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AG-348 (mitapivat), an allosteric activator of red blood cell pyruvate kinase, increases enzymatic activity, protein stability, and adenosine triphosphate levels over a broad range of PKLR genotypes

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ABSTRACT

Pyruvate kinase (PK) deficiency is a rare hereditary disorder affecting red blood cell (RBC) glycolysis, causing changes in metabolism including a deficiency in adenosine triphosphate (ATP). This affects red cell homeostasis, promoting premature removal of RBC from the circulation. In this study, we characterized and evaluated the effect of AG-348, an allosteric activator of PK that is currently in clinical trials for treatment of PK deficiency, on RBC and erythroid precursors from PK-deficient patients. In 15 patients, *ex vivo* treatment with AG-348 resulted in increased enzymatic activity in all patients' cells after 24 hours (h) (mean increase: 1.8-fold; range: 1.2-3.4). ATP levels increased (mean increase: 1.5-fold; range: 1.0-2.2) similar to control cells (mean increase: 1.6-fold; range: 1.4-1.8). Generally, PK thermostability was strongly reduced in PK-deficient RBC. *Ex vivo* treatment with AG-348 increased residual activity from 1.4- to >10-fold more than residual activity of vehicle-treated samples. Protein analyses suggest that a sufficient level of PK protein is required for cells to respond to AG-348 treatment *ex vivo*, as treatment effects were minimal in patient cells with very low or undetectable levels of PK-R. In half of the patients, *ex vivo* treatment with AG-348 was associated with an increase in RBC deformability. These data support the hypothesis that drug intervention with AG-348 effectively up-regulates PK enzymatic activity and increases stability in PK-deficient RBC over a broad range of PKLR genotypes. The concomitant increase in ATP levels suggests that glycolytic pathway activity may be restored. AG-348 treatment may represent an attractive way to correct the underlying pathologies of PK deficiency. (AG-348 is currently in clinical trials for the treatment of PK deficiency. Registered at *clinicaltrials.gov* identifiers: NCT02476916, NCT03853798, NCT03548220, NCT03559699).

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Introduction

Pyruvate kinase (PK) deficiency is a rare hereditary disorder affecting red blood cell (RBC) glycolysis, resulting in life long chronic hemolytic anemia.¹ It is the most common cause of chronic hereditary non-spherocytic hemolytic anemia, although its exact prevalence is unknown.²⁻⁴ PK deficiency is an autosomal recessive disease that occurs worldwide. It is caused by mutations in *PKLR*, the gene encoding the RBC and liver specific isoforms PK-R and PK-L, respectively.^{5,6} Humans express two additional PK isoforms, PK-M1 and PK-M2, both encoded by the *PKLR* gene. PK-M2 is expressed in early fetal tissues and most adult tissues, including early stage

erythroblasts, whereas expression of PK-M1 is confined to muscle.

To date, more than 370 mutations have been reported in PKLR associated with PK deficiency (*van Wijk, manuscript in preparation*). Most of these mutations are missense mutations encoding single amino acid changes that affect the enzyme's structure, stability or catalytic function.^{7,8} Only a few mutations occur relatively frequently, implying that the majority of patients harbor a unique combination of mutations.⁹

Pyruvate kinase is an allosterically regulated homotetrameric enzyme that catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate in the final step of glycolysis, thereby producing energy in the form of adenosine triphosphate (ATP). PK-deficient RBC are characterized by changes in metabolism associated with defective glycolysis, including a build-up of the upstream metabolite 2,3-disphosphoglycerate (2,3-DPG) and deficiency in the PK product ATP. Since RBC rely totally on glycolysis for ATP production it is hypothesized that insufficient energy production affects red cell homeostasis, promoting premature removal of PK-deficient RBC from the circulation by the spleen. Increased RBC dehydration resulting from increased activity of the Gardos channel, and consequent potassium efflux,¹⁰⁻¹² as well as ATP depletion-mediated changes in RBC membrane integrity have been proposed as mechanisms triggering this premature clearance.^{13,14} In addition, ineffective erythropoiesis may contribute to the pathophysiological consequences of PK deficiency.¹⁵⁻¹⁷

Clinically, PK deficiency manifests as chronic hemolytic anemia of highly variable clinical severity. Patients may present with severe neonatal anemia requiring exchange transfusion, whereas at the other end of the spectrum patients are diagnosed later in life with fully compensated hemolysis. The ongoing Pyruvate Kinase Deficiency Natural History Study showed that, apart from anemia, the most frequently reported complications were iron overload and gallstones. However, other complications such as aplastic crises, osteoporosis, extramedullary hematopoiesis, post-splenectomy sepsis, pulmonary hypertension, and leg ulcers are not uncommon. Typically, worsening of hemolysis occurs during infections, stress, and pregnancy.¹⁸

Current treatment for PK deficiency is generally supportive, with splenectomy, blood transfusion and iron chelation as the main therapies, focusing on the anemia and iron overload state.¹⁹ Splenectomy is effective for the majority of PK-deficient patients, in that it reduces transfusion burden and improves baseline hemoglobin levels post splenectomy by an average of 1.6 g/dL. Fourteen percent of patients who underwent splenectomy, however, continued to require regular transfusions.¹⁸ Curative treatment possibilities include stem cell transplantation (SCT) or experimental gene therapy.^{20,21}

Recently, it was shown that AG-348 is an allosteric activator of PK-R. This small molecule directly targets PK-R by binding in a pocket at the dimer-dimer interface, distinct from the allosteric activator fructose-1,6-bisphosphate binding domain (FBP). This induces the active R-state conformation of the PK-R tetramer, resulting in enhanced activity of both wild type and mutant PK-R.²² Phase I studies in healthy volunteers demonstrated glycolytic pathway activation upon treatment with AG-348 demonstrating its potential for treating PK deficiency.²³

Data from a phase II study in PK deficiency patients treated with AG-348 showed that approximately half of treated subjects experienced a rise in hemoglobin (Hb).²⁴

In this study, we further evaluated the effect of *ex vivo* treatment with AG-348 on PK deficient red cells with a broad range of patient genotypes, including effects on enzyme activity and ATP levels. In particular, for the first time we report the effects of AG-348 on PK thermostability in red cell lysates, PK-R protein level, RBC deformability and the effect of AG-348 during *in vitro* PK-deficient erythropoiesis.

Methods

This study was approved by the ethical committee of the University Medical Center Utrecht (Protocol 14-571/M).

Metabolic profiling

Frozen whole blood samples were extracted with hot 70% ethanol, dried and resuspended in water. Relative abundance of central carbon metabolites was performed by ultra performance liquid chromatography-mass spectrometry (UPLC-MS) with high resolution accurate mass detection on a QExactive™ Orbitrap mass spectrometer (Thermo Fisher Scientific).²⁵ Peak identification and integration was carried out with Maven software.²⁶ Quantitative analysis of ATP and 2,3-DPG was performed as described previously.²⁷

Pyruvate kinase activity, protein levels, and thermostability

Red blood cells were purified using microcrystalline α -cellulose columns.^{28,29} PK and hexokinase activity was measured as described.^{28,29} PK thermostability was measured as described³⁰ after 0, 5, 10, 20, 40 and 60 minutes (min) of incubation at 53°C.

Pyruvate kinase activity after *ex vivo* treatment with AG-348 was measured using low substrate (0.5 mM). PK-R protein levels were determined by Mesoscale Assay (MesoScale Discovery) as described²² using goat anti-PKLR antibody (Aviva) and mouse anti-PKLR antibody (Abcam). SULFO-TAG goat anti-mouse (MesoScale Discovery) was used as detection antibody. Protein level was determined by normalizing light intensity of the SULFO-TAG electrochemiluminescence to lysate protein concentration.

Western blot of PK-R and PK-M2 was performed as described³¹ using polyclonal antibodies against PK-L³² and monoclonal antibodies against PK-M2 (PodiCeps). Alexa Fluor® 680-conjugated goat anti-mouse and goat anti-rabbit antibodies (Li-Cor, Invitrogen) were used as detection antibodies.

Ex vivo treatment with AG-348

Purified RBC or whole blood of patients and controls were incubated for 3, 6 and 24 h at 37°C in presence or absence of AG-348 in phosphate-buffered saline containing 1% glucose, 170 mg/L adenine, and 5.25 g/L mannitol (AGAM, pH 7.40). RBC were incubated with different dosages of AG-348 (up to 10 μ M) for up to 24 h, at 37°C. After 6 and 24 h, PK-R activity and ATP levels (CellTiterGlo; Promega, Madison, WI, USA) were measured. For thermostability, RBC lysates were incubated for 2 h with AG-348.

Osmoscan and deformability

Osmoscan and deformability measurements were performed using the Lorca (RRMechatronics).^{33,34} AGAM buffer was added to whole blood (1:10), and supplemented with either AG-348 (20 M) or DMSO (untreated). Samples were incubated for 24 h at 37°C before measurements.

AG-348 treatment during *in vitro* erythropoiesis

Erythroid cells were produced from peripheral blood mononuclear cells (PBMC).³⁵ For proliferation, cells were cultured 10 days in CellQuin medium (Sanquin), supplemented with Stem Cell Factor (100 ng/mL, Amgen), erythropoietin (EPO) (2 U/mL, EPREX), dexamethasone (1 μ M, Sigma), and 1 ng/mL IL-3 (R&D Systems). After 10 days, cells were reseeded (2E6 cells/mL) in CellQuin medium supplemented with EPO (10 U/mL, Eprex), heparine (5 U/mL, Leo Pharmacy), human AB plasma (3%, Sigma). At day 5, CellQuin was replaced by RetQuin medium (Sanquin). AG-348 (2 μ mol/L) was added at days 0, 1, 3-10 during proliferation and days 0, 2, and 4 during differentiation.

Statistical analysis

Statistical analysis was performed using Graphpad Prism (v7.04). *t*-test, Mann-Whitney test or Wilcoxon test was used when appropriate. One-way ANOVA was carried out or a Kruskal-Wallis test, followed by Dunn's tests for *post-hoc* analysis. Pearson's correlation was used to determine correlations of laboratory parameters with enzyme activity measurements.

Results

Fifteen adult, transfusion independent, PK-deficient patients were enrolled for this study (median age: 44.0 years; range: 20.0-51.5). PK deficiency was confirmed by demonstrating compound heterozygosity or homozygosity for mutations in *PKLR* (Sanger sequencing). Whole blood from 15 healthy volunteers was used as control samples.

Baseline patients' characteristics displayed varying degrees of anemia (Table 1). In addition, most patients had strongly elevated reticulocyte counts, a prominent feature of PKD patients, in particular after splenectomy.¹¹ Most patients showed reduced red cell PK activity (Figure 1A) and reduced PK thermal stability (Figure 1C). Since the activity of many red cell enzymes is red cell age-dependent,³⁶ we also compared PK activity to hexokinase (HK) activity (Table 1 and Figure 1B). This PK/HK ratio was clearly decreased in all patients, indicating a relatively strong decrease in activity of PK ($r=-0.677$, $P<0.01$) (Figure 1B). When causative mutations were classified as missense (M) or non-missense (NM) a genotype to phenotype correlation was identified.¹⁸ All four patients without splenectomy were of the M/M genotype, which is in line with the lower likelihood of splenectomy in this group.¹⁸ Similarly, these patients had generally higher Hb levels and lower reticulocyte counts (Table 1).

PK-R protein levels correlate with pyruvate kinase activity

Protein levels of PK-R were measured by both western blot and Meso Scale Discovery (MSD). PK-R protein levels were variable between patients (Figure 1C and D). In general, PK-activity correlated well positively with PK-R protein levels. The most notable exception was patient 4. RBC from this patient contained very low levels of protein, as measured by both western blot and ELISA, but repeated measurements showed high levels of PK activity (Table 1 and Figure 1A, D and E). Low levels of PK-R protein in this patient could result from combined instability of the p.Arg510Gln³⁷ mutant as well as the truncated p.(Arg488*) PK-R nonsense mutant. In line with this, PK thermostability was also strongly reduced in this patient

(Figure 1C). However, the reason for the high PK activity in this patient remains unknown.

Increased PK-M2 expression, that could perhaps compensate for the loss in PK-R38 activity, was not detected except for patient 3 and, to a lesser degree, in RBC from patient 1 (Figure 1D).

Pyruvate kinase-deficient red blood cells show glycolytic intermediates levels that are consistent with decreased pyruvate kinase activity

Metabolic profiling of PKD patients was performed by liquid chromatography-mass spectrometry (LC-MS/MS) on whole blood. Consistent with a decrease in PK activity at the final step of glycolysis, levels of PEP were significantly increased ($P<0.0001$) whereas levels of pyruvate and ATP were significantly decreased (both $P<0.0001$) (Figure 2). In addition, dihydroxyacetone phosphate (DHAP) and 2,3-DPG levels were increased ($P<0.001$ and $P<0.0001$), indicative of retrograde accumulation of upstream glycolytic intermediates.

Ex vivo incubation with AG-348 increases pyruvate kinase activity and adenosine triphosphate in a dose-dependent manner

Ex vivo treatment of PK-deficient and healthy control RBC with increasing dose of AG-348 showed an increase in PK-activity and ATP levels. Illustrative PK activity dose response curves of two patients are shown in Figure 3A and B. Incubations with 10 μ M AG-348 showed a mean 1.3-fold increase (range: 1.0-2.0) in PK activity in PK-deficient RBC after 6 h ($P<0.0001$) and of 1.8 (range: 1.2-3.4) after 24 h ($P<0.0001$) (Figure 3E). Similar values were obtained from control cells: 1.4 after 6 h (range: 0.9-1.9; $P<0.01$) and 2.3 (range: 1.2-7.1) after 24 h ($P<0.0001$).

Adenosine triphosphate levels increased accordingly in a dose-dependent manner (Figure 3C and D). AG-348 treatment significantly increased mean ATP levels in PK-deficient RBC to 1.4-fold (range: 1.0-2.3) after 6 h ($P<0.01$) and 1.5-fold (range: 1.0-2.2) after 24 h ($P<0.0001$). The mean increase in ATP levels in control cells was 1.5-fold (range: 1.3-1.7) after 6 h ($P<0.001$) and 1.6-fold (range: 1.4-1.8) after 24 h ($P<0.0001$). We observed no differences in PK activity and ATP response between PK-deficient patients with M/M and M/NM genotypes (Table 1 and Figure 3E and F).

Since glycolysis in RBC is known to be regulated by band 3,³⁹ we verified RBC band 3 expression levels by western blot analysis. We observed no differences in band 3 expression levels of PK-deficient patients compared to healthy controls (*Online Supplementary Figure S1*).

Collectively, these results are consistent with those of previous reports,²² and illustrate the ability of AG-348 to stimulate PK activity in patient samples with a range of genotypes.

Ex vivo treatment with AG-348 restores pyruvate kinase thermostability

At baseline, PK-R thermostability was strongly reduced in all PK-deficient patients (Figure 4C). Whereas RBC from healthy controls showed a mean residual activity of 81% (range: 64-108%) after incubation at 53°C for 60 min, the mean residual PK-R activity in PK-deficient patients was significantly decreased under these conditions to only 30% (range: 6-54%) ($P<0.0001$). This confirms earlier observations³⁰ and shows that PK thermostability is a

Table 1. Genotypes, amino acid change, splenectomy status and laboratory parameters are shown for all 15 pyruvate kinase (PK)-deficient patients included in this study.

N.	Sex	PKLR mutation	Amino acid change	Genotype	Splenectomy	Hb (g/dL)	Ret. (%)	PK (U/gHb)	HK (U/gHb)	PK/HK ratio
P1	F	c.1121T>C/c.1456C>T	p.(Leu374Pro)/p.(Arg486Trp)	M/M	Yes	9.7	10.0	9.0	5.0	1.8
P2	M	c.331G>A/c.1456C>T	p.(Gly111Arg)/p.(Arg486Trp)	M/M	No	11.2	7.0	3.1	1.7	1.8
P3	M	c.1178A>G/c.1456C>T	p.(Asn393Ser)/p.(Arg486Trp)	M/M	Yes	9.4	>35.0	16.9	6.2	2.7
P4	F	c.1462C>T/c.1529G>A	p.(Arg488*)/p.(Arg510Gln)	M/NM	Yes	6.6	>34.0	15.9	7.4	2.1
P5	F	c.1121T>C/c.1706G>A	p.Leu374Pro/p.(Arg569Gln)	M/M	No	10.9	7.0	2.1	1.5	1.4
P6	F	c.507+1G>A/c.1436G>A	p.[=; 0]/p.(Arg479His)	M/NM	Yes	7.7	75.0	1.9	6.0	0.3
P7	M	c.376-2A>C/c.1529G>A	p.(Glu125_Tyr126ins32; Tyr126Alafs*83)/p.(Arg510Gln)	M/NM	Yes	8.0	55.0	1.7	5.6	0.3
P8	F	c.331G>A/c.1456C>T	p.(Gly111Arg)/p.(Arg486Trp)	M/M	No	11.9	5.0	2.2	1.4	1.6
P9	M	c.331G>A/c.1492C>T	p.(Gly111Arg)/p.(Arg498Cys)	M/M	Yes	10.3	40.0	2.5	4.1	0.6
P10	M	c.1154G>A/c.1529G>A	p.(Arg385Lys)/p.(Arg510Gln)	M/M	No	13.4	15.0	2.7	2.0	1.3
P11	M	c.721G>T/c.1529G>A	p.(Glu241*)/p.(Arg510Gln)	M/NM	Yes	8.2	85.0	2.2	6.1	0.4
P12	M	c.142_159del/c.494G>T	p.(Thr48_Thr53del)/p.(Gly165Val)	M/NM	Yes	12.9	20.0	10.0	3.5	2.8
P13	M	c.1269G>A/c.1654G>A	p.([Met373_Ala423del; 0] /p.(Val552Met)	M/NM	Yes	9.7	55.0	1.8	5.8	0.3
P14	F	c.401T>A/c.1529G>A	p.(Val134Asp)/p.(Arg510Gln)	M/M	Yes	9.6	60.0	1.2	5.2	0.2
P15	M	c.1529G>A/c.1529G>A	p.(Arg510Gln)/p.(Arg510Gln)	M/M	Yes	2.8	40.0	1.6	2.8	0.6
Contr.					No	14.1 (1.3)	1.18 (0.3)	12.7 (2.3)	1.1 (0.2)	11.9 (2.1)
Reference range values:						M: 13.8-17.2 1.0-2.0 9.2-16.9 0.9-1.4 9.0-16.6 F: 11.9-15.5				

Enzyme activity of PK was decreased or normal. Hexokinase (HK) activity was almost always increased due to reticulocytosis. PK/HK ratio was strongly decreased in all cases, indicative of PK deficiency; Mean values (standard deviation) of 15 healthy controls are shown. Reference range values of the UMC Utrecht population are also included. M: male; F: female; Hb: hemoglobin; Ret.: reticulocytes; PK: pyruvate kinase; U: units; H, hexokinase; M/M: missense/missense; M/NM: missense/nonmissense.

common feature of many different mutant forms of PK-R, and not just restricted to known unstable variants like p.Arg510Gln.

Treating PK-deficient RBC *ex vivo* with AG-348 (2 μ M) prior to this assay showed a clear effect on stability of the enzyme (Figure 4C; illustrative examples of RBC from patients 12 and 9 are shown in Figure 4A and B). *Ex vivo* treatment with AG-348 lead to a mean residual PK activity of 58% (range: 0-115%; $P < 0.001$) in RBC from PK-deficient patients and 106% in controls (range: 99-111%; $P = 0.13$). We observed no differences in PK thermostability response between PK-deficient patients with M/M and M/NM genotypes (Table 1 and Figure 4C).

Effect of *ex vivo* AG-348 treatment on deformability of pyruvate kinase-deficient red blood cells

Because RBC deformability is partly ATP dependent,^{40,41} we investigated the effect of *ex vivo* AG-348 treatment on this important RBC functional property by osmotic gradient ektacytometry (osmoscan) and deformability measurements. Figure 5A shows osmoscan curves of the 15 healthy control samples (gray) and that of patient 7 (red) as an example. At baseline, maximum deformability (EL_{max}) of PK-deficient RBC was significantly decreased compared to controls, although the difference was small ($P < 0.01$) (Figure 5D). Similarly, Omin and Ohyper, reflecting RBC surface-to-volume ratio and hydration status,⁴² respectively, were significantly different in PK-deficient patients ($P = 0.039$ and 0.047) (Figure 5C and E). This implies that PK-deficient RBC are somewhat less deformable than nor-

mal RBC, possibly due to a slightly lower surface-to-volume ratio and a slight increase in RBC hydration.

When PK-deficient patients were divided according to genotype (Table 1), there was a significant difference between M/M and M/NM genotypes, indicating that a more severe genotype results in lower maximal RBC deformability (EL_{max} , $P = 0.039$) (Figure 5B).

After 24 h of *ex vivo* treatment with AG-348, an improvement in RBC deformability was observed in about half of the PK-deficient patients (Figure 5E; patients 1, 3, 4, 6, 7, 8, and 9) compared to their untreated RBC. The effect of these changes were similar in magnitude to the baseline difference in deformability observed between PK-deficient RBC and control RBC (Figure 5C). Taken in aggregate, however, there was no overall significant improvement of deformability.

Effect of *ex vivo* AG-348 treatment on PK-R-deficient erythroid development

During erythropoiesis, PK-M2 levels are gradually replaced by PK-R^{43,44} and AG-348 has been described to also activate PK-M2.²² Because PK deficiency has been associated with ineffective erythropoiesis,¹⁵⁻¹⁷ we next investigated the effect of *ex vivo* AG-348 treatment on erythroid development from four PK-deficient patients and controls. Morphologically there were no major abnormalities in erythroid development between healthy controls or PK-deficient patients (Figure 6A). This was supported by there being no difference in erythroid surface marker expression³⁵ (Online Supplementary Figure S2A). There was

no difference in erythroid differentiation with or without AG-348 treatment. This was observed in both healthy control cells and in PK-deficient cells (*Online Supplementary Figure S2B and C*). In presence of AG-348 (2 μ M), we did note, however, a small increase in cell number in both normal controls and patients after proliferation for 10 days, although the difference was not significant ($P=0.059$) (*Figure 6B*).

Western blot analysis of PK-R and PK-M2 protein levels of healthy control samples clearly showed an increase in expression of PK-R and a decrease in PK-M2 during differ-

entiation (*Online Supplementary Figure S3*). The decrease in PK-M2 was also observed in all patient samples, whereas a clear increase in PK-R expression was seen only in samples from patients 7 and 15 (*Online Supplementary Figure S3*). PK-R protein levels were low or inconclusive in patients 3 and 10. Presence of AG-348 during *ex vivo* erythropoiesis was associated with higher levels of PK-R in patients 7 and 15. AG-348 was not seen to have any effect on PK-M2 expression.

On the level of enzymatic activity, the PK/HK ratio from cultured red cells was higher in presence of AG-348 in

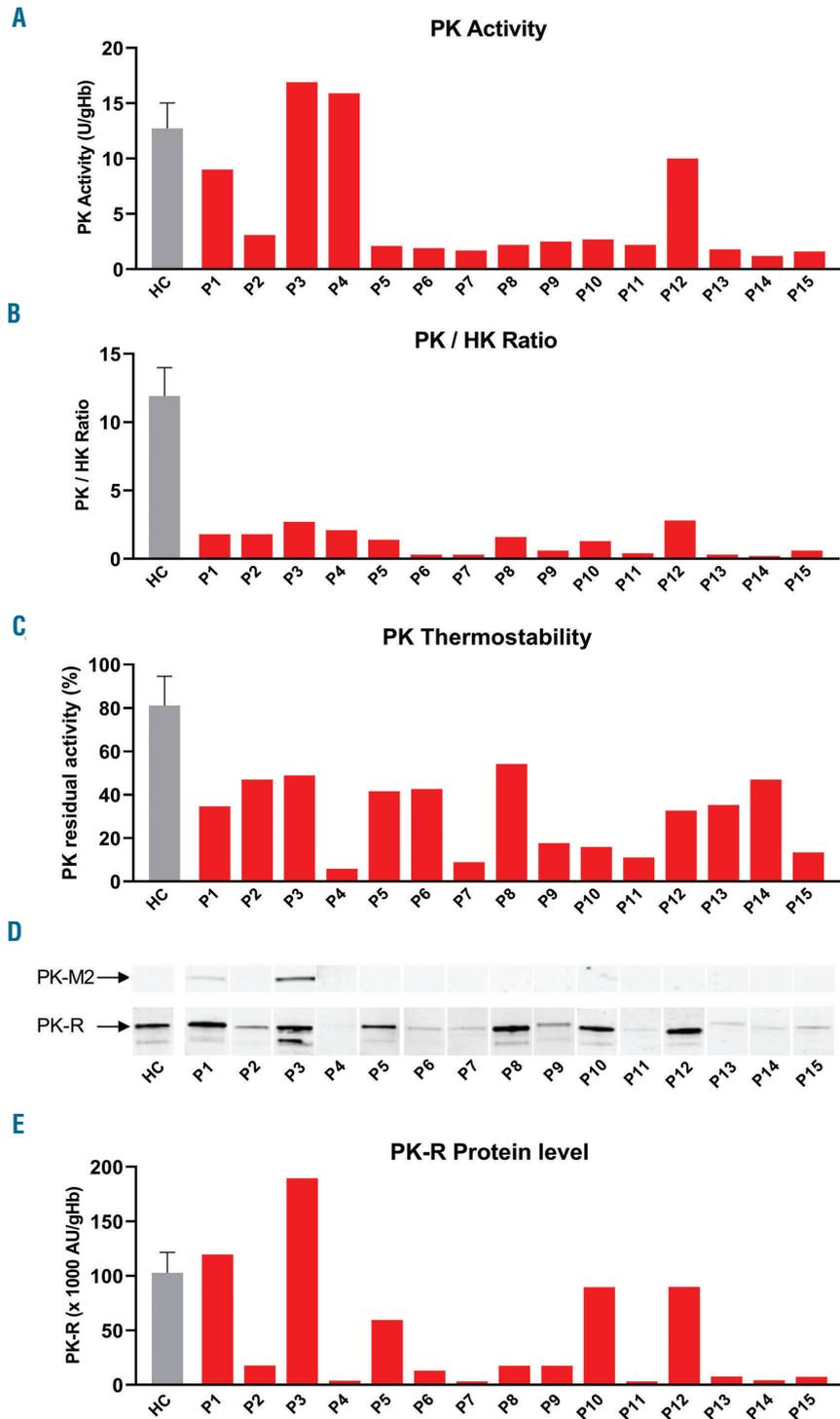


Figure 1. Baseline levels of pyruvate kinase (PK) activity, PK/Hexokinase (HK) ratio, PK thermostability, and PK-R protein levels in PK-deficient patients and controls. (A) PK activity of PK deficient patients (red) and healthy controls (gray). (B) PK/HK ratio in patients (red) and healthy controls (gray), as a means to evaluate PK activity in the presence of high number of reticulocytes, reflected by increased HK activity. (C) PK thermostability of patients (red) and controls (gray). (D) PK-R protein levels of patients (P#) and controls (HC) measured by western blot. (E) PK-R protein levels of patients (red) and controls (gray) measured by Meso Scale Discovery. ECL: electrochemiluminescence.

both patients and healthy controls, although this effect did not reach statistical significance ($P=0.064$) (Figure 6C).

Discussion

In this study, we characterized and evaluated the effect of AG-348, a recently reported first-in-class allosteric activator of PK-R^{22,23} that is currently in clinical trials for the treatment of PK deficiency (*clinicaltrials.gov* identifiers: NCT02476916, NCT03853798, NCT03548220, NCT03559699). Our find-

ings indicate that *ex vivo* treatment with AG-348 effectively increases PK-R activity over a broad range of PKLR genotypes as assessed by enzymatic activity as well as cellular ATP levels. Our findings are consistent with those previously reported by Kung *et al.*,²² and further extend those findings through the evaluation of a substantially larger cohort of PK-deficient blood samples, including a comprehensive baseline metabolomics analysis, and assessment for the first time of the effects of PK deficiency on parameters such as red cell deformability and *ex vivo* expansion of erythroid progenitors.

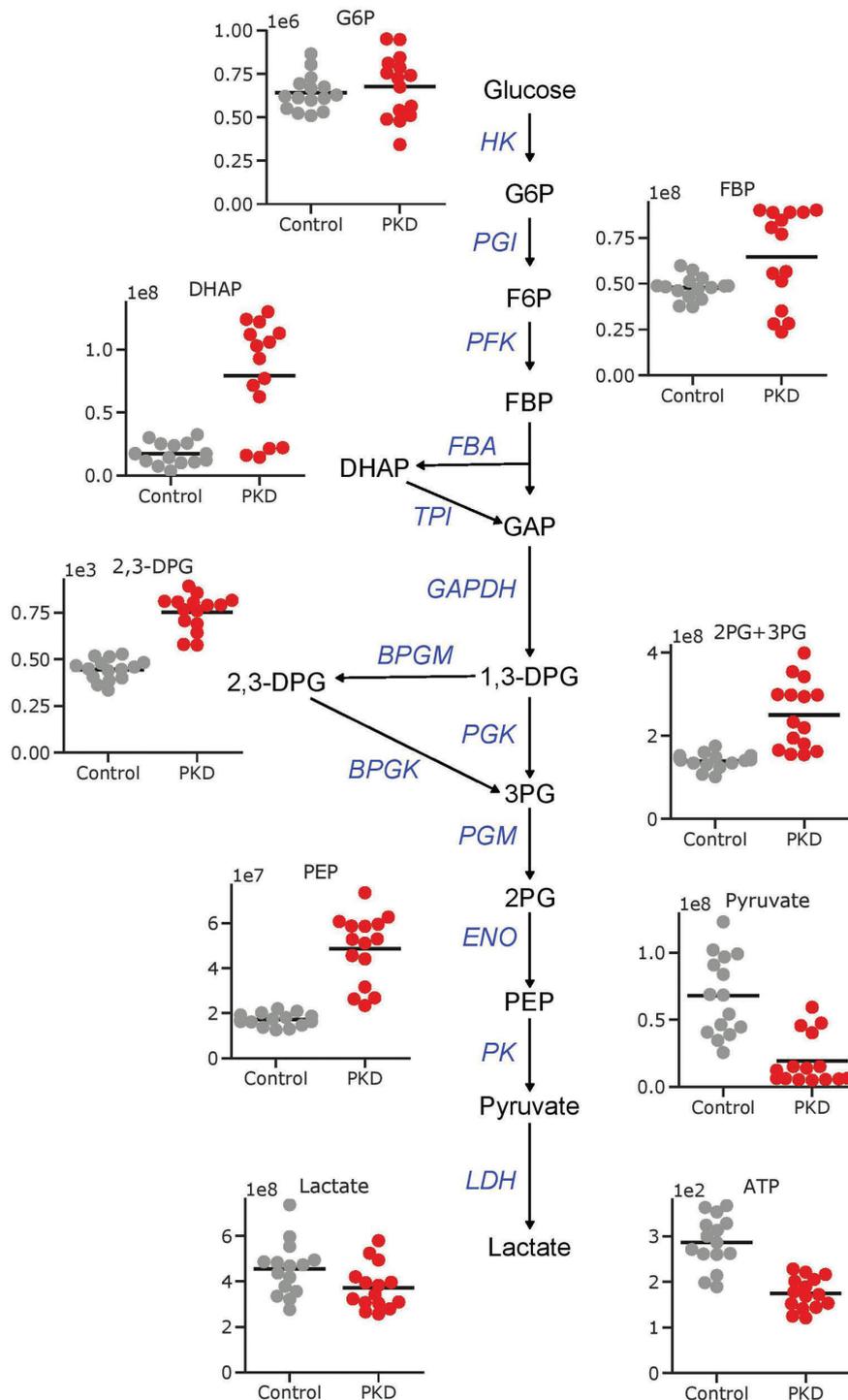


Figure 2. Pyruvate kinase (PK)-deficient patients show glycolytic intermediates levels that are consistent with decreased PK activity. Upstream intermediates of PK of PK-deficient patients (red) are significantly increased compared to values of health controls (gray), with, for example, an almost 2-fold increase in 2,3-DPG. Downstream targets, pyruvate and adenosine triphosphate (ATP), are significantly decreased compared to healthy controls. PKD: pyruvate kinase deficiency; G6P: glucose-6-phosphate; DHAP: dihydroxyacetone phosphate; 2,3-DPG: 2,3-diphosphoglycerate; PEP: phosphoenol pyruvate; FBP: fructose biphosphate; ATP: adenosine triphosphate; HK: hexokinase; PGI: phosphoglucose isomerase; PFK: phosphofructokinase; FBA: fructose biphosphate aldolase; TPI: triosephosphate isomerase; GAPDH: glyceraldehyde phosphate dehydrogenase; BPGM: bisphosphoglycerate mutase; BPGK: bisphosphoglycerate kinase; PGK: phosphoglycerate kinase; PGM: phosphoglycerate mutase; ENO: enolase; LDH: lactate dehydrogenase.

Our cohort shows an overall mean 1.8-fold increase in PK activity in response to *ex vivo* treatment with AG-348 (range: 1.2-3.4) (Figure 3E). This is very similar to the previously reported mean fold increase of 2.1 (range: 1.3-3.4). Similarly, the 1.5-fold increase in ATP levels we observed in PK-deficient RBC (range: 1.0-2.2) was comparable to previously reported results (mean fold increase: 1.7; range: 1.3-2.4).

PK thermostability was found to be significantly improved upon *ex vivo* treatment with AG-348 even though there was a high variability in response among the different genotypes (Figure 4). An interesting subset was composed of six PK-deficient patients (patients 4, 7, 10, 11, 14, and 15) who had the common PK-R mutation c.1529G>A p.(Arg510Gln), previously shown to have little defect in catalytic activity but to be highly unstable. Five of these

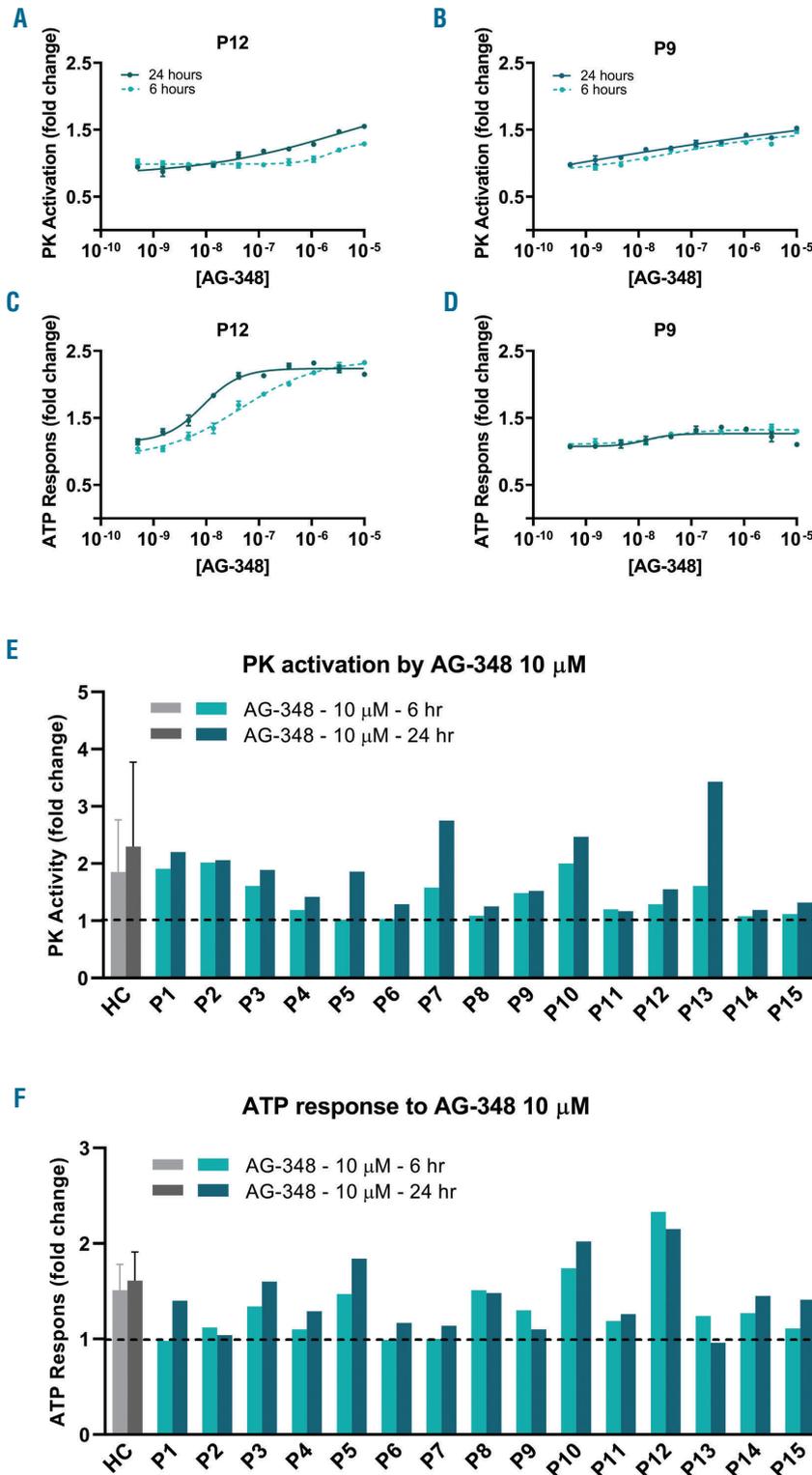


Figure 3. *Ex vivo* AG-348 treatment of red blood cells (RBC) increase pyruvate kinase (PK) activity and adenosine triphosphate (ATP) levels in a dose dependent manner. (A and B) Representative patient dose response curves of PK activity, expressed as fold activation compared to incubation without AG-348, as measured in RBC of patients 12 (A) and 9 (B) after 6- (turquoise) and 24-hour (h) (dark green) incubation with AG-348. (C and D) representative patient ATP dose response curves, expressed as fold activation compared to incubation without AG-348, as measured in RBC of patients 12 (C) and 9 (D) after 6- (turquoise) and 24-h (dark green) incubation with AG-348. (E) PK activation in RBC of 15 PK deficient patients after 6- (turquoise) and 24-h (dark green) incubation with 10 μM AG-348. Mean PK fold activation of healthy controls after 6- (light gray) and 24-h (dark gray) incubation with AG-348 10 μM is shown on the left of the graph. (F) ATP response (fold activation) of 15 PK deficient patients after 6- (turquoise) and 24-h (dark green) incubation with 10 μM AG-348. Mean ATP response of healthy controls is shown on the left of the graph (gray bars). Error bars represent standard deviation. Dashed turquoise line represents 6-h incubation with AG-348, solid dark green line represents 24-h incubation.

patients had unmeasurably low PK activity in the thermostability test, and the activity of four could be restored by pre-incubating RBC with AG-348 (Figure 4, patients 7, 10, 11 and 14). These four patients carried the p.(Arg510Gln) mutation in trans to a splice site mutation (patient 7), a missense mutation (patients 10 and 14), and a nonsense mutation (patient 11). A significant outlier was patient 15, who was homozygous for p.(Arg510Gln), suggesting the possibility that it may be more difficult to stabilize this mutation in the homozygous condition. Another outlier was patient 4, who had p.(Arg510Gln) paired with the nonsense mutation p.(Arg488*) yet had a relatively modest loss of activity in the thermostability test. Collectively these data illustrate that AG-348 has the potential to stabilize a diverse range of different mutant PK-R molecules *in vitro*, but also demonstrate the complex variability that is introduced by the compound heterozygous state of most patients.

One surprising result was that the effects of AG-348 were variable across the different patient samples, and that overall increases in PK activity, ATP and thermostability were not observed together in some patients. For example, patient 13 shows a clear response in terms of PK activity after AG-348 treatment for 24 h whereas the ATP response was present at 6 h and back to baseline by 24 h. However, we did observe that residual PK-R protein levels

at baseline are important for the response to AG-348. When grouping patients according to their response in PK activity or ATP levels (i.e., >1.5-fold vs. <1.5-fold response), PK-R protein levels at baseline (Figure 1E) were found to be higher in the patients that showed a >1.5-fold increase upon *ex vivo* treatment with AG-348 (Figure 7). This suggests that the amount of PK-R protein is an important determinant for the *in vitro* activity of AG-348, and is consistent with its mechanism of action *via* direct binding and stimulation of mutant enzyme.

While the *in vitro* assessments as presented in this study provide clear evidence that AG-348 can stimulate the activity of mutant PKR enzymes in a variety of contexts, the central question is whether changes in these parameters would be associated with increased likelihood of clinical response to AG-348. Recently, data from the phase II study of AG-348 in PKD patients have been published.²⁴ Analysis of those results suggests some parallels between our data and the response observed in treated patients. For example, the clinical manuscript demonstrates that higher baseline PK-R protein levels in patients is associated with Hb increases, which parallels the findings described here. Similarly to the effects on thermostability noted above in patients with the p.(Arg510Gln) mutation, such patients in phase II had variable Hb responses.²⁴

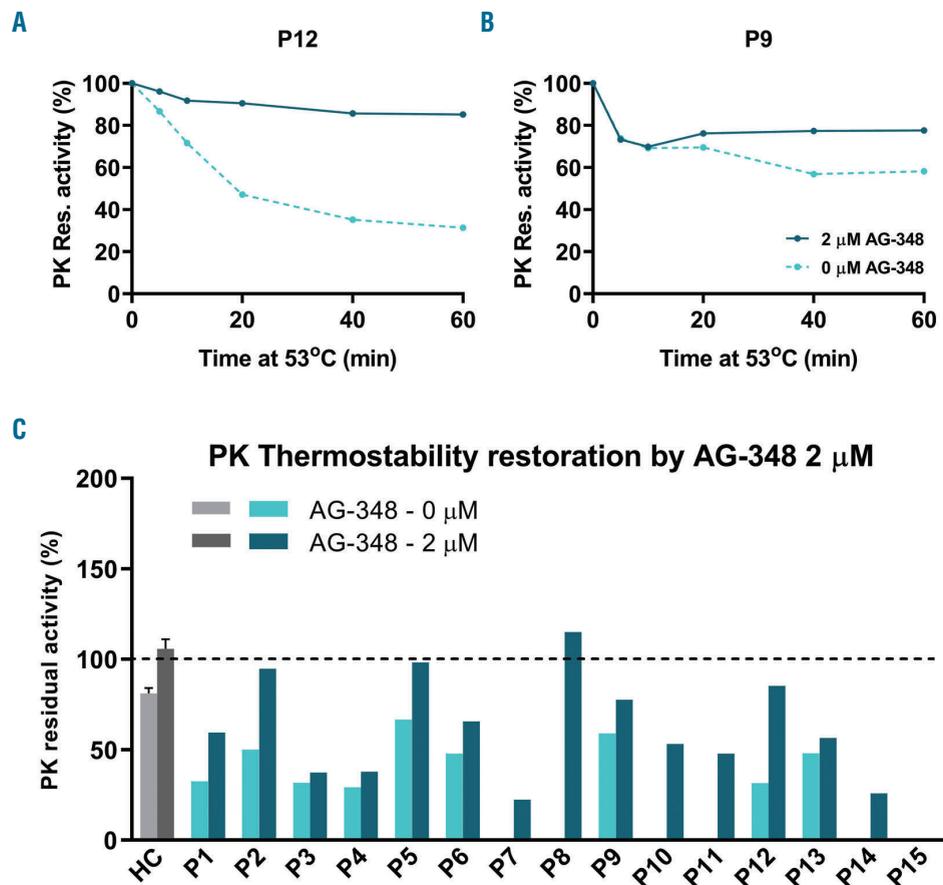


Figure 4. Pyruvate kinase (PK) thermostability is restored upon *ex vivo* treatment with AG-348. (A and B) Representative PK-deficient (PKD) patients' PK thermostability of red blood cell (RBC) lysates of patients 12 (A) and 9 (B) pre-incubated in absence (dashed line) or presence (solid line) of 2 μM AG-348 for 2 hours (h) at 37 °C. For PK thermostability, residual PK activity (%) is measured after 5, 10, 20, 40 and 60 minutes (min) of heat treatment at 53 °C. (C) Residual PK activity (%) after 60 min of heat treatment of 15 PKD pre-incubated in absence (light turquoise) and presence (dark turquoise bars) of 2 μM AG-348. Levels are normalized to baseline levels of PK-activity before heat treatment. Absence of bars indicate that PK residual activity was below the detection limit. Error bars represent standard deviation.

Here, for the first time, we report on the effects of AG-348 on *ex vivo* produced PK-R deficient RBC. In presence of AG-348 we observed a trend towards a slightly improved proliferation of erythroid progenitor cells in both healthy controls and PK-deficient patients of various PKLR genotypes (Figure 5B). Further studies are warranted to establish this more firmly, but it could indicate that, apart from acting on mature RBC in the circulation, AG-348 could have a potential beneficial effect on both normal and PK-deficient early stage erythroid development. In addition, we show that *ex vivo* produced (mutant) PK-R can be activated to a similar degree as (mutant) PK-R from RBC from patients and healthy controls (Figure 6C). This was particularly evident for

patients 7 and 15, who also showed the most pronounced effect of AG-348 on PK-R protein levels (*Online Supplementary Figure S3*).

At the same time, based on cell count, morphology, and erythroid differentiation markers, we did not observe major differences between normal and PK-deficient *ex vivo* erythroid proliferation. This is in contrast with previous reports that suggest ineffective erythropoiesis is a pathological feature of PK deficiency, based on increased numbers of apoptotic early erythroid progenitor cells in the spleen of one PK-deficient patient,¹⁶ and similar findings in the spleen from a mouse model of PK deficiency.¹⁵ Regarding this observed discrepancy between our findings and previous studies, a possible confounder is that we

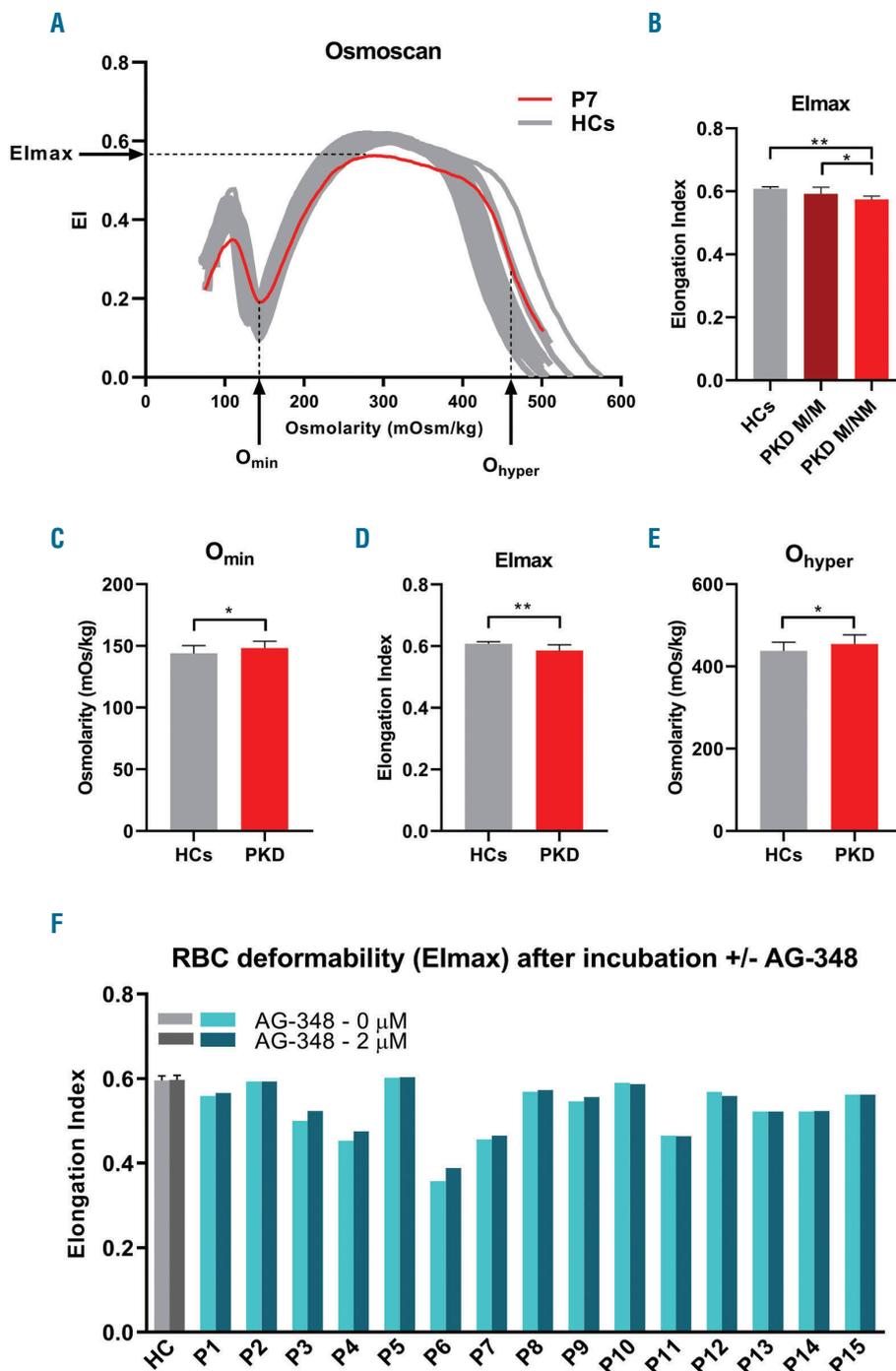


Figure 5. Red blood cell (RBC) deformability in pyruvate kinase (PK)-deficient patients is slightly decreased. (A) Osmoscan curve of patient 7 (red) and 15 healthy controls (HC) (gray). (B) Maximum deformability (Elmax) is slightly decreased in PK deficiency compared to HC. When grouped according to genotype, i.e., missense/missense M/M versus missense/non-missense (M/NM), there is a significant difference, indicating that a more severe genotype results in lower maximal RBC deformability. (C) Omin is slightly increased in PKD patients compared to HC. (D) Elmax is slightly decreased in PKD patients compared to HC. (E) Ohyper is slightly increased in PKD patients compared to HC. (F) After 24 hours of *ex vivo* treatment with 20 μM AG-348 in about half of the PKD patients an improvement in RBC deformability was observed (patients 1, 3, 4, 6, 7, 8, and 9) compared to their untreated RBC. Error bars represent standard deviation. **P<0.01, *P<0.05.

grew erythroid cells from a fixed number of PBMC. PBMC composition will likely vary between patients and controls, in particular the number of CD34⁺ cells. This may influence the eventual outcome of cellular proliferation. More importantly, genotype-phenotype correlations could explain these conflicting results as different genotypes result in different complications,¹⁸ indicating that different

mutations could result in a large variability in the occurrence of ineffective hematopoiesis.

A limitation of this study is the absence of a functional assay that could directly measure the effect of ATP increase. We did observe an improvement in RBC deformability as measured by osmotic gradient ektacytometry after *ex vivo* treatment with AG-348 in half of the

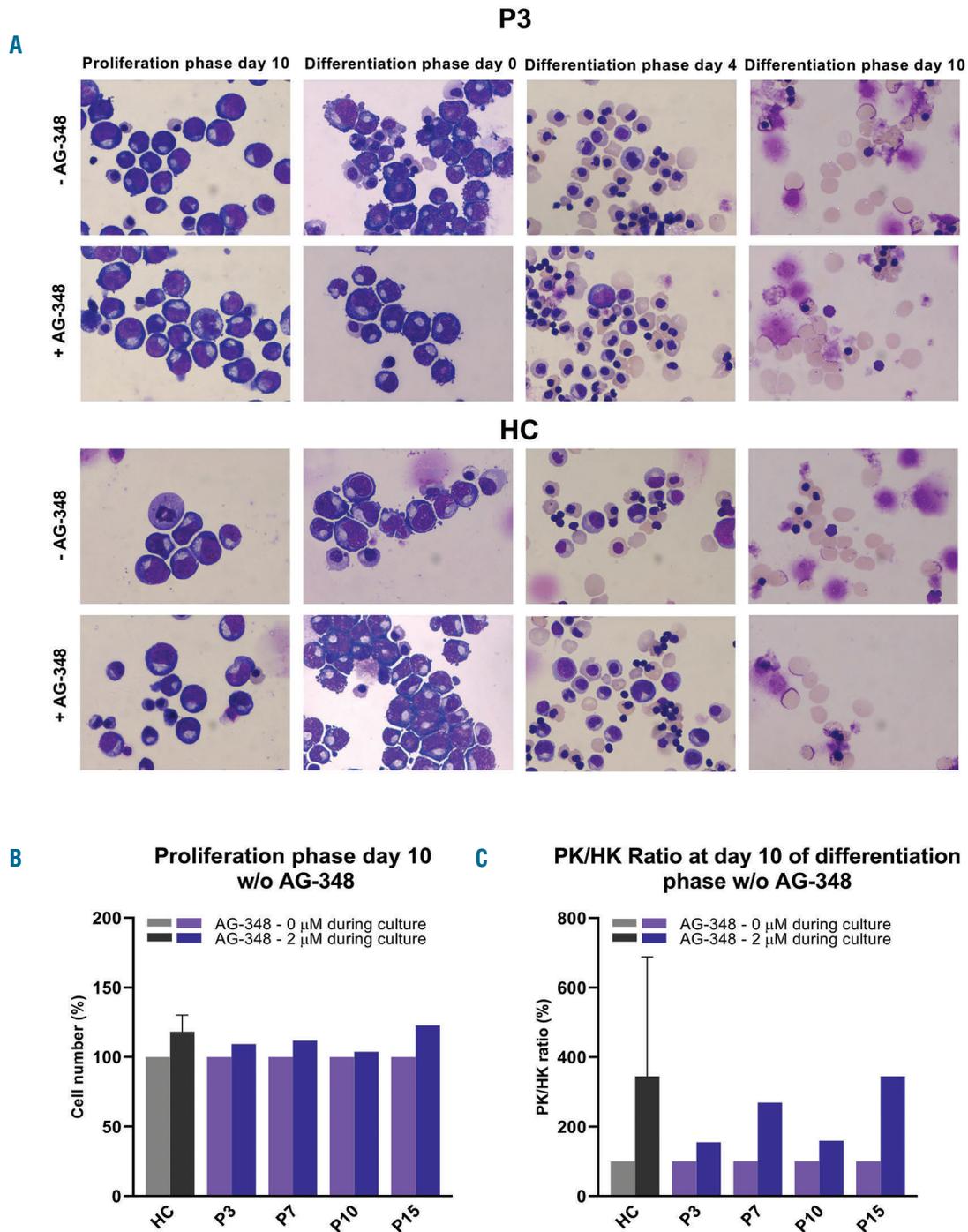


Figure 6. Ex vivo treatment with AG-348 does not evidently affect pyruvate kinase (PKD) ex vivo erythroid proliferation and differentiation. (A) Morphology of *ex vivo* cultured erythroid cells of a PKD patient (P3) and a healthy control (HC) at the final stage of proliferation (day 10) and on various days during differentiation (days 0, 4 and 10). (B) *Ex vivo* erythroid proliferation (cell numbers in %) was slightly higher when cells were cultured in presence of 2 μM AG-348 (dark purple) compared to 0 μM AG-348 (light purple). This was also observed for HC. (C) PK/HK ratio of erythroid cells of PKD patients (purple) and HC (gray) cultured in presence (dark) or absence (light) of 2 μM AG-348 respectively was increased compared to cells cultured without AG-348. W/o: with/without. Error bars represent standard deviation.

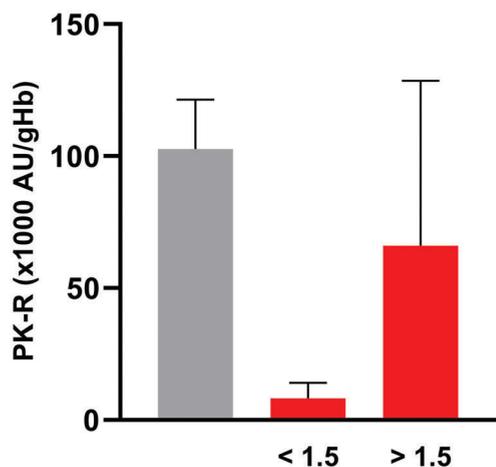


Figure 7. Pyruvate kinase (PK)-deficient red cells with higher PK-R protein levels are more likely to show an increase in adenosine triphosphate (ATP) levels and/or PK-R activity after *ex vivo* treatment with AG-348. Base line PK-R protein levels in patients showing a response in PK activity or ATP levels (i.e., >1.5-fold response vs. <1.5-fold response, red bars) upon *ex vivo* treatment with AG-348 PK-R levels of healthy controls are shown in gray. ECL: electrochemiluminescence; gHb: gram hemoglobin. Error bars represent standard deviation.

patients (Figure 5F). However, this technique likely underestimates the effect of ATP increase by AG-348. Although ektacytometry is an important and established method in the diagnosis of RBC membrane and hydration disorders,^{33,34,45,46} it does have some limitations. Ektacytometry reflects the passive deformation of RBC in contrast to filter-based assays or microfluidics which forces RBC to actively deform; the latter is thought to be more dependent on ATP.^{14,41} This could explain the finding of only a small correlation with ATP levels and deformability measured in RBC concentrates intended for transfusion.⁴⁰

Our results show only a slight difference in RBC deformability between PK-deficient patients and healthy controls, with the lowest deformability observed in M/NM patients (Figure 5B). This is in line with recent findings⁴⁵ using the same technique, but contrasts with earlier findings based on a filtration-based assay.⁴⁷ We also

observed normal deformability in the splenectomized PK-deficient patients compared to a small decrease in EImax in non-splenectomized patients, which correlated inversely to reticulocyte count ($r=-0.740$, $P=0.0016$). These results are in line with previous studies that show that reticulocytes of PK-deficient patients are preferentially removed by the spleen,¹¹ probably due to their compromised deformability as a result of insufficient ATP levels.⁴⁷ It has been suggested that such a decreased deformability could be due to the decreased hydration as a result of K^+ efflux through PIEZO1-mediated Ca^{2+} influx and activation of Gardos channels.¹² However, the osmoscan data show no signs of dehydration of RBC (i.e., decreased Ohyper), which is clearly measurable on RBC of patients with hereditary xerocytosis, where dehydration is thought to occur through the same mechanism.⁴⁸

Collectively, the data presented in this study support the hypothesis that drug intervention with AG-348 effectively up-regulates PK-R enzymatic activity and increases stability in PK-deficient RBC over a broad range of PKLR genotypes. The concomitant increase in ATP levels suggests that the glycolytic pathway activity may be restored. AG-348 treatment may, therefore, represent an attractive way to correct the underlying pathologies of PK deficiency.

Disclosures

RW and EJB are consultant for Agios Pharmaceuticals.

Contributions

CK, BAO, PAK and RW designed the experiments; BAO, SS, KJ, VC and PAK recruited, and collected samples and/or performed experiments; PAK, JH, LD and CK provided AG-348; MAER and RW analyzed the data and wrote the manuscript with input and revisions of BAO, PAK, GP, VC, WWS, EJB and CK.

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