Alkaline Phosphatase Pathophysiology with Emphasis on the Seldom-Discussed Role of Defective Elimination in Unexplained Elevations of Serum ALP – A Case Report and Literature Review

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Abstract: While serum alkaline phosphatase activity has become a routine clinical measurement, we have found that physicians’ knowledge of the pathophysiology of this enzyme is almost solely limited to the concept that an elevated serum alkaline phosphatase suggests disease of liver or bone. For example, physicians at all levels of training had no understanding of such basic physiological information as the function of alkaline phosphatase in the liver or how this enzyme is eliminated from the serum. Based on a patient with an enormously elevated alkaline phosphatase, this report provides a review of existing clinically relevant information concerning the pathophysiology of alkaline phosphatase with emphasis on the mechanisms involved in the homeostasis of this enzyme. A novel aspect of this paper is the discussion of the previously neglected concept that defective enzyme elimination could play a major role in the pathogenesis of serum alkaline phosphatase elevations.

Keywords: alkaline phosphatase catabolism, alkaline phosphatase isoforms, galactose receptor, galactomannan

Introduction
We recently encountered a patient with a markedly elevated serum alkaline phosphatase (ALP) concentration of 3350 IU/L. Discussions of this patient revealed that physicians at all levels of training know that serum ALP elevations occur in cholestatic liver disease or bone conditions but otherwise are unfamiliar with the most rudimentary aspects of the physiology of this enzyme. This report uses the data of this patient with a very high serum ALP as the springboard for a review of the pathophysiology of ALP, with particular emphasis on the seldom discussed role of ALP elimination in the homeostasis of this enzyme.

Case Report
A 77 year-old male with a history of left ventricular thrombus, coronary disease with heart failure, type 2 diabetes, chronic obstructive pulmonary disease, and rheumatoid arthritis on rituximab presented with para-sternal chest wall drainage. A C-T study showed a subternal abscess with probable sternal osteomyelitis, and surgical exploration revealed an infectious process involving the sternum and the cartilage of several adjacent ribs. A small portion of the left edge of the sternum along with adjacent cartilages of ribs 4–7 was resected, and pathological exam revealed Aspergillus fumigatus. Treatment with voriconazole was initiated on 8/27/2020 at a dosage of 300 mg bid (see Figure 1), which, after 12 days, was reduced to 200 mg bid. Two days later a routine assessment of liver function revealed an ALP concentration of 3348 IU/L with a repeat measurement of 3358 IU/L (upper limit of normal: 140 IU/L). The results of the multiple serum ALP measurements obtained before and during the present illness (see Figure 1) show that the peak level
declined steadily over the ensuing month to a concentration of about 800 IU/L and then remained persistently elevated at 800–1800 IU/L. Electrophoretic analysis of a serum sample obtained near the time of the peak level showed that the ALP was comprised of 51% liver, 23% bone and 26% macrohepatic isoforms of the enzyme. GGT was markedly elevated (>1540). During the present illness serum bilirubin and aminotransferases were measured on 22 occasions. Bilirubin never exceeded 1.0 mg/dl and the vast majority of measurements were <0.5 mg/dl; 20 of 22 measurements of AST and ALT were within normal limits, while two sets of measurements were minimally (50 to 65 IU/L) elevated. The patient had no pruritus, nausea, vomiting, nor right upper quadrant pain; a right upper quadrant ultrasound showed a normal appearing liver and no biliary dilatation; MRCP revealed no abnormalities of the liver parenchyma or collecting system. Anti-nuclear and anti-mitochondrial antibodies were not detected. Liver biopsy yielded a small specimen, but the visualized tissue was normal. The Aspergillus infection appeared to be under good control, but 4 months after the onset of the present illness, the patient suddenly expired at home, presumably secondary to a coronary event. Written consent was obtained from the decedent’s next of kin (wife) to publish this history and data. The Veterans Administration does not require institutional approval of case reports.

**Overview of ALP**

ALP is a membrane associated enzyme that catalyzes the release of phosphate via cleavage of the phosphate ester bond. The enzyme is found in virtually all forms of life, spanning bacteria to mammals, and in all species the active site of the enzyme contains two Zn$^{2+}$ and one Mg$^{2+}$, possibly explaining why zinc and magnesium deficiency have been associated with abnormally low serum ALP activity. As indicated by its name, the pH optimum of this enzyme seemingly is in the alkaline range (pH 8.0 to 10). However, this non-physiological pH optimum is an “artifact” of the high substrate concentrations employed in laboratory assays; at low, physiological concentrations, the pH optimum is approximately 7.4. ALP has activity against a wide variety of biologically important substrates such as pyrophosphate, pyridoxal phosphate, and phosphoethanolamine, all of which accumulate in the plasma of patients with hypophosphatemia, a condition with defective synthesis of an ALP isoenzyme. A non-physiological substrate, p-nitrophenol phosphate, is commonly employed in the assay of ALP activity (1 IU of activity releases 1 µm of p-nitrophenol/ min). Thus, each liter of our patient’s serum (ALP activity of 3350 IU/L) had the potential to release about 0.2 M (20 g) of phosphate/hour.
roughly 500 time the phosphate content of a liter of normal plasma. Surprisingly, there seems to be no evidence of hyperphosphatemia or other deleterious effects of a very high serum ALP concentration.

**ALP Isoenzymes**

In humans, ALP is a family of four isoenzymes with differing amino acid sequences indicating that four different genes code for ALP. These four isoenzymes commonly are identified by their primary tissue of origin: placenta, intestine, germ cells, and “tissue non-specific”, so named because high concentrations of this isozyme are found in liver, bone, and kidney. Despite similar amino acid composition, the nonspecific isoenzymes can be differentiated from each other due to differences in carbohydrate content, a post-translational modification. While bone and liver ALP commonly are referred to as isoenzymes, strictly correct nomenclature identifies them as isoforms of the tissue non-specific isoenzyme. ALP is anchored to the membrane via the molecule, phosphoinositol. The mechanism via which the enzyme detaches from the membrane to enter the serum enhances the number of potential serum isoforms. ALP may circulate in multiple forms: as the basic (“soluble”) enzyme that has a molecular weight of about 140 kDa, the enzyme attached to the anchoring molecule, or in a high molecular weight form in which the enzyme remains attached to cell membrane material in the form of a vesicle. The vesicular form is known as macrohepatic ALP, which should not be confused with the rare globulin-bound ALP, which frequently is referred to as macro-ALP. As might be expected from the above, sophisticated separatory techniques may demonstrate up to 15 different serum ALP isoforms.

**Sources of ALP in the Serum**

The vast majority of ALP activity of normal serum is derived from bone and liver. In bone, the enzyme is attached to the outer surface of the cell membrane of osteoblasts and release into the serum increases with osteoblastic activity such as is observed in growing children, fractures, bone neoplasms, osteomalacia, and most notably Paget’s disease. Of interest relative to this case report, osteomyelitis has not been associated with an increase in serum ALP.

The release of ALP into the blood in cholestatic liver disease has been extensively studied, primarily after bile duct ligation in rats. Within 12 hours of interruption of bile flow, the total ALP activity of the liver increased by about 7-fold. This increase was 250 times greater than the amount of enzyme normally excreted in bile in 12 hours, thus the increase in liver ALP resulted from increased synthesis rather than decreased biliary excretion. Histochemical studies of human liver show that ALP normally is present in high concentrations in the endothelium of the hepatic arterioles, venules, sinusoids, and canalicular pole of hepatocytes, but not the biliary ducts. Cholestasis causes a rapid increase in the ALP staining of the hepatocyte membranes, which spreads from the canalicular area to involve the entire hepatocyte membrane, a phenomenon possibly mediated by cyclic AMP. The detergent action of high concentrations of bile acids that accumulate in the liver with cholestasis is thought to damage the membranes releasing ALP into the blood.

Intestinal ALP is present in very low concentration in fasting serum but ingestion of a lipid containing meal results in the release of this enzyme into the intestinal lymphatics with a subsequent increase of up to 20% in the total plasma ALP activity. The placental isozyme, which usually is undetectable in serum, rises during pregnancy, and near term this enzyme may be the predominant form of plasma ALP, producing several-fold increases in the total serum ALP activity.

**Elimination of Plasma ALP**

While elimination rate plays a major role in determining the plasma concentration of ALP, this process has received very little attention. For example, an authoritative, 94 page review of ALP physiology contains only one unreferenced comment relative to elimination: that the ½ time of placental ALP is 4 days.

The rise in serum ALP in cholestasis initially was thought to result from interruption of biliary excretion of the enzyme, as is the case with bilirubin. However, bile flow accounts for only a small fraction of the elimination of endogenous or exogenously infused enzyme. Rather the major mechanism of elimination involves binding of terminal galactose moieties of the enzyme to a galactose receptor (primarily on hepatic cells), which leads to endocytosis and catabolism in the lysosome. Binding to the receptor is rate limiting since co-infusion of ALP and a galactose receptor blocker, asialofetuin, markedly reduced the rate of disappearance of ALP infused into serum. The serum ALP concentration declined at a rate of about 2% per hour when catabolism was inhibited indicating a 2%/hour maximal
rate of distribution of the enzyme to the extravascular pools. In contrast, the serum concentration declined by about 15% per hour when catabolism was intact. Given that the catabolism of ALP is extravascular, this raises the question as to how the enzyme can be catabolized faster than the rate it can exit the bloodstream. The answer is that unlike capillaries, the sinusoids of the liver are freely permeable to ALP. Thus, metabolism in the liver is not limited by the slow rate that ALP distributes across capillaries to the other extravascular pools.

Sialic acid binding to the terminal galactose moieties of ALP blocks receptor binding and slows elimination. Since various isoforms of ALP contain variable amounts of galactose and sialic acid, isoforms may be eliminated at very different rates. For example, literature values suggest that placental ALP, which is fully sialylated has serum half-time 200 times greater than that of intestinal ALP which is non-sialylated.

The standard means of determining the elimination rate of a compound involves analysis of the serum disappearance curve of an intravenously infused bolus of the compound. This analysis is straightforward for small molecules that rapidly achieve diffusion equilibrium between plasma and the extra-vascular pool. However, determining the elimination rate is less clear-cut for large proteins such as ALP that distribute very slowly to the extracellular space via a convective flow (rather than diffusion) and return to plasma via the lymphatic drainage of the extra-vascular space. As will be evident, estimations of clearance vary depending on the technique used to analyze the disappearance curve.

Two published studies provide sufficient data to calculate elimination rates of exogenous ALP in healthy controls. Pickkers et al infused calf-intestinal ALP in 103 healthy subjects. While the authors report a clearance of roughly 100 mL/min, we reanalyzed their data using our pharmacokinetic software (PKQuest) and estimated a clearances of 150 to 200 mL/min. In a second study, Peters et al. infused a recombinant ALP consisting of human intestinal isoenzyme modified such that the crown domain (a 60 amino acid segment) was replaced by the crown domain of human placental ALP, a manipulation seemingly employed to improve enzyme stability. The authors estimated an ALP clearance of 40 mL/min, while our clearance estimate ranged from 20 to 25 mL/min, depending on the ALP dosage. While neither of the two isoforms employed in these studies is “physiologic”, it is evident that different isoforms of ALP may be eliminated at very different rates and that relevant clinical information requires study of the endogenous serum ALP present in a given disease state.

Application of the above clearances to our patient with a serum ALP activity of 3350 IU/L yields the following estimations of plasma ALP elimination rate, which equals plasma delivery rate at homeostasis. A 200 mL/min clearance (ie, all ALP is eliminated from 200 mL of plasma/min) yields the astronomical elimination rate of about 1 million IU/day. Given the ALP concentration of normal liver tissue is only about 1 IU/g (1500 IU/total liver), liver ALP would have to turn over about 650 times per day to supply ALP at the requisite rate. At the lower 20 mL/min clearance, the turnover rate of liver ALP would still be a seemingly impossible 65 times per day. Thus, to maintain a serum ALP of 3350 IU/L, it seems necessary to implicate a clearance far below that determined in the above infusion studies.

The only potentially useful data on elimination of human endogenous isoforms is provided by ½ time measurements, ie, the time required for a markedly elevated serum concentration to decline by ½ after alleviation of the pathological state causing the increased input into the serum. For this ½ time to reflect elimination rate, ALP release must immediately normalize, which occurs in only a limited number of situations such as after delivery of the placenta or amputation of a limb containing a bone ALP secreting tumor (but almost certainly not after alleviation of biliary obstruction). A second major problem with converting ½ times to elimination rates is the influence of unknown variables such as the rates of distribution between plasma and extravascular pools and the volume of the pools. Half-time values cited are 1, 50, and 200 hours for ALP isozymes of intestinal, bone and placental origin, respectively. Assuming the different isoforms distribute in roughly similar fashion, these very diverse 1/2 times probably reflect the enormous potential differences in elimination rates of varying isoforms of ALP.

**Physiological Function of ALP**

Given that cleavage of the phosphate ester linkage is the sole activity of AP, it follows that the biological activity of this enzyme involves the provision of phosphate to tissue or the activation/inactivation of substrates via dephosphorylation. Most information concerning the function of ALP has been obtained from studies of subjects with genetic defects in ALP synthesis or from ALP knockout models in mice. Hypophosphatasia is a condition in which a variety of mutations in the
two alleles of the gene coding for tissue nonspecific ALP cause a variable deficiency of this isoenzyme in bone, liver, kidney, and serum. The primary deleterious effect of this deficiency is defective mineralization of bone and teeth that ranges in severity from death in utero with virtually no bone calcification to early childhood onset of severe osteomalacia to a more benign presentation as osteomalacia in adults. Similar defective bone mineralization is observed with knockout of the non-specific ALP gene in mice. While the precise pathophysiology underlying this defect is not clear, the high serum values of pyrophosphate characteristic of hypophosphatasia suggest that defective release of phosphate from pyrophosphate could play a role in the calcification defect.

Given the close relationship between cholestasis and serum ALP, one might assume that this enzyme plays an important role in bile formation. Surprisingly, however, liver function studies remain normal in hypophosphatasia in both humans and knockout models. In fact, the only data linking ALP and bile formation suggests that ALP activity actually inhibited bile formation when cholestasis was induced via bile duct ligation in the rat. The role of ALP in the kidney also is unclear in that renal malfunction is not a prominent problem in hypophosphatasia in humans or ALP knock out mice.

Multiple functions for intestinal ALP have been proposed based on studies of knockout models. Prior to the development of knockouts, the consistent rise of serum ALP following ingestion of a fatty meal suggested that this enzyme facilitates lipid absorption. However, to the contrary, lipid absorption rate actually is increased in ALP knockout mice, suggesting that ALP inhibits fat absorption. Multiple studies have suggested that intestinal ALP might diminish gut inflammation, possibly via dephosphorylation of toxic bacterial lipopolysaccharides or enhancement of the barrier function of the intestinal epithelium.

In summary, despite the ubiquitous distribution of ALP in nature, bone is the only organ shown to be critically dependent on ALP function. Of interest, while ALP isolated from E. coli has been the source of much of our knowledge of the structure and biochemistry of this enzyme, ALP knockout in these bacteria had no discernible effect on their viability in culture.

Therapeutic Use of ALP

Intravenous infusion of various isoforms of ALP have been used to treat a variety of disease states. The only approved use of this enzyme is an ALP modified to improve its “bone seeking potential” in children with hypophosphatasia. Other conditions in which ALP has been employed with variable/questionable benefit include: infant cardiopulmonary bypass (to enhance the conversion of adenosine monophosphate to adenosine), acute kidney injury, sepsis, and inflammatory bowel disease.

Clinical Application of ALP Measurements

Only selective aspects of this topic will be discussed since the practicing physician generally is well acquainted with the clinical utility of the ALP measurement. In adults, the primary purpose of the near-routine assessment of serum ALP activity is to detect cholestasis; subtle bone problems seldom are uncovered via this assay. If other liver studies (bilirubin, aminotransferases) are abnormal, an elevated ALP is assumed to indicate liver pathology of the cholestatic type. While ALP tends to be higher in extra versus intra-hepatic cholestasis, additional studies are required to identify the origin of the cholestatic problem. Occasionally, cholestasis causes elevations of plasma ALP while other liver function tests remain normal, raising the question of a bone rather than liver source of the enzyme. This differentiation is readily obtained via measurement of serum gamma glutamyl transeptidase (GGT), which similar to ALP is a hepatic membrane bound sialoglycoprotein, which usually is shed into the serum in concert with ALP in cholestasis but is not released from bone. Thus, a normal GGT indicates that an ALP elevation is of bony rather than hepatic origin, and the more expensive ALP isoform measurement seldom is required. While a more specific marker of liver malfunction than ALP, GGT rises with relatively minor hepatic insults (alcohol or drug ingestion) such that non-specific elevations of this enzyme preclude its usefulness as a first-line indicator of cholestasis.

In some forms of hereditary cholestasis, GGT remains normal or near normal despite appreciable increases in bilirubin and ALP, leading to the designation of these conditions as “low GGT familial intrahepatic cholestasis.” In this condition defective function of the bile acid exporter results in markedly diminished bile flow and retention of bile.
acids (and bilirubin). There appears to have been no literature discussion as to why this form of cholestasis should cause a discordant effect on serum ALP versus GGT. A possible explanation is that, in contrast to ALP, GGT is present in high concentration in bile duct epithelium. Release of GGT in cholestasis possibly requires damage to the biliary epithelium secondary to the increased intra-biliary pressure present in most types of cholestasis. No such increased pressure will occur with failure to export bile acids, hence there is no rise in GGT. As discussed, ALP is primarily located on the hepatocyte membrane and shed into the serum as a result of the detergent action of retained hydrophobic bile acids. Like ALP, GGT is cleared by the galactose transporter mechanism, and inhibition of this receptor would be expected to result in elevation of GGT as well as ALP.

Occasionally patients with liver disease present with an elevation of liver ALP while the bilirubin remains normal. This anomaly commonly can be explained by the different mechanisms accounting for elevations of ALP versus bilirubin in cholestasis. ALP rises because of the increased synthesis and membrane shedding of ALP from hepatocytes exposed to the increased biliary pressure of cholestasis whereas bilirubin rises due to a failure of bilirubin excretion. Thus, when cholestasis involves only a fraction of the liver tissue, the obstructed fraction releases excessive ALP while the non-obstructed fraction maintains a normal serum bilirubin concentration. This situation is most commonly observed with infiltrative diseases (tumors, granulomas, etc.) of the liver. As will be discussed defective elimination of ALP theoretically also could lead to an increase in ALP with normal serum bilirubin concentrations.

Bone ALP is shed into the serum during increased osteoblastic activity most prominently observed with Paget’s disease and to a lesser extent with fractures, osteomalacia, and primary or metastatic bone neoplasms and hyperparathyroidism. Serum levels of bone ALP are not elevated in osteomyelitis and the elevations with bone fracture tend to be relatively mild - the average maximal increase in total serum ALP activity was about 30% for patients with femoral neck fractures and 100% for trochanteric fractures, values well below those observed in our patient with a resected sternum.

Intestinal ALP released after ingestion of fatty meals may result in up to 20% increases in the total serum activity. Given the rapid clearance of this isoenzyme, intestinal ALP usually is negligible after an overnight fast, and evaluation of minor increases in serum ALP requires analysis of fasting levels to rule out a contribution by intestinal ALP.

Relatively minor elevations of ALP not clearly attributable to liver or bone disease are relatively common in healthy and hospitalized subjects. In hospitalized patients, such elevations were associated with renal failure, pyelonephritis, congestive heart failure, and various malignancies. In patients with benign disease, the majority of the elevations normalized during a 3 month follow-up; most patients with persistent, appreciable ALP elevations had obvious severe medical problems and extensive evaluation for subtle causes of elevated ALP seldom was not required. Unexplained, persistent elevations of ALP have been reported in healthy subjects, sometimes with a familial pattern, and such subjects have remained healthy during long-term follow-up.

Low serum ALP values have been reported in a host of relatively common conditions including hypothyroidism, celiac disease, zinc and magnesium deficiencies, and malnutrition, but the low sensitivity and specificity of this finding limits the diagnostic value of ALP in these conditions. However, a low serum ALP is a sine qua non of hypophosphatasia and also is useful to identify Wilson’s disease as the cause of acute liver failure - a ratio of ALP: total serum bilirubin of <4.0 indicates Wilson’s disease with a >90% sensitivity and specificity.

**Discussion of Case**

The peak ALP concentration of our patient (3350 IU/L) was unique for our patient population in that the next highest value recorded in the preceding year in our Veterans Administration Hospital laboratory (roughly 30,000 measurements) was 1900 IU/L. During this time period, 15 patients had multiple ALP values between 1000 and 1900 IU/L. Thirteen of these patients had clearly abnormal serum bilirubin and /or aminotransferase values and the other two had widespread metastatic prostatic carcinoma to bone. Thus, our patient also was unusual in that there was no evidence of associated liver disease or a bone neoplasm to account for the ALP elevation.

An ALP isofrom analysis showed that the massive ALP elevation consisted of 51% liver, 23% bone, and 26% macro-hepatic isoforms. For the peak ALP concentration, these percentages translate into serum ALP concentrations of 1712 IU/l, 770 IU/l, and 873 IU/l, respectively, for the three isoforms, all of which are at least 10-fold greater than
normal. Thus, the pathogenesis of our patient’s ALP requires an explanation of why multiple isoforms were elevated. The voriconazole received by our patient potentially could cause elevations of both bone and liver ALP since this drug has been associated with periostitis as well as liver function abnormalities. However, periostitis occurs only after many months of voriconazole therapy, and the liver function abnormalities virtually always take the form of aminotransferase and/or bilirubin elevations in association with ALP elevations, rather than the isolated ALP in this case.

While a small portion of the sternum was infected with Aspergillus, the bulk of the infection was in the adjacent cartilage and soft tissues. Given that study of patients with extensive osteomyelitis found no increase in serum bone ALP, there was no obvious explanation for an increased release of the bone isoenzyme into the blood of our patient. The bulk of the serum ALP activity (77%) was derived from the liver with 51% in the “soluble” form and 26% as the membrane associated, vesicular (macro) form. The usual explanation for elevation of these serum hepatic isoforms is “cholestasis”, i.e., inadequate flow of bile. However, there was no evidence of inadequate bile flow in that all other liver function measurements including bilirubin and aminotransferases, radiological studies (MRI/MRCP), and liver biopsy were normal, and there was no pruritus. Thus, if the massive increase in the serum liver isoforms was due solely to increased release of the enzyme due to liver pathology, this pathology seemingly reflected a yet to be described problem with the anchoring of ALP to the hepatocyte and osteoblast membranes. As discussed previously, if the liver ALP isoforms have an elimination rate comparable to the values obtained in experimental ALP infusion studies, the ALP would have had to turn over at an enormous rate to maintain the serum ALP at 3350 IU/L.

Given the above, we hypothesize that the massive unexplained ALP elevation in our patient was caused, at least in part, by defective elimination of the enzyme - a concept that, surprisingly, seems never to have been previously postulated to play pathogenetic role in the maintenance of elevated ALP elevations. Such defective elimination would explain the marked elevations of both the bone and liver isoforms in the absence of bone and liver pathology known to increase the release of ALP. The declining serum ALP concentration concurrent with the successful treatment of the Aspergillus raises the possibility that the active infection might have played a role in the putatively defective ALP clearance. As discussed, experimental administration of a compound that competes for the galactose receptor (such as asialofetuin) markedly reduces the clearance of ALP. Galactomannan, a component of the cell wall of Aspergillus, is released into the serum in Aspergillus infections, such that measurement of this compound is widely used for diagnostic purposes. Galactomannan, which consists of a mannose backbone with multiple galactose side groups, presumably could bind to the galactose receptor and serve as a competitive inhibitor for ALP uptake. Of interest, in a report describing 31 patients with serum ALP >1000 U/L, sepsis (bacterial and fungal) was the most common diagnosis, and the majority of these patients had a normal serum bilirubin. It seems possible that reduced ALP elimination secondary to the release of bacteria glycoproteins could play a role in isolated elevations of ALP of sepsis patients.

**Conclusions**

Admittedly, the concept that defective elimination plays a pathogenic role in otherwise unexplained ALP elevations is totally speculative and requires further supportive studies. The hypothesis that galactomannan interferes with ALP clearance could be readily tested in small animal experiments. However, direct proof of diminished ALP clearance in patients with isolated elevations of ALP will require quantitation of ALP elimination rate in appropriate patients, a somewhat complicated measurement requiring infusion of labelled exogenous ALP. Less direct, but more readily obtained evidence would be the demonstration of concurrent elevations of other circulating compounds that are eliminated via the galactose receptor in patients with unexplained ALP elevations.

**Abbreviations**

ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; MRCP, magnetic resonance cholangiopancreatography; IU, international units.

**Disclosure**

The authors declare that they have no conflicts of interest for this work.
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