haematologica
Journal of the Ferrata Storti Foundation

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Received: December 16, 2024.
Accepted: February 25, 2025.

Citation: Jane Koo, Alana J. Snyder, Kathleen Campbell, Erica Miller, Allison O'Conner, Markus Grompe, Parinda A. Mehta, Kasiani C. Myers and Stella M. Davies. Hepatocyte senescence and persistent liver injury in Fanconi anemia. Haematologica. 2025 Mar 3. doi: 10.3324/haematol.2024.287096 [Epub ahead of print]

Hepatocyte senescence and persistent liver injury in Fanconi anemia

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Authors' Contributions: JK and SMD performed data analysis, wrote the manuscript and revised the manuscript. AJS, EM and AO performed data collection and revised the manuscript. MG, PAM, and KCM reviewed and revised the manuscript. All authors approved the final manuscript in its current form.
Running head: Cellular senescence in FA liver injury

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Disclosures: None

Data sharing statement: Requests for data can be submitted to jane.koo@cchmc.org

Letter to the Editor:

Fanconi anemia (FA) is the most common inherited bone marrow failure syndrome, characterized by bone marrow failure, congenital anomalies and cancer predisposition[1]. The only cure for progressive bone marrow failure with pancytopenia is hematopoietic stem cell transplantation (HSCT)[2]. More individuals with FA are surviving into adulthood so additional phenotypes of FA are becoming recognized and are beginning to be characterized. One of these manifestations is chronic liver damage[3,4]. We previously described a high frequency (32%) of persistent liver injury (PLI), i.e. prolonged transaminitis without elevation of bilirubin and no hepatic structural abnormalities in a large cohort of patients with FA[5]. Interestingly, we identified a high frequency of liver injury in FA patients irrespective of transplantation status, although multivariate analysis did identify total-body irradiation-based transplant preparative regimens, but not prior treatment with androgens, as a risk factor for PLI. Limited data from this cohort suggested no improvement in transaminitis with medications trialed, including corticosteroids, immune suppressive medications or ursodeoxycholic acid. The mechanism behind PLI in individuals with FA has not previously been described.

Oxidative stress is an important factor in the pathogenesis of bone marrow failure, leukemia progression and other manifestations observed in FA[6]. Cellular senescence is an adaptive, time-limited response to acute cellular stresses, including oxidative damage. Senescent cells are arrested in the G1/S cell cycle transition, displaying a G1 DNA content and upregulated checkpoint inhibitors like p21 and p16[7]. Chronic senescence leads to accumulation of senescent cells in tissues, promoting a senescence-associated secretory phenotype (SASP). SASP induces a pro-inflammatory environment through cytokine, chemokine, and growth factor production, which contribute to tissue remodeling and fibrosis[8]. Chronic SASP activation is implicated in liver diseases such as steatosis, fibrosis, and hepatocellular carcinoma[9]. We hypothesized cellular senescence plays a significant role in liver injury in FA, contributing to fibrosis and cholestasis which appear irreversible and can terminate in liver failure.

We performed immunohistochemical analysis on available liver biopsy samples for markers of hepatocyte proliferation and cellular senescence in three patients diagnosed with PLI and three FA controls without PLI

Information regarding FA controls from the pathology department was limited to anonymized data, with no access to patient-identifying information, including transplant status. All available FA controls from the research pathology core were utilized for comparison. All tissue samples were stained with proliferating cell nuclear antigen (PCNA), CD68 (marker of Kupffer cells, liver tissue-resident macrophages), cyclin-dependent kinase inhibitor 1 (p21), cyclin-dependent kinase inhibitor 2A (p16) and cytokeratin 7 (CK7). This study was conducted in accordance with all applicable ethical guidelines and regulations of the United States where the research took place.

Figure 1 summarizes the clinical course of PLI for each patient. Table 1 further details the demographic and transplant characteristics of all patients in the cohort. All three patients had protracted courses of PLI persisting for 14-18 years. The median time of PLI presentation was 9 years of age (range 1-11 years), but one patient was identified with PLI during infancy at one year of age. Transaminitis is asymptomatic so the duration of PLI may be longer in patients not getting regular laboratory testing. All three patients had PLI onset prior to receiving allogeneic HSCT, identified between 0.5 to 8 years earlier. None of the patients had clinical symptoms of liver disease at the time of identification of PLI. Patients 1 and 2 developed clinical symptoms of jaundice, scleral icterus and abdominal distension later in their course. Liver imaging at initial identification of PLI was normal in all patients. All patients underwent extensive evaluation to determine the etiology of their liver disease, including ruling out infection and autoimmune disease. Therapies used to attempt to mitigate patients' transaminitis included ursodiol, cholestyramine, immune suppression (steroids, mycophenolate mofetil) and antibiotics (vancomycin, rifaximin). None of these treatments had any effect on the overall course of liver injury. Patient 2 ultimately died of cholestatic end-stage liver disease.

The three patients with FA underwent liver biopsy to determine the etiology of PLI. One control patient is represented in Figure 2, but all controls showed similar findings. Patients 1 and 2 underwent repeat biopsies and had two liver tissue samples available for analysis. In all three cases, we observed widespread moderate-to-strong positivity of PCNA of hepatocyte nuclei (Figure 2A-F) compared to the FA controls. For patients 1 and

2 with repeat biopsies, the subsequent liver biopsy showed increased PCNA staining in hepatocytes (Figure 2C, E). Staining for CD68, a marker for Kupffer cells, showed Kupffer cells from all three FA patients with PLI were enlarged compared with controls, suggesting increased phagocytic activity. The CD68 positive Kupffer cells in the repeat biopsies of patients 1 and 2 appear further enlarged (Figure 2 I, K). Immunohistochemistry staining for two markers of cellular senescence (p21 and p16) demonstrated widespread positivity localized to the portal triads in nuclei and cytoplasm of hepatocytes, and likely cholangiocytes, in all three patients with FA and PLI (Figure 2M-X). Similar to other markers, staining for p21 (Figure 2O, Q) and p16 (Figure 2U, W) was more intense and widespread in the repeat liver biopsies of patients 1 and 2. IHC staining for CK7, typically a marker of cholangiocyte injury, demonstrated moderate, widespread perivenular, hepatocyte and ductular staining in patients 1 and 2 and is positive only in the bile duct of the portal triad. Patient 3 has scattered and sparse positivity for CK7 (Figure 2DD), suggesting earlier stage biliary tract injury compared to the biopsies of patients 1 and 2.

Herein, we demonstrate a previously undescribed potential mechanism driving the pathogenesis of an emerging phenotype of persistent and chronic liver damage in patients with FA. We observed markers of cellular senescence (p21 and p16) in liver biopsies from FA patients with PLI, indicating that cellular senescence may be a significant driver of persistent liver injury in FA. Cellular senescence is a known response to DNA damage and oxidative stress[8], both of which are hallmark features of FA due to the inherent defect in DNA repair in FA. In FA patients with chronic oxidative stress, senescence may become maladaptive, leading to persistent senescent cell accumulation in liver tissue and the inability to regenerate. It clearly takes many years of PLI before clinical decompensation occurs (Fig.1). Likely hepatocyte regeneration was initially able to compensate for the chronic injury manifested by transaminitis. It is well known that telomere shortening is associated with decreased hepatocyte proliferative capability and induction of p21 and other markers of cellular senescence[10,11]. Hence, a plausible scenario for the progression of liver disease in our patients may be inability to compensate for ongoing liver injury and hepatocyte senescence by hepatocyte regeneration.

The role of senescence in liver pathology is well-documented in other liver diseases, including non-alcoholic steatohepatitis and liver fibrosis[12,13]. Senescent cells not only fail to proliferate and regenerate but also produce

pro-inflammatory cytokines (SASP), which can perpetuate local inflammation and contribute to fibrosis[12]. Additionally, the increased number of CD68-positive Kupffer cells, liver-resident macrophages, observed in liver biopsies from FA patients with PLI indicate a heightened inflammatory state and potential immune-mediated component to liver injury. Kupffer cells, the liver-resident macrophages, play a crucial role in maintaining hepatic homeostasis but can also contribute to inflammation and tissue remodeling under stress conditions. The interplay between senescent hepatocytes and Kupffer cell activation may create a self-sustaining cycle of inflammation and cellular stress in FA patients leading to continual activation of Kupffer cells and chronic inflammation. This inflammatory microenvironment could make FA patients more susceptible to liver injury progression and may play a role in liver fibrosis or cholestatic liver disease, as seen in our 2 more advanced cases.

The data presented in this report are important for clinicians taking care of patients with FA as modest chronic transaminitis in patients with FA is a familiar clinical finding and has for many years been regarded as of little clinical consequence. The progression to terminal liver failure of patient 1 in this study, and likely irreversible liver injury already present in patient 2 suggest important clinical consequences of PLI in FA. The mechanism of injury we propose, with excess senescence and associated inflammation occurring over many years suggests that traditional therapies such as steroids or ursodiol would be ineffective, and indeed this has been the case in our limited clinical experience. Identifying the onset of PLI is difficult as this depends on a clinician performing liver function tests in an asymptomatic child. However, we can report elevation of liver enzymes in a child with FA as early as age one years, suggesting that the process starts early and proceeds slowly over many years. Once cholestasis and fibrosis are established the process is unlikely to be reversible so it may be optimal to initiate therapy as early as possible in persons with FA and PLI. Much of the phenotype of FA is driven by increased oxidative stress so chronic long-term administration of an antioxidants such as quercetin or resveratrol, which have been tested as a protectant in other senescence-driven liver diseases, might be considered[14,15]. However, it is far from known whether oxidative stress is the predominant causative problem driving the liver phenotype in these patients and further exploration into other mechanistic pathways (e.g. aldehyde-induced DNA damage) is warranted. Future research could also focus on the role of targetable proinflammatory cytokines, particularly those implicated in fibrosis, such as serum amyloid A, interleukin-15

and TGFα[16-18]. A deeper understanding of these cytokines' contributions to fibrotic processes may reveal novel therapeutic targets, paving the way for innovative treatments that mitigate fibrosis-associated complications, Increased awareness of liver injury in FA in the hematology community is of great importance as a larger cohort of FA adults mature and encounter new aspects of the complex FA phenotype.

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Table 1. Patient demographics and transplant characteristics

	Patient 1	Patient 2	Patient 3
Age at PLI* diagnosis (years)	9	11	1
Sex	Female	Female	Male
FA[@] Complementation Group	FANCL	FANCP	FANCB
Prior Androgen Use (Yes/No)	Yes	Yes	No
HSCT[#] (Yes/No)	Yes	Yes	Yes
Age at HSCT (years)	10	11	7
Transplant type	MUD[^]	MMUD[&]	MUD
Preparative Regimen	ATG/Cy/Flu/TBI[§]	ATG/Bu/Cy/Flu[§§]	ATG/Bu/Cy/Flu
GVHD[$] prophylaxis	CSA[°]/Prednisone/T-cell depletion	T-cell depletion	T-cell depletion
Acute GVHD (Yes/No)	No	No	No
Chronic GVHD (Yes/No)	Yes	Yes	No
Chronic GVHD organ involvement	Skin	Liver	NA
VOD[%] (Yes/No)	No	No	No
Onset of PLI from HSCT (years)	-1	-0.5	-8
PLI symptoms at presentation	None	None	None
Evolving PLI symptoms	Jaundice, pruritus, abdominal pain, fatigue, portal hypertension, ascites	Jaundice, abdominal distension, scleral icterus	None
Liver imaging findings at presentation (modality)	Normal liver (US[^])	Normal liver (US)	Normal liver (US)
Duration of PLI (years)	18	13	16
Treatments for PLI	Ursodiol, oral vancomycin, rifaximin, cholestyramine	Ursodiol, steroids, mycophenolate	Ursodiol
Cause of death	Liver failure	Alive	Alive

*PLI: persistent liver injury; [@]FA: Fanconi anemia; [#]HSCT: hematopoietic stem cell transplant; [$]GVHD: graft-vs-host disease; [%]VOD: veno-occlusive disease; [^]MUD: matched unrelated donor; [&]MMUD: Mismatched unrelated donor; [§]ATG/Cy/Flu/TBI: Anti-thymocyte globulin/Cyclophosphamide/Fludarabine/Total body irradiation; [§§]ATG/Bu/Cy/Flu: Anti-thymocyte globulin/busulfan/cyclophosphamide/fludarabine; [°]CSA: cyclosporine; [^]US: ultrasound

Figure 1. Clinical time courses of patients in the liver biopsy cohort with persistent liver injury, demonstrating progressive liver disease.

Figure 1. A visual panel of the clinical course of each patient is depicted, including the progression of liver enzyme testing, the evolution of clinical symptoms, liver imaging findings, and treatments administered. The table summarizes the clinical characteristics of each patient. HSCT: hematopoietic stem cell transplant; ALT: alanine aminotransferase; AST: aspartate aminotransferase; PLI: persistent liver injury; MUD: matched unrelated donor; MMF: mycophenolate mofetil; MMUD: mismatched unrelated donor; GVHD: graft-vs-host-disease; Cy: cyclophosphamide; Flu: fludarabine; Bu: busulfan; CSA: cyclosporine; Pred: prednisone; CT: computed tomography; US: ultrasound.

Figure 2. Immunohistochemistry (IHC) staining of liver biopsies for multiple markers with a proposed role in the pathogenesis of persistent liver injury from three separate patients with FA display important differences in the patients with liver injury and are negative in a matched FA control without transaminitis.

Figure 2. (A-F) IHC for proliferating cell nuclear antigen (PCNA) demonstrates widespread moderate to strong positivity while it is negative in the FA control. Increased positivity is observed in subsequent samples for patients 1 and 2. **(G-L)** IHC staining of Kupffer cells marked by CD68 shows diffusely scattered positive cells in FA cases while negative in the FA control. **(M-R)** Staining for cyclin-dependent kinase inhibitor 1 (p21), a marker of cellular senescence, demonstrates strong positivity for all patients with FA, with increased positivity for subsequent liver biopsies obtained for patients 1 and 2. **(S-X)** Cyclin-dependent kinase inhibitor 2A (p16) staining likewise shows widespread positivity centralized around the portal truads for all three patients with FA and is scantly positive in the FA control. **(Y-DD)** IHC for cytokeratin 7 (CK7) demonstrates moderate, widespread perivenular/hepatocyte and ductular staining in patients 1 and 2. Patient 3 had intermittent, mild staining for CK7. Magnification 40x throughout. Both positive and negative control tissue stained appropriately.

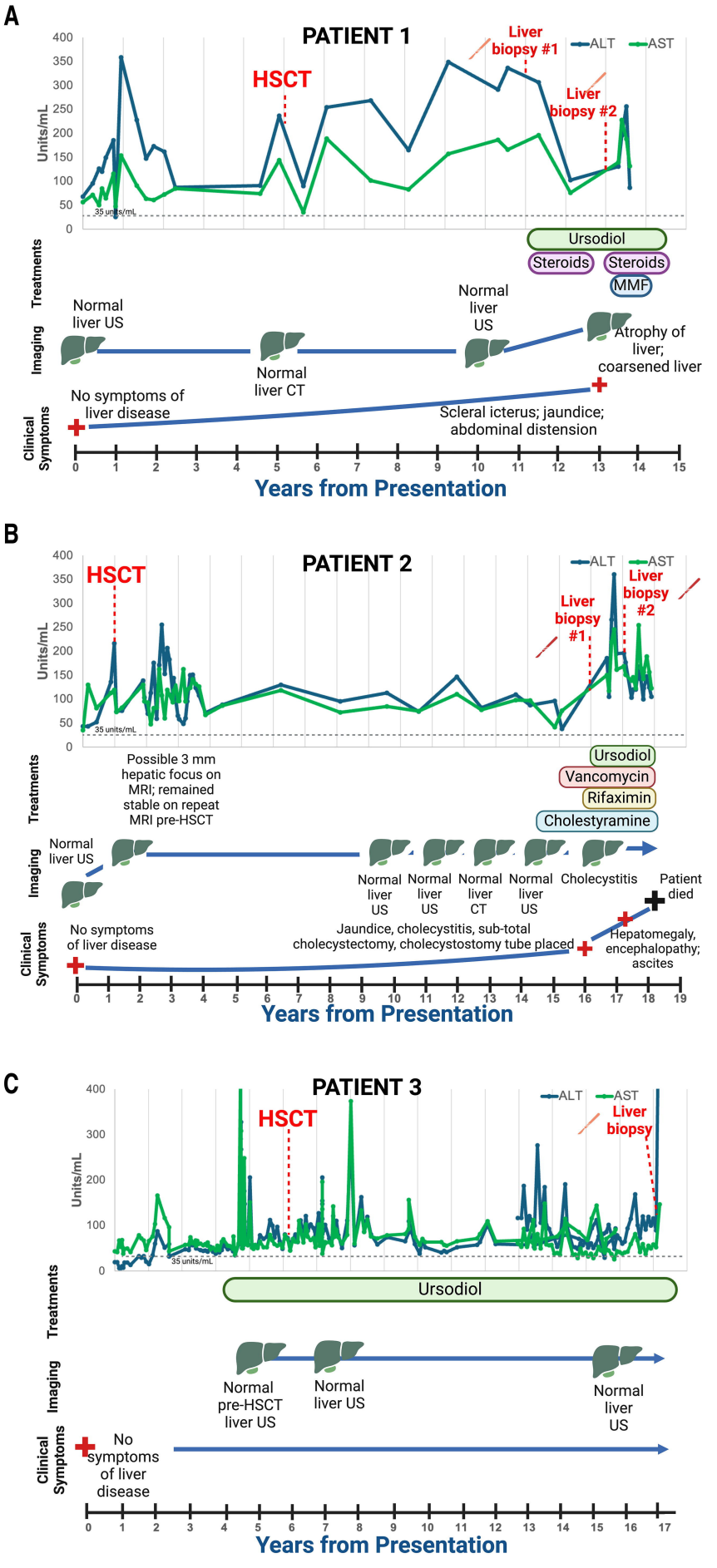
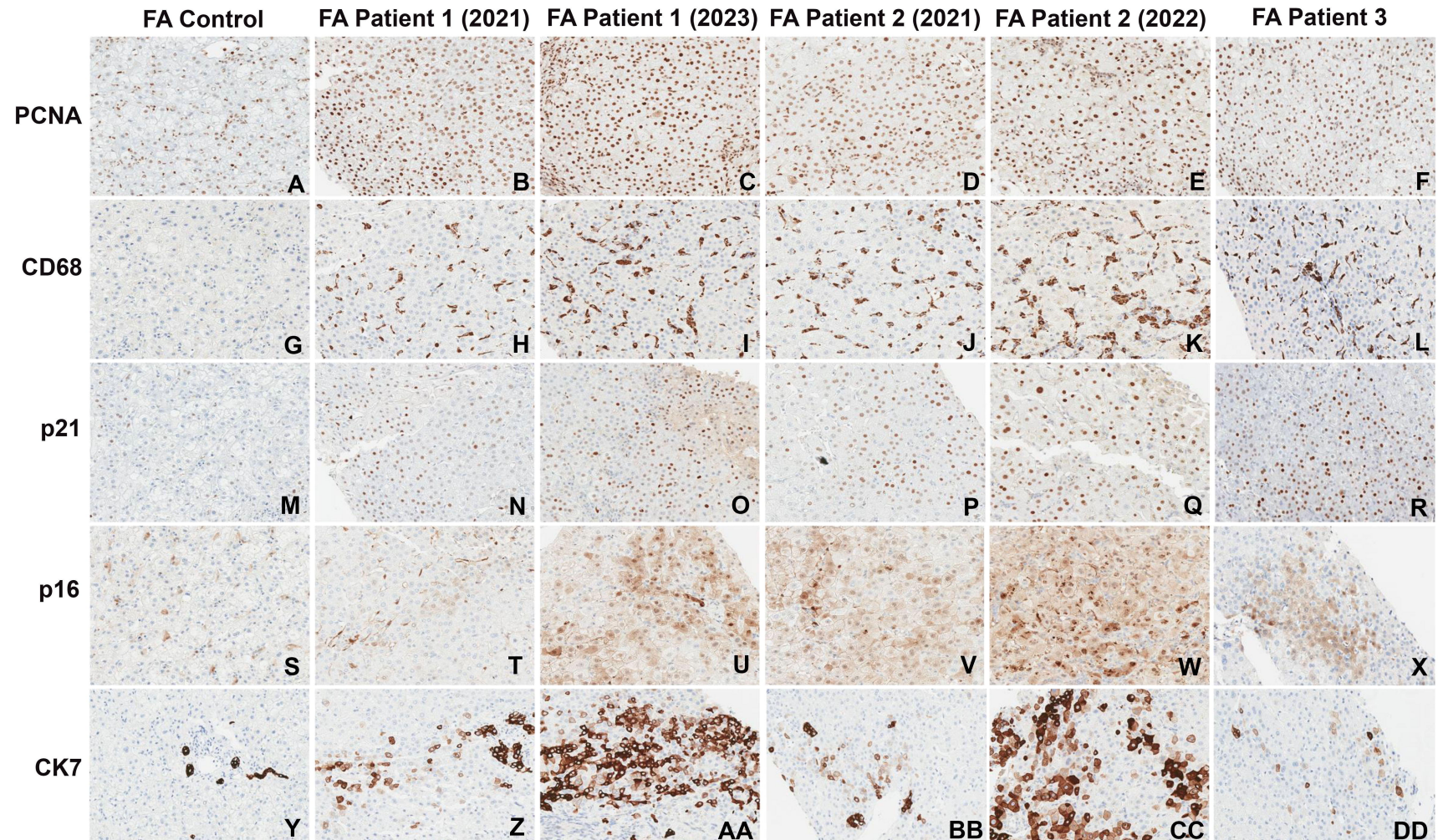

A
PATIENT 1
HSCT
Liver biopsy #1
Liver biopsy #2
ALT
AST
Units/mL
35 units/mL
Treatments
Ursodiol
Steroids
Steroids
MMF
Imaging
Normal liver US
Normal liver CT
Normal liver US
Atrophy of liver; coarsened liver
Clinical Symptoms
No symptoms of liver disease
Scleral icterus; jaundice; abdominal distension
Years from Presentation
B
PATIENT 2
HSCT
Liver biopsy #1
Liver biopsy #2
ALT
AST
Units/mL
35 units/mL
Treatments
Possible 3 mm hepatic focus on MRI; remained stable on repeat MRI pre-HSCT
Ursodiol
Vancomycin
Rifaximin
Cholestyramine
Imaging
Normal liver US
Normal liver US
Normal liver US
Normal liver CT
Normal liver US
Cholecystitis
Patient died
Clinical Symptoms
No symptoms of liver disease
Jaundice, cholecystitis, sub-total cholecystectomy, cholecystostomy tube placed
Hepatomegaly, encephalopathy; ascites
Years from Presentation
C
PATIENT 3
HSCT
Liver biopsy
ALT
AST
Units/mL
35 units/mL
Treatments
Ursodiol
Imaging
Normal pre-HSCT liver US
Normal liver US
Normal liver US
Clinical Symptoms
No symptoms of liver disease
Years from Presentation

Figure 2


FA Control
FA Patient 1 (2021)
FA Patient 1 (2023)
FA Patient 2 (2021)
FA Patient 2 (2022)
FA Patient 3
PCNA
CD68
p21
p16
CK7
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Z
AA
BB
CC
DD