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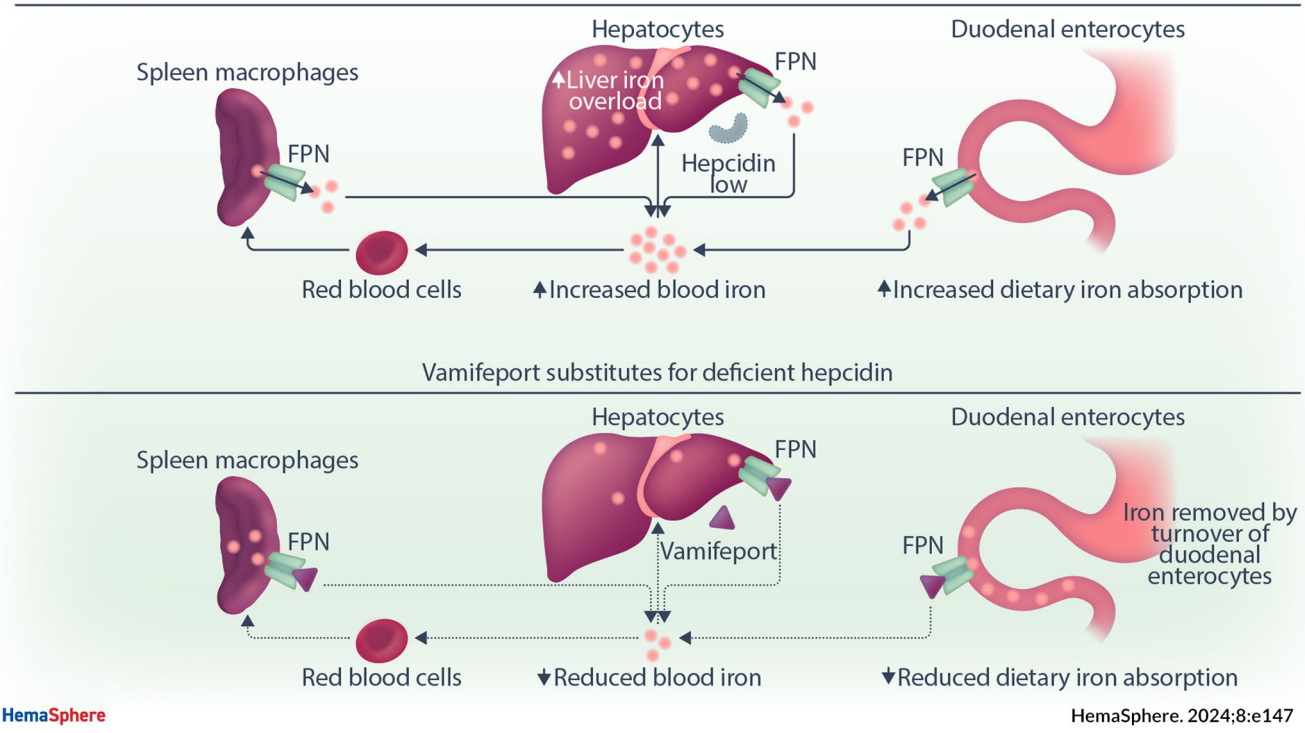
Graphical Abstract



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The oral ferroportin inhibitor vamifeport prevents liver iron overload in a mouse model of hemochromatosis

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Abstract

Hemochromatosis is an inherited iron overload condition caused by mutations that reduce the levels of the iron-regulatory hormone hepcidin or its binding to ferroportin. The hepcidin–ferroportin axis is pivotal to iron homeostasis, providing opportunities for therapeutic intervention in iron overload disorders like hemochromatosis. The aim of this study was to evaluate the efficacy of the oral ferroportin inhibitor vamifeport in the *Hfe* C282Y mouse model, which carries the most common mutation found in patients with hemochromatosis. A single oral dose of vamifeport lowered serum iron levels in *Hfe* C282Y mice, with delayed onset and shorter duration than observed in wild-type mice. Vamifeport induced transient hypoferremia by inhibiting ferroportin and resulted in a feedback regulation of liver *Hamp* in wild-type mice, which was absent in *Hfe* C282Y mice, reflecting the dysregulated systemic iron sensing in this hemochromatosis model. Chronic dosing with vamifeport led to sustained serum and liver iron reductions in *Hfe* C282Y mice, as well as markedly reducing liver *Hamp* expression in *Hfe* C282Y mice, suggesting distinct regulation of liver *Hamp* expression following acute or continuous iron restriction via vamifeport. At the tested dose, vamifeport retained its activity when combined with phlebotomy and did not significantly interfere with liver iron removal by phlebotomy in *Hfe* C282Y mice. These data demonstrate that chronic vamifeport treatment significantly reduces serum iron levels and prevents liver iron loading in the *Hfe* C282Y mouse model of hemochromatosis, thus providing preclinical proof of concept for the efficacy of vamifeport in hemochromatosis with or without phlebotomy.

INTRODUCTION

Hemochromatosis is an inherited iron overload disorder that is common in Northern Europeans.[1] Hemochromatosis is characterized by excessive dietary iron absorption and pathologically high iron deposition in organs such as the liver, pancreas, and heart, which can lead to the formation of reactive oxygen species and ultimately to organ damage.[2] The high iron levels occur either as a result of abnormally low levels of the iron regulatory hormone hepcidin or due to decreased binding of hepcidin to ferroportin,[2] which is the sole transporter of iron out of cells.[3–5] Hepcidin regulates iron levels by binding to ferroportin on the membranes of intestinal cells, hepatocytes, and macrophages, occluding its iron-exporting domains, and causing its internalization and subsequent degradation,[1,5,6] thereby reducing iron transport into the plasma.[2] The hepcidin–ferroportin axis is pivotal to iron homeostasis and so is a rational therapeutic target for conditions associated with iron overload.

The most common form of hemochromatosis (type 1) is usually caused by homozygous C282Y mutations in the *HFE* gene encoding the hemochromatosis (HFE) protein, which is involved in the regulation of hepcidin.[2] More than 80% of patients with hemochromatosis are homozygous for this mutation.[7,8] The C282Y mutation disrupts a key disulfide bond in the α3 domain of the HFE protein and prevents binding of mutant HFE to β2-microglobulin, leading to impaired intracellular trafficking and accelerated degradation of HFE.[9,10] Hemochromatosis can also be caused by mutations in other genes, including *HAMP*, *HJV*, *TFR2*, and *FPN1*, which encode hepcidin, hemojuvelin, transferrin receptor 2, and ferroportin, respectively.[2,11] However, non–*HFE*-associated hemochromatosis is much rarer than *HFE*-associated hemochromatosis. The *HFE* C282Y mutation and the non-*HFE* mutations (except for the *FPN1* mutation) associated with hemochromatosis lead to inappropriately low levels of hepcidin relative to iron status and increased iron release into the plasma.[12] This results in high transferrin saturation (TSAT) and the formation of non–transferrin-bound iron, which can ultimately lead to iron

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overload in key organs.[2] If left untreated, iron overload can lead to serious complications, including liver fibrosis, cirrhosis and cancer, cardiomyopathy and heart failure, arthritis, and diabetes.[13] Phlebotomy, which remains the standard of care for patients with hemochromatosis, aims to reduce serum ferritin levels to ~50 ng/mL and to improve TSAT to ~50%.[1,14,15] Newly diagnosed patients often undergo frequent phlebotomies for many months during the so-called 'induction phase' of therapy.[1,14] Although phlebotomy is currently the only treatment option for patients with hemochromatosis, patient compliance with maintenance therapy steadily declines over time, and a proportion of hemochromatosis patients are refractory or intolerant to phlebotomy.[16] Therefore, there is a need for novel therapies that target the underlying cause of the disease or that reduce the frequency of phlebotomies.

Vamifeport (previously known as VIT-2763) is a small-molecule ferroportin inhibitor that competes with hepcidin for binding to ferroportin, leading to its internalization, ubiquitination, and degradation, thereby reducing cellular iron efflux.[17] Vamifeport is in development as an oral treatment for hemoglobinopathies, such as sickle cell disease and conditions associated with dysregulated iron metabolism.[17–21] The aim of this study was to evaluate the efficacy of vamifeport in reducing serum iron levels and/or preventing liver iron deposition in the *Hfe* C282Y mouse model of hemochromatosis, alone and in combination with phlebotomy.

MATERIALS AND METHODS

Animal models

Mice were housed under pathogen-free conditions in the CSL Vifor animal facility (Schlieren, Switzerland), according to Swiss veterinary law. Mice were bred in the facility and acclimatized for at least 5 days before study start. Animals were group-housed (2–5 mice/cage) under a 12-h reverse dark/light cycle and provided with nesting material, enrichment materials, and water/food ad libitum. Both acute and chronic studies were conducted in the experimental room, with animals taken to the holding room after each procedure in the chronic studies. There was no treatment blinding in these studies.

Acute pharmacodynamic effects of a single oral dose of vamifeport in the Hfe *C282Y mouse model of hemochromatosis and in 129S2 wild-type mice*

The acute effects of vamifeport were assessed in 8–9-week-old female ($n = 42$) and male ($n = 39$) *Hfe* C282Y mice[22] (JAX stock #005063; Jackson Laboratory). The hypomorphic allele was generated by targeting exon 4 with a c.881G>A substitution, which causes a cysteine to tyrosine missense mutation at codon 294 (p.C294Y). This mutation is the equivalent of the p.C282Y mutation found in patients with hemochromatosis. Cages of *Hfe* C282Y mice and strain- and age-matched wild-type female ($n = 45$) and male ($n = 45$) 129S2/SvPasCRL mice (Charles River Laboratories) were randomly assigned to the relevant treatment groups and time points. Mice (8–9 weeks old) received a single dose of either vehicle (0.5% methylcellulose) or vamifeport 60 mg/kg (10 mL/kg) by oral gavage. After dosing, the animals had access to a standard rodent diet (Granovit SA) and drinking water ad libitum. Groups of animals were euthanized by complete exsanguination after terminal anesthesia with isoflurane at 0.5, 1, 3, 6, and 16 h after dosing. Blood and livers were collected for analysis of serum iron and liver hepcidin (*Hamp*) expression.

Chronic pharmacodynamic effects of vamifeport dosed in the drinking water in the Hfe *C282Y mouse model of hemochromatosis*

To study the chronic effects of vamifeport, *Hfe* C282Y mice were distributed to cages at weaning (3 weeks of age), and each cage was allocated to a treatment group/timepoint according to a stratified randomization. Mice were fed a low iron diet (LID) (#2039, Fe = 10.4 mg/kg; Granovit SA) and had access to drinking water ad libitum. As we primarily aimed to assess the treatment effects of vamifeport versus vehicle in the chronic studies, no wild-type mice were used, in line with the principles of the 3Rs. Vamifeport was administered in drinking water rather than by oral gavage as in the acute study to avoid animal manipulation and potential stress related to long-term twice-daily oral gavage (i.e., over several weeks of treatment). Four-week-old female ($n = 15$) and male ($n = 26$) *Hfe* C282Y mice (129-Hfetm1.1Nca/J) were provided with ad libitum access to autoclaved mineral water containing vehicle or vamifeport 1.0 mg/mL (base weight of vamifeport, corresponding to ~110 mg/kg daily oral dose) for up to 8 weeks. The formulation of vamifeport in drinking water was freshly prepared weekly, and water intake per cage was measured at 3- or 4-day intervals by weighing the water bottles. The average daily intake of water was 2.7 ± 0.3 mL/mouse. The mineral water was supplemented with 0.5 mM Fe(II)SO_4 containing ^{58}Fe (12% of total iron content; Vifor Pharma, batch no. ROR 3171), 1% glucose, and 10 mM ascorbic acid. The vamifeport dose was selected based on the oral dose that was shown to be efficacious in other mouse models of chronic vamifeport treatment (120 mg/kg; 60 mg/kg given by oral gavage twice daily).[17] Weekly small-volume blood samples were taken by tail vein incision for the analysis of hemoglobin levels. At the predefined timepoints (2, 4, 6, and 8 weeks), animals were euthanized by complete exsanguination after terminal anesthesia with isoflurane followed by cervical dislocation. Blood, spleens, duodena, and livers were collected to analyze the effect of vamifeport on the serum and organ iron levels and liver hepcidin (*Hamp*) expression.

Chronic effects of vamifeport dosed in the drinking water on the liver de-ironing effect of phlebotomy in the Hfe *C282Y mouse model of hemochromatosis*

A pilot study was performed to investigate the potential of combining vamifeport with phlebotomy, in which 9–10-week-old female ($n = 7$) and male ($n = 6$) *Hfe* C282Y mice received vehicle or 0.3 mg/mL (~40 mg/kg) or 1 mg/mL (~110 mg/kg) vamifeport in the drinking water, as described above. Mice were fed a standard diet ad libitum (#3432, Fe = 170 mg/kg; Granovit SA) from weaning (3–4 weeks of age) to start of experiment (9–10 weeks of age; iron loading) and then switched to a LID (Fe = 13.4 mg/kg) at study week 0. Mice were anesthetized with isoflurane and phlebotomized (removing ~20% of their total blood volume) by sublingual vein bleeding every 2 weeks. Non-phlebotomized *Hfe* C282Y mice (four males/four females) and wild-type 129S2 mice (four males/five females) treated with vehicle served as controls. At the end of the study (week 4), mice were pre-terminally anesthetized with isoflurane, and blood was collected by retro-orbital bleeding. Mice were then sacrificed by cervical dislocation, and their livers and spleens were harvested and used to analyze the effect of vamifeport combined with phlebotomy on organ iron accumulation.

Analysis of iron-related parameters

Serum iron levels were determined in triplicate using a MULTIGENT Iron assay (Abbott Diagnostics) at 0.5, 1, 3, 6, and 16 h in the acute study and at 2, 4, 6, and 8 weeks in the chronic study.

Relative liver hepcidin (*Hamp*) expression was analyzed by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) using TaqMan Gene Expression Assays (#Mm04231240_s1; Thermo Fisher Scientific) on a LightCycler 480 II instrument (Roche Diagnostics), according to manufacturer's instructions. *Hamp* transcript levels were calculated by comparison with a reference gene *Gusb* (TaqMan: #Mm01197698_m1; Thermo Scientific). Relative *Hamp* expression and fold-change between treatment and control groups were calculated using the ΔC_t *Hamp-Gusb* and $2^{-\Delta\Delta C_t}$ methods, respectively. *Hamp* expression levels were analyzed at 0.5, 1, 3, 6, and 16 h in the acute study and at 2, 4, 6, and 8 weeks in the chronic study. In the acute study, the ratio of *Hamp* expression levels to liver iron concentration was also calculated as ΔC_t (*Hamp-Gusb)*/liver iron concentration (µg/g).

In the chronic and phlebotomy studies, hemoglobin concentrations were determined weekly in tail vein blood (HemoCue AB). Complete blood counts were measured using the veterinary ProCyte blood analyzer (Idexx Bioresearch).

Organs were snap frozen in liquid nitrogen, and total iron and/or ^{58}Fe concentrations were determined using inductively coupled plasma–optical emission spectrometry and inductively coupled plasma–mass spectrometry, respectively. Organ iron levels (total and ^{58}Fe) were analyzed at 4 weeks in the phlebotomy study and at 2, 4, 6, and 8 weeks in the chronic study. Duodena were fixed in 10% buffered formalin and embedded in paraffin. Deparaffinized tissue sections were stained for nonheme ferric iron deposition with diaminobenzidine (DAB)-enhanced Perls' stain. Consecutive sections were stained with hematoxylin and eosin. Images were acquired with ×40 objective (NA 0.95) using an Olympus VS120 Virtual Slide Microscope.

Statistical analysis

As part of the animal license application (ZH108/2017) for the chronic studies, a priori sample size calculations were performed with an estimated effect size for several parameters, target power of 0.8, and $\alpha = 0.05$. Analysis of hemoglobin data used two-way analysis of variance (ANOVA) with repeated measures for time-course effects. Where statistically significant effects were observed, Bonferroni multiple comparison test was performed. Analysis of serum iron, *Hamp* gene expression, and organ iron concentrations utilized one-way ANOVA with Dunnett's multiple comparison test. Statistical analyses were conducted using Prism software (GraphPad Prism version 9.4.1, GraphPad).

RESULTS

The effects of a single dose of vamifeport on serum iron and liver *Hamp* expression are attenuated in the *Hfe* C282Y mouse model of hemochromatosis versus 129S2 wild-type mice

Mice homozygous for the C282Y mutation (*Hfe* C282Y mice) have limited *Hfe* expression and show similar trends in iron metabolism-related parameters to those seen in HFE-deficient ($Hfe^{-/-}$) mice. Both mouse models develop iron overload due to defects in iron-regulated *Hamp* expression and recapitulate well the pathologies observed in human hemochromatosis.[22,23]

To assess the pharmacodynamic effects of vamifeport on systemic iron levels in the hemochromatosis model, a single oral dose of vamifeport (60 mg/kg) was administered to *Hfe* C282Y mice and to corresponding wild-type controls (129S2 mice), and the kinetics of serum iron and liver *Hamp* expression changes was assessed. A schema overviewing the experimental design of the studies assessing the acute effects of vamifeport treatment is presented in Figure 1A. As expected, vehicle-treated *Hfe* C282Y mice had lower liver *Hamp* expression than their wild-type counterparts (ΔC_t *Gusb-Hamp* 6.6 ± 0.9 vs. 8 ± 0.5) at all time points (Table 1). Furthermore, liver *Hamp* expression relative to liver iron content was inappropriately low in *Hfe* C282Y mice compared with 129S2 wild-type mice (Figure 1A). In agreement with published data,[22] vehicle-treated *Hfe* C282Y mice had slightly higher steady-state serum iron levels than their wild-type 129S2 counterparts (51 ± 4 vs. 45 ± 5 µM, respectively) at all time points (Figure 1B). In both *Hfe* C282Y and 129S2 wild-type mice, serum iron levels started to reduce 30 min after a single oral dose of vamifeport (60 mg/kg). However, response to vamifeport was delayed, less pronounced, and shorter lasting in *Hfe* C282Y mice than in their wild-type counterparts, with the reduction only reaching significance at 1 h (compared with 30 min in wild-type mice). In vamifeport-treated *Hfe* C282Y mice, serum iron levels were significantly lower than those seen after vehicle treatment at 1 and 3 h post-dose, whereas in 129S2 wild-type mice, serum iron levels were significantly reduced following vamifeport treatment at 30 min, 1, 3, and 6 h post-dose, compared with vehicle. At 16 h post-dose, serum iron levels did not differ between vamifeport- and vehicle-treated groups in either *Hfe* C282Y or 129S2 mice.

Interestingly, there were no significant changes in liver *Hamp* expression following a single oral dose of vamifeport (60 mg/kg) in *Hfe* C282Y mice, but significant reductions were observed at 3 and 6 h after vamifeport treatment in 129S2 wild-type mice (Figure 1C). This clearly demonstrates that liver *Hamp* expression induced by acute iron restriction is differentially regulated in *Hfe* C282Y and 129S2 wild-type mice.

Chronic iron restriction by vamifeport results in sustained reductions in serum and liver iron in the *Hfe* C282Y mouse model of hemochromatosis

To study the long-term effects of chronic vamifeport treatment on serum iron, liver *Hamp*, hemoglobin, and organ iron concentrations, cohorts of *Hfe* C282Y mice received vamifeport 1.0 mg/mL (corresponding to a daily dose of 110 mg/kg) in the drinking water for up to 8 weeks. The experimental design of this study is depicted in Figure 2A.

Serum iron levels started to decline at week 2 of vamifeport administration in *Hfe* C282Y mice but were not significantly different from those seen with vehicle treatment at this time point (Figure 2B). Chronic vamifeport intake led to a sustained reduction in serum iron levels in *Hfe* C282Y mice—serum iron concentrations continued to decline at weeks 4, 6, and 8 of vamifeport treatment and were significantly lower than the levels observed with vehicle treatment at all of these timepoints.

In contrast to observations in the acute study (in which *Hamp* expression was measured up to 16 h after a single vamifeport dose), liver *Hamp* expression was significantly lower in *Hfe* C282Y mice at weeks 2, 4, 6, and 8 of chronic vamifeport administration than in the vehicle-treated group (Figure 2B). These data suggest that sustained iron restriction due to chronic vamifeport treatment regulates *Hamp*

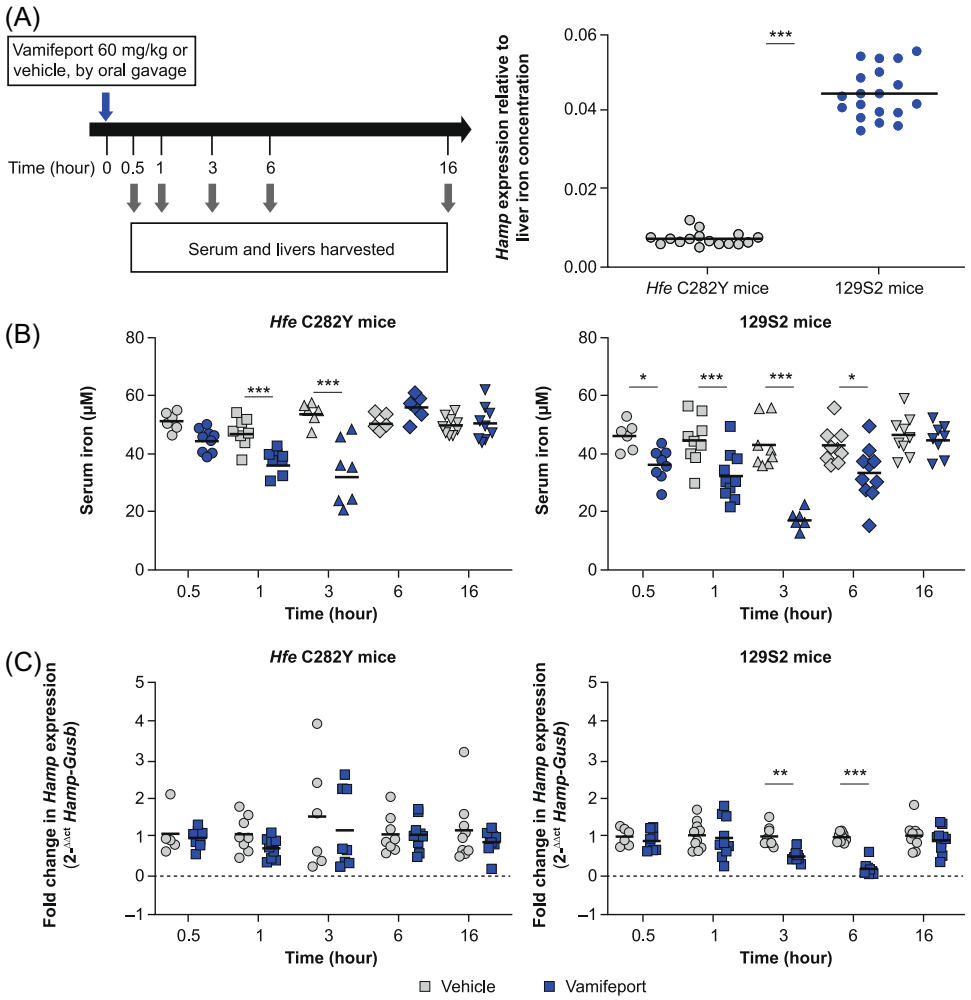

FIGURE 1 Kinetics of pharmacodynamic changes following a single oral dose of vamifeport in the *Hfe* C282Y mouse model of hemochromatosis and 129S2 wild-type mice. Experimental design (left) and liver *Hamp* expression relative to liver iron concentration (right) (A); serum iron concentration (B); liver *Hamp* expression in *Hfe* C282Y (left) and 129S2 wild-type (right) mice after treatment with vehicle or vamifeport. Data are expressed as a fold change of the normalized gene expression compared to the vehicle-treated group at each time point (C). For all scatter plots, data are presented as individual values with means ($n = 6–10$ animals per timepoint/treatment). Significant differences compared with the vehicle-treated group are indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

TABLE 1 *Hamp* expression levels (ΔC_t *Gusb-Hamp*) in *Hfe* C282Y and wild-type 129S2 mice in the acute study.

	Hfe C282Y		129S2	
Time point	Vehicle	Vamifeport	Vehicle	Vamifeport
30 min	6.2 ± 0.7***	6.1 ± 0.4	8.0 ± 0.3	7.8 ± 0.4
1 h	6.5 ± 0.7***	6.0 ± 0.6	7.9 ± 0.5	7.7 ± 0.9
3 h	6.0 ± 1.6***	5.7 ± 1.4	8.4 ± 0.4	7.4 ± 0.3
6 h	6.7 ± 0.6***	6.7 ± 0.6	8.0 ± 0.2	5.0 ± 1.2
16 h	7.0 ± 0.8*	6.7 ± 0.8	7.7 ± 0.5	7.5 ± 0.6
Average ± SD	6.6 ± 0.9***	N/A	8.0 ± 0.5	N/A

Note: Significant differences between *Hfe* C282Y and wild-type mice are indicated as $^{*}p < 0.05$ and $^{***}p < 0.001$.

Abbreviation: N/A, not applicable.

expression differently from that resulting from acute vamifeport treatment.

With the exception of week 7, from week 1 until study end (week 8), hemoglobin levels were significantly lower during chronic vamifeport dosing than those observed in vehicle-treated *Hfe* C282Y mice (Figure 2C), reflecting the iron-restricted erythropoiesis induced by vamifeport.

The mutation present in *Hfe* C282Y mice results in inappropriate *Hamp* expression, leading to low hepcidin levels and excessive iron absorption and storage. Total liver iron concentration (reflecting the liver iron accumulated before and during the study) remained significantly lower in *Hfe* C282Y mice following chronic vamifeport treatment than following vehicle treatment. Differences in total liver iron concentration were observed from week 2, and the levels remained lower in the vamifeport-treated animals over the study period, reaching statistical significance from week 4 (Figure 2D).

Chronic vamifeport intake via drinking water prevented the accumulation of ^{58}Fe in the livers of *Hfe* C282Y mice that was seen following vehicle administration. At 2, 4, 6, and 8 weeks, ^{58}Fe liver iron concentrations were significantly lower in vamifeport-treated mice than in vehicle-treated mice (Figure 2D), demonstrating that iron restriction by vamifeport efficiently prevented de novo iron accumulation.

Total spleen iron concentration increased over time in both the vehicle- and vamifeport-treated groups, but levels at week 6 were significantly higher in mice receiving chronic vamifeport treatment

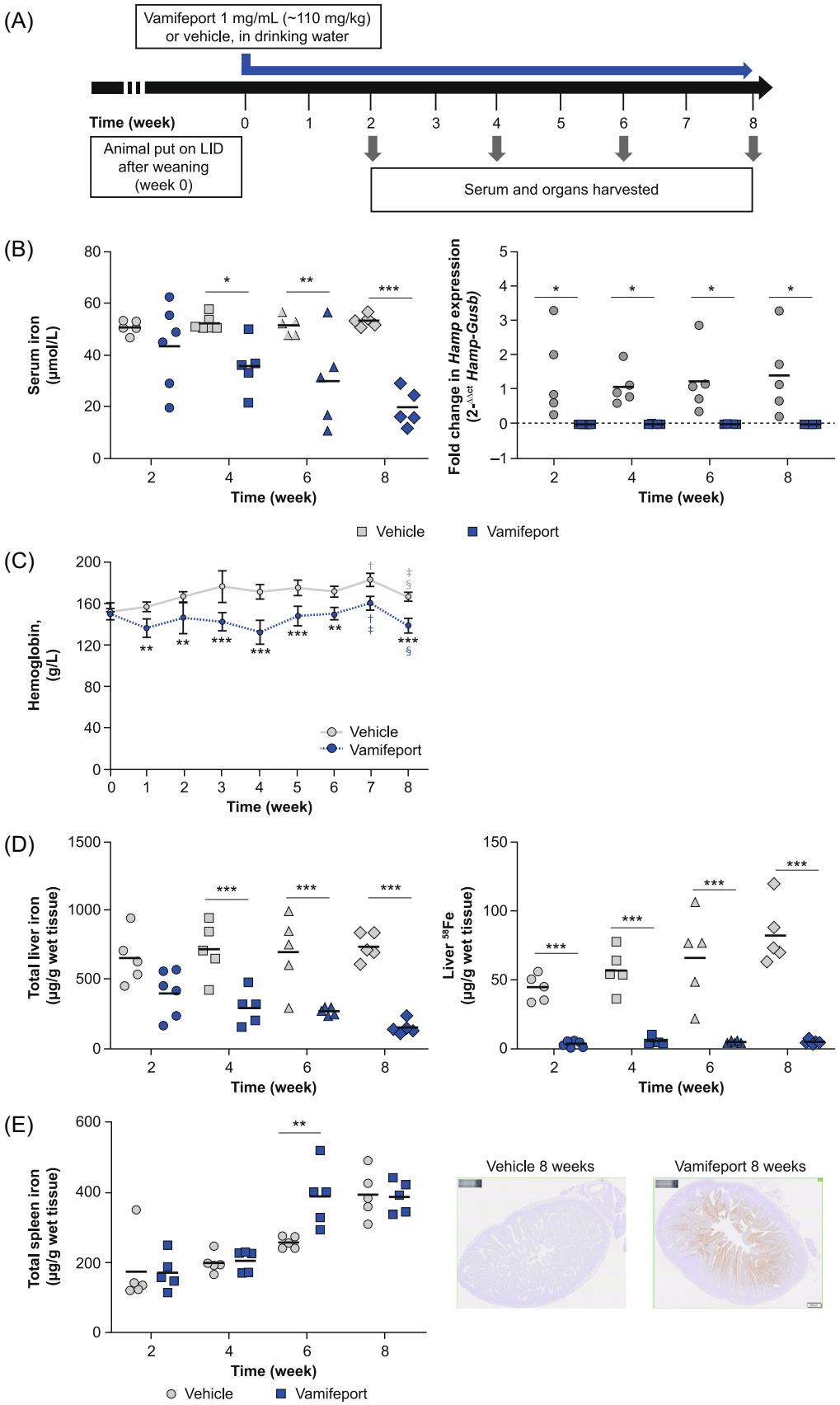

FIGURE 2 (See caption on next page).

than in those receiving vehicle treatment, reflecting the retention of iron in spleen cells as a result of ferroportin inhibition (Figure 2E). DAB-enhanced Perls' staining of duodenal cross-sections from *Hfe* C282Y mice showed iron accumulation in duodenal enterocytes after 8 weeks of vamifeport treatment; no duodenal iron staining was apparent in mice receiving vehicle treatment (Figure 2E). These data demonstrate that by inhibiting ferroportin, vamifeport prevents iron export from spleen cells and duodenal enterocytes.

Red blood cell numbers were significantly increased at 6 weeks, and significant increases in reticulocytes were observed at 2 and 4 weeks in *Hfe* C282Y mice treated with vamifeport compared with vehicle (Figure 3A,B). Leukocyte and platelet levels were similar in these treatment groups (Figure 3C,D). Levels of hematocrit, mean corpuscular hemoglobin, mean corpuscular volume, and reticulocyte hemoglobin were significantly lower in *Hfe* C282Y mice treated with vamifeport at all time points, due to the induction of iron-restricted erythropoiesis during vamifeport treatment (Figure 3E–H).

Vamifeport does not significantly interfere with liver iron removal by phlebotomy in the *Hfe* C282Y mouse model of hemochromatosis

Phlebotomy is the current standard of care for the clinical management of patients with hemochromatosis and usually occurs weekly during the induction phase, decreasing to three to four times a year during the maintenance phase.[1,14] To investigate the possibility of combining vamifeport treatment with phlebotomy in hemochromatosis, a pilot study was performed to assess the effects of vamifeport in combination with bi-weekly phlebotomy versus phlebotomy alone (i.e., alongside vehicle treatment) in 9–10-week old *Hfe* C282Y mice fed a standard iron diet before study start. The experimental design of this pilot study is included in Figure 4A.

Vamifeport was provided in the drinking water at concentrations of 0.3 and 1.0 mg/mL, aiming for daily vamifeport doses of ~40 and ~110 mg/kg, respectively. The intake of water containing vamifeport at 0.3 mg/mL was similar to that of vehicle-containing water in phlebotomized *Hfe* C282Y mice (Table 2). However, phlebotomized *Hfe* C282Y mice drank 56% less of the water containing the higher concentration of vamifeport (vs. vehicle). Surprisingly, control, non-phlebotomized *Hfe* C282Y mice treated with 1 mg/mL vamifeport consumed only about 20% less water than the non-phlebotomized vehicle-treated group, suggesting that the intake of water containing 1 mg/mL vamifeport is reduced in sublingually phlebotomized animals. Based on the measured intake of vamifeport-containing water, daily doses of vamifeport in this study were ~40 mg/kg for 0.3 mg/mL and ~60 mg/kg for 1 mg/mL. Due to the disproportionally reduced intake of vamifeport (and therefore also Fe(II)SO_4 from the water) in the 1 mg/mL dose group, only the data obtained for the 0.3 mg/mL group were considered reliable. Therefore, results are reported for the 0.3 mg/mL vamifeport dose only.

Serum iron concentration and liver *Hamp* expression were unchanged with phlebotomy alone; nonsignificant reductions were seen in both parameters with vamifeport plus phlebotomy treatment (Figure 4B). Phlebotomy alone significantly reduced the total liver iron concentration in the *Hfe* C282Y mouse model of hemochromatosis but did not affect ^{58}Fe liver iron concentration compared with that in vehicle-treated, non-phlebotomized mice (Figure 4C). At the dose tested, vamifeport did not significantly interfere with the liver de-ironing effect of phlebotomy in *Hfe* C282Y mice receiving phlebotomy plus vamifeport. Furthermore, there was a trend toward lower total liver iron levels in *Hfe* C282Y mice receiving phlebotomy plus vamifeport than in mice receiving phlebotomy alone. In addition, the reduction in total liver iron levels observed with phlebotomy plus vamifeport versus no phlebotomy ($p < 0.001$) had a higher level of statistical significance than that seen with phlebotomy alone versus no phlebotomy ($p < 0.05$). There was no significant difference in ^{58}Fe liver iron levels between *Hfe* C282Y mice receiving phlebotomy alone and those not receiving phlebotomy. Combining vamifeport with phlebotomy significantly decreased ^{58}Fe liver iron concentration in *Hfe* C282Y mice compared with phlebotomy alone, showing that vamifeport prevented the absorption of ^{58}Fe from drinking water and thereby prevented iron accumulation in the liver. Similar spleen iron (Figure 4D), hemoglobin, and erythropoietin levels (Figure 4E) were observed during phlebotomy plus vamifeport treatment and treatment with phlebotomy alone.

DISCUSSION

There are currently no approved oral drugs for patients with hemochromatosis, and for many years, the standard of care has been phlebotomy.[1,24] While phlebotomy is effective, simple, and inexpensive, it remains an invasive treatment that requires medical assistance and is not without complications. Adverse effects were found to occur “most” or “all of the time” in 37% of patients receiving maintenance therapy and 52% of those in the induction phase.[25] The adverse effects included fatigue, fainting, pain at the venous access site, and hematomas. Weekly phlebotomies received during the induction phase may lead to anemia, as well as being inconvenient or intolerable to patients; in some cases, it may be difficult to gain veinous access.[24,25] Moreover, patient compliance with phlebotomy in the maintenance phase generally decreases over time,[16] and 16% of patients would ‘definitely’ or ‘probably’ decide to stop phlebotomy if alternative treatment options became available.[25] Targeting the ferroportin–hepcidin axis, which regulates the body's iron homeostasis, may provide a therapeutic opportunity for patients with hemochromatosis.

The present preclinical studies aimed to evaluate the efficacy of the oral ferroportin inhibitor vamifeport in reducing serum iron levels and preventing liver iron deposition in the *Hfe* C282Y mouse model of hemochromatosis, alone and in combination with phlebotomy. In these studies, *Hfe* C282Y mice showed elevated serum iron levels compared with control 129S2 wild-type mice, which is consistent with lower liver expression of the iron regulatory peptide hepcidin in

FIGURE 2 Kinetics of pharmacodynamic effects following chronic oral dosing of vamifeport in the *Hfe* C282Y mouse model of hemochromatosis. Experimental design (A); serum iron concentration and liver *Hamp* expression. *Hamp* data are expressed as a fold change of the normalized gene expression compared to the vehicle-treated group at each time point (B); hemoglobin levels over time (presented as mean values with standard deviations) (C); total liver iron and ^{58}Fe liver iron concentrations; (D) total spleen iron concentration and representative images from diaminobenzidine-enhanced Perls' staining of duodenal cross sections from vehicle- or vamifeport-treated *Hfe* C282Y mice (E). For all scatter plots, data are presented as individual values with means ($n = 5–6$ animals per timepoint/treatment). Significant differences compared with the vehicle-treated group are indicated in black as: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Significant within-treatment differences in the vehicle group are indicated in gray as $^{\dagger}p < 0.01$ week 1 versus week 7; $^{\ddagger}p < 0.01$ week 1 versus week 8; $^{\S}p < 0.05$ week 7 versus week 8. Significant within-treatment differences in the vamifeport group are indicated in blue as $^{\dagger}p < 0.05$ week 3 versus week 7; $^{\ddagger}p < 0.05$ week 4 versus week 7; $^{\S}p < 0.05$ week 7 versus week 8. LID, low iron diet.

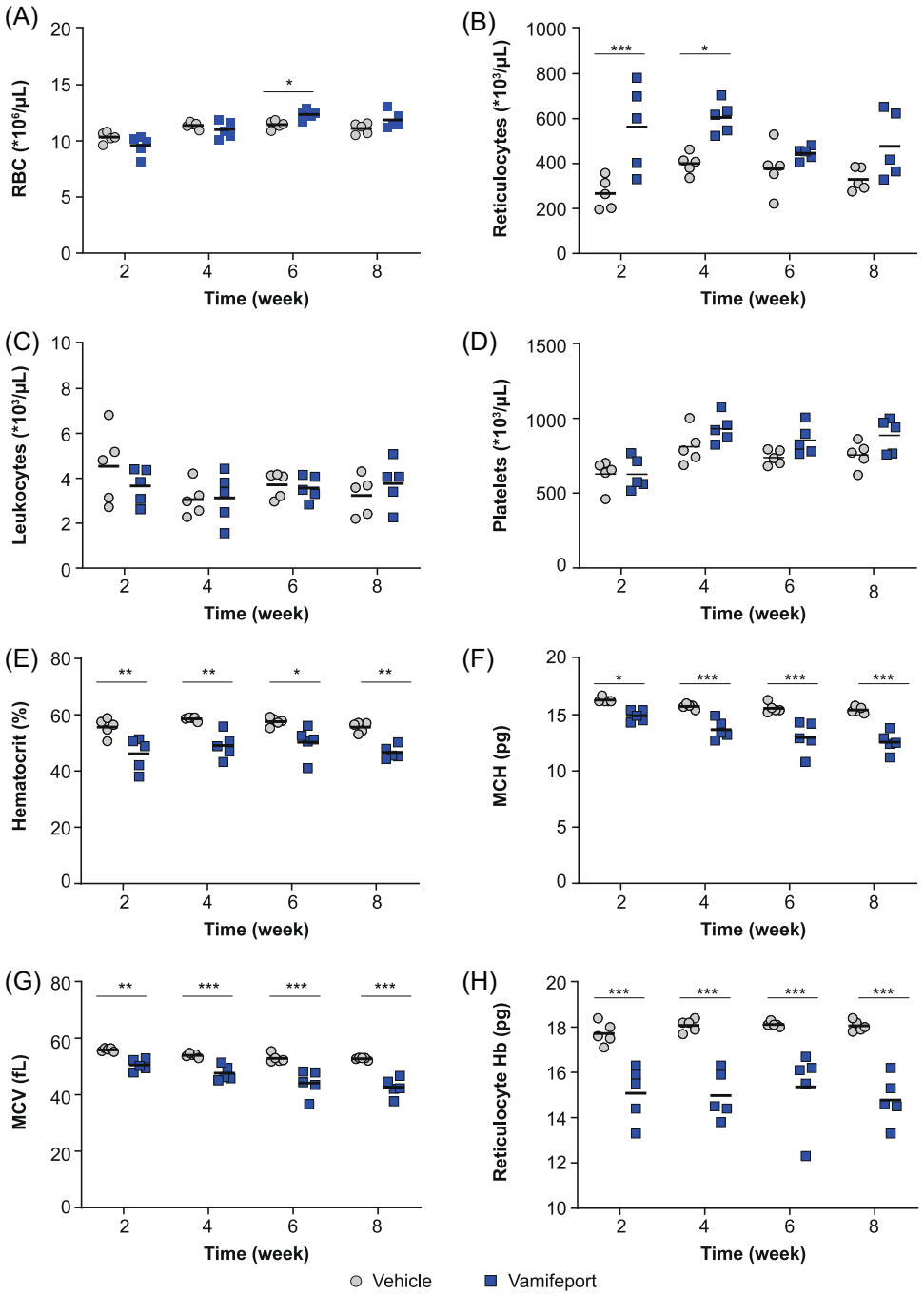

FIGURE 3 Kinetics of hematological effects following chronic oral dosing of vamifeport in the *Hfe* C282Y mouse model of hemochromatosis. Red blood cell count (A); reticulocyte count (B); leukocyte count (C); platelet count (D); hematocrit level (E); mean corpuscular hemoglobin (F); mean corpuscular volume (G); reticulocyte hemoglobin content (H). For all scatter plots, data are presented as individual values with means (n = 5–6 animals per timepoint/treatment). Significant differences compared with the vehicle-treated group are indicated as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

the *Hfe* C282Y group. Serum iron levels were transiently reduced during acute vamifeport treatment in *Hfe* C282Y mice. Chronic oral vamifeport treatment significantly reduced serum iron levels in this mouse model, demonstrating the potential of ferroportin inhibition by vamifeport to correct the elevated systemic iron levels in hemochromatosis, despite chronically reduced hepcidin levels. In the chronic study, the lowered serum iron levels resulting from vamifeport treatment attenuated iron accumulation in the liver. Vamifeport has previously been shown to reduce serum iron levels in mouse models of β-thalassemia,[17,19] polycythemia vera,[26] and sickle cell disease,[20] as well as in healthy volunteers[27] and patients with non–transfusion-dependent thalassemia[28] in phase 1 and 2 studies, respectively.

While *Hamp* expression was downregulated in response to acute hypoferremia at 3 and 6 h after vamifeport administration in 129S2 wild-type mice, liver *Hamp* expression levels did not significantly change in *Hfe* C282Y mice in the acute study. These findings are consistent with the presence of nonfunctional HFE in *Hfe* C282Y

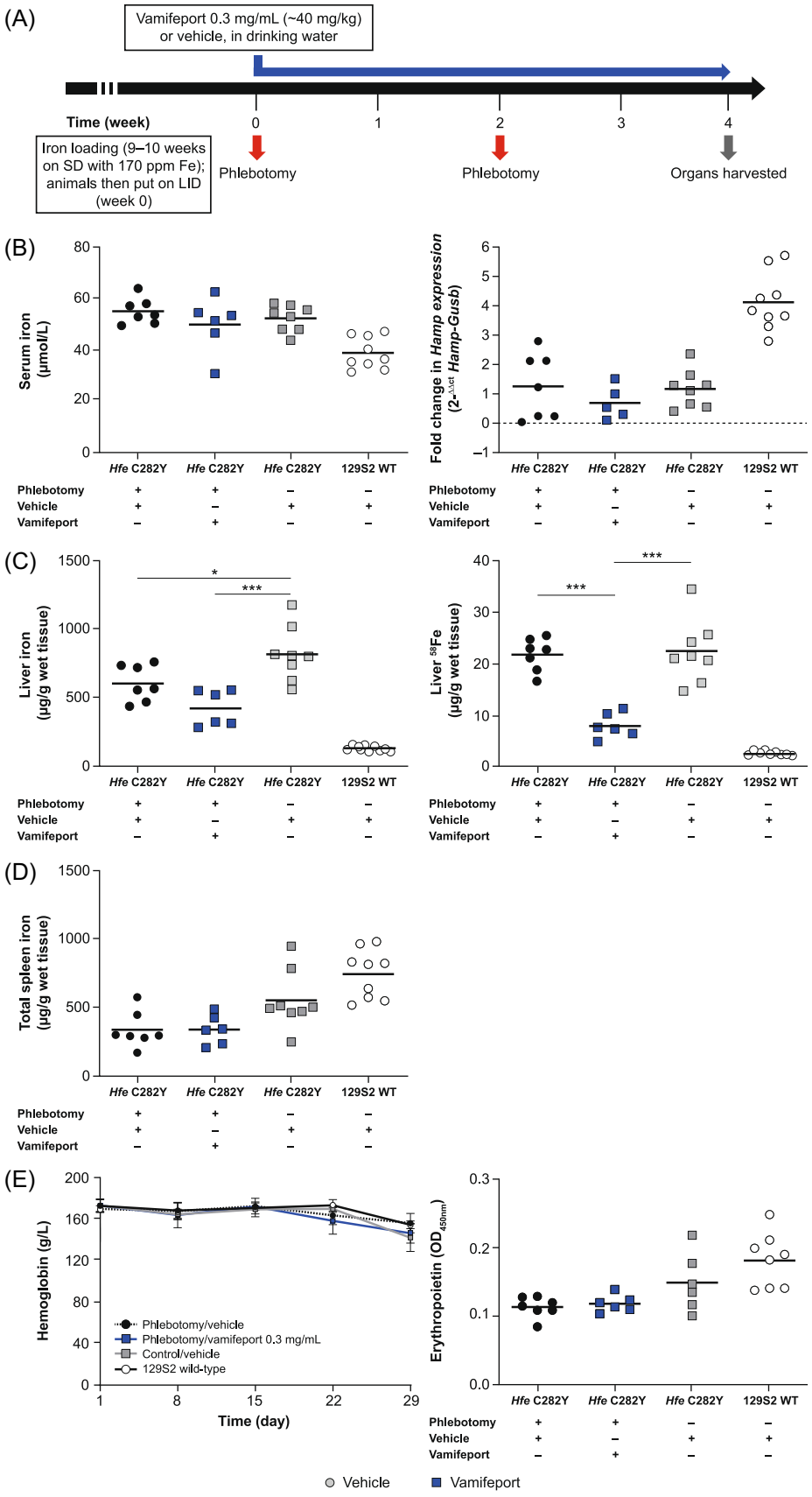

FIGURE 4 (See caption on next page).

TABLE 2 Drinking water consumption and the calculated vamifeport dose in the phlebotomy study in *Hfe* C282Y mice.

	Treatment group	Mean daily water consumed, per mouse (mL)	Reduction in water consumption vs. vehicle (%)	Calculated daily vamifeport dose (mg/kg)
Phlebotomized	Vehicle	3.4 ± 0.7	N/A	N/A
	Vamifeport 0.3 mg/mL	3.2 ± 0.5	6	~40
	Vamifeport 1 mg/mL	1.5 ± 0.4	56	~60
Non-phlebotomized	Vehicle	2.9 ± 0.4	N/A	N/A
	Vamifeport 1 mg/mL	2.3 ± 0.9	21	~90

Abbreviation: N/A, not applicable.

mice, which prevents appropriate endogenous hepcidin inhibition in the presence of acute hypoferremia.[22,29] Another possible explanation for the lack of hepcidin downregulation in the acute study is that serum iron levels may still have been above the threshold where hepcidin is inhibited following acute vamifeport treatment in *Hfe* C282Y mice. Although acute vamifeport treatment did not significantly affect hepatic *Hamp* levels in *Hfe* C282Y mice, chronic treatment did markedly reduce *Hamp* expression. The differential regulation of *Hamp* observed during acute (reduced serum iron and persisting liver iron overload) and chronic (reduced serum and liver iron concentrations) vamifeport treatment may reflect the two postulated mechanisms of hepcidin regulation.[30] The detection of changes in liver iron stores is mediated mostly by hepatic BMP6, which in turn interacts with BMPRI/II and a multiprotein complex on the hepatocyte membrane to regulate hepcidin expression. In contrast, changes in serum iron (which is bound to transferrin) regulate hepcidin expression via HFE and TFR2 signaling.[31]

Hemoglobin levels during chronic vamifeport treatment were significantly lower than those observed in vehicle-treated *Hfe* C282Y mice; nevertheless, hemoglobin levels remained in the normal range for this parameter in mice. Vamifeport also induced iron-restricted erythropoiesis in *Hfe* C282Y mice, as evidenced by the reductions in mean corpuscular hemoglobin content, mean corpuscular volume, and hematocrit levels (likely as a function of lowered mean corpuscular volume). Although vamifeport-treated mice had lower total blood hemoglobin levels, red blood cell counts were unaffected or slightly increased at Week 6. Chronic oral dosing of vamifeport also significantly reduced both total and ^{58}Fe liver iron concentrations, suggesting that vamifeport prevents liver iron loading in *Hfe* C282Y mice. Furthermore, ferroportin inhibition by vamifeport resulted in an increase in iron sequestration in iron-exporting organs such as the spleen and duodenum.

Importantly, at the dose tested, vamifeport did not significantly interfere with the de-ironing process of phlebotomy in the pilot preclinical study. However, the dose of vamifeport used (~40 mg/kg) was lower than that shown to be optimally effective in other rodent studies (120 mg/kg).[17] Although vamifeport at a dose of ~40 mg/kg did not significantly improve liver iron removal compared with phlebotomy alone, it did significantly prevent the uptake of ^{58}Fe in the livers of *Hfe* C282Y mice receiving phlebotomy, suggesting that vamifeport could potentially have a role in improving the effectiveness of venesection in patients with hemochromatosis. Overall, these data suggest that vamifeport does not interact with phlebotomy in reducing liver iron accumulation but that it prevents further iron uptake in the liver. Nonetheless, additional studies using higher vamifeport doses are needed to confirm these preliminary findings. Although phlebotomy is currently the standard of care for patients with hemochromatosis, it only removes excess iron without correcting iron homeostasis. As well as vamifeport, other therapeutic approaches such as hepcidin mimetic peptides, are under investigation that target the underlying pathophysiology of HFE-related hemochromatosis (i.e., decreased endogenous hepcidin). However, these agents do not actively remove excess organ iron. Based on their modes of action, hepcidin mimetics shall be administered following phlebotomy, as recently demonstrated in a clinical study of rusfertide.[32] Once iron levels have been reduced by induction phlebotomy, hepcidin mimetics might have the potential to replace phlebotomy in the maintenance phase of treatment.[14]

Hepcidin deficiency or downregulation is the underlying cause of iron overload in most types of hemochromatosis, and an expanding body of evidence from other investigational drugs supports targeting the hepcidin–ferroportin axis as a potential therapeutic strategy in patients with hemochromatosis. Approaches that have been investigated include restoring hepcidin levels with the administration of hepcidin mimetics (e.g., rusfertide [PTG-300][32–34] and the rusfertide analog PN23114[35]), synthetic hepcidins (e.g., LJPC-401[36]), and minihepcidins (e.g., PR65[37] and the oral minihepcidin PN20076[34]). Agents are also being evaluated that target TMPRSS6, a protein that inhibits hepcidin transcription (e.g., lipid nanoparticle-formulated small interfering RNAs [siRNAs],[38] the GalNAc–siRNA conjugate SLN124,[39] and antisense oligonucleotides [e.g., IONIS-TMPRSS6-LRx/sapablursen[40]], all of which target *Tmprss6* expression). However, most of the available data come from preclinical studies of subcutaneously administered agents in the more severe *Hfe*$^{-/-}$ knockout mouse model of hemochromatosis,[33,35,38–40] and so comparisons with the current study are difficult. Rusfertide and LJPC-401 are currently the only agents targeting the hepcidin–ferroportin axis that have published clinical data for hemochromatosis.[32,36] However, clinical development of LJPC-401 has now ceased.[41]

Orally administered treatments such as vamifeport could have several potential advantages over parenteral therapies. These include

FIGURE 4 Vamifeport does not interfere with liver iron removal by phlebotomy in the *Hfe* C282Y mouse model of hemochromatosis. Experimental design (A); serum iron concentration and liver *Hamp* expression. *Hamp* data are expressed as a fold change of the normalized gene expression compared to the vehicle-treated group at each time point (B); total liver iron and ^{58}Fe liver iron concentrations; (C) total spleen iron concentration (D); hemoglobin levels over time (presented as mean values with standard deviations) and erythropoietin concentrations (E). For all scatter plots, data are presented as individual values with means (n = 5–6 animals per timepoint/treatment). The 129S2 wild-type group is included to show the normal levels of these parameters but is excluded from the statistical analysis. Significant differences between treatment groups are indicated as $^{*}p < 0.05$ and $^{***}p < 0.001$. LID, low iron diet; OD, optical density; SD, standard diet; WT, wild type.

improved convenience and ease of administration for patients, dosage/formulation flexibility, obviation of the risk of injection-site reactions/infection,[17] and, in general, easier storage and supply chain management compared with injectable agents. Although absorption and onset of action are generally slower for oral drugs, vamifeport is absorbed relatively quickly, with levels detectable 15–30 min post-dose and serum iron levels reducing to basal levels 4–8 h after administration in healthy volunteers.[27] Notably, orally administered drugs tend to have shorter half-lives and lower bioavailability than those given parenterally, meaning that higher and more frequent doses are often required.[42] The absorption of oral drugs can also be affected by food.[43]

The results of the current preclinical studies in a mouse model of hemochromatosis support the clinical testing of vamifeport in patients with this condition to determine if the observed results translate to clinically meaningful effects. A strength of the present preclinical studies is that they were conducted in an animal model that reflects the most common mutation found in patients with hemochromatosis.[2] Other *HFE* mutations and mutations in non-*HFE* genes are much rarer; therefore, the *Hfe* C282Y mouse model most closely resembles the hemochromatosis patient population seen in clinical practice. A potential limitation of the current studies is that the genetic backgrounds of the *Hfe* C282Y and 129S2 wild-type mice used in this study are not completely identical; as such any variations in basal levels of the measured parameters may not solely be due to the *Hfe* gene mutation. Therefore, wild-type littermates obtained by back-crossing homozygous *Hfe* C282Y mice with 129S2 mice could be a more appropriate wild-type control for any future studies. It would also be of interest to repeat these studies in a different, more severe model of hemochromatosis that has more pronounced iron overload than that seen in *Hfe* C282Y mice. Nonetheless, due to its mode of action, vamifeport may also be active in other forms of hemochromatosis where hepcidin is downregulated.

In summary, these preclinical proof-of-concept studies demonstrate that the ferroportin inhibitor vamifeport significantly reduces serum iron levels and prevents liver iron loading following chronic dosing in the *Hfe* C282Y mouse model of hemochromatosis, thus supporting future clinical development in this indication. If vamifeport proves to be efficacious in patients with hemochromatosis, it could potentially have a role alongside phlebotomy in patients with hemochromatosis who are in the induction phase and could theoretically obviate the need for phlebotomy for those in the maintenance phase of treatment.

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AUTHOR CONTRIBUTIONS

Naja Nyffenegger, Franz Dürrenberger, and Vania Manolova designed the studies. Anna Flace, Ahmet Varol, Patrick Altermatt, Cédric Doucerain, and Hanna Sundstrom performed the research. Naja Nyffenegger, Patrick Altermatt, Hanna Sundstrom, Franz Dürrenberger, and Vania Manolova analyzed and/or interpreted the data. All authors reviewed the manuscript, approved the final draft, and agreed to be accountable for the accuracy and integrity of its content.

CONFLICT OF INTEREST STATEMENT

Naja Nyffenegger, Anna Flace, Ahmet Varol, Patrick Altermatt, Cédric Doucerain, Hanna Sundstrom, Franz Dürrenberger, and Vania Manolova are all current or past employees of CSL Vifor and may own equities. Naja Nyffenegger, Vania Manolova, and Franz Dürrenberger are inventors in patents related to this publication.

DATA AVAILABILITY STATEMENT

Data can be shared upon request to the corresponding author.

ETHICS STATEMENT

The animal experiments described herein complied with Swiss law and associated guidelines for animal experimentation and were approved by the responsible authority (Veterinary Department, Canton Zurich; license numbers ZH108/2017 and ZH121/2014). Studies were performed in compliance with the Vifor Pharma Group Code of Conduct and complied with the principles of the 3Rs (replace, reduce, and refine).

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