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Liver kidney microsome antibodies. Analysis of a laboratory series

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ABSTRACT

Objectives: The objectives were to characterize the liver kidney microsome (LKM) antibody profile of a 14-month-old girl with autoimmune hepatitis and analyze the laboratory prevalence of LKM positivity.

Design and methods: This is retrospective analysis of the LKM antibody immunofluorescence tests performed by the Immunology Laboratory of Johns Hopkins Hospital from September 8, 2020 to July 31, 2022. LKM positive sera were also tested by an ELISA for LKM1 antibodies, which recognize the cytochrome P450 2D6 antigen. In silico analysis of 2D6 mRNA expression across anatomical sites was performed using Bgee and GTEx Portal databases.

Results: Of the total of 1598 patients (893 F, 705 M, ages 0.8–94 years) tested for LKM antibodies, 3 were positive, yielding a 0.2% period prevalence. The clinical diagnosis was autoimmune hepatitis in the index case, acute viral hepatitis in a 3-yo male, and hepatocellular carcinoma in a 54-yo male. LKM antibodies yielded the classical homogenous staining pattern in the liver cytosol and proximal kidney tubular cells. The first two patients were also positive for LKM1 antibodies, whereas the third was negative. 2D6 mRNA was expressed highly in the liver, moderately in the duodenum, and minimally in other tissues.

Conclusions: Overall, LKM antibodies are rare. They contribute to establish a diagnosis of autoimmune hepatitis, although they are also found in other liver diseases. The cytochrome P450 2D6 is one of the antigens recognized by LKM antibodies, but other antigens are likely targeted considering that 2D6 is minimally expressed in the kidney and yet LKM antibodies bind to kidney tubuli.

1. Introduction

Autoimmune hepatitis (AIH) is a rare but increasingly recognized disease, with a prevalence of about 18 cases per 100,000 persons and incidence of about 1 case per 100,000 persons per year [1]. AIH is more common in females (male:female ratio between 1:3 and 1:9) and typically diagnosed during the 4th or 5th decade of life, although it can occur at any age. Symptoms are not specific, often including fatigue, malaise, jaundice, abdominal pain, and arthralgias. Severity varies greatly, ranging from asymptomatic cases to advanced liver fibrosis and cirrhosis, to acute liver failure. AIH is diagnosed by excluding viral hepatitis and including a combination of

Abbreviations: AIH, Autoimmune Hepatitis; LKM, Liver Kidney Microsome; ANA, Anti-nuclear Antibodies.

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liver function tests, histopathologic examination of a liver biopsy (showing lympho-plasmocytic infiltrate with either centrilobular or interface hepatitis), imaging studies, increased serum levels of total immunoglobulin G (IgG) antibodies, and appearance of autoantibodies. These antibodies are directed against nuclear antigens (ANA), smooth muscle antigens, endoplasmic reticulum antigens (also known as microsomal antigens) such as the Liver Kidney Microsome antibody 1 (LKM-1) and 2 (LKM-2), and liver cytosolic proteins, such as the LC-1 antibody. Autoantibodies contribute to classify AIH into two types. AIH type 1 is the most common type (about 95% of the AIH cases in the US are type 1), affects patients of all ages, responds to medical treatment in most patients, and features smooth muscle antibodies and often ANA positivity. AIH type 2 is rare, develops mainly in young patients (teenagers or younger), responds extremely well to immunosuppression (although some patients follow a fulminant course that requires liver transplantation), and features LMK-1 antibodies [2].

Serum antibodies directed against antigens located in the endoplasmic reticulum in patients with chronic hepatitis were first reported by Mario Rizzetto and colleagues in 1973 using indirect immunofluorescence, a technique still used nowadays. LKM antibodies are characterized by a homogenous staining in the cytosol of the hepatocytes and proximal (cortical) tubules of the kidney, while the distal (medullary) tubules are negative [2]. Immunofluorescence is not highly specific because LKM antibodies can be found in a variety of other conditions besides AIH. For example, they have been reported in patients with chronic viral hepatitis [3], alcoholic cirrhosis, halothane hepatitis, and hepatitis induced by drugs such as dihydralazine, tienilic acid, and anticonvulsants, and autoimmune polyglandular syndrome type 1. Immunofluorescence also does not inform about the identity of the antigens recognized by the patient antibodies. Starting in the late 1980s, it has been shown that the antigens targeted by the LKM antibodies are components of the cytochrome P450 enzyme family or other enzymes involved in basal metabolism. For example, the LKM-1 antibody recognizes the cytochrome P450 2D6 antigen, allowing the development of antigen-specific antibody assays. Most clinical laboratories, however, measure LKM antibodies by indirect immunofluorescence, thus they measure overall, "histological" LKM antibodies rather than specific "biochemical" antibodies. Several dominant epitopes have been identified within the amino acid sequence of CYP2D6. Manns et al. first reported the recognition of a linear heptamer (DPAQPPRD) comprised between amino acids 263 and 270 in 11 of 26 sera that were positive for LKM-1 antibodies [4]. Kerkar et al. then identified a hexamer (RLLDLA), which is also found in the hepatitis C virus and cytomegalovirus, suggesting molecular mimicry as an important pathway in the development of liver autoimmunity [5].

LKM antibodies have been described in children with acute liver failure secondary to AIH. In a series of 986 children with acute liver failure, 63 (6%) caused by AIH (33 females and 30 males, age range between 0.6 and 17.5 years, median 9.9), LKM antibodies were found in 17 of 63 (27%), either alone (12 cases) or in combination with ANA or smooth muscle antibodies [6]. The authors reported that liver transplantation was more likely in subjects with LKM antibodies than in those with ANA and/or smooth muscle antibodies, overall highlighting the predictive utility of LKM antibodies as well as their pathogenic role. LKM antibodies have indeed been shown to inhibit the activity of CYP2D6 and activate liver-infiltrating T cells [7].

Goals of this report was to describe the clinical picture and immunological characterization of a 14-month-old girl with AIH and review our experience with the LKM antibody test.

2. Case presentation and methods

2.1. Index case

A 14-month-old healthy female, born at 40-week gestation via C-section, presented to the pediatric emergency room for weight loss, generalized pruritus, and acholic stools. Her liver function tests showed marked elevation in total bilirubin (4.8 mg/dL, normal

Table 1
Key demographic and laboratory characteristics of the 3 patients who tested positive for liver kidney microsome (LKM) antibodies by indirect immunofluorescence.

Sex	Age (yr)	IgG (450–1250 mg/dL)	Total bilirubin (<1.2 mg/dL)	Alanine AT (<31 U/L)	Aspartate AT (<31 U/L)	LKM antibodies (absent)	LKM1 antibody (<15 U/mL)	Clinical Outcome
F	1.2	609	4.8	2542	1851	≥1: 320	239	Patient diagnosed with autoimmune hepatitis 2. She has not had another flare since discharge. Remains on CELLCEPT 250 mg BID and is pending GI outpatient follow up.
M	3	892	4.3	2156	2648	≥1: 320	270	Patient diagnosed with acute viral hepatitis, and has completely recovered from this single episode of liver injury. Genetic testing found him to be carrier for cystic fibrosis and Gilberts on the cholestasis panel.
M	54	1607	1.3	106	743	1:160	12	Patient diagnosed with HCC secondary to non-alcoholic steatohepatitis Completing cycle 5 of bevacizumab/ atezolizumab.

value < 1.2), alanine amino transferase (2542 U/L, normal <31), aspartate amino transferase (1,851U/L, normal <31), and alkaline phosphatase (376 U/L, normal range between 100 and 320) (Table 1). An abdominal ultrasound showed trace of ascites in the right upper quadrant. Histological examination of the liver biopsy showed marked portal inflammation with lymphocytes, neutrophils, eosinophils, and scattered plasma cells. There was parenchymal collapse (best appreciated on the reticulin stain), with scattered swollen hepatocytes and rosette formation. These morphologic findings were considered consistent with autoimmune hepatitis once viral hepatitis or drug-induced hepatitis were excluded. Serum was sent to the immunology laboratory to measure LKM and SMA antibodies by indirect immunofluorescence. LKM were strongly positive, at a ≥1:320 titer, overall supporting the diagnosis of type 2 AIH. The liver substrate showed the classical binding of these antibodies, which stain homogenously the cytosol while leave the nuclei unstained (Fig. 1A). The kidney substrate showed cytosolic positivity in the proximal tubules (Fig. 1B). Serum IgG levels were normal for age (609 mg/dL), and smooth muscle antibodies were borderline positive at a 1:20 titer (data not shown). The patient was started on prednisolone (7.5 mg by mouth twice a day). Clinical signs and symptoms, as well liver function tests, markedly improved. The glucocorticoid was tapered, and she was discharged with mycophenolate mofetil (Cellcept™ 250 mg by mouth twice a day) and a close gastroenterology follow up. An aliquot of her serum was retained to be tested using a commercial ELISA to detect antibodies against the cytochrome P450 2D6 antigen (LKM1 antibody, see below).

2.2. LKM antibodies by immunofluorescence

This assay was performed using the NOVA Lite ANA KSL kit (Inova Diagnostics, San Diego, CA), according to the manufacturer's recommendations. The kit uses tissue sections prepared from mouse liver, kidney, and stomach and allows the detection of LKM antibodies, as well as antibodies against gastric parietal cells, smooth muscles, mitochondria, and ANA. Slides were read using the QUANTA-Lyser 3000 (from Inova Diagnostics), a fully automated, walk-away instrument that eliminates human error in sample preparation, pipetting, and washing. Cover-slipping is the only step that is still required to be performed manually. Digital images were

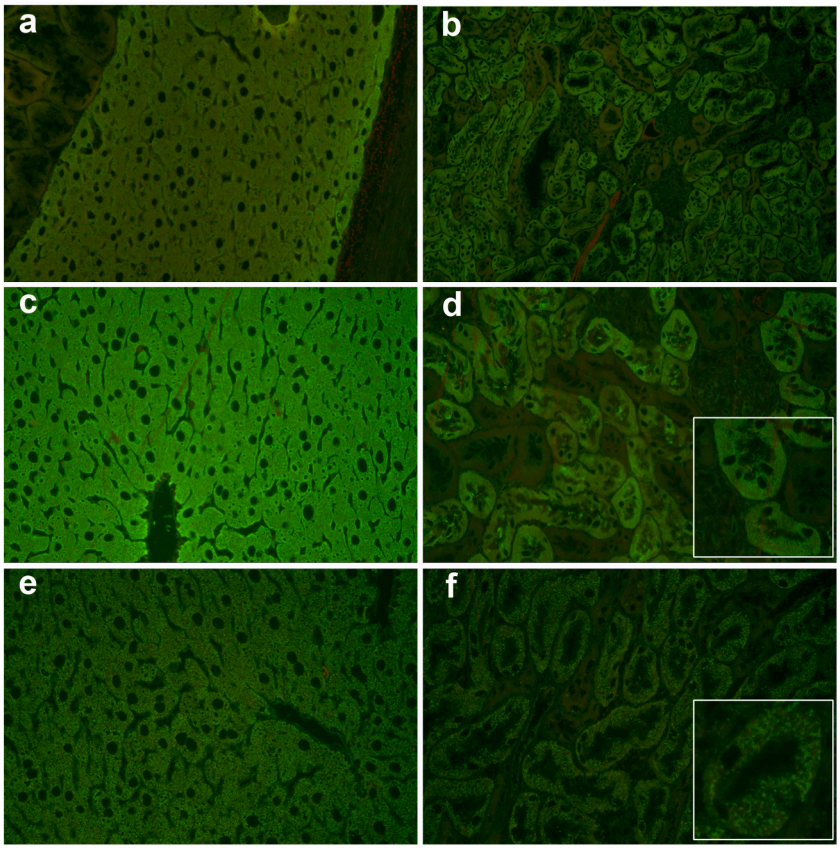

Fig. 1. Liver Kidney Microsome (LKM) antibodies in the 3 patients. Antibodies were assessed by indirect immunofluorescence using a mouse liver kidney stomach substrate. **A and B)** Index case (patient 1): antibodies stain homogenously the cytosol, but not the nuclei, of hepatocytes (A) and renal proximal tubules. The distal tubules (to the far left) and muscularis mucosa of the stomach wall (to the far right of the field) are unstained**. C and D)** Patient 2: Similar staining appearance as that of the index case in liver (C) and kidney (D). **E and F)** Patient 3: although the subcellular location of the staining was the same (cytosol of hepatocytes and renal proximal tubules), the staining was distinctively granular in the liver (E) and especially kidney (F) substrate. The insets in panels D and F emphasize the different quality of the staining: diffusely homogeneous in D versus granular in F.

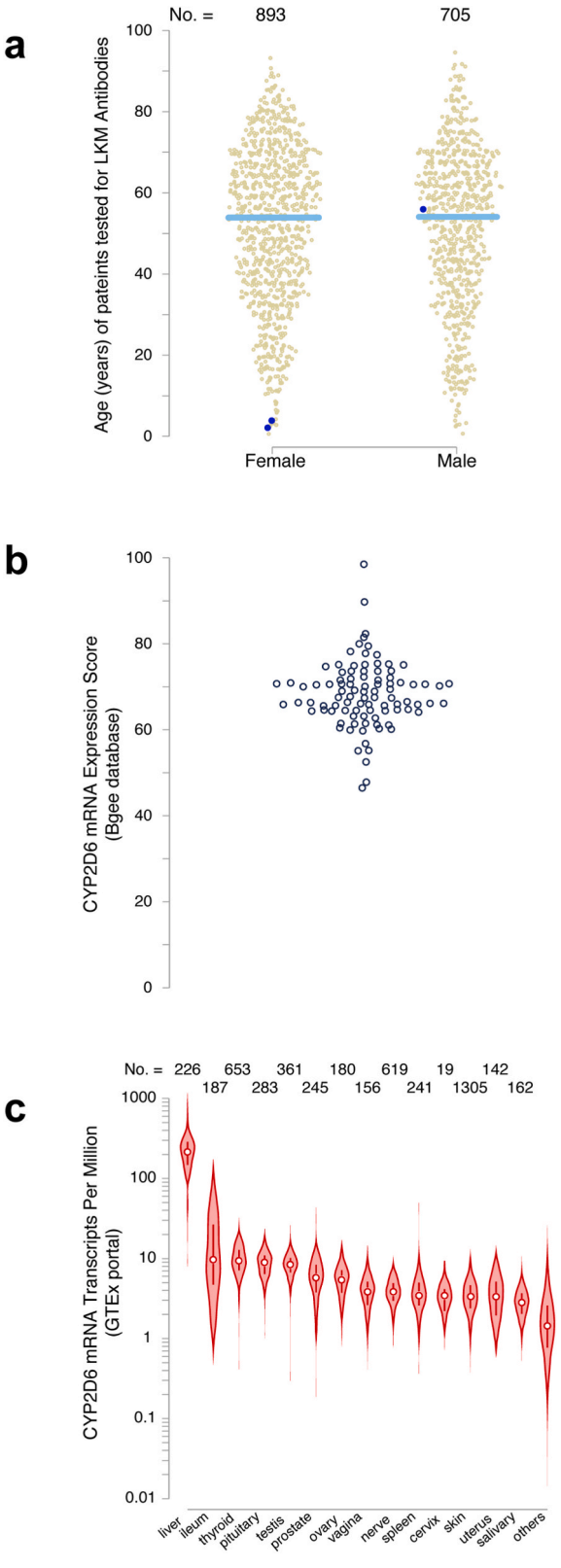

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Fig. 2. A) Age distribution by gender of the 1598 patients who were tested for LKM antibodies by immunofluorescence during the September 2020–July 2022 period. The filled blue circles indicate the 3 patients who tested positive; the horizontal bar represents the median age. **B)** RNA expression score of the human CYPD6 gene from the Bgee database. **C)** RNA expression (in Transcripts per Million) of the human CYP2D6 gene across different organs from the GTEx portal. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

interpreted by a laboratory technologist and confirmed by two of the authors (LAR and DC).

2.3. LKM1 antibody by ELISA

The presence of LKM1 antibody in the serum of the 3 LKM positive patients was assessed using a commercial ELISA assay (Eagle Biosciences, Nashua, NH), according to the manufacturer's instructions. Optical absorbance was read at 450 nm using a SpectraMax ABS microplate reader and the SoftMax Pro software (both from Molecular Devices, San Jose, CA).

2.4. In silico analysis of 2D6 mRNA expression

We searched the Bgee database (from the Evolutionary Bioinformatics groups of the University of Lausanne) and GTEx Portal (Office of the Director, National Institutes of Health) for accession number ENSG00000100197, corresponding to the human cytochrome P450, subfamily D, member 6 mRNA. The Bgee database uses non-parametric statistics to normalize gene expression and make it comparable across genes, conditions, and species. It reports results, called expression score, as a continuous scale from 0 to 100, with 0 indicating lack of expression and 100 maximal expression. The GTEx Portal, now in its 8th version, has compiled the gene expression from 52 different anatomical locations from a large number of post-mortem donors. Not every donor contributes a sample from each of the 52 locations because samples that are not considered normal by pathologists or yield poor quality RNA are excluded. Results are expressed as number of mRNA transcript per million transcripts (TPM).

3. Results

3.1. Key characteristics of the patients tested for LKM antibodies

The Clinical Immunology Laboratory of the Johns Hopkins began offering the LKM immunofluorescence test on September 8, 2020 and performed 1676 LKM tests in 1598 distinct patients as of July 31, 2022. The patients included 893 (56%) females and 705 (44%) males, and varied greatly in age, from a minimum of 0.8 years to a maximum of 94 years. The age was similar in the sexes with a mean and median of 50.8 and 53.2 in females, and 50.6 and 53.5 in males (Fig. 2A). The broad age range was reflected in the diversity of the ordering locations: LKM orders were placed in 277 different hospital locations from 810 different physicians. Seventy-nine patients were tested two (No. = 71), three (No. = 7) or four (No. = 1) times, all with consistent results. Of the total 1598 patients tested for LKM, 3 were positive, one being the index case described in the case presentation section. The period prevalence of LKM positivity over the course of 2 years, was therefore 0.2% (3 of 1,1598).

3.2. Clinical summary of the two additional patients who tested positive for LKM antibodies

Patient 2 was a 3-year-old, healthy male presented to the pediatric emergency department as a transfer from an outside hospital. A day prior to this visit, the patient was experiencing vomiting, loose stools, decreased energy and jaundiced skin/eyes. At the outside hospital, abnormal liver function values were detected (AST 2359 U/L, ALT 1881 U/L, total bilirubin 5.2 mg/dL and alkaline phosphatase 381 U/L). The patient also tested positive for a rhinovirus and enterovirus infection. A liver duplex ultrasound showed diffuse decrease in hepatic echogenicity with accentuation of venous walls. A liver biopsy demonstrated acute hepatitis, with a differential diagnosis of acute viral infection, drug-related liver injury, or autoimmune hepatitis, with viral infection being the diagnosis favored by the clinical team. The patient was started on intravenous Vitamin K (Phytonadione, 2.5 mg daily for three days) for his coagulopathy, began to improve spontaneously, and was discharged with a close gastroenterology follow up. Immunology serum tests showed normal IgG levels (892 mg/dL), negative SMA, and strongly positive (≥1:320) LKM antibodies (Table 1). The immunofluorescence appearance of these LKM antibodies was similar to that described for the index case: a diffusely homogenous staining in the cytosol of hepatocytes (Fig. 2C) and renal proximal tubules (Fig. 2D).

Patient 3 was a 54-year-old male with no history of autoimmune liver disease and a history of diverticulosis presented with weight loss, fatigue, abdominal pain, dyspnea, and abnormal liver function tests (AST 830 U/L, ALT 124 U/L, total bilirubin 1.1 mg/dL, alkaline phosphatase 581 U/L). A right upper quadrant ultrasound showed an enlarged liver with very heterogeneous echotexture with nodular outlines and a possible mass. A fine needle aspiration of the possible liver mass revealed a poorly differentiated hepatocellular carcinoma with lympho-vascular invasion. The patient was started on bevacizumab/atezolizumab and discharged with a close gastroenterology and oncology follow up. Immunology serum tests showed polyclonal elevation in IgG (1607 mg/L), presence of SMA 1:80 titer, and presence of LKM 1:160 titer. The LKM immunofluorescence appearance was somewhat different from that observed in the first two, pediatric cases: the staining was still localized in the cytosol of hepatocytes and renal proximal tubules, but had a distinctive granular, rather than diffusely homogenous appearance. The granularity was seen in the liver (Fig. 2E) and especially in the

kidney (Fig. 2F) substrates, although evident when focusing up and down on the section and thus difficult to reproduce in a 2D format. Nevertheless, the difference microscopic appearance suggested to us the recognition of different cytosolic antigens. It was not possible to test for other LKM antibodies besides LKM1 due to the lack of serum, a test that would have been useful to establish whether these antibodies were induced by the patient 's current treatment.

3.3. LKM1 antibody results in the 3 LKM positive patients

The index case and patient 2 tested strongly positive for LKM1 antibodies, with levels of 239 and 270 U/mL, respectively (Table 1). Patient 3, on the contrary, was negative with values below 15 U/mL. These results indicate that that index case (autoimmune hepatitis type 2) and patient 2 (acute viral hepatitis) mounted an immune response against an antigen predominantly expressed in hepatocytes.

3.4. Expression profile of the CYP2D6 gene

The Bgee database included mainly RNA Seq data for CYP2D6 (https://bgee.org/gene/ENSG00000100197). It showed that 2D6 had an expression score of 99 in the liver, followed by 90 in duodenum, and then averaged around 68 (±7) in a variety of other tissues including the kidney, which had a score of 69 (Fig. 2B). The GTEx portal database confirmed and extended these observations: the mean (±SD) TPM for liver was 232 (±136) from 226 donors, an expression significantly higher ($p < 0.0001$) than that observed in the small intestine where the mean (SD) TPM was 19 (±22) (Fig. 2C). In all the other anatomical locations the mean TPM was below 10, thus much lower than the hepatic TPM. In kidney, the mean (SD) TPM was only 3 (±2).

4. Discussion

This study reports the prevalence of LKM antibodies in a laboratory series and offers new insights into the nature of the tissue antigens targeted by these antibodies.

LKM antibody prevalence was low in our study, around 0.2%, a value that is lower than that reported in previous studies. For example, Bai and colleagues measured serum LKM-1 antibodies by ELISA in 59 patients with autoimmune hepatitis and 360 patients with chronic hepatitis C and, reporting positivity in 2 (3.4%) and 9 (2.5%) patients, respectively [8]. Narkewicz et al. characterized 63 children with acute liver failure caused by AIH and found LKM antibodies in 15 of them (24%), 12 having LKM alone, 3 LKM and ANA, and 2 LKM and smooth muscle antibodies [6]. Muratori et al. studied 21 patients with type 2 AIH and demonstrated that 7 of those were positive for LKM1 (33%), 9 for LC1 and 5 for both [13]. More recently, Khayat et al. found LKM antibodies in 2 of 10 (20%) patients with autoimmune liver disease and 1 of 14 (7%) patients with autoimmune liver disease plus non-alcoholic fatty liver disease [9]. The lower prevalence reflects the different nature of the studies, ours being laboratory-centered while the others diagnosis-centered. In this study the LKM orders came from a very diverse patient population, ranging in age from newborn to nearly centenarian and from different clinical services. These are factors that contribute to decrease the predictive value of a laboratory test. This diversity is evident in the diagnoses of our three LKM positive patients, one having AIH type 2, another acute viral hepatitis, and the other one hepatocellular carcinoma.

"LKM antibodies" is an umbrella term that comprises a family of different antibody specificities that, although yielding similar or identical immunofluorescence staining pattern differ in the antigen(s) they recognize. The best characterized antigenic target is the CYP2D6, a member of the cytochrome P450 family of enzymes. In particular, it has been shown that AIH patients preferentially produce antibodies binding to an octapeptide within the CYP2D6 protein [4]. Patients with chronic hepatitis C infection can produce LKM antibodies that have a comparable staining pattern to that seen in AIH patients and yet recognize either different epitopes within CYP2D6 or different liver antigens [10]. Furthermore, the kidney substrate is an integral part of the LKM antibodies but CYP2D6 protein is not expressed in adult human kidneys [11]. We confirmed these data through the analysis of the GTEx portal repository, established from adult, human, post-mortem donors, and found a very small number of CYP2D6 mRNA transcripts in the kidney transcriptome. Review of the LKM immunofluorescence staining pattern of the in the 3 patients featured in this study uncovered an interesting detail: while the renal tubular fluorescence in the two pediatric cases (the index case and patient 2) was strong and linear, in the adult case (patient 3) it was weak and granular. These features could perhaps reflect different expression levels of CYP2D6 in the renal proximal tubules by age, with high expression, and thus visibility for the immune system, in young ages and low expression or absence in adults. Although it is known that the expression of drug metabolizing enzymes varies between children and adults [12], no study has specifically analyzed the expression of CYP2D6 in pediatric renal tissues.

Overall, the immunological profile of AIH appears complex and incompletely defined. The disease is more commonly sporadic but given that AIH type 2 patients are typically children (in contrast to most autoimmune diseases), genetic causes have been investigated. Only a handful of autoimmune diseases are caused by a single gene defect [13], such as the autoimmune polyglandular syndrome type 1, caused by mutations in the AIRE gene [14]. This syndrome, characterized by the triad of chronic mucocutaneous candidiasis, hypoparathyroidism, and Addison disease, also features AIH type 2 in about a quarter of the cases [15–17].

Statement of ethics

Ethics approval was not required.

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Clinical Immunology Laboratory at Johns Hopkins Hospital.

Author contributions

Sandra Sanchez and Patrizio Caturegli designed the study, gathered, analyzed, and interpreted the data, and wrote the manuscript. Diana Fang contributed to the discussion and observed the differences in immunofluorescence pattern between the adult patient versus the two children. Shaoming Xiao wrote an R code that converted the data extracted from GTEx into Stata. Lu Ann Rezavi and Daniela Cihakova interpreted the digital immunofluorescent images. Lu Ann Rezavi also performed the LMK1 antibody ELISA assay at the Johns Hopkins Immunology lab. Brittney M. Howard reviewed the report and ordered the ELISA kit.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Data availability

Data will be made available on request.

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