Research Article

JOURNAL OF HEPATOLOGY

Phase I open label liver-directed gene therapy clinical trial for acute intermittent porphyria

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Background & Aims: Acute intermittent porphyria (AIP) results from porphobilinogen deaminase (PBGD) haploinsufficiency, which leads to hepatic over-production of the neurotoxic heme precursors porphobilinogen (PBG) and delta-aminolevulinic acid (ALA) and the occurrence of neurovisceral attacks. Severe AIP is a devastating disease that can only be corrected by liver transplantation. Gene therapy represents a promising curative option. The objective of this study was to investigate the safety of a recombinant adeno-associated vector expressing PBGD (rAAV2/5-*PBGD*) administered for the first time in humans for the treatment of AIP.

Methods: In this phase I, open label, dose-escalation, multicenter clinical trial, four cohorts of 2 patients each received a single intravenous injection of the vector ranging from 5×10^{11} to 1.8×10^{13} genome copies/kg. Adverse events and changes in urinary PBG and ALA and in the clinical course of the disease were periodically evaluated prior and after treatment. Viral shedding, immune response against the vector and vector persistence in the liver were investigated.

Results: Treatment was safe in all cases. All patients developed anti-AAV5 neutralizing antibodies but no cellular responses against AAV5 or PBGD were observed. There was a trend towards a reduction of hospitalizations and heme treatments, although ALA and PBG levels remained unchanged. Vector genomes and transgene expression could be detected in the liver one year after therapy.

Conclusions: rAAV2/5-*PBGD* administration is safe but AIP metabolic correction was not achieved at the doses tested in this trial. Notwithstanding, the treatment had a positive impact in clinical outcomes in most patients.

Lay summary: Studies in an acute intermittent porphyria (AIP) animal model have shown that gene delivery of *PBGD* to hepatocytes using an adeno-associated virus vector (rAAV2/5-*PBG*) prevent mice from suffering porphyria acute attacks. In this phase I, open label, dose-escalation, multicenter clinical trial we show that the administration of rAAV2/5-*PBGD* to patients with severe AIP is safe but metabolic correction was not achieved at the doses tested; the treatment, however, had a positive but heterogeneous impact on clinical outcomes among treated patients and 2 out of 8 patients have stopped hematin treatment.

Clinical trial number: The observational phase was registered at Clinicaltrial.gov as NCT 02076763. The interventional phase study was registered at EudraCT as n° 2011-005590-23 and at Clinicaltrial.gov as NCT02082860.

Keywords: Gene therapy; Acute intermittent porphyria; AAV/PBGD; Adeno-associated virus.

Received 16 March 2016; received in revised form 9 May 2016; accepted 10 May 2016; available online 17 May 2016

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Abbreviations: AIP, acute intermittent porphyria; PBGD, porphobilinogen deaminase; ALAS, 5-aminolevulinic acid synthase; ALA, aminolevulinic acid; PBG, porphobilinogen; AAV, adeno-associated virus; rAAV2/5-*PBGD*, recombinant adeno-associated vector expressing PBGD; PCR, polymerase chain reaction; SF-36, 36-Item short form health survey; BDI-II, Beck depression inventory II; BAI, Beck anxiety inventory.

Introduction

Acute Intermittent Porphyria (AIP) is inherited as an autosomal dominant disorder of the heme biosynthesis pathway [1,2]. AIP is caused by a defect in porphobilinogen deaminase (*PBGD*) gene which spans 10 kb in chromosome 11q23 [3]. More than 370 different mutations of *PBGD* have been described, including missense, nonsense and splicing mutations, as well as deletions and insertions [4,5].

Heme is synthesized in all body cells but mainly in erythroid cells and the liver. In AIP, PBGD enzymatic activity is reduced to about 50% of normal leading to limited capacity to enhance heme synthesis upon increased biosynthetic demands in the liver [1–3]. 5-aminolevulinic acid synthase (ALAS) is the initial and rate-limiting enzyme of heme biosynthesis. In the liver, the ALAS1 enzyme mediates the reaction of glycine with succinyl-CoA to yield aminolevulinic acid (ALA), which is transformed into porphobilinogen (PBG) by aminolevulinic acid dehydratase. PBGD mediates the condensation of PBG to hydroxymethylbilane, which is processed in a stepwise manner to heme, and negatively controls ALAS1 expression. In AIP subjects the heme deficiency taking place under conditions of augmented heme requirements enhances hepatic ALAS1 activity leading to ALA and PBG accumulation [1–3]. These compounds are believed to be responsible for the complex set of neurotoxic symptoms exhibited by AIP patients [6].

AIP is characterized by acute episodes and asymptomatic periods [1,2,6]. AIP patients commonly show high ALA and PBG blood and urinary levels and their concentrations further increase during acute attacks. These episodes are triggered by factors that activate hepatic heme synthesis including exposure to drugs (like barbiturates, sulfonamides), hormonal changes, infections or starvation [1,2,7]. Clinical disease occurs with very low prevalence (1 in 185,000) [8], but epidemiologic figures based on the incidence of acute attacks greatly underestimate the number of individuals with the genetic defect, which in Sweden is as high as 1 in 10,000 [9,10] and 1 in 1675 in France [11], indicating that a large proportion of affected individuals exhibit an asymptomatic form of the disease, in some cases with high ALA and PBG levels in urine [12,13].

Abdominal pain, frequently accompanied by vomiting, diarrhea or constipation, is the most common symptom of acute attacks. Paresthesia and paralysis also occur, and death may result from respiratory paralysis. Other symptoms include seizures, psychotic episodes, tachycardia and hypertension [1,2,7,14]. Current treatment of acute attacks involves intravenous heme (heme arginate–Normosang® in Europe and lyophilized hematin–Panhematin® in USA) infused and/or a high-carbohydrate diet [15].

Most symptomatic patients have only one attack, but approximately 5% women and 3% men with AIP suffer recurrent and frequent attacks, which persist for many years [6]. This form of severe AIP is a devastating condition that significantly affects the quality of life and demands repeated courses of treatment with heme. Although heme represses ALAS, thus blocking heme biosynthesis, it also activates hemeoxygenase-1 (EC:1.14.99.3), which in turn promotes acute attack recurrences and the decline of the therapeutic efficacy [16,17]. Thromboembolic disease and iron overload (a dose of 250 mg of heme arginate contains 22.7 mg of iron) are also side effects associated with repeated courses of this therapy [18]. Even though prophylactic heme appears to be beneficial in patients with recurrent attacks, life-long exposure to drugs for the control of symptoms may cause considerable adverse events that greatly impair quality of life [16,17]. Thus alternative therapies for severe AIP are needed.

Complete biochemical and symptomatic resolution of AIP was observed in all patients after liver transplantation [19]. This observation supports our working hypothesis that therapies aimed at supplementing hepatocytes with the normal version of the *PBGD* gene may correct the disease. Confirming this notion, our studies in murine AIP models showed that liver-directed gene therapy using an AAV vector encoding PBGD under the control of a liver-specific promoter (rAAV2/5-*PBGD*) was able to restore hepatic PBGD activity to normal values and prevented the occurrence of acute attacks [20]. Toxicology studies in mice (unpublished results) and in non-human primates [21] showed that the vector could be administered safely even at high doses. In 2009 the European Medicines Agency granted Orphan Drug Designation to rAAV2/5-*PBGD* for the treatment of AIP. Subsequently we designed and performed a phase I clinical trial in patients with severe AIP to assess feasibility, safety and efficacy of rAAV2/5-*PBGD*. Here, we report the results of this clinical study, which is the first gene therapy trial performed in patients with AIP, and the first to employ an AAV5-based gene therapy product.

Materials and methods

Gene therapy vector

Vector design and production methodologies have already been described [20,21]. The titer of the virus was determined by quantitative PCR and expressed as genome copies/ml (gc/ml) [21].

Trial design and objectives

The study was designed as a phase 1, open label, dose-escalation clinical trial. Since AIP is a rare disease and its clinical presentation very heterogeneous, each patient served as his/her own control. Thus, the study comprised two different phases (Fig. 1); one pre-therapy and the other post-therapy (observational and

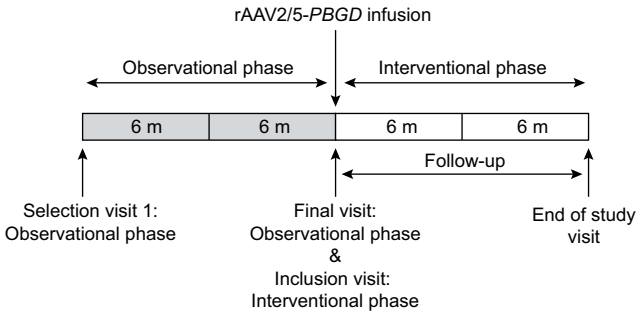

Fig. 1. Study design.

interventional phases, respectively). The safety and the efficacy of the treatment were assessed comparing clinical and biochemical parameters of the observational and interventional phases.

The primary objective was to assess the safety of the treatment. Secondary objectives were to assess the effect of the treatment on ALA and PBG urinary levels, the clinical evolution of the disease, health-related quality of life, and psychological disturbances.

Patients were included and followed-up in two Spanish centers, Clínica Universidad de Navarra (Pamplona) and Hospital 12 de Octubre (Madrid). All patients received the treatment at Clínica Universidad de Navarra.

Inclusion and exclusion criteria

Only patients with at least 2 hospital admissions caused by AIP acute attacks or 4 admissions for AIP-specific treatment in the year prior to the initiation of the trial were included. Exclusion criteria were the presence of liver disease including hepatitis C or B viral infection, cirrhosis and hepatocellular carcinoma, advanced renal failure, and presence of neutralizing antibodies against the vector. Participation in the observational phase for at least 6 months immediately prior to treatment was required in order to be included in the interventional phase. Complete inclusion and exclusion criteria are provided in Supplementary Table 1.

Study procedures

Observational phase

At the selection visit, patients were evaluated by complete medical history including AIP-specific events such as hospital admissions in the 12 months prior inclusion and AIP-specific therapies, physical examination, blood tests (blood cell count, liver and renal function tests, ferritin and α-fetoprotein), urinary ALA and PBG levels, antibodies against vector and transgene, and genetic confirmation of AIP. A liver ultrasound was performed to exclude liver malignancies. Patients were followed-up every 2 months for a minimum of 6 months until their inclusion in the interventional phase. At each follow-up visit, patients were questioned about the occurrence of AIP-specific symptoms, concomitant medication, in particular AIP-specific therapies (intravenous heme replacement therapy or glucose infusions) and the number and duration of AIP-related hospital admissions. Patients also had routine physical examination and laboratory tests (including blood cell count, liver and renal function tests, and ALA and PBG urinary levels). Moreover, patients were asked to collect a urine sample for ALA and PBG measurement between visits in case of acute attack or prior to any AIP-specific treatment. Finally, health-related quality of life and psychological symptoms were assessed every 2 months using 36-Item Short Form Health Survey (SF-36) version 2 (v2), Beck Depression Inventory II (BDI-II) and Beck Anxiety Inventory (BAI) questionnaires.

Interventional phase

Recruitment into this phase took place 2 to 4 weeks before administration of the gene therapy vector and all the assessments performed upon inclusion in the observational phase were repeated at this time point. Patients were divided into 4 cohorts following the same order of inclusion as in the observational study. rAAV2/5-*PBGD* was administered as follows: cohort A (n = 2): 5×10^{11} gc/kg of body weight; cohort B (n = 2): 2×10^{12} gc/kg of body weight; cohort C (n = 2): 6×10^{12} gc/kg of body weight; cohort D (n = 2): 1.8×10^{13} gc/kg of body weight.

The corresponding dose of rAAV2/5-*PBGD* was suspended in 0.9% NaCl solution in a final volume of 20 ml, which was slowly infused through a peripheral vein of the arm over 20 minutes. To better evaluate (acute) safety, patients remained hospitalized for 48 h following vector administration. After discharge patients were followed-up weekly during the first 8 weeks, fortnightly until week 12, and then monthly until week 48.

Viral shedding was analyzed in biological fluids (blood, urine, semen, oral and nasal swabs, and stool), as described [21], at 8, 24, 48 h and then at each visit until viral clearance was confirmed in two consecutive samples. Humoral and cellular immune responses against the vector and the transgene were assessed, as described [21] at the selection visit (S) and in visits 1, 2, 3, 4, 8 and 10 and 2, 4, 8 and 10, respectively, after therapy. Blood cell analysis as well as liver and renal function tests were performed 48 h after the treatment and at each follow-up visit. As during the observational phase, ALA and PBG urinary levels were determined at each programmed visit and between visits if the patients experienced an acute attack or received AIP-specific treatment. Medical history and a record of symptoms, concomitant medication (especially AIP-specific therapies), AIP-related number and duration of hospital admissions as well as routine physical examination were obtained at each follow-up visit. AIP patients were requested to fill out SF-36, BAI and BDI questionnaires every month after the treatment. Additionally, a liver ultrasound was performed at the inclusion in the trial and at the end of the follow-up. Patients will have a liver ultrasound performed every year for 10 years after the gene therapy.

Laboratory studies

ALA and PBG determination in urine sample were performed in a centralized laboratory at Porphyria Centre Sweden, Karolinska University Hospital (Stockholm, Sweden). Routine blood tests were performed at local laboratories (Hospital 12 de Octubre and Clínica Universidad de Navarra).

Detection of vector DNA sequence in liver samples

Needle liver biopsies were obtained from 6 patients 1 year after treatment. Biopsies were frozen in isopentane cooled with liquid nitrogen. Genomic DNA and total RNA was isolated from liver biopsies using the DNeasy Blood and Tissue Extraction Kit (Qiagen) and QIAamp RNA Tissue Extraction mini kit. Primers specific for the boundary between the *coPBGD* sequences and the polyA element were used to amplify a sequence specific for the vector DNA and messenger RNA (mRNA) to differentiate it from the endogenous gene and nRNA as previously described [21]. To determine endogenous *PBGD* gene and mRNA copy number human PBGD specific primers were used. Real-time PCR-based quantification was performed using SYBR Green master mix (Applied Biosystems, Foster City, CA). Results were expressed as copy number per μg of total DNA or RNA.

Ethics

Participants gave separate written informed consent for the observational and interventional phases after the nature and possible consequences of the studies were explained. The study protocols conform to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the local Ethics Committees in Pamplona and Madrid and by the Spanish Agency of Medicines and Medical Devices. The observational phase was registered at Clinicaltrial.gov as NCT 02076763. The interventional phase study was registered at EudraCT as n° 2011-005590-23 and at Clinicaltrial.gov as NCT02082860.

Statistical analysis

Differences in clinical and biochemical parameters between the observational and interventional phases were analyzed using a non-parametric test for paired values (Wilcoxon test). Aiming to minimize a possible bias due to the different duration of the follow-up in the observational phase, the changes in ALA and PBG values and in SF-36, BAI and BDI-II scores were analyzed considering the data obtained in the first and last semester of the observational phase only. This applied to all patients except for one belonging to cohort D, who participated in the observational phase for 6 months only. To assess the effect of the gene therapy on the clinical course of the disease (AIP-hospitalizations and heme infusions), the number of AIP-hospitalizations per month, the number of days of hospitalization per month and the number of days on heme therapy per month during the observational and interventional phases were compared in each patient. $p < 0.05$ was considered statistically significant.

Results

Patients

Nine patients, 2 males and 7 females, aged between 33 and 62 years, suffering severe AIP were evaluated. One female patient was excluded at the initial screening visit because of being positive for neutralizing antibodies against AAV5. The remaining 8 patients had genetic confirmation of AIP and were recruited to the observational and the interventional phases of the trial (Fig. 2).

Six patients were receiving AIP-specific treatment on a scheduled manner for the control of chronic symptoms while 2

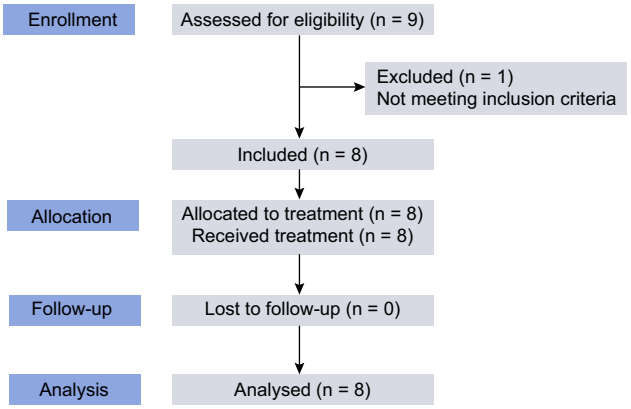

Fig. 2. Participant's flow diagram.

Table 1. Severe adverse events after treatment.

Type of SAE	Time from gene therapy	Outcome
Pilonidal cyst surgery	11 weeks	Resolved
Hospital admission for insulin-pump infusion placement	27 weeks	Resolved
Myocarditis	28 weeks	Resolved
Upper abdominal pain	28 weeks	Resolved

received therapy for acute attacks only. Patients were receiving hematin treatment for a significant period of time prior study inclusion (median 4.8 years; range 1.5–12.8). The 8 patients were followed in the observational phase for a median of 14.8 months (range 6–18 months). They all received rAAV2/5-*PBGD* and completed the 48 weeks of follow-up. Clinical data of the patients are given in Supplementary Table 2.

Safety

rAAV2/5-*PBGD* administration was well tolerated and no treatment-related adverse events were observed during follow-up. Serum transaminases remained within the normal range throughout the study time except in patient 8 (belonging to cohort D) who experienced a transient and mild increase of liver enzymes (alanine aminotransferase (ALT) less than 3 x upper normal limit) one week after therapy. It coincided with an acute AIP attack and normalized once the attack had subsided (data not shown). A total of 4 severe adverse events were observed in 2 patients, however, none of them was related to either gene therapy or study procedures (Table 1).

Immune responses

Patients were analyzed for total and neutralizing antibodies against AAV5 and total antibodies against the recombinant PBGD protein at different time points after vector administration. All developed antibodies against the AAV5 protein capsid capable of neutralizing AAV5 infectivity (Supplementary Fig. 1). In general, the response was dose dependent, except for patient 1 who despite receiving the lowest vector dose, developed the highest antibody titer, interestingly this patient showed a positive, although very week, signal in total antibodies against AAV5 at the initial visit of the observational study that was no detected again. Noteworthy, none of the patients developed antibodies against PBGD recombinant protein (data not shown). No cellular immune response against the vector or transgene was detected in any of the patients (Supplementary Table 3).

Viral shedding

We investigated the presence of the virus in serum, urine, stool, nasal secretion, saliva and semen. Vector shedding analysis in serum showed maximum vector concentrations 8 h after therapy (Supplementary Fig. 2). Titers declined thereafter to become undetectable by day 30 post-injection. Very low levels of rAAV2/5-*PBGD* could be transiently detected in saliva, urine, nasal secretion and faeces, but was undetectable in all patients by day 30. Importantly, no vector was detected in semen samples, excluding the risk of germ line transmission in those patients (Supplementary Fig. 2).

ALA and PBG levels, requirement of AIP-specific therapies and hospitalizations

Patients showed high levels of urinary ALA and PBG both during the observational and interventional phases of the study and no significant changes were observed after rAAV2/5-*PBGD* administration (Fig. 3A, B).

However, a trend towards decreased heme replacement therapy was observed in the interventional phase compared to the observational period (Fig. 4A). In parallel the number and duration of hospitalizations also decreased after therapy (Fig. 4B, C). A noticeable improvement in the symptomatology occurred in 2 patients. One female patient from cohort A, patient 2, who during the observational phase was receiving an average of 1–2 heme doses per month for recurrent attacks, stopped hematin promptly after therapy because of cessation of acute episodes. However, she continued on oral analgesics and received intravenous glucose on 4 occasions for control of milder symptoms. Also, a male patient, from cohort B, patient 3, who was receiving hematin infusions every 3 weeks during the observational phase because of persistent symptoms, stopped hematin 5 weeks after gene therapy.

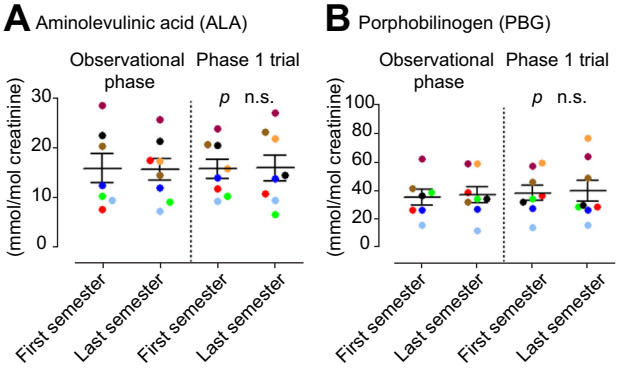

Fig. 3. Aminolevulinc acid (ALA) (A) and porphobilinogen (PBG) (B) urinary levels before and after rAAV2/5-*PBGD* administration. Mean values of the first six months and the last six months of the observational and interventional phases are shown. Normal urinary levels are <1.5 mmol PBG/mol creatinine and <3.9 mmol ALA/mol creatinine, respectively.

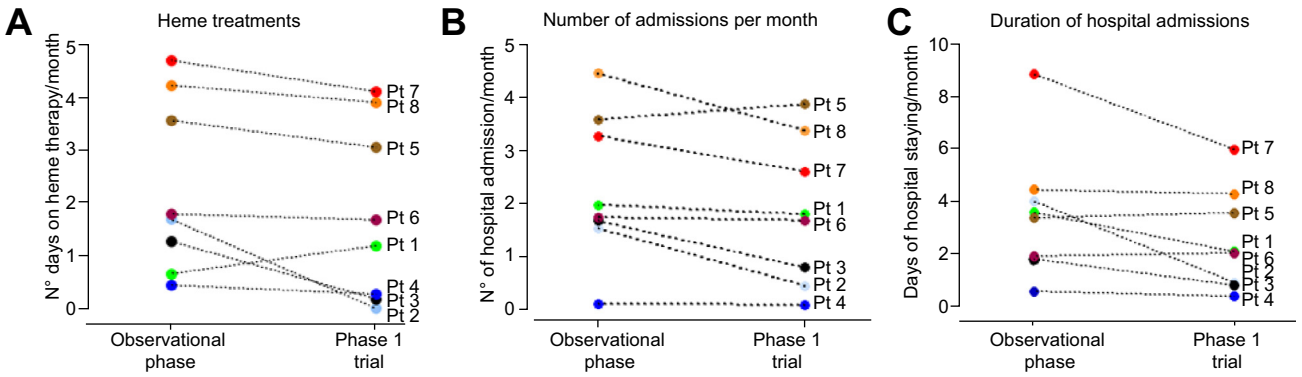

Fig. 4. AIP-related treatments and hospitalizations. Courses of heme infusions (A), number of hospitalizations due to AIP symptomatology (B), and duration of hospital admissions (C) before and after rAAV2/5-PBGD therapy. Comparison of the median of each variable during the observational and interventional phases.

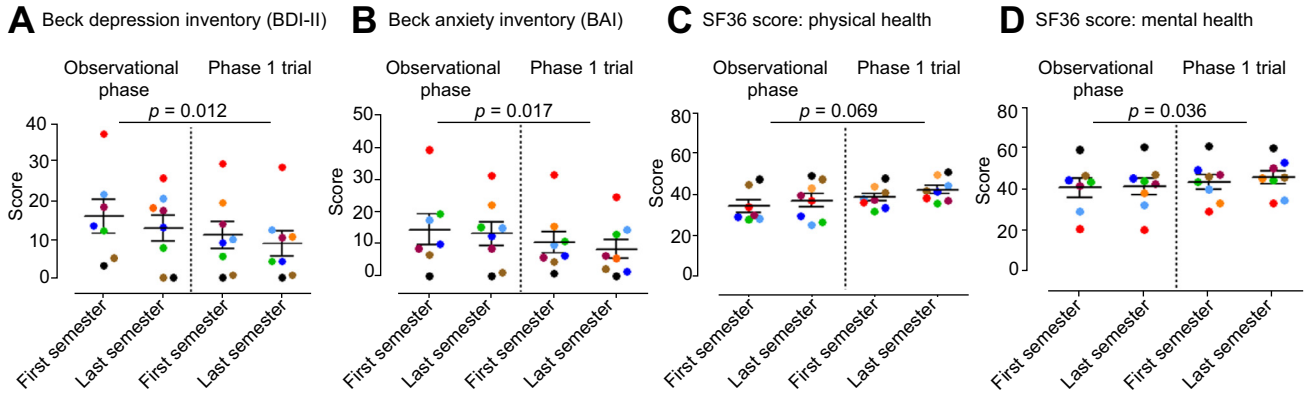

Fig. 5. Changes of Beck Depression Inventory II (BDI-II), Beck Anxiety Inventory (BAI), and the two main components of SF36 v2 health-related quality of life questionnaire, physical and mental health, after rAAV2/5-*PBGD* administration. Each dot represents the mean values of BDI-II (A) and BAI (B) in the first six months and the last six months of the observational and interventional phases for each patient. The standard cut-off scores of BDI-II are: minimal depression (0–9), mild depression (10–18); moderate depression (19–29) and severe depression (30–63). The standard cut-off scores of BAI are low anxiety (0–21), moderate anxiety (22–35) and severe anxiety (36–63). (C, D) Each dot represents the mean values of the two main components of SF36 v2 health-related quality of life questionnaire, physical and mental health in the first six months and the last six months of the observational phase and of the post-therapy period, respectively, for each patient. According to the SF36 v2 scoring, the lower the score the more disability, the higher the score the less disability. The median and the standard deviation of the patients' mean values have been represented and the differences between the observational and interventional phases were analyzed using a non-parametric test for paired values each variable during the two periods: the observational and interventional phases.

Depression, anxiety and quality of life

Patients showed a significant improvement of the depression score (BDI-II) after therapy in comparison to the observational phase (Fig. 5A) and the anxiety score (BAI-I questionnaire) was ameliorated in 7 patients and remained unchanged in one case, patient 3, (Fig. 5B). Moreover, the SF 36 questionnaire, which evaluates patient health status and overall quality of life, revealed a significant amelioration of the mental status following therapy and a better score for physical parameters in 7 patients (Fig. 5C and D).

Liver transduction

One year after vector administration 6 out of 8 patients volunteered to have a liver biopsy for the evaluation of tissue transduction. Vector genomes were detected in the liver of all tested patients albeit the copy number was unrelated to the vector dose (Table 2). Thus, patient 2 from cohort A showed more vector copies in the liver than patients from cohort B and the number of vector copies in the liver from the tested patient from cohort C (patient 5) was higher than in the biopsied patient from cohort D (patient 7). These findings might reflect sampling variability due non-homogenous liver transduction or inter-individual differences in the susceptibility of the liver to be transduced with AAV5 vector. As expected no significant differences were observed in endogenous PBGD gene copy number among patients. More importantly, vector-derived mRNA expression was detected in the patients bearing the higher copy number of vector genomes, patients 2, 5, and 7. The expression of the recombinant mRNA in those patients is equivalent to 5–7% of the expression of the endogenous *PBGD* mRNA.

Discussion

In AIP the liver is responsible for excessive production and accumulation of toxic heme precursors, thus playing a key role in the development of clinically active disease. This notion is supported by the fact that liver transplantation results in complete

Table 2. Liver transduction by rAAV2/5-*PBGD* one year after vector administration.

Patient ID	coPBGD DNA exogenous (copies/μg)	PBGD DNA endogenous (copies/μg)	coPBGD mRNA exogenous (copies/μg)	PBGD mRNA endogenous (copies/μg)
Pt 1	62.45	29,481.87	ND	1351.36
Pt 2	3394.47	35,440.51	24.98	480.42
Pt 3	109.21	30,333.25	ND	391.90
Pt 4	660.93	47,966.17	ND	179.14
Pt 5	14,280.4	46,166.66	25.64	352.83
Pt 7	2239.7	28,747.73	34.28	686.13

metabolic correction of AIP [19]. However, transplantation is associated with many complications and requires life-long immunosuppressive therapy [19]. There clearly is a need for patients with severe AIP for less invasive therapeutic options before neurological sequelae become irreversible. In AIP mice transduction of hepatocytes with gene therapy vectors encoding PBGD prevented acute attacks upon phenobarbital administration, suggesting gene therapy as a promising approach for the treatment of AIP [20,22–24]. Based on these observations we tested rAAV2/5-*PBGD* in increasing intravenous doses in 8 AIP patients divided into 4 cohorts (2 subjects each) from 5×10^{11} (cohort A) to 1.8×10^{13} gc/kg (cohort D). All patients included in the study had at least 2 hospital admissions and/or 4 courses of AIP-specific therapies for acute attacks in the year previous to the inclusion visit. Most trial participants had a very severe form of the disease with chronic symptoms and frequent bouts of symptomatic aggravation requiring heme replacement therapy. Our study shows that the therapy was well tolerated. No serious adverse events related to the AAV5-based vector administration were observed. As expected all patients generated neutralizing anti-AAV5 antibodies after therapy but, notably, we could not detect any cellular immune response - neither against the transgene nor against the vector capsid. In accordance with the absence of a cellular immune response against the vector capsid proteins, no transaminase elevation was observed. This was in contrast to findings in two previous liver-directed gene therapy trials using AAV serotypes 2 [25,26] and 8 [27] in which the patients experienced elevations of serum transaminases between 6 to 8 weeks after therapy, likely due to T cell response against AAV capsid antigen present in transduced hepatocytes. This led to abrogation or reduction of transgene expression in the first study and to the use of steroid therapy to preserve the transduced cells in the second study. Interestingly, the doses of vector employed in the hemophilia trials were nearly 10-fold lower that the ones employed in our study, the highest dose being 2×10^{12} gc/kg while in our trial the highest dose was 1.8×10^{13} gc/kg. The absence of liver damage and anti-capsid T cell immunity in the present AIP gene therapy trial could be due to scant immunogenicity of AAV5 compared to AAV2 and AAV8 or to low liver transduction at the doses used. There are structural differences between AAV5 and other serotypes, that can explain the different antigenicity of this vector. Indeed, according to amino acid sequencing data, AAV5 shares less than 60% of the sequences of AAV2 and AAV8, while the last two serotypes share more than 80% of amino acid sequence identity [28].

An important safety issue of AAV-based gene therapy is the potential genotoxicity of the vector. This point has been strongly debated. Donsante *et al.* [29] showed that treatment of beta-glucuronidase-deficient mice with a therapeutic AAV vector in the neonatal period of life was followed by increased incidence of HCC. Similarly, Chandler *et al.* reported a high incidence of HCC in a mouse model of methyl-malonic acidemia, however, they also showed that vector dose, the type enhancer/promoter, and the timing of gene delivery are critical factors for determining HCC incidence [30]. Furthermore, several other independent and larger studies in adult mice showed lack of carcinogenicity of AAV therapeutic vectors [31]. However, recently Nault *et al.* reported the presence of integrated wild type (wt) AAV genomes in HCC samples in 11 out of 193 patients [32]. In our trial AAV vector was detected in liver biopsies in the six patients tested one year after therapy but integration analysis using an improved Multiplex LAM-PCR covering internal and external vector breakage sites showed that integrations were scarce and randomly distributed [33] and none of them involved the oncogenic regions reported by Nault *et al.* [32].

At the vector doses used in the present trial we did not succeed in reducing ALA and PBG levels. Preclinical studies in AIP mice showed that therapeutic efficacy with rAAV2/5-*PBGD* was attained only at the dose of 1.25×10^{13} gc/kg [20] which was similar to the highest one used in the present study. On the other hand, data from non-human primates [21] indicated that in order to raise PBGD enzymatic activity in liver tissue over endogenous levels it was necessary to use a rAAV2/5-*PBGD* dose of 5×10^{13} gc/kg. Thus, seemingly even the doses administered to cohort D (1.8×10^{13} gc/kg) were below the therapeutic range, in fact in those patients in which we could detect transgenic mRNA in liver tissue, the levels were equivalent to 5–7% of the endogenous mRNA. New studies with AAV vectors in AIP patients are clearly warranted. Future trial should employ higher vector doses, optimized vector design or new serotypes with enhanced liver transduction efficiency.

Interestingly, the effect of the treatment on clinical outcomes was very variable among rAAV2/5-*PBGD*-treated patients. While two patients, treated in the first and in the second dose cohorts, experienced a significant clinical improvement leading to marked reduction in intravenous heme requirements (patients 2 and 3), among the remaining six patients the beneficial effect on the clinical outcomes was not so clear at least in terms of AIP treatment requirement. However, most of the participants in the study scored better in psychometric tests following therapy. These observations were in contrast with unchanged PBG and ALA levels. Although this paradox might be explained by a placebo effect, that may improve the psychometric test scores and also may lead to a better pain control, it could also be speculated that the different genetic background resulting in different endogenous PBGD activity may be the reason of this heterogeneity. The differences in the residual endogenous PBGD activity among the participants may also explain the lack of a dose-effect relation

observed in this clinical trial. It should also be noted that some AIP patients may be asymptomatic despite marked elevation of heme precursors [12,13].

In summary, the safety and the signs of clinical benefit in a first in human clinical trial of rAAV2/5-PBGD warrant consideration for future gene therapy trials for AIP.

Financial support

European Commission 7th Framework Programme; AIPGENE Grant 261506. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The investigators are solely responsible for the content and the decision to submit the manuscript for publication.

Conflict of interest

Nadina Grosios, Jaap Twisk and Harald Petry are Uniqure employees. Astrid Pañeda, María Paz and Juan Ruiz are DIGNA Biotech employees. The rest of the authors have no conflict of interest to declare.

Authors' contributions

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Acknowledgments

The authors thank the patients, nurses, and hospital staff who participated in the study. The authors are grateful to the Expert Advisory Board involved in this project for its guidance in the design of the study an interpretation of the result, Dr Christer Andersson, Dr Michael Badminton, Dr Jean Charles Deybach, Dr Eliane Sardh, Dr Paul Wilson, as well as to EPNET for their support.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2016.05.012.

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