haematologica
Journal of the Ferrata Storti Foundation

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Received: January 15, 2026.
Accepted: June 22, 2026.

Citation: Vincenzo La Mura, Niccolò Bitto, Alessandro Ciavarella, Paola Dongiovanni, Paola Gruarin, Miriam Longo, Laura Napoli, Marco Maggioni, Michela Ripolone, Anna Maria Ierardi, Anna Ludovica Fracanzani, Sergio Abrignani and Flora Peyvandi. Biopsy-proven immune-mediated hepatitis after valoctocogene roxaparvovec.
Haematologica. 2026 July 2. doi: 10.3324/haematol.2026.300542 [Epub ahead of print]

Biopsy-proven immune-mediated hepatitis after valoctocogene roxaparvovec

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RUNNING HEAD: Hypertransaminasemia after gene therapy

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AUTHORS CONTRIBUTION

V.L.M. and F.P. conceived and supervised the study. V.L.M., N.B., A.C. collected and analyzed the clinical data. P.D., M.L., L.N. M.L. contributed to the electron microscopy analysis. M.M. conducted the histopathological evaluations. P.G. and S.A. performed the immunophenotype analyses. V.L.M. organized and supervised the writing of the manuscript with input from all authors. A.L.F., S.A, F.P. provided revision of the manuscript for the most critical issues. All authors reviewed and approved the final version.

DATA-SHARING STATEMENT

For original data contact the corresponding author, Prof. Flora Peyvandi (flora.peyvandi@unimi.it).

ACKNOWLEDGEMENTS

Our gratitude to Daniela Papa and Bojan Vodovar for technical assistance.

FUNDING

This work was partially supported by the Italian Ministry of Health (Bando Ricerca Corrente). The Department of Pathophysiology and Transplantation, University of Milan, is funded by the Italian Ministry of Education and Research (MUR): Dipartimenti di Eccellenza Program 2023 to 2027. The Hemostasis & Thrombosis Unit of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico is member of the European Reference Network on Rare Haematological Diseases EuroBloodNet-Project ID No 101157011. ERN-EuroBloodNet is partly co-funded by the European Union within the framework of the Fourth EU Health Programme.

CONFLICT OF INTEREST STATEMENT

V.L.M.: Advisory boards: CSL Behring, Biomarin, Pfizer, Alfasigma. Symposia/educational meetings: Biomarin, Gore, Alfasigma. Travel grant: Takeda, Sanofi, Bayer

N.B.: travel grant from Biomarin.

A.C.: honoraria from Bayer (advisory board) and Roche (congress).

P.D.: no competing interests.

P.G.: no competing interests.

M.L.: no competing interests.

L.N.: no competing interests.

M.M.: no competing interests.

M.R.: no competing interests.

A.M.I.: no competing interests.

A.L.F.: no competing interests.

S.A.: no competing interests.

F.P.: Advisory boards: CSL Behring, Biomarin, Roche, Sanofi, Sobi, Pfizer. Symposia/educational meetings: Takeda, Sanofi, Sobi

ABSTRACT

Background & Aims: Valoctogene roxaparvovec is an approved and efficacious gene therapy (GT) for severe hemophilia A (HA), however alanine aminotransferase (ALT) elevation after dosing raises safety concerns. Corticosteroids are the first-line therapy for ALT>1.5X the baseline value, but this indication requires further pathological characterization in humans. We explored mechanisms of ALT elevation to personalize corticosteroid therapy. **Methods:** Follow-up data on real-life experience with valoctogene roxaparvovec were collected. Transjugular liver biopsy for histology, transmission electron microscopy and immunophenotype characterization were compared with positive/negative controls. **Results:** Five severe HA patients were treated with valoctogene roxaparvovec. Four (80%) had ALT>1.5X the baseline value but two had transaminase above the normal range (59 U/L) and lowering of transgene expression and were treated with prednisone. Liver biopsy from these two cases before starting prednisone revealed acute immune-mediated hepatitis and abnormal enlargement of the smooth endoplasmic reticulum of hepatocytes. ALT normalized along with preservation of transgene expression after a personalized schedule of oral prednisone. High T-cytotoxic and low T-regulatory activity was observed during acute hepatitis. The median transgene expression at the end of the follow-up was 83(12-206)% (chromogenic assay), but it was highly variable regardless the exposure to prednisone: 48(12-83)% in patients treated with prednisone and 194(20-206)% in patients not treated with prednisone.

Conclusions: Immune-mediated hepatitis can cause ALT elevations in HA after valoctogene roxaparvovec and, in selected cases, a biopsy-proven diagnosis could support a safer and more effective therapeutic course of prednisone to maintain optimal transgene expression.

KEY WORDS: hepatitis, transaminases, liver, hemophilia, gene therapy, safety, efficacy

INTRODUCTION

Valoctocogene roxaparvovec is the first gene therapy (GT) approved for the treatment of severe hemophilia A (HA), a congenital bleeding disorder characterized by the plasma deficiency of coagulation factor VIII (FVIII).[1] It is an adeno-associated virus (AAV)-5 therapy based upon a B-domain-deleted human FVIII transgene (AAV5-hFVIII-SQ) targeting the liver which has been engineered with a specific promoter for hepatocytes. After a single intravenous dose, valoctocogene roxaparvovec provides long-term expression of FVIII which reduces the annualized rates of exogenous factor use, bleeding episodes and the burden of replacement therapy.[2] These important results could be associated with elevation of the liver enzyme alanine aminotransferase (ALT),[3] that has been observed across all clinical trials involving AAV5 GT in hemophilia and, in a small number of cases, reached the grade 3 scale of adverse events (Common Terminology Criteria for Adverse Events).[4] At today, the experience deriving from the trials suggests the use of corticosteroids as the first-line therapy[5]. Specifically, all patients having ALT>1.5X the baseline value should receive 60 mg of prednisone, subsequently tapered over at least two months. This treatment aims to preserve transgene expression but has some counterintuitive aspects: it exposes patients to high corticosteroid doses even with normal transaminase levels, is empirical, untested against placebo, and based on preclinical studies not confirmed in humans due to lack of liver-biopsy based analyses during acute transaminase elevation. Unfortunately, ALT elevation is unpredictable and, although this adverse event has been until now largely transient and not associated with life-threatening complications, the intervention of the hepatologist may be crucial for safety and efficacy reasons.[6,7] Indeed, this adverse event has been observed also in other monogenic disorders treated with AAV-based gene therapies[8] and, in few cases, the event resulted in acute severe hepatic insufficiency.[9] Importantly in those experiences the pre-existing liver damage and the higher vector doses influenced the severity of this adverse effect[8] but

altogether the above observations reinforce the importance of better characterizing the mechanisms behind ALT elevation after GT in hemophilia.[4,7,10] Along this line a call to action was launched in 2023 to clarify liver-related aspects regarding safety and efficacy of AAV5-based GT in hemophilia through the promotion of a strict collaboration between hematologists and hepatologists.[11,12] Importantly, experts in both fields and patient associations emphasized the potential utility of data stemming from liver biopsies during ALT elevation for a better characterization of hepatocellular damage, immune system participation, transgene expression and distribution.[4,5,10]

This observational study reports our pilot experience as a hub centre for GT in hemophilia following its approval in HA. We collected histological, cytological, and immunological data related to ALT elevations to guide corticosteroid therapy and to highlight the role of hepatologists in clinical decisions based on pathology and immunology, rather than on fixed treatment schedules.

METHODS

Design of the study, patients, analysis of factor VIII activity

Hepatological and hematological characteristics at baseline and at the end of the follow-up of five consecutive patients with severe HA treated with valoctogene roxaparvovec were collected. A multiple comparison analysis was conducted by starting with the description of the first case (index case) who was addressed to a transjugular liver biopsy because of ALT elevation above the normal laboratory range after GT. Data from the index case were compared with clinical, laboratory and/or histological and cytological data of the other cases who underwent GT and, when appropriate, with other controls as summarized in Figure 1. Specifically, liver histology and cytology were compared with the second consecutive case of ALT elevation above the normal laboratory range after GT. Moreover, the cytology at electron microcopy analysis was compared with 3 liver samples from other

3 patients managed at our liver unit (clinical details in Supplementary Table 1 and relative comments), one with normal liver (control no.1), one with a history of autoimmune hepatitis triggered by pegylated-interferon therapy for chronic hepatitis B and with a biochemical response to corticosteroids at the time of liver biopsy (control no.2), and one with active primary biliary cholangitis/autoimmune hepatitis and pharmacological induction by triptans (hepatotoxicity), therefore resembling drug induced liver injury (control no.3). The immunophenotype of the index cases was compared with that of 2 healthy donors and the two other severe HA patients of this series treated with valoctogene roxaparvovec who had ALT elevation 1.5X the baseline value but within the normal laboratory range and did not receive corticosteroids.

Plasma FVIII was measured by one-stage clotting and chromogenic assay (the latter containing reagents of bovine origin, Coamatic, Chromogenix) and presented as percentage of activity.[13]

Patient management was conducted in agreement with the protocols of good clinical practice adopted by our institution. All patients with ALT elevation were studied to exclude other causes than GT for this adverse effect (alcohol consumption, medical or herbal products, viral infections).

All subjects involved in this description gave their informed written consent for storage and use of their biological material for scientific purposes as approved by the Ethical Committee at Fondazione IRCCS Cà Granda, Ospedale Maggiore Policlinico Milano and conformed to the 1975 Declaration of Helsinki (Ethics Committee no. 164_2019).

Data were reported as median and range or means with standard errors, when appropriate.

Transjugular liver biopsy

An intravenous infusion of recombinant FVIII (turoctocog alfa pegol - 40 IU/kg) was performed before liver biopsy. Hepatic vein catheterization and transjugular liver biopsy were performed by an experienced radiologist as previously described.[14] Briefly, after local anesthesia (2% subcutaneous lidocaine), ultrasound-guided puncture of the right jugular vein was performed. Under fluoroscopic guidance, the right hepatic vein was catheterized and controlled using angiography. After heparinization of the catheter, 20 ml of blood were collected from the hepatic vein for laboratory analysis. Finally, a transvenous liver biopsy was performed using the core-biopsy system (Cook Medical, LABS-100, 18G). After biopsy, hemostasis of the jugular venous access was achieved by local compression. An additional dose of FVIII (30 IU/Kg of turoctocog alfa pegol) was administered one day after the procedure with no complications.

Histopatology of the liver, transmission electron microscopy, gene expression and immunophenotype analysis

See supplementary materials.

RESULTS

Hepatological and hematological characteristics at baseline and at the end of follow-up of 5 consecutive patients with severe HA were dosed with valoctogene roxaparvovec are summarized in Table 1. One single patient had metabolic dysfunction associated steatotic liver disease as defined by overweight and steatosis. All patients had normal ALT level at baseline and no signs of advanced fibrosis/cirrhosis as per entry criteria of GT.[15] The median follow-up of the series was 45(21-67) weeks after dosing and three of five had a follow-up over 6 months.

Four patients (80%) had ALT elevation 1.5X the baseline value, but two had ALT above the normal laboratory range reaching the highest level of 633 IU/L at week 10 (index case) and

174 IU/L at week 9, respectively. Both patients had initial loss of transgene expression during ALT elevation and started oral prednisone 60 mg daily. These two patients were addressed to liver biopsy as described into details below. The other two cases had ALT elevation 1.5X the baseline value but within the normal laboratory range and were not treated with prednisone. Just one patient had stable ALT over time.

The median transgene expression reached at the end of the follow-up was 83(12-206)% (chromogenic assay), no life-threatening complications were detected and the transgene expression was not associated with thrombotic nor hemorrhagic events over time.

Index case: clinical and laboratory follow-up until 67 weeks after dosing

The index case was a 25-year-old man diagnosed with HA at the age of one. Figure 2 summarizes the trend of FVIII and ALT levels over time after dose (measurements of FVIII and ALT at any time point are detailed in Supplementary Table 2). Transaminases increased 1.5X the baseline value at week 5 post-infusion. At that time FVIII was 21% (chromogenic assay). He declared having consumed milk-derived products to increase the muscle mass, and we prompted him to withdraw these products to avoid any potential adverse effect on the liver, as recommended.[10] Due to the persistence of ALT elevation, we then proposed corticosteroids, as suggested,[5] but the patient denied consent because of the modest increase of transaminases, the satisfactory level of transgene expression and fear of corticosteroid-related adverse effects. To rule out an immune response causing liver damage and potentially the loss of transgene expression, we also proposed a liver biopsy, that was refused. By week 7 ALT rapidly increased to reach the peak of 633 U/L at week 10. Cholestasis markers and indexes of hepatic synthesis remained consistently within the normal limits across all measurements. Major and minor causes of acute liver damage were excluded (Supplementary Table 3). No exposure to alcohol, new medications nor to potentially hepatotoxic drugs were registered. Liver ultrasound was negative and stiffness

measurement by Fibroscan® was 5.2 KPa. Due to the rapid worsening of ALT levels and the concomitant additional loss of FVIII expression from 29 to 17% (chromogenic assay), at week 10-11, respectively, the patient accepted to start oral prednisone at 60 mg/day but not a liver biopsy, which was proposed again to exclude drug induced liver injury or any other immune-mediated acute liver damage and to tailor immunosuppression therapy as per good clinical practice[16]. Following the onset of prednisone, ALT progressively decreased, with a concomitant reduction of FVIII to 13% (chromogenic assay). During full dose of prednisone, the patient experienced mild acne. As per-schedule protocol, from week 14 to 22 after GT infusion prednisone was gradually tapered to a dose of 2.5 mg/day. However, this reduction was followed at week 25 by a second ALT peak of 104 U/L and FVIII decline from 31% at week 23 to 18% at week 25 (chromogenic assay). Therefore, prednisone was increased to 10 mg/day. Liver biopsy was then accepted by the patient and performed at week 27 without complications. Prednisone was increased immediately to 25 mg daily still unaware of the histological results. The final diagnose, based on liver biopsy and laboratory data, was immune-mediated seronegative acute hepatitis (see below for histological details). HLA haplotype analysis showed the presence A*02 and DRB*04*14, a finding not typical for autoimmune hepatitis.[17]

Prednisone 25 mg daily was enough to achieve a slow normalization of ALT. Importantly FVIII values stabilized around 10-12% (chromogenic assay). Then prednisone was gradually tapered and stopped at week 60. No adverse effects of corticosteroids were observed during this second challenge with prednisone. The hepatological evaluation at one year after GT dosing detected a normal morphology of the liver at ultrasound exploration and a Fibroscan® value of 5.4 KPa, along with persistent normalization of transaminases. At week 67, the patient remained with normal transaminases and stable transgene expression despite prednisone withdrawal.

Index case: histopathology of the liver and comparison with the second case of ALT elevation above the normal range after GT

Biopsy of the index case included four liver fragments overall 27 mm in length, with 14 complete portal spaces. Histopathology showed a normal lobular architecture (Figure 3 panel A) with mild lymphocytic infiltration presenting as focal mild interface hepatitis in the lobule and portal spaces (Figure 3 panel B-C). A few foci of spotty necrosis were observed in the lobule, occasionally in the perivenular zone 3 areas with an infiltrate of plasma cells at immunohistochemistry analysis (Figure 3 panel D). The morphological and immunohistochemical features were consistent with mild active hepatitis with an immuno-mediated pattern (Ishak score grade 4, stage 0).

We had a second consecutive case of ALT elevation above the normal range after infusion of valoctogene roxaparvovec (70 U/L at week 7; 174 U/L at week 9) in a 38-year-old man who had a prior history of HCV infection eradicated in 2011 with peg-interferon plus ribavirin and no signs of advanced fibrosis/cirrhosis nor additional risk factors of acute or chronic liver damage. The most important clinical data before gene therapy and during the follow-up are summarized in Table 1 (named “case 4”) (measurements of FVIII and ALT at any time point are detailed in Supplementary Table 2). At week 9 the patient started 60 mg of oral prednisone, the liver biopsy was performed at the same week. Figure 4 shows ALT, FVIII expression and prednisone dose up to the end of the follow-up. The laboratory work-up excluded causes of ALT elevation other than GT (Supplementary Table 3). Liver histology disclosed a pattern of immune-mediated hepatitis like that observed in the index case. Indeed, the lobular architecture was preserved, but there was evidenced lymphocytic infiltration causing mild interface hepatitis in the lobule and portal spaces (Figure 3 panel E-G) along with few clusters of plasma cells at immunohistochemistry analysis (Figure 3 panel H).

Transmission electron microscopy of the index case and comparison with control cases, genetic analysis

The index case had no abnormality in mitochondria, peroxisomes and nuclei (Figure 5 panel A). Conversely, there was a marked proliferation of the smooth endoplasmic reticulum (SER), which appeared extensively distributed throughout the hepatocyte cytoplasm while the rough endoplasmic reticulum (RER) showed no significant increase with occasional dilatations (Figure 5 panel B). In some hepatocytes, the SER alterations were particularly severe, the cytoplasm being almost entirely occupied by dilated structures (Figure 5 panel C). This morphology was also found in the second case of ALT elevation above the normal range (Figure 5 panels D-F).

The above cytological alterations were not found in hepatocytes from the case with a normal liver biopsy (negative control) (Figure 5 panel G) nor in the hepatocytes from the case with a history of autoimmune hepatitis after pegylated-interferon therapy for chronic hepatitis B, who, at the time of liver biopsy, had a biochemical response to corticosteroids (Figure 5 panel H). Conversely, another analyzed case presenting active portal biliary cholangitis/autoimmune hepatitis and pharmacological induction by triptans (hepatotoxicity), thus resembling a drug induced liver injury, showed hepatocytes with a milder proliferation of SER, organized in isolated clusters whose pattern was like those observed in our cases of ALT elevation after GT (Figure 5 panel I).

In the two cases with mild immune-mediated hepatitis after GT we further evaluated the expression of ER-stress markers related to the unfolded protein response (UPR), which is the main mechanism involved in ER proliferation and it is orchestrated by three major signaling branches: ATF6, IRE1, and PERK. Due to the very limited size of the liver biopsies, we restricted the analysis to transcriptional targets representative of UPR activation compared their expression with the control sample. In particular, we assessed

BiP, the spliced XBP1 isoform (XBP1s) and CHOP, which are downstream modulated from ATF6, IRE1, and PERK pathways, respectively. All three markers were significantly rose in both patients receiving gene therapy compared to normal liver, and the major increase was found for BiP and CHOP genes (BiP: p=0.002, XBP1: p=0.035 and CHOP: p=0.034 vs control no.1; Figure 5 panel J-L), supporting the activation of the UPR and consistently with the marked ER expansion observed by TEM analysis.

Immunophenotype characterization of CD4 and CD8 T-cells in the index case in comparison with controls

Immunophenotype characterization was carried out in the index case during ALT elevation above the normal laboratory range and after response to prednisone. Data were compared to the two HA patients with ALT 1.5X the baseline value but within the normal laboratory range after valoctogene roxaparvovec (Supplementary Figure 1 shows the trend of FVIII and transaminases over time after GT dosing and timing of immunophenotype characterization of these two patients) and two healthy donors. At time of liver biopsy the index case showed no differences in the frequency of total CD4 T-cells (Figure 6 panel A) in comparison with controls. In contrast, subset analysis for CD4 revealed that he had the highest representation of CD39PD-1 positive CD4 T cells (Figure 6 panel B), characterizing exhausted CD4 T-cell, and had also the lowest frequency of regulatory T-cells (Treg) and circulating follicular T helper cells (cTFh) (Figure 6 panel C-D), key-regulators of the humoral immune response. Interestingly, the highest frequency of Treg and cTFh was observed in the two HA cases who had ALT within the normal laboratory range but, the difference with the index case disappeared when the latter achieved normalization of ALT.

CD8 T-cells frequency is showed in Figure 6, panel E-F. Notably, the index case at time of liver biopsy had the highest frequency of CD8 cells, in particular CD8 cells that secrete granzyme B, suggesting an active cytotoxic activity during ALT elevation after GT. This difference was blunted after prednisone induced normalization of ALT. The two HA cases who maintained the ALT within the normal laboratory range and did not receive prednisone also showed high levels of CD8 cells that secrete granzyme B.

Remarkably, in this pilot analysis, the characterization of the immunophenotype on peripheral blood and hepatic vein of the index case gave the same comparative results.

DISCUSSION

ALT elevation after GT for hemophilia is a frequent adverse event. Generally, it is not associated to severe liver dysfunction but needs hematologists and hepatologists intervention. It remains a concern for patients due to potential safety, efficacy, and GT outcomes. In our real-life experience, five consecutive patients were treated with valoctogene roxaparvovec. Four (80%) had ALT ≥1.5X the baseline value, although in two of them ALT remained within the normal laboratory range, while for the other two patients ALT reached the high value of 633 U/L (index case) and 174 U/L (case 4 in the series), respectively. The two cases with ALT within the normal laboratory range did not receive corticosteroids and FVIII expression was preserved. Indeed, both maintained levels of FVIII>5% throughout the follow-up: one had a FVIII level of 206% and the other of 20% (chromogenic assay) at week 51 and 45 post dose, respectively, thus confirming the high unpredictable heterogeneity of transgene expression after GT regardless of the exposure to corticosteroids.[18] At variance, the index case had two episodes of ALT elevation above the normal range: the first at week 6 after dosing along with a parallel reduction of FVIII at the time of starting oral prednisone (60 mg), the second at week 22 at the time of prednisone tapering. When this second peak occurred, the patient underwent liver biopsy

and the histology revealed an interface hepatitis along with plasma cell infiltrates, i.e., a pattern of a low grade immune-mediated hepatitis that justified the choice to prolong corticosteroids. Furthermore, immunophenotype characterization confirmed an immune disbalance toward a T-cytotoxic cell activation. Serial assessment showed that after stable ALT normalization following prednisone withdrawal, the immunophenotype was similar to that in two severe HA cases treated with valoctogene roxaparvovec, who had only a slight ALT increase within normal range. The histological and laboratory data of the index case drove the prolongation of prednisone with two objectives: turning down the hepatocellular damage and attenuating the loss of transgene expression. The information collected on the first three cases treated with GT, suggested us to adopt a biopsy-proven approach for the decision on corticosteroids following significant elevation after GT. This strategy was implemented in our internal protocols and applied to the second consecutive case of immune-mediated hepatitis after GT with valoctogene roxaparvovec. This last patient is now successfully tapering corticosteroids, preserving FVIII expression.

At today, experimental investigations on ALT elevation after GT in hemophilia have postulated three pathogenic mechanisms: (a) an anti-AAV capsid peptide cytotoxic T-cell response [19], (b) endoplasmic reticulum stress with subsequent hepatocyte apoptosis due to high clotting factor expression, particularly in hemophilia A [20], and (c) a direct effect of vector particle load.[21] Our analyses found an immune imbalance during ALT elevation and signs of endoplasmic reticulum stress at electron microscopy, also confirmed by the molecular analysis, demonstrating that immune activation and subcellular stress can coexist during the acute event of ALT elevation after GT. If reproduced on a large scale, these observations have the potential to fill the gap of knowledge between human and pre-clinical investigations.[20,22,23] Furthermore, if independently confirmed, our findings would support the introduction of valoctogene roxaparvovec in the list of the 40 drugs potentially associated with a condition similar to drug-induced autoimmune-like hepatitis (DI-ALH).[24]

This nomenclature has been recently proposed by the International Autoimmune Hepatitis Group and EASL DILI Consortium to reduce the risk of adverse effects from immunosuppressive medications and associated healthcare costs. Indeed, DI-ALH rarely require long-term immunosuppression whereas patients with autoimmune hepatitis mostly require long-term immunosuppression due to the risk of developing chronic hepatitis and cirrhosis. Adopting this nomenclature would remark the transitory effect of the hepatitis after GT and reinforce the importance of discussing a tailored approach for treating patients with ALT over the range of normality for safety and efficacy purposes.

The information stemming from our experience has some limitations. First, the match between liver biopsy, electron microscopy analysis and immunophenotype was made just on a single case of marked ALT elevation after GT, thus precluding generalization of the results. However, the comparison of the histological and cytological data with our second consecutive case of immune hepatitis after GT may in part overcome this limitation, although it is restricted to only two biopsy-proven cases from a single-centre experience, making our work necessarily exploratory and hypothesis-generating. In addition, the rarity of the congenital bleeding disorder and the low number of patients treated up to now with GT outside the pivotal trials enhance the clinical importance of the present analysis.[25] Furthermore and importantly, our findings demonstrate that investigations based on transjugular liver biopsy from people with hemophilia are affordable, safe and able to bring useful knowledge contribution. The adoption of the transjugular approach for liver biopsy is, in expert hands, the most advisable technique to conduct these studies in people at risk for procedure-related bleeding complications, as already demonstrated in other clinical settings.[26] A drawback is the reduced length and thickness of the liver sample and its fragmentation, but this limitation can be in part overcome by two- three serial passages of the needle in liver parenchyma.[26,27] In the present study, the adoption of positive and negative comparison cases for the findings of subcellular damage and immunophenotype

characterization represents an attempt to demonstrate the causality of the immune response despite the risk of a selection bias for controls, as TEM analysis is not routinely performed. We finally diagnosed an acute immune-mediated hepatitis, allowing us to consider a tailored approach for immune suppression. This approach led to a full biochemical response to corticosteroids, a stable liver stiffness measurement by Fibroscan®, at least for the index case, for more than 6 months after GT. Nevertheless, considering the limited one-year observation time after the dosing regimen for the index case with biopsy-proven immune-mediated hepatitis, long-term data are mandatory to draw safety conclusions. Indeed, the development of advanced liver fibrosis generally occurs decades after exposure to any trigger. In this respect, a study including 5 liver biopsies carried out years 2.7-4.1 after valoctogene roxaparvovec dosing detected no long-term adverse effects on liver histology[28] and this has been recently confirmed for the liver biopsy of a single case of hemophilia B treated with etranacogene roxaparvovec, another AAV-5 based GT.[29]

In conclusion, this is the first characterization of a biopsy proven immune-mediated hepatitis early after valoctogene roxaparvovec administration in two patients with severe HA successfully treated with oral prednisone. The histological and cytological alterations observed at the time of ALT elevation, along with the immunophenotypic characterization before and after achieving biochemical enzymatic response, confirm that immune suppression with corticosteroids is a valid and appropriate therapeutical tool in this clinical setting. The high variability of transgene expression, together with the favourable safety and efficacy profile observed in the two comparison cases who did not received prednisone, owing to more modest ALT elevation, suggest that the general strategy employed to manage ALT elevation may warrant revision as we propose in Figure 7. Accordingly, the use of immune suppression only when ALT is clearly above the normal laboratory range and/or associated with loss of transgene expression seems to be the

most advisable schedule of treatment. Moreover, a personalized corticosteroid therapy could avoid the loss of transgene expression at this interval of ALT increase.

ABBREVIATIONS

AAV: Adeno-associated virus

AIF: Agenzia Italiana del Farmaco

ALT: alanine aminotransferase

AAV5-hFVIII-SQ: B-domain-deleted human FVIII transgene

cTFh: circulating follicular T helper cells

iNKT: Circulating invariant NK T-cells

CTCA: Common Terminology Criteria for Adverse Events

EM: effector memory

EMRA: effector memory RA

FVIII: Factor VIII

GT: Gene therapy

HA: Hemophiia A

PBS: phosphate- buffered saline

Treg: regulatory T-cells

RER: rough endoplasmic reticulum

SER: smooth endoplasmic reticulum

Th1: T helper 1

Th17: T helper 17

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Table 1: hepatological and hematological characteristics of patients included in the study at baseline and at the end of the follow up

	Case 1 *Index case*	**Case 2**	**Case 3**	**Case 4**	**Case 5**
Age at time of GT (years)	24	38	48	38	46
BMI before GT (Kg/m^2)	29.4	24.6	22.2	18.8	22.9
steatosis at liver ultrasound before GT (yes/no)	yes	no	yes	no	no
Prior history of viral hepatitis (HCV/HBV)	no	yes	yes	yes	yes
liver stiffness before GT (KPa)	4.8	4.9	5.2	4.0	3.5
CAP (dB/m)	326	205	283	205	165
baseline ALT value (IU/L)	20	27	19	13	27
ALT above 1.5X the baseline value (yes/no)	yes	yes	yes	yes	no
ALT above the normal range (yes/no)*	yes	no	no	yes	no
Maximum ALT level (IU/L) during the follow-up	633	51	49	174	37
Week of detection of the ALT peak after dosing	10	15	11	9	9
Liver biopsy after GT (yes/no)	yes	no	no	yes	no
Prednisone after GT:					
• yes/no	yes	no	no	yes	no
• starting week after infusion of GT	10	---	---	9	---
• number of weeks of treatment at the end-of the follow-up	50 *(withdrawn)*	---	---	13 *(ongoing)*	---
FVIII expression at the end of the follow-up (% of activity-chromogenic assay)	12	206	20	83	194
Weeks of follow-up	67	51	45	22	21

* upper limit of normal range for our laboratory: 59 IU/L (all measurements were performed at our hub center)BMI: body mass index; CAP: Controlled Attenuation Parameter, GT: gene therapy; FVIII: factor VIII

FIGURE LEGENDS

Figure 1: flow chart of the comparative study centred on index case data. AIH: autoimmune hepatitis. PBC: primary biliary cholangitis. DILI: drug induced liver injury. HA: hemophilia A.

Figure 2: alanine transferase **(**ALT), factor VIII (FVIII) levels (chromogenic and one-stage assay) and prednisone dose over time in the patient who experienced an ALT increase over the normal range after gene therapy for hemophilia A (index case).

Figure 3: histopathology of the liver in two consecutive patients with severe HA with ALT elevation after gene-therapy, first case (panels A-D), second case (panels E-H). Panel A: overview of liver architecture. Panels B-C: portal spaces with mild lympho-plasmacellular infiltrate with focal interface necrosis (arrows). Panel D: focal intralobular spotty necrosis with clusters of plasma-cells in portal space highlighted by MUM1 immunostaining. Panel E-G: mild interface hepatitis in the lobule and portal spaces. Panel H: few clusters of plasma cells at immunohistochemistry analysis. All images were acquired by 200X fold increase by using DMD108 Digital Micro-Imaging Device, Leica Microsystem (Microcope objective HCX PL Fluotar 20x/0.50, /0.17/D).

Figure 4: alanine transferase (ALT), factor VIII (FVIII) levels (chromogenic and one-stage assay) and prednisone dose over time in the second consecutive case of a patient who experienced an ALT increase over the normal range after gene therapy for hemophilia A (index case).

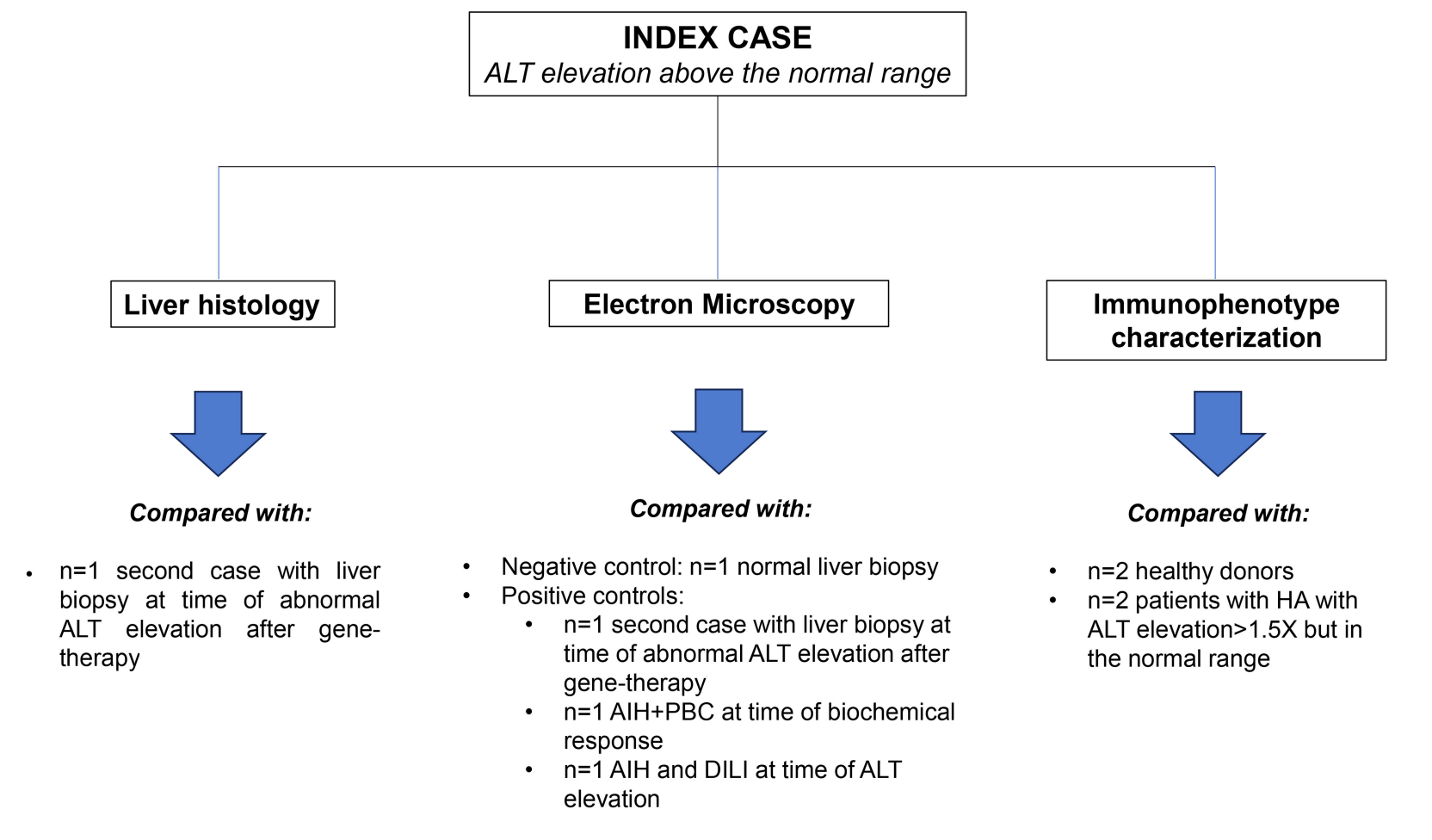
Figure 5: substructural hepatocyte abnormalities explored by transmission electron microscopy. Panels A-C: patient with severe HA with abnormal elevation of alanine aminotransferase after gene therapy (index case). Panel A: representative hepatocytes exhibiting normal ultrastructural features, with no evident alterations in mitochondria, peroxisomes or nuclear morphology (4000X magnification). Panels B-C: hepatocytes showing rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER) morphology (black arrows). In panel B, a marker proliferation of SER is evident, extensively distributed through the cytoplasm, while RER is not significantly increased and shows occasional dilatation (10000X magnification). In panel C, severe SER alterations are observed, with the cytoplasm almost entirely occupied by dilated membranous structures (2000X magnification). Panels D-F: hepatocytes from a second consecutive patient with severe HA and abnormal elevation of alanine aminotransferase after gene therapy, showing similar ultrastructural features. Panel D: representative hepatocytes exhibiting normal ultrastructural features, with no evident alterations in mitochondria, peroxisomes or nuclear morphology (4000X magnification). Panels E-F: RER and SER morphology (black arrows) with marked SER proliferation and dilatation (10000X and 2000X magnification, respectively). Panel G: normal hepatocytes from control no.1 (no liver disease-4000X magnification). Panel H: normal hepatocytes from control no.2 (autoimmune hepatitis after Peg-interferon therapy for chronic hepatitis B-4000X magnification). Panel I: SER abnormalities in control no.3 (portal biliary cholangitis/autoimmune hepatitis and pharmacological induction by triptans-4000X magnification) showing a milder proliferation of SER, organized in isolated clusters. In the inset, the re-arrangement of SER cisternae in hepatocytes was highlighted at 10000X magnification. All images were acquired HITACHI microscope (model HT7800 120kV), TEM CMOS camera (XAROSA 20 Megapixel). N: nucleus; M: mitochondria; RER: rough endoplasmic reticulum; SER: smooth endoplasmic reticulum (Panel A-I). BIP, XBP1 and

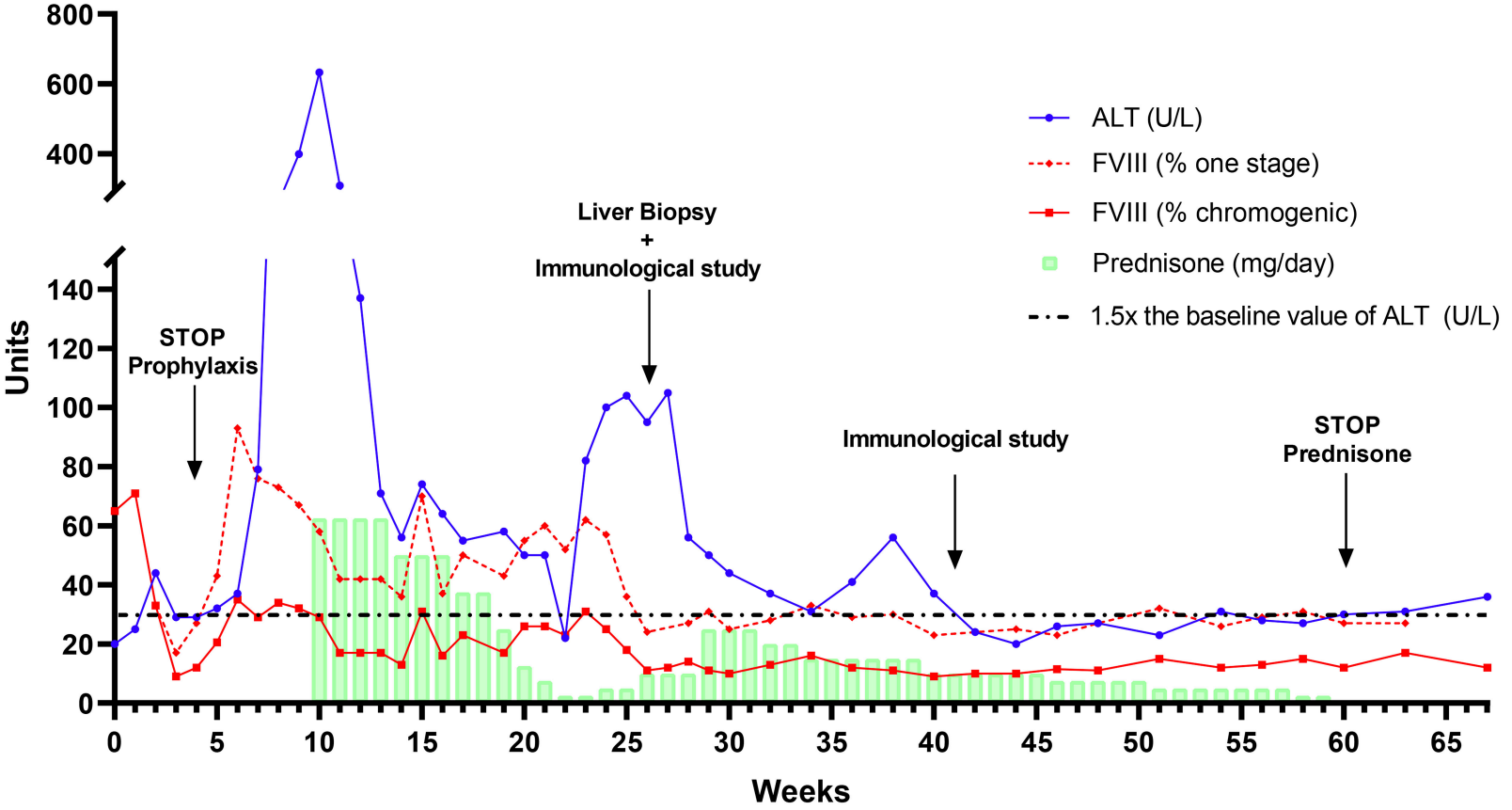
CHOP mRNA levels were assessed by qRT-PCR in liver biopsies of patients who underwent gene therapy (GT) and of control n.1. Data was normalized to GAPDH housekeeping gene and results were expressed as fold increase arbitrary units (AU) ± standard deviation (SD) (Panel J-L).

Figure 6: analysis of CD4 (panels A-D) and CD8 (panels E-F) T-cell compartment**.** Panel A: frequency of total CD4 T-cells. Panel B: CD39PD-1 positive CD4 T cells frequency. Panel C: frequency of CD4 T cells regulatory (Treg). Panel D: circulating follicular T helper cells (cTFh) subpopulations. Panel E: CD8 T-cell frequency. Panel F: CD8 T-cells producing granzyme.

HD: blood from peripheral vein of two heathy donors (bar represents mean and standard error); HA index case: patients with hemophilia A who had ALT elevation over the range of normality after gene therapy (HV-ALT+: blood from the hepatic vein at time of ALT elevation, PV-ALT+: blood from peripheral vein at time of ALT elevation, PV-ALT - : blood from peripheral vein at time of ALT normalization); HA-ALT normal: blood from peripheral vein of two subjects with hemophilia A who had ALT in the normal range over the follow-up after gene therapy (bar represents mean and standard error).

Figure 7: potential new algorithm to manage alanine transferase (ALT) elevation in patients after AAV-5 based gene-therapy for hemophilia A.


INDEX CASE
ALT elevation above the normal range
Liver histology
Electron Microscopy
Immunophenotype characterization
Compared with:
n=1 second case with liver biopsy at time of abnormal ALT elevation after gene-therapy
Compared with:
Negative control: n=1 normal liver biopsy
Positive controls:
n=1 second case with liver biopsy at time of abnormal ALT elevation after gene-therapy
n=1 AIH+PBC at time of biochemical response
n=1 AIH and DILI at time of ALT elevation
Compared with:
n=2 healthy donors
n=2 patients with HA with ALT elevation>1.5X but in the normal range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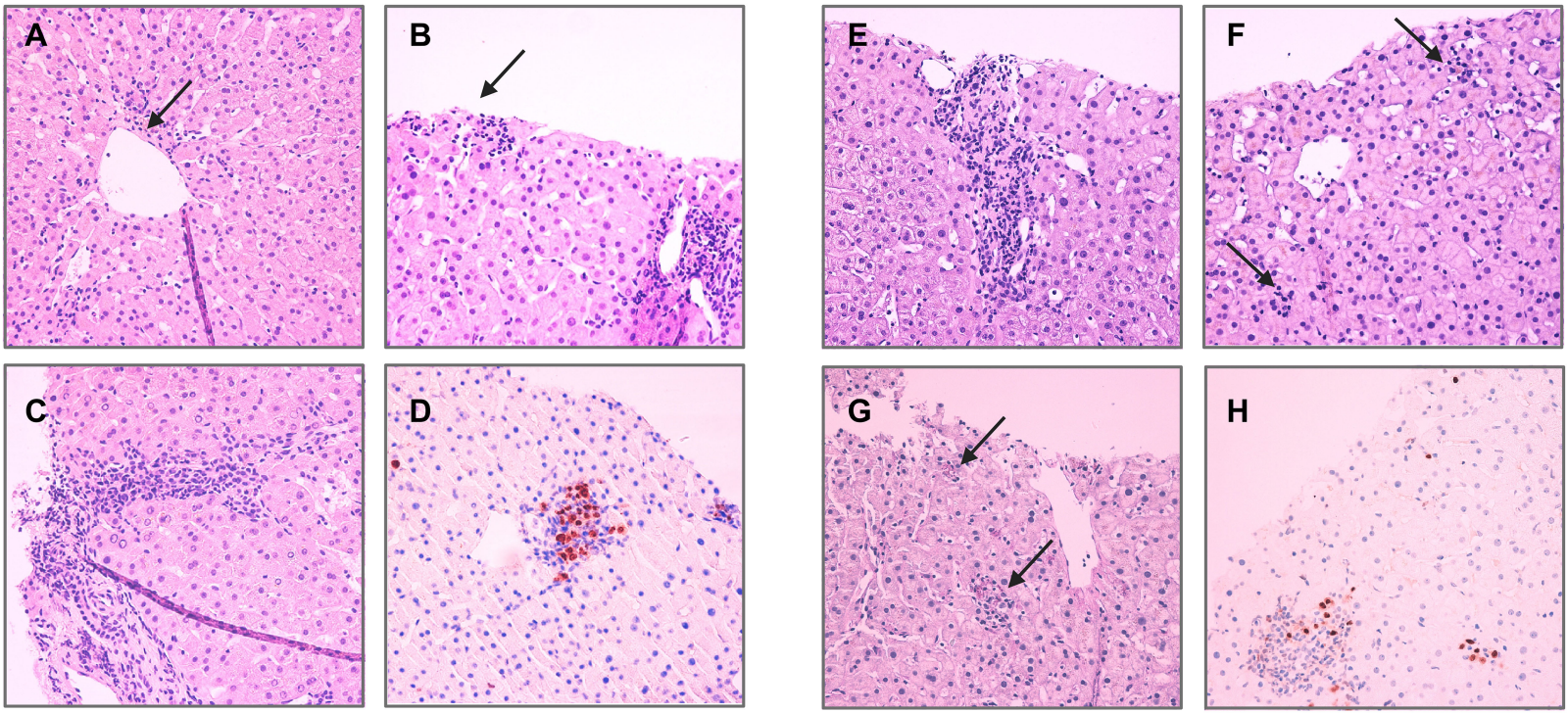
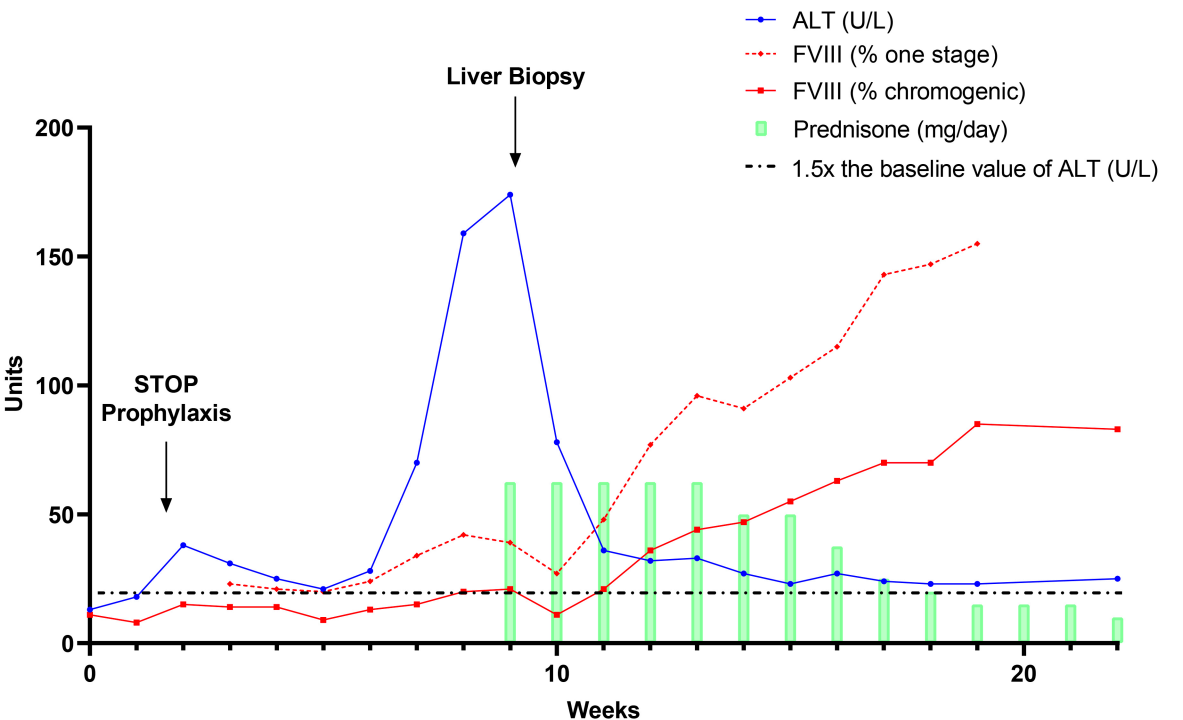
40
45
50
55
60
65
Weeks
STOP
Prophylaxis
Liver Biopsy
+
Immunological study
Immunological study
STOP
Prednisone
ALT (U/L)
FVIII (% one stage)
FVIII (% chromogenic)
Prednisone (mg/day)
1.5x the baseline value of ALT (U/L)


Index case
Second case
A
B
C
D
E
F
G
H


ALT (U/L)
FVIII (% one stage)
FVIII (% chromogenic)
Prednisone (mg/day)
1.5x the baseline value of ALT (U/L)
Liver Biopsy
STOP
Prophylaxis
Units
200
150
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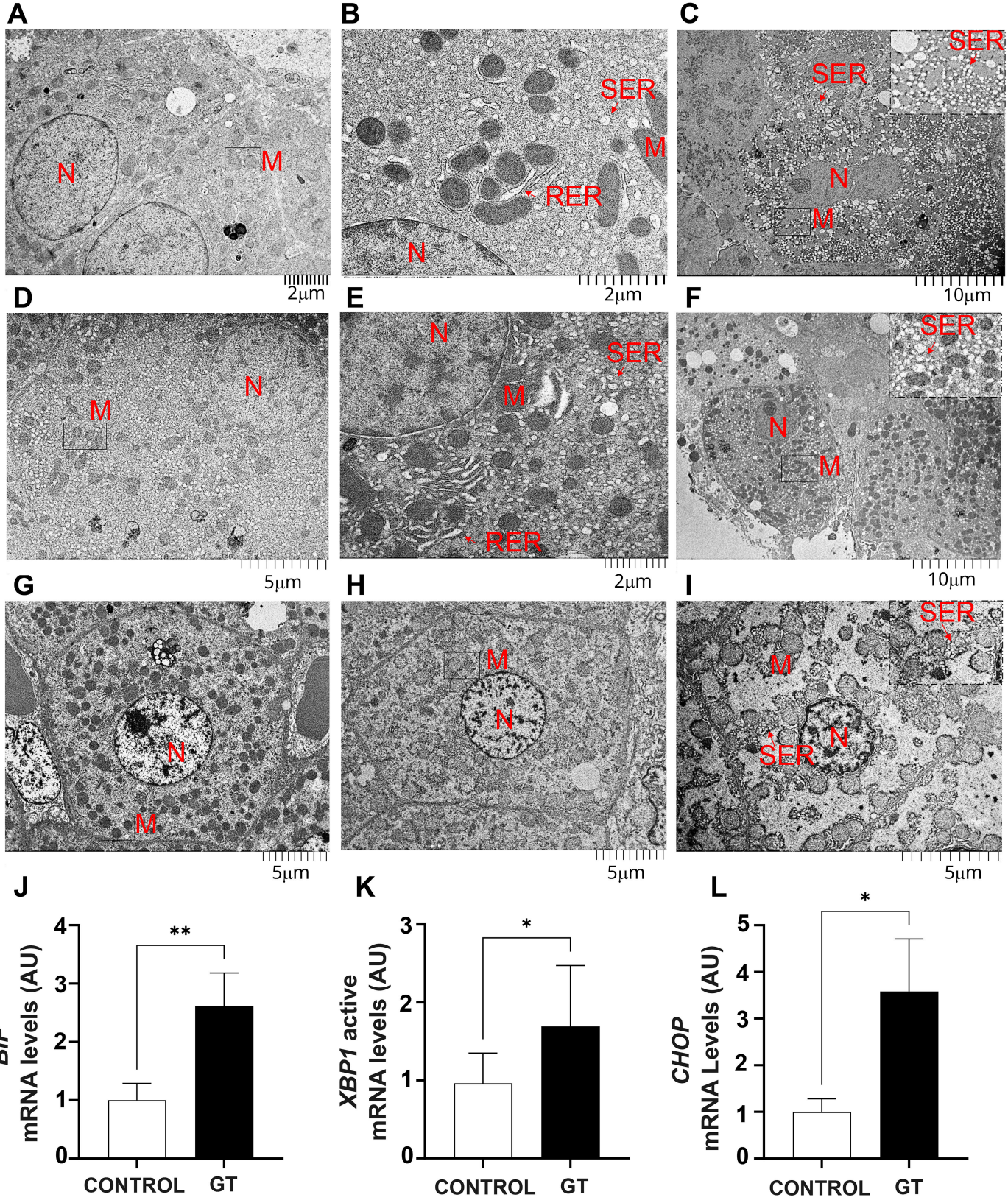
Weeks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mRNA Levels (AU)
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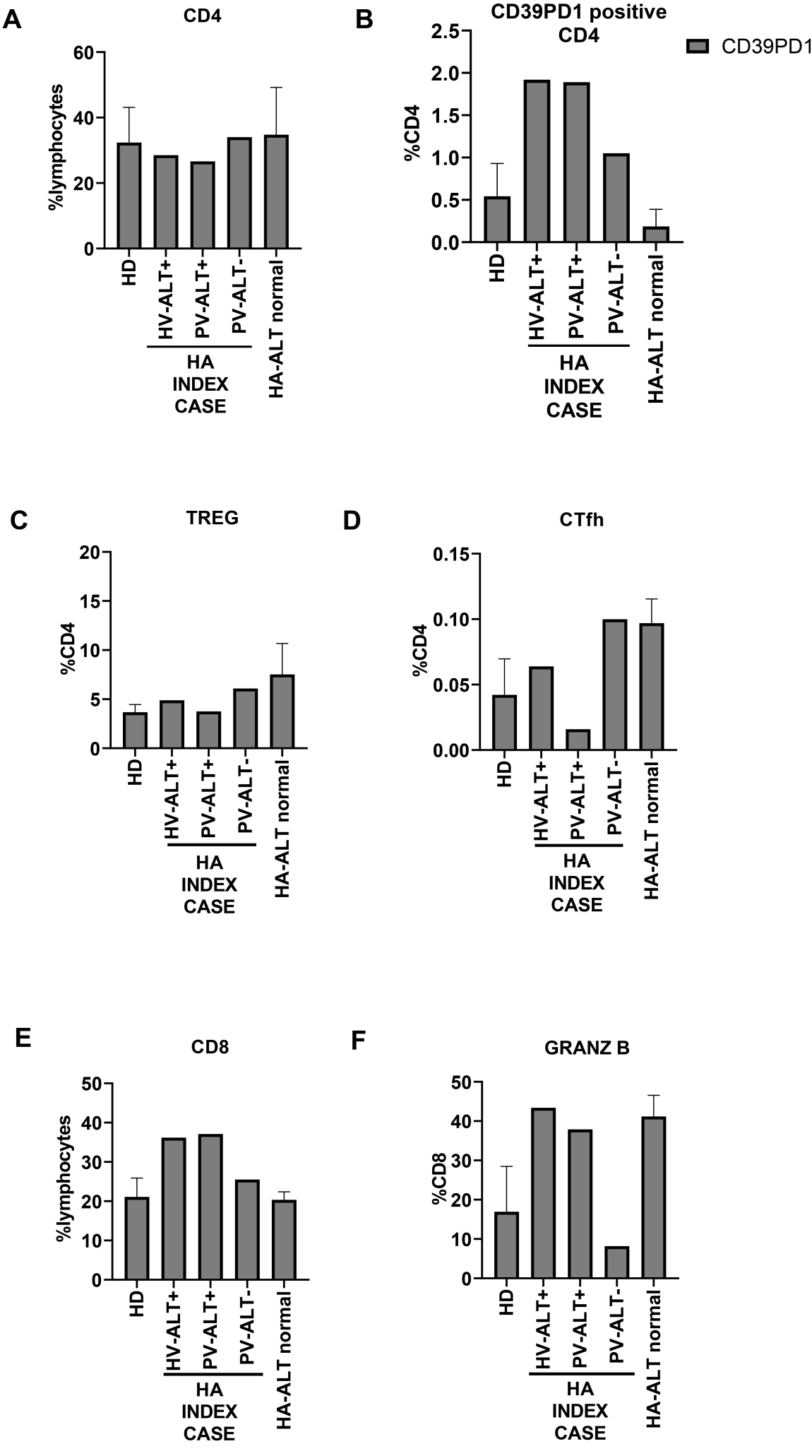
CONTROL
GT


A
CD4
%lymphocytes
60
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HD
HV-ALT+
PV-ALT+
PV-ALT-
HA INDEX CASE
HA-ALT normal
B
CD39PD1 positive CD4
CD39PD1
%CD4
2.5
2.0
1.5
1.0
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HD
HV-ALT+
PV-ALT+
PV-ALT-
HA INDEX CASE
HA-ALT normal
C
TREG
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HV-ALT+
PV-ALT+
PV-ALT-
HA INDEX CASE
HA-ALT normal
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HD
HV-ALT+
PV-ALT+
PV-ALT-
HA INDEX CASE
HA-ALT normal
E
CD8
%lymphocytes
50
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HD
HV-ALT+
PV-ALT+
PV-ALT-
HA INDEX CASE
HA-ALT normal
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GRANZ B
%CD8
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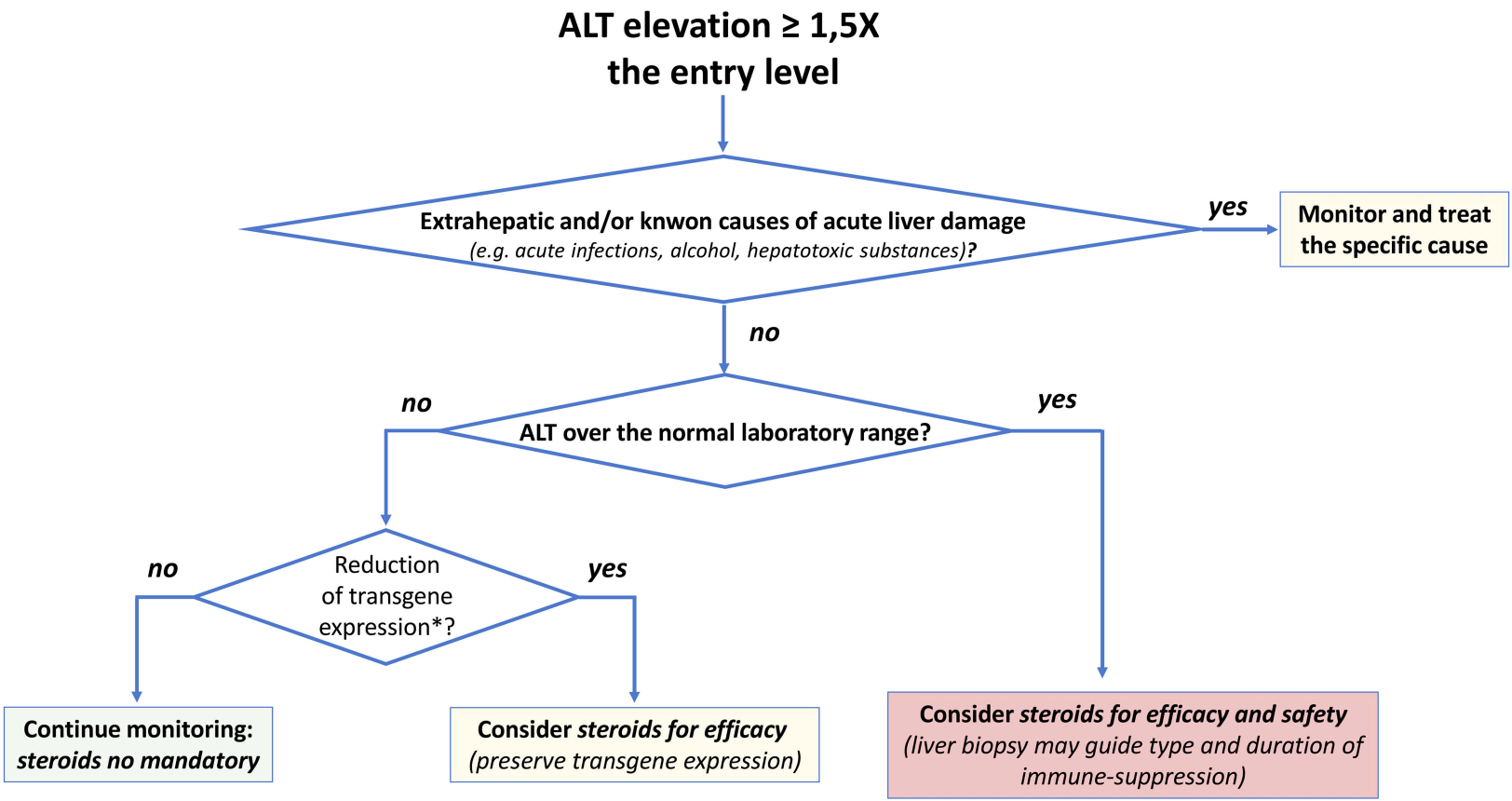
20
10
0
HD
HV-ALT+
PV-ALT+
PV-ALT-
HA INDEX CASE
HA-ALT normal



*chromogenic method for factor VIII

TITLE: Biopsy-proven immune-mediated hepatitis after valoctocogene roxaparvovec

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SUPPLEMENTARY MATERIALS

SUPPLEMENTARY METHODS

Histopathology of the liver

Four-micron sections were obtained from routinely fixed-paraffin embedded tissue. Hepatitis was graded and staged according to the Ishak score system.[1] Immunostaining for MUM1 (EP190) was automatically performed on BenchMark Ultra Immunostainer (Ventana, Roche).

Transmission electron microscopy (TEM)

Liver tissues were fixed in 2.5% glutaraldehyde (pH 7.4) (Electron Microscopy Sciences EMS, Hatfield, PA, USA) for 1 h at room temperature and overnight at 4 °C. The specimens were washed in 0.1 M, pH 7.4 cacodylate buffer (EMS), post-fixed for 1 h in 2% osmium tetroxide (EMS) and dehydrated using a graded series of ethyl alcohol. Finally, ultrathin sections (80 nm thick slices) were prepared using an ultramicrotome Power Tome XL (RMC, Tucson, AZ, USA). The grids, stained with 0.5% lead citrate (EMS) and uranyl acetate replacement/methanol 1:1 were examined with Hitachi HT7800 transmission electron microscope (Hitachi, Japan).

Immunophenotype analysis

Peripheral blood mononuclear cells from patients and healthy donors were isolated by density- gradient centrifugation following the Ficoll-paque plus standard protocol. Sample containing 100.000 cells was washed in phosphate- buffered saline (PBS) 1x and stained for 15 minutes at room temperature with Fixable Viability Stain 780 (BD Horizon, cat. no. 565388) diluted 1:2000 in PBS in the dark and then washed in magnetic-activated cell

sorting buffer. For surface marker detection, cells were stained in Brilliant Stain Buffer (BD Horizon, cat. no. 566349) diluted 1:2 in PBS supplemented with antibodies for 30 min at room temperature in the dark. Cells were then washed in PBS and fixed for 15 min at 4°C using eBioscience FOXP3 staining kit according to the manufacturer protocol (eBioscience, cat. no. 00-5523). To detect intracellular proteins cells were permeabilized and stained in Permeabilization reagent (eBioscience, cat. no. 00-833) supplemented with antibodies for 30 min at 4°C. Samples were acquired on a BD FACSymphony A5 flow cytometer (BD Biosciences) equipped with 5 lasers (UV, 350 nm; violet, 405 nm; blue, 488; yellow/green, 561 nm; red, 640 nm**.** The antibodies used for immunophenotype analyses are below reported.

List of antibodies used for immunophenotypic analyses.

Antibody target (eventual alias in brackets)	**Fluorochrome**	**Clone**	**Company**	**Catalog #**
CD127	BB700	HIL-7R-M21	BD	566398
CD137 (41BB)	BUV615	4B4-1 LIGAND	BD	751492
CD183 (CXCR3)	PECY7	1C6/CXCR3	BD	560831
CD185 (CXCR5)	APC-CY7	J252D4	BioLegend	356926
CD19	APC-Vio 770	LT19	Miltenyi	130-128-022
CD195 (CCR5)	FITC	HEK/1/85a	BioLegend	313705
CD196 (CCR6)	APCR700	11A9	BD	565173
CD197 (CCR7)	BV711	150503	BD	566602
CD21	PECY5	B-ly4	BD	551064
CD138	Pe-CF594	MI15	BD	564606
CD25	Pecy5	M-A251	BD	555433
CD27	BUV737	L128	BD	564301
CD27	Vio Bright FITC	M-T271	Miltenyi	130-128-022
CD279 (PD1)	BV650	EH12.1	BD	564104
CD3	BUV 805	UCHT1	BD	612895
CD4	BUV 395	SK3	BD	563550
CD4	BUV737	SK3	BD	612748
CD40L	PECY5	cl.24-31	BioLegend	310808
CD45RA	BUV496	HI100	BD	750258
CD45RO	BV605	UCHL1	BD	562791

CD69	BV650	FN50	BD	563835
CD8	BUV 563	RPA-T8	BD	612914
FOXP3	PE-CF594	259D/C7	BD	562421
GRANZYME B	BV421	GB 11	BD	563389
GRANZYME K	Alexa Fluor647	G3H69	BD	566655
IgD	BV480	IA6-2	BD	566138
IgG	VioBlue	IS11-3B2.2.3	Miltenyi	130-128-022
IgM	APC	PJ2-22H3	Miltenyi	130-128-022
LIVE AND DEAD	FIX VIAB 780	-	BD	565388
LIVE AND DEAD	Aqua	-	thermofisher	L34957

SUPPLEMENTARY TABLES

Supplementary Table 1: clinical and laboratory issues of controls included for the comparative analysis of transmission electron microscopy (TEM) data: demographic, anthropometric, and clinical features of the patients with suspected metabolic disease (Control 1) or autoimmune hepatitis (Control 2, Control 3) (see the details of the clinical history below).

	Control no.1*	**Control no.2***	**Control no.3***
Sex	Male	Male	Female
Age, years	46	56	61
BMI, kg/m2	28.37	24.8	21.26
IFG/T2D	no	no	no
Glucose, mg/dL	82	93	/
Total cholesterol, mg/dL	194	148	212
non-HDL cholesterol, mg/dL	144	108	116
HDL cholesterol, mg/dL	50	40	81
Triglycerides, mg/dL	134	70	74
Total Bilirubin, mg/dL	1.05	0.62	0.9
Creatinin, mg/dL	0.96	0.71	0.88
Alcaline phosphatase (U/L)	45	130	150
Albumin (g/dL)	4.65	4.6	4.2
Alanine transferase, IU/l	22	52	67
Aspartate transferase, IU/l	26	41	60
Gamma-glutamyltransferase, IU/l	27	64	156
White blood cells, 10^9/L	7.59	6.36	6.3
Haemoglobin, g/dL	15	14.6	13.1
Platelets, 10^9/L	259	187	195

BMI: body mass index; IFG: impaired fasting glucose; T2D: type 2 diabetes

Clinical data on controls included in the TEM analysis:

Control no.1: The patient was followed for severe hyperferritinemia (760 mg/mL) and increased transferrin saturation since 2019. At liver biopsy (2021), iron overload was not markedly detected, and hepatic parenchyma showed a preserved lobular structure. For the absence of hypertransaminasemia, metabolic etiology and any other cause of chronic liver damage, the definitive diagnosis was of normal liver. The patient was enrolled in this study as negative control.

Control no.2: The patient was followed for B virus hepatitis (HBV) infection from 2014. In 2015, he received antiviral treatment with pegylated-interferon, which was interrupted and replaced with tenofovir after the fifth week for hypertransaminasemia and rise of total bilirubin level. At that time liver biopsy confirmed the presence of regenerated nodules associated with the chronic HBV infection and plasma cells, likely suggestive of autoimmune hepatitis (AIH) triggered by Peginterferon therapy. The patient was enrolled in this study as positive control.

Control no.3: The patient was recruited at liver unit where she was followed for history of hypertransaminasemia, elevated cholestatic index and mild hypercholesterolemia. At the time of visit, she reported suffering from headaches, for which he had recently started the treatment with triptans. At that time, she had a rise in transaminases and the liver biopsy was proposed. The histopathological evaluation ruled out the presence of fibrosis, while it highlighted signs of hepatic enzyme induction, potentially ascribable to triptans. Moreover, focal areas consistent with both cholestatic disorder at initial stage and immune-mediated etiology were also detected, suggesting an overlap of primitive biliary cholangitis (PBC) and autoimmune hepatitis (AIH) in the context of a drug induced liver injury (DILI).

Supplementary Table 2: Measurements of FVIII and ALT at any time point

		baseline	**Weeks after dose**											
#case	**variable**		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1 *(index case)*	FVIII (%) chromogenic	65	71	23	9	12	20,5	35	29	34	32	29	17	17	17
	FVIII (%) one stage		33	24	17	27	43	93	67	72	67	58	42	44	42
	ALT (U/L)	20	25	44	29	29	32	37	79	258	399	633	309	137	71
2	FVIII (%) chromogenic		13	8	10	9	15	28	31	43	82	61	114	96	170
	FVIII (%) one stage		15	15	20	20	33	48	63	89	148	138	208	217	348
	ALT (U/L)	27	22	16	20	16	13	12	8	13	15	15	14	23	40
3	FVIII (%) chromogenic	0	0	3	3	5	8	10	9	10	12	16	20	22	18
	FVIII (%) one stage														
	ALT (U/L)	19	12	21	21	18	18	21	18	21	19	21	49	46	37
4	FVIII (%) chromogenic	11	8	15	14	14	9	13	15	20	21	11	21	36	44
	FVIII (%) one stage		8	15	23	21	20	24	34	42	39	27	48	77	96
	ALT (U/L)	13	18	38	31	25	21	28	70	159	174	78	36	32	33
5	FVIII (%) chromogenic	53	9	17	8	21	29	42	69	88	109	150	142	213	238
	FVIII (%) one stage		13	19	17	37	58	79	106	155	171	203	280	337	382
	ALT (U/L)	27	20	16	18	16	18	21	21	33	37	29	25	24	24

		baseline	Weeks after dose											
#case	variable		**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**
1 *(index case)*	FVIII (%) chromogenic		13	31	16	23		17	26	26	23	31	25	18	11
	FVIII (%) one stage		28	70	37	50		43	55	60	52	62	57	36	24
	ALT (U/L)		56	74	64	55		58	50	50	22	82	100	104	95
2	FVIII (%) chromogenic		179	164	176	181	156	180	209	213	217	77	256	123	234
	FVIII (%) one stage		358	322	265	336	293	308	311	341	328	128	445	295	365
	ALT (U/L)		48	51	49	48	47	50	42	42	46	40	47	35	29
3	FVIII (%) chromogenic		17	15	18	13	12	23	19	22	17	18	20	21	28
	FVIII (%) one stage														
	ALT (U/L)		33	41	39	35	32	37	30	27	25	28	23	22	24
4	FVIII (%) chromogenic		47	55	63	70	70	85			83	end			
	FVIII (%) one stage		91	103	115	143	147	155			157	end			
	ALT (U/L)		27	23	27	24	23	23			25	end			
5	FVIII (%) chromogenic		253	192	207	230	232	215		194	end				
	FVIII (%) one stage		399	335	349	328	382	342		276	end				
	ALT (U/L)		28	31	34	30	28	23		29	end				

		baseline	**Weeks after dose**											
#case	variable		**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**
1 *(index case)*	FVIII (%) chromogenic		12	14	11		10		13		16		12		11
	FVIII (%) one stage		24	27	31		25		28		33		29		30
	ALT (U/L)		105	56	50		44		37		31		41		56
2	FVIII (%) chromogenic		197		341	288		260		470		189		234	
	FVIII (%) one stage		312		541	502		313		593		280		349	
	ALT (U/L)		27		22	24		24		39		17		20	
3	FVIII (%) chromogenic			20		17		16		25				20	
	FVIII (%) one stage													45	
	ALT (U/L)			20		18		19		20				20	
		baseline	**Weeks after dose**											
#case	Variable		**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**	**51**	**52**
1 *(index case)*	FVIII (%) chromogenic			9		10		10		11,5		11		15	
	FVIII (%) one stage			23		26		25		23		27		32	
	ALT (U/L)			37		24		20		26		27		23	
2	FVIII (%) chromogenic		190		205				243			223		206	end
	FVIII (%) one stage		305		333				374			356		381	end
	ALT (U/L)		18		22				20			22		21	end
3	FVIII (%) chromogenic			25				20	end						
	FVIII (%) one stage			54				39	end						
	ALT (U/L)			21				20	end						

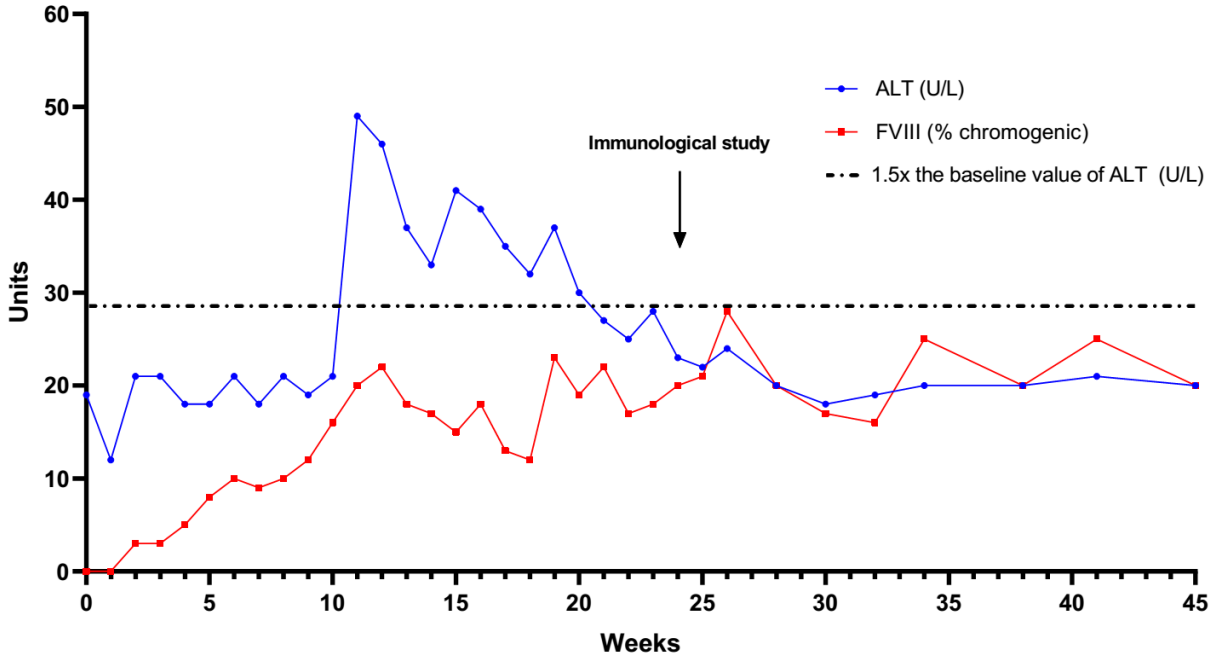
#case	variable		**54**	**56**	**58**	**60**	**63**	**67**	
1 *(index case)*	FVIII (%) chromogenic		12	13	15	12	17	12	end
	FVIII (%) one stage		26	29	31	27	27	27	end
	ALT (U/L)		31	28	27	30	31	36	end

Supplementary Table 3 – Viral and autoimmune markers assessed during ALT elevation after GT to evaluate alternative causes of liver damage

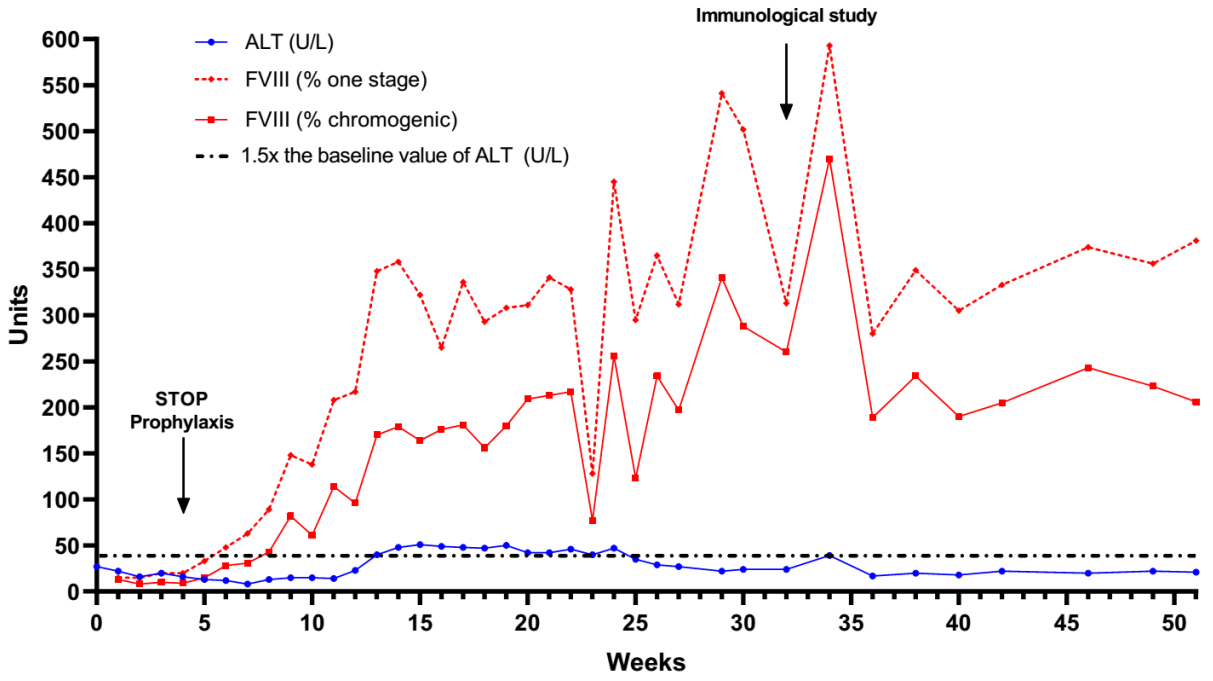
	Case 1 (index case)	Case 4	Reference Range
Autoimmunity			
Immunoglobulin A	170	145	70 - 400 mg/dL
Immunoglobulin G	947	1373	700 - 1600 mg/dL
Immunoglobulin M	79	62	40 - 230 mg/dL
Anti-smooth muscle antibodies	Negative	Negative	<1/40
Anti-nuclear antibodies	Negative	Negative	<1/80
Anti-mitochondrial antibodies	Negative	Negative	<1/40
Anti-Liver Kidney Microsomal antibodies	Negative	Negative	<1/40
Anti-neutrophil cytoplasmic antibodies	Negative	Negative	<1/20
Viral markers			
Hepatitis B Virus (HBV) surface Antigen (HBsAg)	Negative	Negative	Negative
Antibody against HBsAg (HBsAb)	2.0	743	> 10 IU/L
Antibody against HBV core antigen (HBcAb)	Negative	Negative	Negative
HBcAb IgM	0.06	0.05	< 1.0 Index
Antibody against hepatitis A virus (HAV Ab) Total	Positive	Negative	Negative
HAV Ab IgM	Negative	Negative	Negative
Antibody against Hepatitis C Virus (HCVAb)	0.04	102	< 1.0 S/CO
Cytomegalovirus (CMV) DNA	Not detected	N.A.	< 109 UI/mL
Antibody against CMV IgG	62	5	-
Antibody against CMV IgM	5	5	-
Antibody against herpes simplex virus (HSV) 1/2 IgG	<0.9	<0.9	0.90 - 1.10 Index
Antibody against herpes simplex virus (HSV) 1/2 IgM	<0.9	<0.9	<0.9 Index
Parvovirus DNA	Not detected	N.A.	<250 IU/L
Varicella IgG	702	1543	Not applicable
Varicella IgM	<0.9	<0.9	<0.9 Index
Antigen/Antibody against human immunodeficiency virus (HIV) 1/2	Negative	Negative	< 1.0 S/CO

SUPPLEMENTARY FIGURE

A)



B)



Supplementary Figure 1: alanine transferase **(**ALT), factor VIII (FVIII) levels (chromogenic and one-stage assay) over time in the two patients who had ALT persistently in the normal range after gene therapy for hemophilia A.

REFERENCES

1. Ishak K, Baptista A, Bianchi L, et al. Histological grading and staging of chronic hepatitis. Journal of Hepatology 1995;22(6):696–699.