RNA was retro-transcribed with VILO random hexamers synthesis system (Life Technologies-ThermoFisher Scientific, Carlsbad, U.S.A). Quantitative real time PCR (qRT-PCR) was performed by an ABI 7500 fast thermocycler (Life Technologies), using the TaqMan Universal PCR Master Mix (Life Technologies, Carlsbad, CA) and TaqMan probes (**Table S1**). The SYBR Green chemistry (Fast SYBR Green Master Mix; Life Technologies) was used for cDNA amplification through human primers (**Table S2**). All reactions were delivered in triplicate. Data were normalized to β-actin gene expression and results were expressed as arbitrary units (AU) or fold increase as indicated in bar graphs.

Western Blot Analysis

Total protein lysates were extracted from cell cultures, using RIPA buffer containing 1 mmol/L Na-orthovanadate, 200 mmol/L phenylmethyl sulfonyl fluoride and 0.02 μg/μL aprotinin. Samples were pooled prior electrophoretic separation and all reactions were performed in duplicate. Then equal amounts of proteins (50 μg) were separated by SDS-PAGE, transferred electrophoretically to nitrocellulose membrane (BioRad, Hercules, CA) and incubated with specific antibodies overnight. At least, three independent lots of

freshly extracted proteins were used for experiments. Antibodies and concentration used are listed in **Table S3**.

Statistical analysis

Differences between two groups were calculated by two -way ANOVA, followed by post hoc *t-test* (two-tailed). Differences among multiple groups were analyzed by two-way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, which corrects for the number of comparisons and controls the False Discovery Rate (FDR). Adjusted *p* values <0.05 were considered statistically significant. Statistical analyses were performed using JMP 16.0 (SAS, Cary, NC) and Prism software (version 9.1, GraphPad Software).

3. Results

3.1 α-Lipoic acid improved heme production in siPBGD cells

At baseline, *PBGD* silencing reduced both PBGD mRNA levels by 60% (p<0.0001 at ANOVA; adjusted p<0.0001 *vs* Scramble; **Figure 1A**) and its enzymatic activity by 42% (p=0.0001 at ANOVA; adjusted p=0.001 *vs* Scramble; **Figure 1B**). siPBGD cells showed lower ALAS1 expression (p=0.003 at ANOVA; p=0.01 *vs* Scramble; **Figure 1C**) and intracellular heme content compared to the scramble probably due to the delay in the heme biosynthetic pathway induced by *PBGD* downregulation (p<0.0001 at ANOVA; adjusted p<0.0001 *vs* Scramble; **Figure 1D**).

Fasting further reduced PBGD activity (p=0.0001 at ANOVA; adjusted p=0.04 *vs* siPBGD untreated (NT) and *vs* fasted Scramble; Figure 1B) and promoted ALAS1 upregulation without increasing heme content (p=0.003 at ANOVA; p=0.03 *vs* siPBGD-NT; **Figure 1C-D**), thereby resembling what occurs in *in vivo* models and symptomatic AIP subjects.

To assess the potential efficacy of α-LA as prophylactic treatment, we exposed siPBGD cells to α-LA 0.5 mM for 24 hours before fasting and its efficacy was compared to glucose supplementation (0.33 mM) at the same timing. Additionally, we explored whether α-LA could enhance glucose efficacy by evaluating the potential synergism between the two treatments.

During fasting, α-LA treatment alone prevented siRNA-induced *PBGD* downregulation in terms of both mRNA levels (p=0.0005 at ANOVA; adjusted p=0.0003; **Figure 1E**) and protein activity (p=0.03 at ANOVA; p=0.005 *vs* siPBGD fasted cells; **Figure 1F**) paralleled by increased heme availability (p<0.0001 at ANOVA; adjusted p<0.0001; **Figure 1G**). Moreover, α-LA showed greater ability than glucose to promote heme biosynthesis by rising PBGD levels (p<0.0001 at ANOVA; adjusted p=0.0002; **Figure 1E-F**) and the amount of heme (p<0.0001 at ANOVA; adjusted p=0.0004; **Figure 1G**).

Although α-LA+Gluc treatment ameliorated PBDG expression (p=0.0005 at ANOVA; adjusted p=0.0002 *vs* siPBGD fasted cells; adjusted p=0.0005 *vs* siPBGD + Gluc), enzymatic performance (p=0.03 at ANOVA; adjusted p=0.005 *vs* siPBGD fasted cells; **Figure 1F**) and intracellular heme synthesis (p<0.0001 at ANOVA; adjusted p<0.0001 *vs* fasted siPBGD cells + glucose; adjusted p=0.002 *vs* fasted siPBGD + α-LA; **Figure 1G**), the effect was quite comparable to those induced by α-LA alone thus supporting that it may efficiently rescue heme production in hepatocytes.
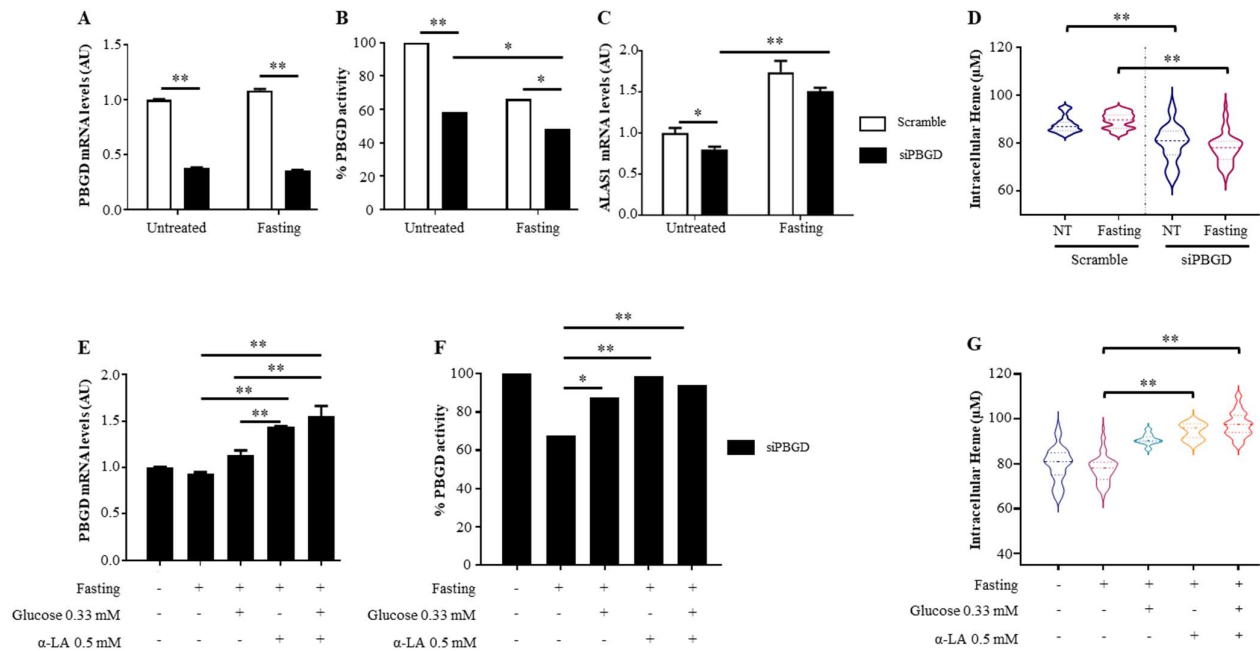
Figure 1: α-LA rescued heme biosynthesis in PBGD-silenced HepG2 cells. **A**) PBGD mRNA expression was assessed by qRT-PCR in scramble and siPBGD cells at baseline and after fasting. **B**) PBGD kinetics was measured in both scramble and siPBGD cell lysates with or without fasting and incubated with PBG substrate for 1h. Data were normalized to mg of proteins and expressed as % of PBGD residual activity. **C**) ALAS1 mRNA levels were assessed by qRT-PCR in scramble and siPBGD cells at baseline and after fasting. **D**) Violin plot showed intracellular heme content (µM), which was colorimetrically measured in cell lysates following the manufacturer's instruction and expressed. **E**) The mRNA expression of PBGD was evaluated by qRT-PCR in siPBGD cells at baseline, after fasting and in presence of glucose, α-LA or both treatments. **F**) The percentage of PBGD residual activity was assessed in siPBGD cells at baseline, after fasting and in presence of glucose, α-LA or both treatments. **G**) Heme concentration (µM) was determined in siPBGD cells and compared to fasting, siPBGD+Gluc, siPBGD+α-LA and to siPBGD plus α-LA+Gluc. For gene expression, data were normalized to β-actin housekeeping gene and expressed as fold increase (Arbitrary Unit-AU) compared to control group. For violin plot, data were expressed as median concentration (thick dashed lines) and interquartile range (dotted lines). At least three independent experiments were conducted. Adjusted *p<0.05 and **p<0.01

3.2 *α-Lipoic acid stimulates glucose utilization and provides energy supplies during fasting*

Alterations in glucose metabolism might precipitate the AIP acute attack and compromise the efficacy of glucose therapy. However, metabolic aberrancies occurring in hepatocytes at baseline have not been described yet and whether they could be refrained through an insulin-mimic agent have not been reported in previous studies.

In siPBGD cells, the expression of glucokinase (GCK, p=0.009 at ANOVA; adjusted p=0.04, **Figure 2A**), phosphofructokinase (PFK-L, p<0.0001 at ANOVA; adjusted p<0.0001; **Figure 2B**) and pyruvate kinase (PK, p<0.0001 at ANOVA; adjusted p=0.005, **Figure 2C**), genes involved in different steps of glycolysis, was lower compared to scramble. Accordingly, total ATP levels were reduced by ~40% in siPBGD cells (p<0.0001 at ANOVA; adjusted p<0.001; **Figure 2D**).

Fasting further delayed glycolysis in siPBGD cells by dramatically downregulating GCK (p=0.009 at ANOVA; adjusted p=0.009, **Figure 2A**), PFK-L (p<0.0001 at ANOVA; adjusted p<0.001; **Figure 2B**) and PK expression (p<0.001 at ANOVA; adjusted p<0.001,

Figure 2C) and by even inducing ATP shortfall (p<0.001 at ANOVA; adjusted p<0.001 *vs* Scramble fasted cells; adjusted p=0.0017 *vs* siPBGD; **Figure 2D**).

As expected, glucose administration regulated the expression of GCK (p=0.004 at ANOVA; adjusted p=0.02 *vs* siPBGD fasted cells, **Figure 2E**), PFK-L (p=0.0002 at ANOVA, adjusted p=0.0001 *vs* siPBGD fasted cells; **Figure 2F**) and PK (p<0.0001 at ANOVA, adjusted p<0.0001 *vs* siPBGD fasted cells; **Figure 2G**) although it resulted ineffective at promoting ATP production, whose levels matched with those produced in siPBGD cells at baseline (p<0.0001 at ANOVA; adjusted p=0.0009 *vs* siPBGD fasted cells; **Figure 2H**) thereby supporting that alterations of glucose metabolism occur in presence of *PBGD* downregulation.

Interestingly, α-LA pre-treatment alone not only upregulated GCK (p=0.004 at ANOVA; adjusted p=0.004 *vs* siPBGD fasted cells; **Figure 2E**), PFK-L (p<0.0001 at ANOVA, adjusted p<0.0001 *vs* siPBGD fasted cells; **Figure 2F**) and PK mRNA levels (p=0.0002 at ANOVA, adjusted p=0.03 *vs* siPBGD fasted cells; **Figure 2G**), but also it hugely rose total ATP compared to either siPBGD fasted cells or to siPBGD+Gluc ones (p<0.0001 at ANOVA; adjusted p<0.0001; **Figure 2H**).

The combined α-LA+Gluc supplementation additively participated to promote gene expression of glycolytic enzymes (p=0.004 at ANOVA; adjusted p=0.0001 *vs* siPBGD fasted cells, adjusted p=0.004 *vs* siPBGD+Gluc, p=0.02 *vs* siPBGD+α-LA; p<0.0001 at ANOVA, adjusted p=0.0001 *vs* siPBGD fasted cells, p=0.001 *vs* siPBGD+ α-LA; p<0.0001 at ANOVA, adjusted p<0.0001 *vs* siPBGD fasted cells, *vs* siPBGD+Gluc and *vs* siPBGD+ α-LA; **Figure 2E-G**). Compared to the single treatments, α-LA+Gluc administration enriched siPBGD cells of ATP resources (p<0.0001 at ANOVA, p<0.0001 adjusted p<0.0001 *vs* siPBGD fasted cells, *vs* siPBGD+Gluc and *vs* siPBGD+α-LA; **Figure 2H**) and enhanced triglyceride secretion (p<0.0001 at ANOVA; p<0.0001 *vs* siPBGD fasted cells, *vs* siPBGD+Gluc and *vs* siPBGD+α-LA; **Figure 2I**). Therefore, it could be speculated that α-LA administration may supply *PBDG*-silenced hepatocytes of energy fuels during energy shortage, by possibly improving glucose utilization.
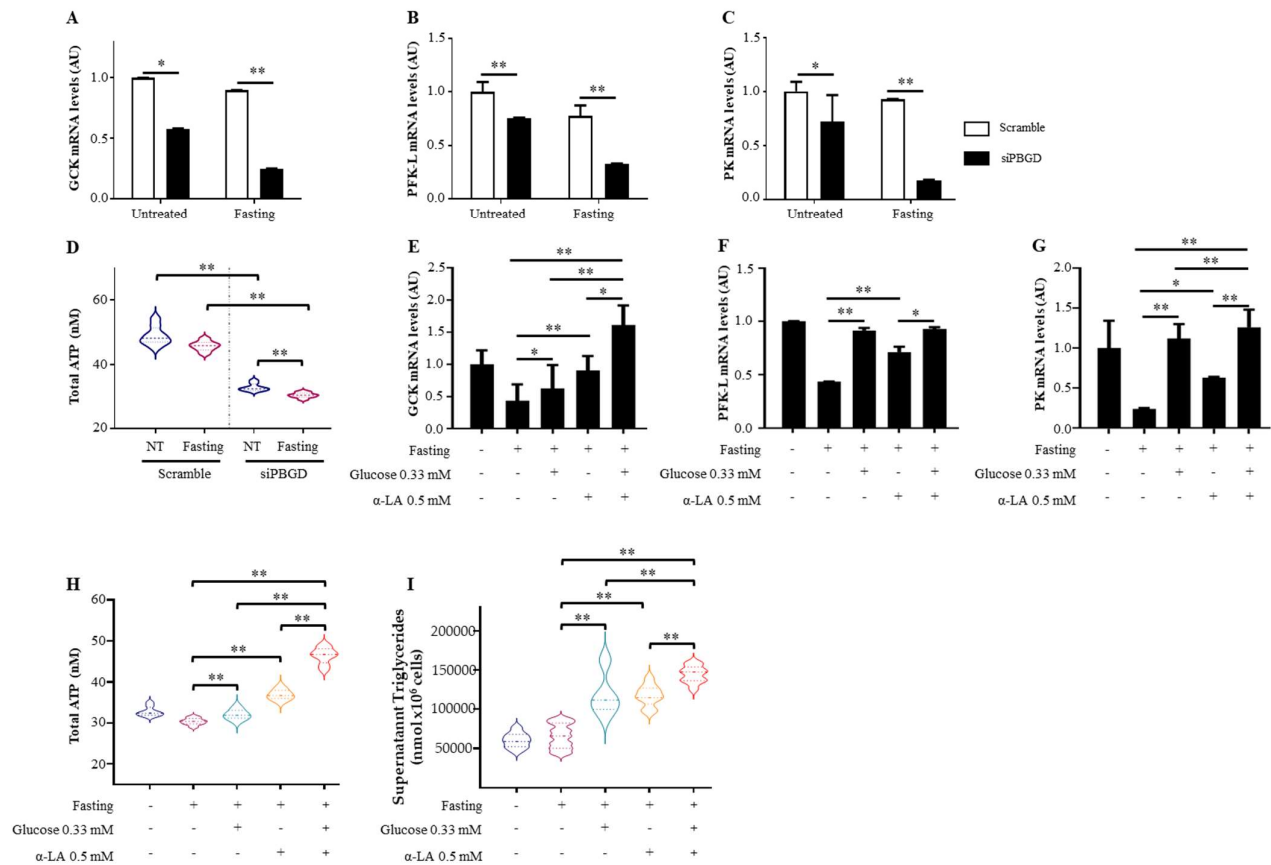
Figure 2: α-LA improved glucose utilization in PBGD-silenced HepG2 cells. **A-C**) GCK, PFK-L and PK mRNA levels were assessed by qRT-PCR in scramble and siPBGD cells at baseline and after fasting. **C**) Mitochondrial ATP concentration was quantified in cell lysates through a fluorometric assay. **E-G**) GCK, PFK-L and PK expression was measured in siPBGD cells at baseline, after fasting and in presence of glucose, α-LA or both treatments. **H**) Intracellular ATP was measured in siPBGD cells and compared to fasting, siPBGD+Gluc, siPBGD+α-LA and to the combined treatment according to manufacturer 'instruction. **I**) Measurement of triglyceride secreted in cell supernatants of siPBGD cells with or without fasting and pre-treated with glucose, α-LA and α-LA+Gluc. For gene expression, data were normalized to β-actin housekeeping gene and expressed as fold increase (Arbitrary Unit-AU) compared to control group. For violin plot, data were expressed as median concentration (thick dashed lines) and interquartile range (dotted lines). At least three independent experiments were conducted. Adjusted *p<0.05 and **p<0.01

3.3 *α-Lipoic acid recovered mitobiogenesis: the dual role of PGC1α*

Mitochondrial dysfunctions, in terms of bioenergetic failure, have been described in AIP mice with overt symptomatology [11, 12], but alterations of mitochondrial dynamics need to be further elucidated.

The mRNA and protein levels of PCG1α, master regulator of mitobiogenesis, were induced by fasting in both scramble and siPBGD cells (p<0.0001 at ANOVA, p=0.008 *vs* siPBGD-NT, **Figure 3A-B**) accompanied by downregulation of Optical Atrophy 1 (OPA1), which mediates fusion of mitochondrial inner membranes, and Mitofusin 2 (MFN2), which joins mitochondrial outer membranes (p=0.01 at ANOVA, p<0.05 *vs* Scramble-NT and *vs* siPBGD-NT, **Figure S1A-B**). Conversely, we found that dynamin 1-like protein (DRP1), which regulates mitochondrial separation, markedly localized in the cytoplasm of siPBGD cells in fasting condition (**Figure 3C**). Since DRP1 activation is usually associated to low OXPHOS capacity and energy shortfall, we assessed the expression of Complex IV of the respiratory chain, which is encoded by mtDNA and represents the core of

OXPHOS functionality. In keeping with previous findings, siPBGD cells showed the lowest levels of COXI and COXII, both subunits composing the mtDNA-encoded Complex IV (p<0.0001 at ANOVA, p<0.0.0001 *vs* siPBGD-NT and p=0.0007 *vs* scramble fasted cells, **Figure 3D**; p<0.05 *vs* siPBGD-NT and p<0.01 *vs* scramble fasted cells, **Figure 3E**), thereby supporting that fasting may activate mitochondrial biogenesis and exacerbate mitochondrial injury in siPBGD cells by shifting towards fission rather than fusion.

In siPBGD cells, pre-treatment with α-LA before fasting enhanced mitochondrial biogenesis by upregulating PCG1α (p<0.0001 at ANOVA, p=0.01 *vs* siPBGD fasted cells, **Figure 3F**) more than glucose administration alone (p<0.0001 at ANOVA, p=0.004, **Figure 3F**). The OPA1 and MFN2 mRNA expression were increased with the α-LA and glucose supplementation compared to siPBGD in fasting condition (p<0.0001 at ANOVA, p<0.0001; **Figure 3G-H**), showing similar levels among the two treatments (**Figure 3G-H**). Unexpectedly, DRP1 expression was strongly pulled down by α-LA treatment (p<0.0001 at ANOVA, p=0.0002 *vs* siPBGD-NT, p=0.0001 *vs* siPBGD fasted cells and p<0.0001 *vs* siPBGD+Gluc, **Figure 3I**) supporting that it may counteract the effect of fasting by switching mitochondrial biogenesis towards fusion rather than fission.

Co-treatment with α-LA+Gluc during fasting had the highest impact of PCG1α upregulation (p<0.0001 at ANOVA, p<0.0001 *vs* siPBGD fasted cells and *vs* siPBGD+Gluc, p=0.009 *vs* siPBGD+ α-LA, **Figure 3F**) along with the overexpression of OPA1 (p<0.0001 at ANOVA, p<0.0001 *vs* siPBGD fasted cells and *vs* single treatments, **Figure 3G**), MFN2 (p<0.0001 at ANOVA, p<0.0001 *vs* siPBGD fasted cells, p=0.001 *vs* siPBGD+Gluc and p=0.0003 *vs* siPBGD+ α-LA, **Figure 3H**) and DRP1 (p<0.0001 at ANOVA, p=0.0003 *vs* siPBGD fasted cells, **Figure 3I**). No differences among glucose, α-LA and α-LA+Gluc were found at improving Complex IV abundance during fasting (p<0.0001 at ANOVA, p<0.0001 *vs* siPBGD fasted cells, **Figure 3L**). Nonetheless, the expression of D-loop, which reflects mitochondrial mass, was around 2.5-fold higher only after α-LA+Gluc treatment compared to siPBGD cells with or without single treatments (p<0.0001 at ANOVA, p<0.0001 *vs* siPBGD fasted cells and *vs* siPBGD+Gluc and vs siPBGD+α-LA, **Figure 3M**). These findings may support that α-LA+Gluc combination may additively rescue the overall mitochondrial dynamics in terms of mitochondrial mass, energy production and mitochondrial turnover.
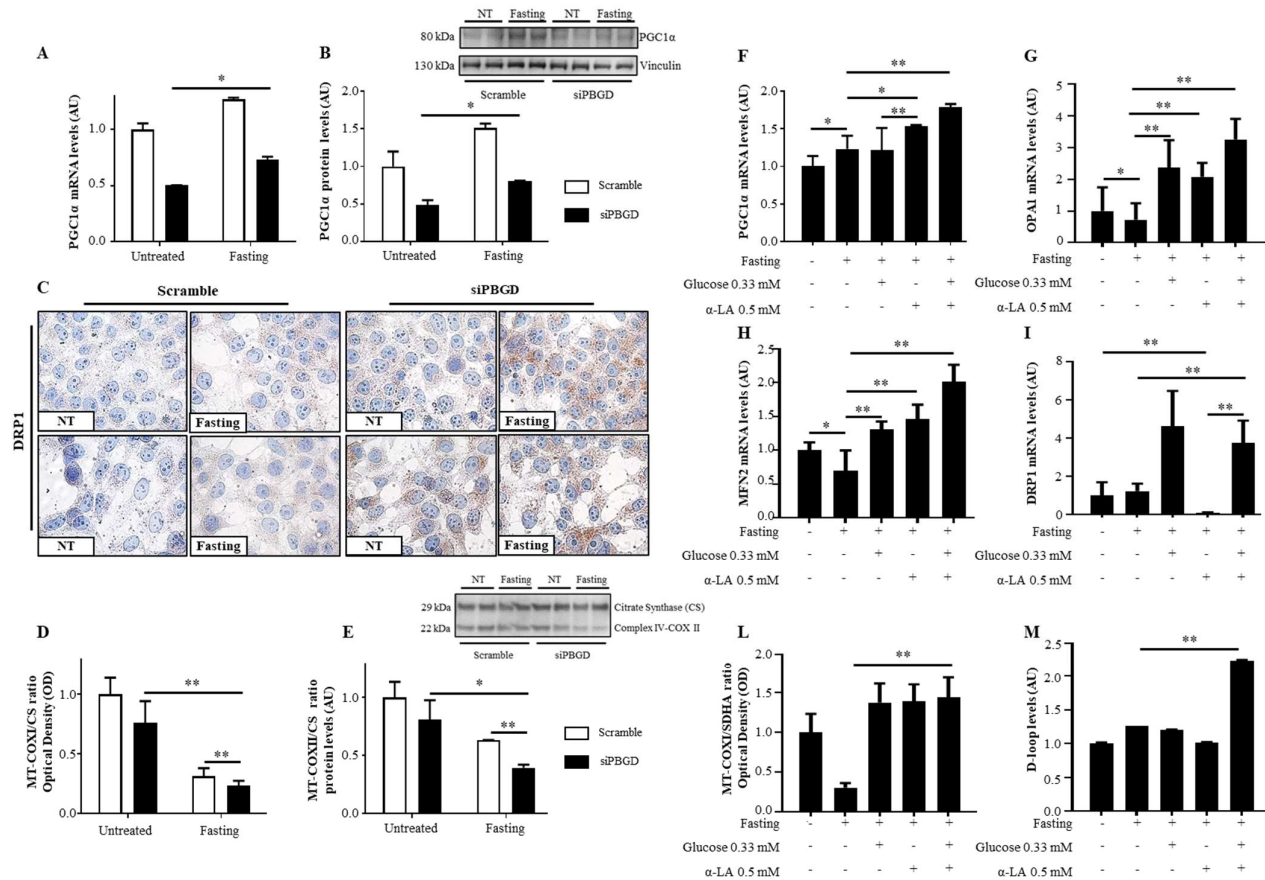
Figure 2: α-LA combined to glucose recovered mitochondrial dynamics in PBGD-silenced HepG2 cells. **A-B**) PGC1α mRNA and protein levels were assessed in both scramble and siPBGD cells at baseline and after fasting by qRT-PCR and Western blot, respectively.   **C**) Cytoplasmatic localization of DRP1 protein assessed at immunocytochemistry in scramble and siPBGD cells in absence or in presence of fasting. **D**) Mitochondrially-encoded subunit I (MT-COXI) of Complex IV was evaluated by ELISA (λ=600 nm) and normalized to nuclear-encoded Citrate Synthase levels (λ=405 nm). **E**) Protein levels of mitochondrially-encoded subunit II (MT-COXII) of Complex IV was assessed by Western blot and normalized to nuclear-encoded Citrate Synthase. **F-I**) PGC1α, OPA1, MFN2 and DRP1 expression was evaluated by qRT-PCR in siPBGD cells at baseline, after fasting and in presence of glucose, α-LA or both treatments. **L**) Intracellular MT-COXI of Complex IV was assessed by ELISA (λ=600 nm) in siPBGD cells with or without fasting and pre-treated with glucose, α-LA and α-LA+Gluc. MT-COXI protein expression was normalized to nuclear-encoded Citrate Synthase levels (λ=405 nm). **M**) D-loop levels was measured in DNA samples extracted from siPBGD cells at basal status and in fasting as well as in those treated with glucose, α-LA and α-LA+Gluc. D-loop expression was normalized to RNAse-P reference gene. For gene expression, data were normalized to β-actin housekeeping gene and expressed as fold increase (Arbitrary Unit-AU) compared to control group. For Western Blot, data were normalized on vinculin or citrate synthase housekeeping genes. At least three independent experiments were conducted. Adjusted *p<0.05 and **p<0.01

4. Discussion

The binomial association between porphyrins and acute neuropsychiatric attacks has been at the basis of AIP pathogenesis for decades. Still, there are several reports which doubt about the exclusive pathogenic role of heme precursors in disease, as AIP patients who accumulate ALA and PBG may not manifest any acute attack throughout their life [17, 23]. Thus, other factors may be involved in the pathophysiology of the acute event. A link may exist between metabolic homeostasis and biosynthesis of heme, as the latter is

an essential precursor for the correct nutrients' handling to meet energy requirements during basal and stressful situations.

Attempting to shed light on this issue, we silenced *PBGD* gene in HepG2 cells (siPBGD) and characterized metabolic alterations occurring at baseline and after glucose deprivation, a condition which could mimic fasting *in vitro*. Transient PBGD silencing in HepG2 cells reduced PBGD and ALAS1 levels paralleled by low hepatocellular heme content thus supporting it may reproduce the slowdown of the hepatic heme biosynthesis observed in carriers of the *HMBS* genetic mutations [24]. We revealed that siPBGD cells at baseline showed low expression of glycolytic enzymes and mitochondrial ATP production, possibly indicating that *PBGD* downregulation may cause early aberrancies of glucose metabolism and OXPHOS functionality. The basal characterization of hepatic metabolism in AIP context has been poorly explored and just one study reported that the hepatic transcriptomic profile was similar between AIP and the congenic wild-type (WT) mice in absence of the stress-induced attack [13]. Nonetheless, characterization of nutritional status of AIP subjects highlighted that the inadequate glucides and carbohydrates consumption less than 45–60% of the total energy intake, was associated with disease severity [25, 26]. In addition, sub-clinical OXPHOS defects, resulting in high circulating lactate levels, were found outside of the crisis in AIP patients in clinical remission [27]. Therefore, there may be some discrepancies in gene expression at baseline between transcriptomic findings by Chen et al [13] and ours,   mainly due to the limitations of transient PBGD silencing *in vitro* which could not fully resemble features of a stable knockout model. However, the downregulation of glycolysis and the biochemical evaluation of energetic status of siPBGD cells could, in broad terms, mirror the energy failure featuring AIP patients at higher risk to develop symptoms [13, 25-27].

Previous evidence has outlined that most dysregulated pathways in the liver which may precipitate the AIP acute attacks are under the transcriptional regulation of PCG1α, a powerful nutrient sensor activated in response to stressful factors [28]. In AIP context, PGC1α attempted to re-establish glucose homeostasis by activating hepatic gluconeogenesis and increasing mitochondrial mass, but it also induced   ALAS1 expression [12, 13, 17], thereby exacerbating the porphyrins 'overproduction. In keeping with these findings, we found that the induction of fasting through glucose deprivation halved PBGD enzymatic activity at 50% and sensitized siPBGD cells to upregulate ALAS1, likely *via* PGC1α, without increasing heme production similar to what has been reported in AIP rodents and patients exposed to precipitating factors [11, 17].

Fasting even downed glycolysis and total ATP levels, possibly aggravating energetic imbalance in siPBGD cells as occurs in AIP mice during an attack which lowers glucose availability and runs into cataplerosis of the Krebs cycle, resulting unable to provide reducing equivalents (NADH, FADH2) to the OXPHOS [29]. Beyond bioenergetic failure, alterations of mitochondrial dynamics, including a cycle of fission and fusion events, could exacerbate metabolic dysfunctions as it regulates the intracellular mitochondrial mass, shape, and metabolic status of these organelles [30].   Here, we firstly revealed that fasting-induced PCG1α activation in siPBGD cells was accompanied by low expression of fusion genes and overexpression of DRP1, the main inducer of mitochondrial separation. Fission of mitochondria is commonly associated with low OXPHOS performance and ATP synthesis consistent with the reduced expression of complex IV which has been observed in siPBGD fasted cells. Homedan et al showed that hepatic complexes I, II and III, but not complex IV, decreased their activities in *PBGD*-deficient mice treated with phenobarbital, a drug which induces the acute episodes [12, 29]. Conversely, morphological alterations of mitochondria presenting para-crystallin inclusions have been described in liver biopsies of AIP patients [30] and lack of complex IV activity alongside collapse of ATP were found in the hippocampus of AIP mice knocked-in for the *Hmbs* c.500G > A (p.R167Q) mutation, resulting in a severe phenotype with neuropsychiatric behavior [31]. Most recently, it has been demonstrated that the deficiency of *ferrochelatase* (*FETCH*), a gene involved in the last step of heme production and responsible for the development of Erythropoietic protoporphyria, damaged both glycolysis and OXPHOS along with a decrease

in mitochondrial fusion [32]. Still, glucose metabolism and mitochondrial dysfunction might precipitate the acute attacks but not all studies share the same alterations possibly reflecting the huge phenotypic variability of AIP symptomatology [12, 29-32]. Overall, our model may support the hypothesis that fasting on one hand worsens glycolytic alterations and on the other unmasks mitochondrial damage by shifting mitochondrial dynamics towards fission rather than fusion and, consequently, contributing to OXPHOS aberrancies.

Life-threating attacks featuring AIP patients represent a crippling issue for subject who suffer from this rare disorder and mostly for those who are predisposed to recurrent crisis. The ongoing therapies aim to modulate ALAS1 activity and even showed several constraints [14, 15, 17, 33]. AIP patients with mild attacks may be treated with glucose solution, but there are no clear clinical data showing beneficial effects in AIP prophylaxis [17, 34], possibly due to an impaired glucose metabolism [10-12]. Conversely, hemin directly represses ALAS1 activity but its long-term exposure has been associated with hepatic iron accumulation, oxidative stress, inflammation and liver fibrosis in AIP patients alongside refractoriness to the attacks [14, 33], growing the need of novel medications. In the recent years, it has emerged that AIP patients showed hyperinsulinemia, a condition which appeared protective against a stress-induced attack [17]. It has been also postulated that reduced insulin levels and C-peptide were associated with AIP disease activity, possibly due to the essential role of insulin for the ALAS1 inhibition and blockage of porphyrin overproduction [35].

Therefore, we assessed whether a treatment with an insulin sensitizer, the α-LA, alone or in combination with glucose may improve metabolic abnormalities when supplemented before fasting-induced stress with the goal to offer a potential preventive approach of acute events.

α-LA acts as co-factor of the α-ketoglutarate dehydrogenase in the Krebs cycle, which bridges glycolytic-mitochondrial respiration network and provides succinyl-CoA substrate for heme biosynthesis [36, 37]. The main findings of this project were that pre-treatment with α-LA, likely boosting mitochondrial Krebs cycle, ameliorated heme biosynthesis in siPBGD cells and their energetic status by promoting ATP and lipid production. Moreover, we revealed that α-LA may counterbalance the harmful effects of fasting through the stimulation of mitochondrial elongation and inhibition of mitochondrial fission attempting to meet energy demand. Glucose administration alone even recovered the expression of glycolytic enzymes and of genes involved in mitochondrial dynamics but it failed at improving ATP content, possibly explaining the reduced efficacy of glucose therapy reported in humans [17]. When α-LA was combined to glucose, we found that total ATP, triglyceride levels and mitochondrial mass were increased in siPBGD cells supporting that α-LA may additively enhance glucose efficacy and rescue the overall mitochondrial dynamics (**Figure 4**). By looking at a translational perspectives, α-LA supplementation may potentially sustain the hepatic absorption and maintenance of glucose homeostasis from dietary carbohydrates [17].

To conclude, emerging studies have highlighted that heme depletion may deeply affect glucose metabolism and mitochondrial dynamics [32]. The present study attempted to dissect the role of metabolic aberrancies occurring in hepatocytes in the context of *PBGD* downregulation, mimicking AIP condition *in vitro*. We proposed, for the first time, a novel compound which may be an appealing for the prevention of AIP crisis through the improvement of hepatocellular bioenergetics. As proof-of-concept, our findings highlighted that α-LA could re-establish glucose employment, thus restoring the cross-talk among cytosolic glycolysis and mitochondrial respiration and, therefore, the hepatocellular homeostasis.
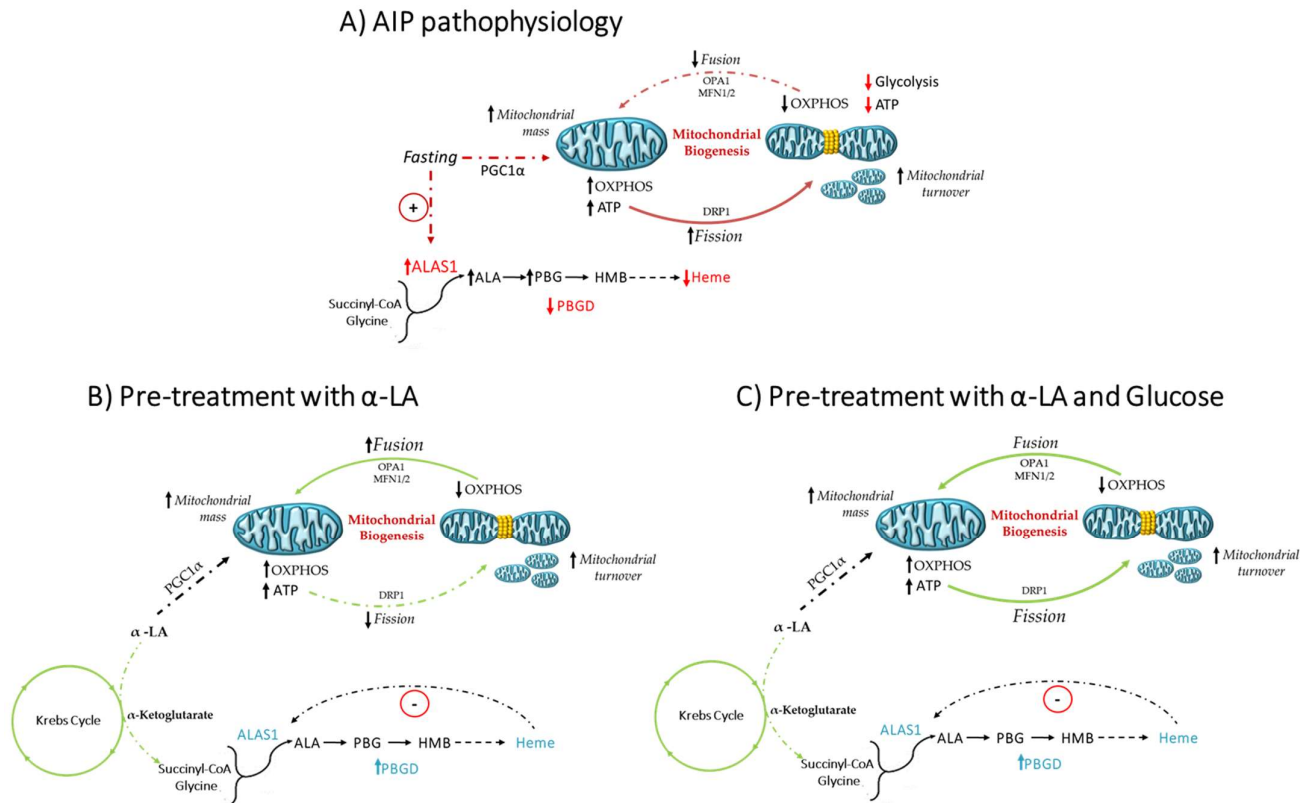
Figure 4: Schematic representation of metabolic alterations and the efficacy of α-LA in siPBGD cells during fasting. **A**) Fasting induced mitochondrial biogenesis through PGC1α, possibly worsening mitochondrial injury by shunting mitochondrial dynamics towards fission, mediated by DRP1, and inhibiting OPA1 and MFN2 which provide fusion of mitochondrial inner and outer membranes. Accumulation of divided mitochondria lowered OXPHOS ability and exacerbated ATP shortfall. **B**) Pre-treatment with α-LA in siPBGD cells even promoted PGC1α activity but it improved OPA1 and MFN2 expression, thereby enhancing mitochondrial fusion, a condition which could support OXPHOS activity during energy demand. **C**) Pre-treatment with α-LA and glucose may recover the overall mitochondrial wellness by acting at multiple levels. On one hand, α-LA may improve heme biosynthesis, a mechanism occurring in mitochondrial matrix. On the other hand, α-LA may enhance glycolysis, ATP production and OXPHOS possibly sustaining Krebs cycle. Restoration of hepatocellular homeostasis could allow to recover mitochondrial dynamics, in terms of fusion, fission and overall mitochondrial mass.

Conflicts of Interest: The authors declare no competing interests

5. References

1. Wang, B.; Rudnick, S.; Cengia, B.; Bonkovsky, H. L., Acute Hepatic Porphyrias: Review and Recent Progress. *Hepatology Communications* **2019,** 3, (2), 193-206.

2. Lenglet, H.; Schmitt, C.; Grange, T.; Manceau, H.; Karboul, N.; Bouchet-Crivat, F.; Robreau, A. M.; Nicolas, G.; Lamoril, J.; Simonin, S.; Mirmiran, A.; Karim, Z.; Casalino, E.; Deybach, J. C.; Puy, H.; Peoc'h, K.; Gouya, L., From a dominant to an oligogenic model of inheritance with environmental modifiers in acute intermittent porphyria. *Human molecular genetics* **2018,** 27, (7), 1164-1173.

3. Stenson, P. D.; Mort, M.; Ball, E. V.; Shaw, K.; Phillips, A. D.; Cooper, D. N., The Human Gene Mutation Database: building a comprehensive mutation repository for clinical and molecular genetics, diagnostic testing and personalized genomic medicine. *Human Genetics* **2014,** 133, (1), 1-9.

4. Pischik, E.; Kauppinen, R., An update of clinical management of acute intermittent porphyria. *Appl Clin Genet* **2015,** 8, 201-214.

5. Monteiro, H. P.; Abdalla, D. S. P.; Augusto, O.; Bechara, E. J. H., Free radical generation during δ-Aminolevulinic acid autoxidation: Induction by hemoglobin and connections with porphyrinpathies. *Archives of Biochemistry and Biophysics* **1989,** 271, (1), 206-216.

6. Brennan, M. J. W.; Cantrill, R. C., δ-Aminolaevulinic acid and amino acid neurotransmitters. In *The Biological Effects of Glutamic Acid and Its Derivatives*, Najjar, V. A., Ed. Springer Netherlands: Dordrecht, 1981; pp 49-58.

7. Weiss, T. S.; Pahernik, S.; Scheruebl, I.; Jauch, K. W.; Thasler, W. E., Cellular damage to human hepatocytes through repeated application of 5-aminolevulinic acid. *Journal of hepatology* **2003,** 38, (4), 476-82.

8. Jaramillo-Calle, D. A.; Solano, J. M.; Rabinstein, A. A.; Bonkovsky, H. L., Porphyria-induced posterior reversible encephalopathy syndrome and central nervous system dysfunction. *Molecular genetics and metabolism* **2019,** 128, (3), 242-253.

9. Chen, B.; Solis-Villa, C.; Hakenberg, J.; Qiao, W.; Srinivasan, R. R.; Yasuda, M.; Balwani, M.; Doheny, D.; Peter, I.; Chen, R.; Desnick, R. J., Acute Intermittent Porphyria: Predicted Pathogenicity of HMBS Variants Indicates Extremely Low Penetrance of the Autosomal Dominant Disease. *Human mutation* **2016,** 37, (11), 1215-1222.

10. Lelli, S. M.; San Martín de Viale, L. C.; Mazzetti, M. B., Response of glucose metabolism enzymes in an acute porphyria model. Role of reactive oxygen species. *Toxicology* **2005,** 216, (1), 49-58.

11. Collantes, M.; Serrano-Mendioroz, I.; Benito, M.; Molinet-Dronda, F.; Delgado, M.; Vinaixa, M.; Sampedro, A.; Enriquez de Salamanca, R.; Prieto, E.; Pozo, M. A.; Penuelas, I.; Corrales, F. J.; Barajas, M.; Fontanellas, A., Glucose metabolism during fasting is altered in experimental porphobilinogen deaminase deficiency. *Human molecular genetics* **2016,** 25, (7), 1318-27.

12. Homedan, C.; Schmitt, C.; Laafi, J.; Gueguen, N.; Desquiret-Dumas, V.; Lenglet, H.; Karim, Z.; Gouya, L.; Deybach, J.-C.; Simard, G.; Puy, H.; Malthièry, Y.; Reynier, P., Mitochondrial energetic defects in muscle and brain of a Hmbs−/− mouse model of acute intermittent porphyria. *Human molecular genetics* **2015,** 24, (17), 5015-5023.

13. Chen, B.; Wang, M.; Gan, L.; Zhang, B.; Desnick, R. J.; Yasuda, M., Characterization of the hepatic transcriptome following phenobarbital induction in mice with AIP. *Molecular genetics and metabolism* **2019,** 128, (3), 382-390.

14. Schmitt, C.; Lenglet, H.; Yu, A.; Delaby, C.; Benecke, A.; Lefebvre, T.; Letteron, P.; Paradis, V.; Wahlin, S.; Sandberg, S.; Harper, P.; Sardh, E.; Sandvik, A. K.; Hov, J. R.; Aarsand, A. K.; Chiche, L.; Bazille, C.; Scoazec, J. Y.; To-Figueras, J.; Carrascal, M.; Abian, J.; Mirmiran, A.; Karim, Z.; Deybach, J. C.; Puy, H.; Peoc'h, K.; Manceau, H.; Gouya, L., Recurrent attacks of acute hepatic porphyria: major role of the chronic inflammatory response in the liver. *Journal of internal medicine* **2018,** 284, (1), 78-91.

15. Balwani, M.; Sardh, E.; Ventura, P.; Peiró, P. A.; Rees, D. C.; Stölzel, U.; Bissell, D. M.; Bonkovsky, H. L.; Windyga, J.; Anderson, K. E.; Parker, C.; Silver, S. M.; Keel, S. B.; Wang, J. D.; Stein, P. E.; Harper, P.; Vassiliou, D.; Wang, B.; Phillips, J.; Ivanova, A.; Langendonk, J. G.; Kauppinen, R.; Minder, E.; Horie, Y.; Penz, C.; Chen, J.; Liu, S.; Ko, J. J.; Sweetser, M. T.;

Garg, P.; Vaishnaw, A.; Kim, J. B.; Simon, A. R.; Gouya, L., Phase 3 Trial of RNAi Therapeutic Givosiran for Acute Intermittent Porphyria. *The New England journal of medicine* **2020,** 382, (24), 2289-2301.

16.   Fontanellas, A.; Ávila, M. A.; Anderson, K. E.; Deybach, J. C., Current and innovative emerging therapies for porphyrias with hepatic involvement. *Journal of hepatology* **2019,** 71, (2), 422-433.

17.   Solares, I.; Izquierdo-Sánchez, L.; Morales-Conejo, M.; Jericó, D.; Castelbón, F. J.; Córdoba, K. M.; Sampedro, A.; Lumbreras, C.; Moreno-Aliaga, M. J.; Enríquez de Salamanca, R.; Berraondo, P.; Fontanellas, A., High Prevalence of Insulin Resistance in Asymptomatic Patients with Acute Intermittent Porphyria and Liver-Targeted Insulin as a Novel Therapeutic Approach. *Biomedicines* **2021,** 9, (3), 255.

18.   Andersson, C.; Bylesjö, I.; Lithner, F., Effects of diabetes mellitus on patients with acute intermittent porphyria. *Journal of internal medicine* **1999,** 245, (2), 193-7.

19.   Ko, C. Y.; Lo, Y. M.; Xu, J. H.; Chang, W. C.; Huang, D. W.; Wu, J. S.; Yang, C. H.; Huang, W. C.; Shen, S. C., Alpha-lipoic acid alleviates NAFLD and triglyceride accumulation in liver via modulating hepatic NLRP3 inflammasome activation pathway in type 2 diabetic rats. **2021,** 9, (5), 2733-2742.

20.   Rahmanabadi, A.; Mahboob, S.; Amirkhizi, F.; Hosseinpour-Arjmand, S.; Ebrahimi-Mameghani, M., Oral α-lipoic acid supplementation in patients with non-alcoholic fatty liver disease: effects on adipokines and liver histology features. *Food & function* **2019,** 10, (8), 4941-4952.

21.   Hosseinpour-Arjmand, S.; Amirkhizi, F.; Ebrahimi-Mameghani, M., The effect of alpha-lipoic acid on inflammatory markers and body composition in obese patients with non-alcoholic fatty liver disease: A randomized, double-blind, placebo-controlled trial. *Journal of clinical pharmacy and therapeutics* **2019,** 44, (2), 258-267.

22.   Hsiao, K. J.; Lee, F. Y.; Wu, S. J.; Chang, W. J., Determination of erythrocyte porphobilinogen deaminase activity using porphobilinogen as substrate. *Clinica chimica acta; international journal of clinical chemistry* **1987,** 168, (2), 257-8.

23.   Stein, P. E.; Badminton, M. N.; Rees, D. C., Update review of the acute porphyrias. *British journal of haematology* **2017,** 176, (4), 527-538.

24.   Phillips, J. D., Heme biosynthesis and the porphyrias. *Molecular genetics and metabolism* **2019,** 128, (3), 164-177.

25.   Storjord, E.; Dahl, J. A.; Landsem, A.; Ludviksen, J. K.; Karlsen, M. B.; Karlsen, B. O.; Brekke, O.-L., Lifestyle factors including diet and biochemical biomarkers in acute intermittent porphyria: Results from a case-control study in northern Norway. *Molecular genetics and metabolism* **2019,** 128, (3), 254-270.

26.   García-Diz, L.; Murcia, M. A.; Gris, J. L.; Pons, A.; Monteagudo, C.; Martínez-Tomé, M.; Jiménez-Monreal, A. M., Assessing nutritional status of acute intermittent porphyria patients. *European journal of clinical investigation* **2012,** 42, (9), 943-952.

27.   Herrick, A. L.; Fisher, B. M.; Moore, M. R.; Cathcart, S.; McColl, K. E. L.; Goldberg, A., Elevation of blood lactate and pyruvate levels in acute intermittent porphyria — A reflection of haem deficiency? *Clinica Chimica Acta* **1990,** 190, (3), 157-162.

28.   Di Pierro, E.; Granata, F., Nutrients and Porphyria: An Intriguing Crosstalk. **2020,** 21, (10).

29.   Homedan, C.; Laafi, J.; Schmitt, C.; Gueguen, N.; Lefebvre, T.; Karim, Z.; Desquiret-Dumas, V.; Wetterwald, C.; Deybach, J.-C.; Gouya, L.; Puy, H.; Reynier, P.; Malthièry, Y., Acute intermittent porphyria causes hepatic mitochondrial energetic failure in a mouse model. *The international journal of biochemistry & cell biology* **2014,** 51, 93-101.

30.   Ostrowski, J.; Kostrzewska, E.; Michalak, T.; Zawirska, B.; Medrzejewski, W.; Gregor, A., Abnormalities in liver function and morphology and impaired aminopyrine metabolism in hereditary hepatic porphyrias. *Gastroenterology* **1983,** 85, (5), 1131-7.

31.   Berger, S.; Stattmann, M.; Cicvaric, A.; Monje, F. J.; Coiro, P.; Hotka, M.; Ricken, G.; Hainfellner, J.; Greber-Platzer, S.; Yasuda, M.; Desnick, R. J.; Pollak, D. D., Severe hydroxymethylbilane synthase deficiency causes depression-like behavior and mitochondrial dysfunction in a mouse model of homozygous dominant acute intermittent porphyria. *Acta Neuropathologica Communications* **2020,** 8, (1), 38.

32.   Shetty, T.; Sishtla, K.; Park, B.; Repass, M. J.; Corson, T. W., Heme Synthesis Inhibition Blocks Angiogenesis via Mitochondrial Dysfunction. *iScience* **2020,** 23, (8), 101391.

33.   Willandt, B.; Langendonk, J. G.; Biermann, K.; Meersseman, W.; D'Heygere, F.; George, C.; Verslype, C.; Monbaliu, D.; Cassiman, D., Liver Fibrosis Associated with Iron Accumulation Due to Long-Term Heme-Arginate Treatment in Acute Intermittent Porphyria: A Case Series. *JIMD reports* **2016,** 25, 77-81.

34.   Balwani, M.; Wang, B.; Anderson, K. E.; Bloomer, J. R.; Bissell, D. M.; Bonkovsky, H. L.; Phillips, J. D.; Desnick, R. J., Acute hepatic porphyrias: Recommendations for evaluation and long-term management. *Hepatology* **2017,** 66, (4), 1314-1322.

35.   Storjord, E.; Dahl, J. A.; Landsem, A.; Fure, H.; Ludviksen, J. K.; Goldbeck-Wood, S.; Karlsen, B. O.; Berg, K. S.; Mollnes, T. E.; E, W. N.; Brekke, O. L., Systemic inflammation in acute intermittent porphyria: a case-control study. *Clinical and experimental immunology* **2017,** 187, (3), 466-479.

36.   Solmonson, A.; DeBerardinis, R. J., Lipoic acid metabolism and mitochondrial redox regulation. *The Journal of biological chemistry* **2018,** 293, (20), 7522-7530.

37.   Daher, R.; Mansouri, A.; Martelli, A.; Bayart, S.; Manceau, H.; Callebaut, I.; Moulouel, B.; Gouya, L.; Puy, H.; Kannengiesser, C.; Karim, Z., GLRX5 mutations impair heme biosynthetic enzymes ALA synthase 2 and ferrochelatase in Human congenital sideroblastic anemia. *Molecular genetics and metabolism* **2019,** 128, (3), 342-351.