Lysophosphatidic Acid Is a Potential Mediator of Cholestatic Pruritus

ANDREAS E. KREMER,* JOB J. W. W. MARTENS,* WIM KULIK,‡ FRANZISKA RUËFF,§ EDITH M. M. KUIPER,‖ HENK R. VAN BUUREN,‖ KAREL J. VAN ERPECUM,¶ JURATE KONDRACKIENE,# JESUS PRIETO,** CHRISTIAN RUST,‡‡ VICTORIA L. GEENES,§§ CATHERINE WILLIAMSON,§§ WOUTER H. MOOLENAAR,‖‖ ULRICH BEUERS,* and RONALD P. J. OUDE ELFERINK*

**Tytgat Institute for Liver and Intestinal Research and ‡Laboratory Genetic Metabolic Diseases, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; §Departments of Dermatology and Allergology, University of Munich, Munich, Germany; ‡‡Internal Medicine II - Grosshadern, University of Munich, Munich, Germany; ‖Department of Gastroenterology & Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands; ¶Department of Gastroenterology and Hepatology, University Medical Center, Utrecht, The Netherlands; #Department of Gastroenterology, Kaunas University of Medicine, Kaunas, Lithuania; **Department of Medicine and Liver Unit, Clinica Universitaria, Medical School and Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain; §§Maternal and Fetal Disease Group, Institute of Reproductive and Developmental Biology, Imperial College London, London, England; and ‖‖Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands*

BACKGROUND & AIMS: Pruritus is a common and disabling symptom in cholestatic disorders. However, its causes remain unknown. We hypothesized that potential pruritogens accumulate in the circulation of cholestatic patients and activate sensory neurons. **METHODS:** Cytosolic free calcium ($[Ca^{2+}]_i$) was measured in neuronal cell lines by ratiometric fluorometry upon exposure to serum samples from pruritic patients with intrahepatic cholestasis of pregnancy (ICP), primary biliary cirrhosis (PBC), other cholestatic disorders, and pregnant, healthy, and nonpruritic disease controls. Putative $[Ca^{2+}]_i$-inducing factors in pruritic serum were explored by analytical techniques, including quantification by high-performance liquid chromatography/mass spectroscopy. In mice, scratch activity after intradermal pruritogen injection was quantified using a magnetic device. **RESULTS:** Transient increases in neuronal $[Ca^{2+}]_i$ induced by pruritic PBC and ICP sera were higher than corresponding controls. Lysophosphatidic acid (LPA) could be identified as a major $[Ca^{2+}]_i$ agonist in pruritic sera, and LPA concentrations were increased in cholestatic patients with pruritus. LPA injected intradermally into mice induced scratch responses. Autotaxin, the serum enzyme converting lysophosphatidylcholine into LPA, was markedly increased in patients with ICP versus pregnant controls ($P < .0001$) and cholestatic patients with versus without pruritus ($P < .0001$). Autotaxin activity correlated with intensity of pruritus ($P < .0001$), which was not the case for serum bile salts, histamine, tryptase, substance P, or μ-opioids. In patients with PBC who underwent temporary nasobiliary drainage, both itch intensity and autotaxin activity markedly decreased during drainage and returned to preexistent levels after drain removal. **CONCLUSIONS: We suggest that LPA and autotaxin play a critical role in cholestatic pruritus and may serve as potential targets for future therapeutic interventions.**

Keywords: Autotaxin; Bile Salts; Cholestasis; Itch.

Chronic pruritus is a disabling symptom accompanying a broad range of systemic disorders such as chronic liver diseases, chronic renal failure, malignancies, infections, and endocrine and hematologic diseases.[1,2] Despite the recent discovery of itch-specific neuronal pathways, including novel itch mediators and their receptors,[1,3,4] the pathogenesis of pruritus remains enigmatic. Regardless of the underlying cause, various cholestatic disorders such as intrahepatic cholestasis of pregnancy (ICP), benign recurrent intrahepatic cholestasis, progressive familial intrahepatic cholestasis, primary biliary cirrhosis (PBC), and primary sclerosing cholangitis often induce pruritus. These cholestatic liver disorders are all characterized by an impairment of hepatocellular and/or cholangiocellular secretory function and bile flow.[5] In these patients, pruritus may become refractory to all medical treatments and can in severe cases be an indication for liver transplantation, even in the absence of liver failure.[6] In the past, enhanced serum levels of both bile salts and μ-opioids have been implicated in the etiology of cholestatic pruritus.[7] However, neither correlations between itch intensity and bile salt or opioid levels nor a causative link have ever been established.

Autotaxin (ATX) was originally identified in the conditioned medium of human A2058 melanoma cells and described as an autocrine cell motility factor.[8] ATX is overexpressed in several other tumor entities and has been linked to tumor cell proliferation, motility, and formation of metastasis.[9] Physiologically, ATX is required for angiogenesis and neuronal development, as indicated by ATX-deficient mice, which are embryonic lethal due to vascular malformation and neuronal abnor-

Abbreviations used in this paper: ATX, autotaxin; $[Ca^{2+}]_i$, cytosolic free calcium; HBSS, Hank's balanced salt solution; ICP, intrahepatic cholestasis of pregnancy; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PBC, primary biliary cirrhosis.

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malities.[10,11] Recently, ATX could be identified as an extracellular secreted enzyme with lysophospholipase D activity, which generates lysophosphatidic acid (LPA) from lysophosphatidylcholine (LPC).[12,13] LPA is a small but potent bioactive phospholipid with a wide variety of effects in many cell types ranging from cytoskeletal (re)organization and cell migration to cytokine production and platelet activation.[9,14] Effects of ATX are believed to be mainly mediated by the enzymatic formation of LPA, which activates at least 6 different G protein–coupled receptors.[9,15] Most interest in ATX has so far been directed toward its functions in cancer and early development. However, it was recently established that LPA plays a crucial role in the induction of neuropathic pain[16] and causes reprogramming of gene expression in different types of afferent nerve fibers.[17]

Here we report that levels of LPA and ATX are markedly increased in serum of patients with cholestatic pruritus. Moreover, serum levels closely correlate with itch intensity, and intradermal injections of LPA induce scratching behavior in mice. We therefore suggest that LPA and ATX play a critical role in cholestatic itch and may serve as potential targets for future therapeutic interventions.

Materials and Methods

Human Subjects

Peripheral venous blood was obtained from healthy donors, pregnant women, and patients with cholestatic disorders after informed consent according to the Declaration of Helsinki. The study was approved by the local medical ethical committees. Blood samples were immediately centrifuged at 4°C, and serum was frozen in aliquots at −80°C. ICP was diagnosed, as previously described,[18] in pregnant women with pruritus who had no rash in conjunction with increased serum liver transaminase and/or bile salt levels. Women were excluded if they had abnormal hepatitis serology (hepatitis A, B, and C) or extrahepatic biliary obstruction following ultrasonographic examination. Pregnant controls had no history of liver dysfunction or any complication in the current or previous pregnancies.

Animals

A Teflon-coated magnet was implanted in each hind paw of female C57BL/6J mice (6–8 weeks of age) 1 week before experiments were performed. Mice were given 120 minutes to acclimate to the chamber surrounded by a magnetic coil before they were briefly removed from the chamber and intradermally injected with saline (50 μL) or LPA (8–200 nmol in 50 μL) in the neck. Movements of the magnets induced an electric current in the magnetic field, which was registered by an oscillograph attached to a computer. The number of scratch bouts was analyzed as previously described.[19] Software was used to count scratch movements with a low cutoff frequency of 10 Hz, a high cutoff frequency of 20 Hz, a threshold level of 300 mV, a minimum of 4 beats per bout, and a maximal coefficient of variation of 40% between the beats of a bout. The analytical procedure was validated with intradermal compound 48/80, showing a positive predictive value of 95% at a sensitivity of 50%.

All mouse experiments were approved by the Institutional Animal Care and Use Committee.

Materials

Cell culture media were from Lonza (Basel, Switzerland); stearoyl-LPA (LPA 18:1) and myristoyl-LPC (LPC 14:0) from Avanti Lipids (Alabaster, AL); choline oxidase, horseradish peroxidase, homovanillic acid, pertussis toxin, and ionomycin from Sigma-Aldrich (St Louis, MO); and Ki16245 and ATX antibody for Western blotting from Cayman (Ann Arbor, MI). Indo-1 AM was from Invitrogen (Carlsbad, CA), and Microcon filters were from Millipore (Billerica, MA).

Cell Culture

SH-SY5Y cells were cultured in Ham's F12K medium containing 10% (vol/vol) fetal bovine serum, penicillin (100 IU/mL), streptomycin (100 μg/mL), and L-glutamine (0.2 mmol/L) at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Fluorometric Measurement of Cytosolic Free Calcium Levels

SH-SY5Y cells were detached, washed twice, and suspended in HEPES-buffered Hank's balanced salt solution (HBSS). Cells were incubated with 10 μmol/L Indo-1 AM for 30 minutes at 37°C, washed, resuspended in HEPES-buffered HBSS, and incubated for another 30 minutes at 25°C to allow dye hydrolysis. After another wash step, cells were resuspended in HEPES-buffered HBSS. Analyses were performed in a NOVOstar analyzer (BMG Labtech GmbH, Offenburg, Germany; excitation, 320 nm; emission, 405 nm and 520 nm). Cell suspensions were allowed to adapt to 37°C for 10 minutes before serum or extracts were added. Receptor blockers were added 10 minutes before addition of serum. Cytosolic free calcium ($[Ca^{2+}]_i$) was calculated after calibration with ionomycin (10 μmol/L) and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (5 mmol/L) according to Grynkiewicz et al.[20]

ATX Activity Assay

ATX activity was analyzed as recently described.[21] In short, serum and bile samples were incubated with a buffer containing 1 mmol/L LPC 14:0, 500 mmol/L NaCl, 5 mmol/L $MgCl_2$, 100 mmol/L Tris (pH 9.0), and 0.05% Triton X-100 for 60 minutes at 37°C. The phosphodiesterase activity of ATX was determined by the amount of liberated choline, as detected by an enzymatic

fluorometric method using choline oxidase (2 U/mL), horseradish peroxidase (1.6 U/mL), and homovanillic acid as substrate for peroxidase. After addition of both enzymes in a buffer (consisting of 20 mmol/L $CaCl_2$, 2 mmol/L homovanillic acid, 50 mmol/L 3-(*N*-morpholino) propanesulfonic acid [pH 8.0], and 0.1% Triton X-100), the increase in fluorescence was monitored at 37°C on a NOVOstar analyzer (excitation, 320 nm; emission, 405 nm).

Quantitative Determination of LPA Levels

A detailed description of the procedure is given in the Supplementary Methods. Briefly, serum lipids were extracted after addition of myristoyl-LPA as internal standard and analyzed by high-performance liquid chromatography/mass spectroscopy.

Determination of Bile Salt Levels

Total serum bile salt levels were determined using Diazyme total bile salts kit (Diazyme Laboratories, Poway, CA) according to the manufacturer's instructions.

Determination of Histamine Levels

Serum histamine concentrations were measured by a competitive enzyme immunoassay (Immunotech, Marseille, France) based on the competition between free acylated histamine and alkaline phosphatase acylated histamine conjugate.

Determination of Tryptase Levels

Serum tryptase concentrations were determined by a fluoroenzyme immunoassay (UNICAP Tryptase; Pharmacia Diagnostics, Freiburg, Germany) detecting both α- and β-tryptase.

Determination of μ-Opioid Activity

Total serum μ-opioid activity was determined as described by Swain et al.[22]

Determination of Substance P Levels

Substance P concentrations were analyzed by a competitive enzyme immunoassay according to the manufacturer's instructions (Bachem, Torrance, CA).

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis and Western Blotting

To concentrate ATX, 50 μL of serum and bile samples was first incubated for 4 hours at 4°C with immunoprecipitating ATX antibody 5E5 (kindly provided by J. Aoki)[10] bound to Sepharose. After washing, Sepharose beads were incubated for 10 minutes at 37°C with sodium dodecyl sulfate–loading buffer containing β-mercaptoethanol and spun down. Equal amounts of supernatant were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, blotted on polyvinylidene difluoride membranes, blocked with 5% skim milk in phosphate-buffered saline/0.05% Tween 20, and incubated with anti-ATX (1:1500; Cayman) overnight. Proteins were visualized with horseradish peroxidase–conjugated immunoglobulins and detected by enhanced chemiluminescence (Amersham, Buckinghamshire, England).

Statistical Analysis

Statistical differences were evaluated for 2 groups by Student *t* test and for 3 or more groups by 1-way analysis of variance with Bonferroni correction using SPSS (version 16.0; SPSS Inc, Chicago, IL). Spearman's correlation coefficient and corresponding *P* values were calculated to assess the relationship between tested parameters. All data are expressed as means ± SD.

Results

Neuron-Activating Serum Factor Identified as LPA

To identify potential pruritogens in cholestasis, we screened sera from pruritic patients for activation in different neuronal cell lines. We chose $[Ca^{2+}]_i$ as an indicator of neuronal activation because it is a key mediator of the neuronal secretory response toward diverse receptor-dependent and -independent stimuli. In the human neuroblastoma cell line SH-SY5Y, we observed a dose-dependent increase in intracellular calcium concentrations with the addition of serum (Figure 1*A*). Interestingly, sera from women with ICP showed a markedly stronger neuronal activation compared with pregnant controls and healthy female controls (Figure 1*B*). Similarly, sera from pruritic patients with PBC tended to induce higher transient increases in $[Ca^{2+}]_i$ levels than sera from patients with PBC without pruritus and healthy controls (Figure 1*C*). We further analyzed the serum samples to identify the neuron-activating serum factor. Pretreatment of serum with 90% ethanol or proteinase K hardly diminished the $[Ca^{2+}]_i$ response, indicating that the serum factor was not a peptide or protein (Figure 2*A*). Serum samples were centrifuged through filters to estimate the molecular size. The serum factor could pass a 100-kilodalton filter, but not a 10-kilodalton filter. Interestingly, pretreatment of serum with 90% ethanol enabled the factor to partially pass through the 10-kilodalton and even a 3-kilodalton filter (Figure 2*B*). This observation could be explained by strong binding of the factor to albumin (~60 kilodaltons). Thus, like unconjugated bilirubin, the substance appeared to be partially forced into solution on ethanol-induced precipitation of albumin. Total recovery of the serum factor through a 10-kilodalton filter was achieved by addition of cholate above its critical micellar concentration, enabling a hydrophobic substance to be completely solved in an aqueous solution (Figure 2*B*). Because those micelles have a size of approximately 4.4 kilodaltons, they barely pass a 3-kilodalton filter. Hence, we were dealing with a

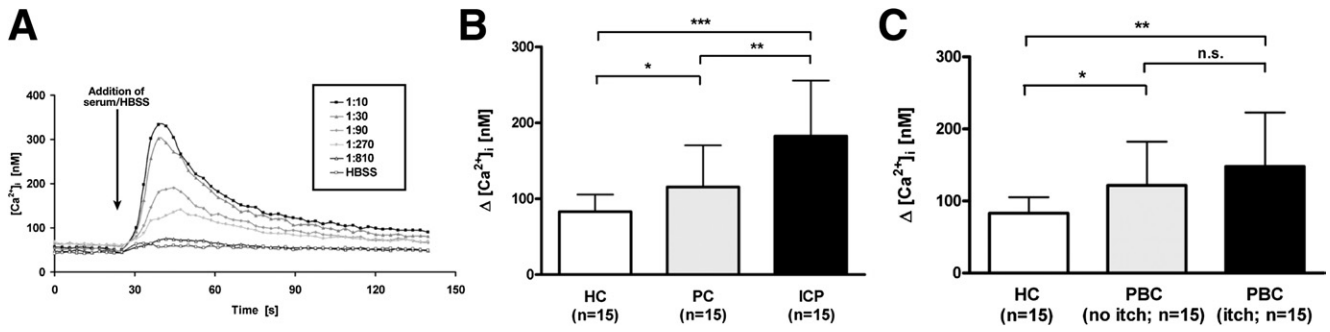

Figure 1. $[Ca^{2+}]_i$ is increased in human neuroblastoma cell line SH-SY5Y by sera from patients with cholestatic pruritus more than by sera from healthy controls. *A* shows that serum induced a transient increase in $[Ca^{2+}]_i$ that was dose dependent. Note that even high serum dilutions of up to 1:320 induced an increase of $[Ca^{2+}]_i$. (*B*) Sera from women with ICP induced higher increases of $[Ca^{2+}]_i$ compared with gestation-matched normal pregnancies (PC) and age-matched female controls (HC). (*C*) Sera from patients with PBC with and without pruritus induced a higher increase in $[Ca^{2+}]_i$ compared with age-matched healthy female controls. $^{*}P < .015$, $^{**}P < .005$. $\Delta[Ca^{2+}]_i$ represents the peak of calcium transient minus basal calcium concentration as shown in *A*: maximal $[Ca^{2+}]_i$ – basal $[Ca^{2+}]_i$. n.s., not significant.

small, hydrophobic substance. Its chemical properties were further analyzed by a 2-phase Bligh and Dyer lipid extraction. At neutral pH, the compound presented an amphiphilic character but dissolved better in the lower aqueous phase. Lowering the pH to 1.0 and thus potentially protonating the serum factor led to a recovery of the substance mainly in the upper lipid phase (Figure 2*C*). The increased hydrophobicity upon acidification could be explained by protonation of phosphate or sulfate groups in the molecule.

These observations suggested that a small phospholipid could be responsible for the activation of neuronal cells. As pertussis toxin diminished the increase in $[Ca^{2+}]_i$, signaling of the unknown substance appeared to occur via a G-protein–coupled receptor (Figure 2*D*). These observations rendered LPA a likely candidate because LPA has been reported to increase calcium levels in neuronal cells.[23] Pretreatment of the neuronal cells with Ki16425, a specific LPA receptor blocker, significantly reduced the increase in $[Ca^{2+}]_i$, indicating that LPA was the major serum factor in our cholestatic serum samples (Figure 2*D*).

Analyzing the LPA content in serum samples by mass spectrometry indeed showed markedly higher concentrations of LPA 18:1 in sera from women with ICP compared with gestation-matched pregnant controls (Figure 2*E*). Similar differences were observed for other LPA species such as LPA 16:0, 18:0, 18:2, 20:3, and 20:4 (data not shown).

ATX Is Enhanced in Pruritus of Cholestasis

LPA is formed in the blood through cleavage of choline from LPC by ATX, which has recently been identified as a lysophospholipase D.[12,13] Because LPC is present in plasma at relatively high concentrations (>100 μmol/L), the amount of LPA (in low micromolar range) in blood primarily depends on ATX activity.[11] Therefore, we analyzed whether ATX activity in blood also correlated with the occurrence of itch. We observed higher ATX activity in sera from women with pruritus due to ICP as compared with pregnant and nonpregnant controls (Figure 3*A*). The enhanced ATX activity correlated with increased ATX protein content in sera from these patients (Figure 3*B*). We studied whether this observation could be extended to other forms of cholestasis. Therefore, sera from patients with different cholestatic disorders with and without pruritus were analyzed. Quite strikingly, we found that, irrespective of the cause of cholestasis, ATX levels were markedly enhanced in patients with pruritus compared with patients without pruritus (Figure 3*C–E*). Recently, in patients with chronic hepatitis C, liver ATX messenger RNA expression[24] and serum ATX levels[25] have been reported to be enhanced. ATX serum levels were also increased in our group of patients with hepatitis C virus when compared with healthy controls but were significantly lower than in pruritic patients (Figure 3*F*).

ATX Activity Correlates With Intensity of Pruritus

Pruritus is a subjective perception that differs between individuals. Quantification of this symptom is difficult but can be achieved using visual analog scales.[1] Patients quantified their itch intensity at the time point of blood drawing on a scale ranging from 0 (no pruritus) to 10 (most severe form of pruritus). Next we analyzed the correlation between itch intensities and the ATX activity in serum from these patients by linear regression analysis. We found a highly significant correlation between enzyme activity and intensity of itch perception (Figure 4*A*). In contrast, other agents proposed as potential pruritogens in cholestasis and other diseases in the past[7,26] did not show any correlation with itch intensity in our patient cohort. This was tested for histamine, tryptase, substance P, and μ-opioid activity (Figure 4*B–F*). Even though cholestatic patients with pruritus as a group showed higher serum bile salt concentrations (Table 1), there was no correlation with itch intensity (Figure 4*D*).

Figure 2. Identification of $[Ca^{2+}]_i$-enhancing serum factor as LPA. *A* shows the effect of pretreatments of serum with 90% ethanol and proteinase K on $[Ca^{2+}]_i$ in neuronal cells. U, untreated; E, supernatant of 90% ethanol precipitation; Prot K, incubation for 24 hours with proteinase K (n = 3). *B* shows the effect of filter experiments on serum. 100 kD, 10 kD, flow through of a 100-kilodalton and 10-kilodalton filter, respectively; E, supernatant of 90% ethanol precipitation (before filtering); CA, resuspension in 2 mmol/L cholate (before filtering) (n = 3). (*C*) Effect of Bligh and Dyer lipid extraction at pH values of 7.4 and 1.0. WP pH 7.4, LP pH 7.4, WP pH 1.0, and LP pH 1.0 indicate the water phase (WP) and lipid phase (LP) of serum after lipid extraction at pH values of 7.4 and 1.0 (n = 3). (*D*) Effect of pertussis toxin (PTX) and LPA receptor blocker Ki16245 on cell activation by serum (n = 3). Δ405/520 represents the change in fluorescence at 405 nm (Ca^{2+}-sensitive signal) divided by that at 520 nm (Ca^{2+}-insensitive signal). (*E*) LPA 18:1 is enhanced in ICP cases compared with gestation-matched noncholestatic pregnant controls. LPA 18:1 is shown as the ratio to LPA 14:0 (added as an internal standard before extraction). $^{*}P < .05$, $^{**}P < .01$.

A direct role of histamine and bile salts as pruritogens has already been questioned in the past.[7,27] An antipruritic effect of μ-opioid antagonists has been reported in some patients.[28] However, μ-opioid activity was not enhanced in patients with ICP compared with regular pregnancies and only very few pruritic patients with PBC had increased μ-opioid levels, questioning a major causative role of opioids for the pathogenesis of pruritus in cholestasis (Figure 4*F*).[29] In some patients with PBC, extensive long-lasting pruritus was intractable and did not adequately respond to any recommended medication.[6,26] These patients underwent nasobiliary drainage for 2 to 7 days as an experimental treatment of most severe pruritus.[27,30] In all 4 procedures, this led to dramatic reduction or complete relief of pruritus within 24 hours that lasted for several days to weeks. Interestingly, concomitant with relief of pruritus, ATX activity decreased and increased back to pretreatment levels when pruritus returned (Figure 5*A*). This effect was not due to direct biliary clearance of ATX because neither ATX activity (Figure 5*B*) nor protein (Figure 5*C*) could be found in bile from these and other patients.

Induction of Pruritus by LPA

To investigate a potential role of LPA in the induction of pruritus in vivo, we used female C57BL/J6

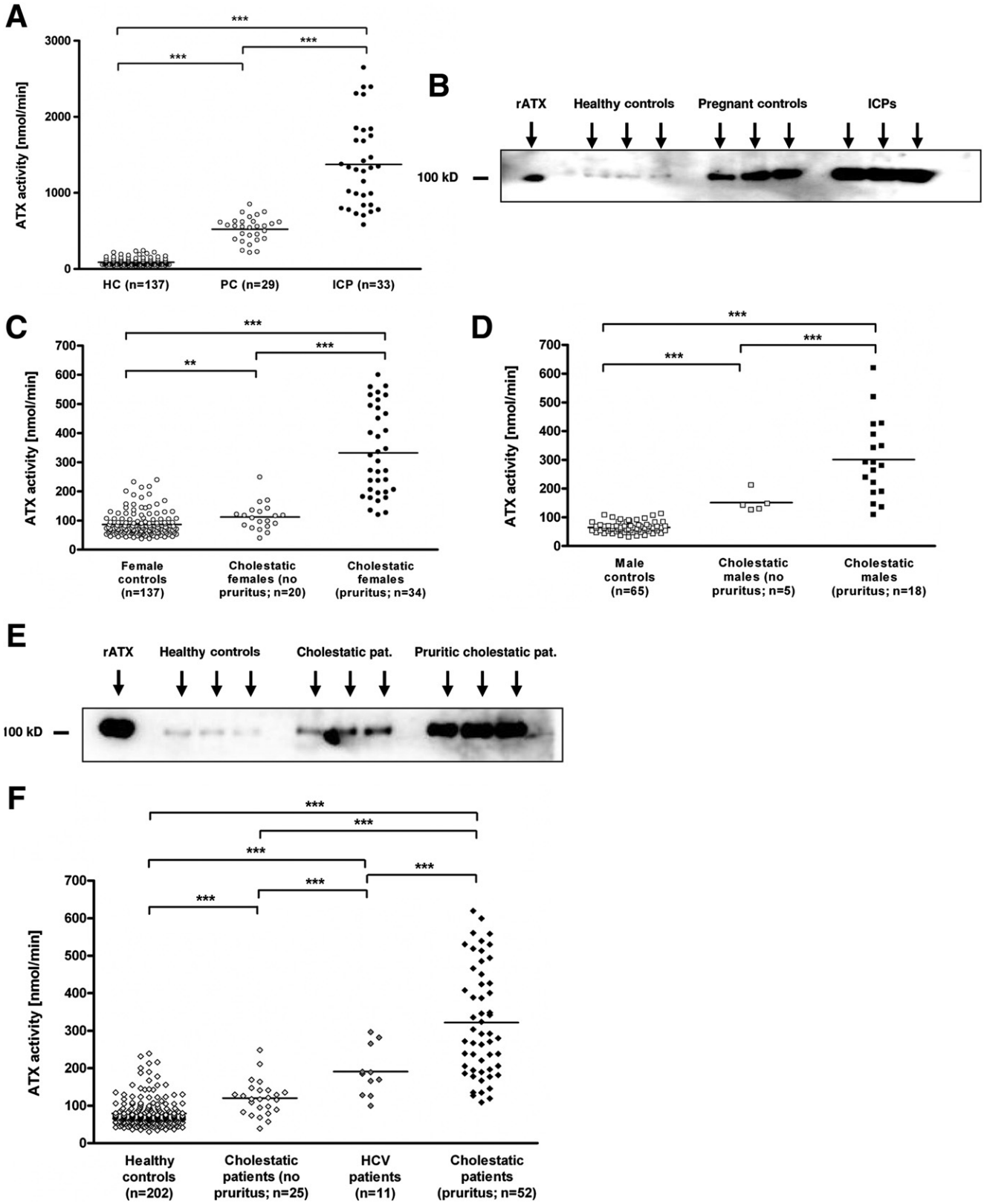

Figure 3. Serum ATX is elevated in cholestatic patients with pruritus irrespective of the cause of cholestasis. (*A*) ATX activity (measured as choline release upon incubation with LPC) is highly enhanced in patients with ICP compared with pregnant controls and healthy female controls. $^{***}P < .0001$. (*B*) Western blot for ATX protein in patient sera. Recombinant ATX (rATX) was used as a positive control. (*C* and *D*) ATX activity was highly enhanced in cholestatic women and men with pruritus compared with nonpruritic cholestatic patients and healthy controls. $^{**}P < .01$, $^{***}P < .0001$. (*E*) Western blot for ATX protein in serum from patients with PBC. rATX was used as positive control. (*F*) ATX activity in patients with chronic hepatitis C was enhanced compared with controls but significantly lower compared with cholestatic patients with pruritus. $^{***}P < .0001$.

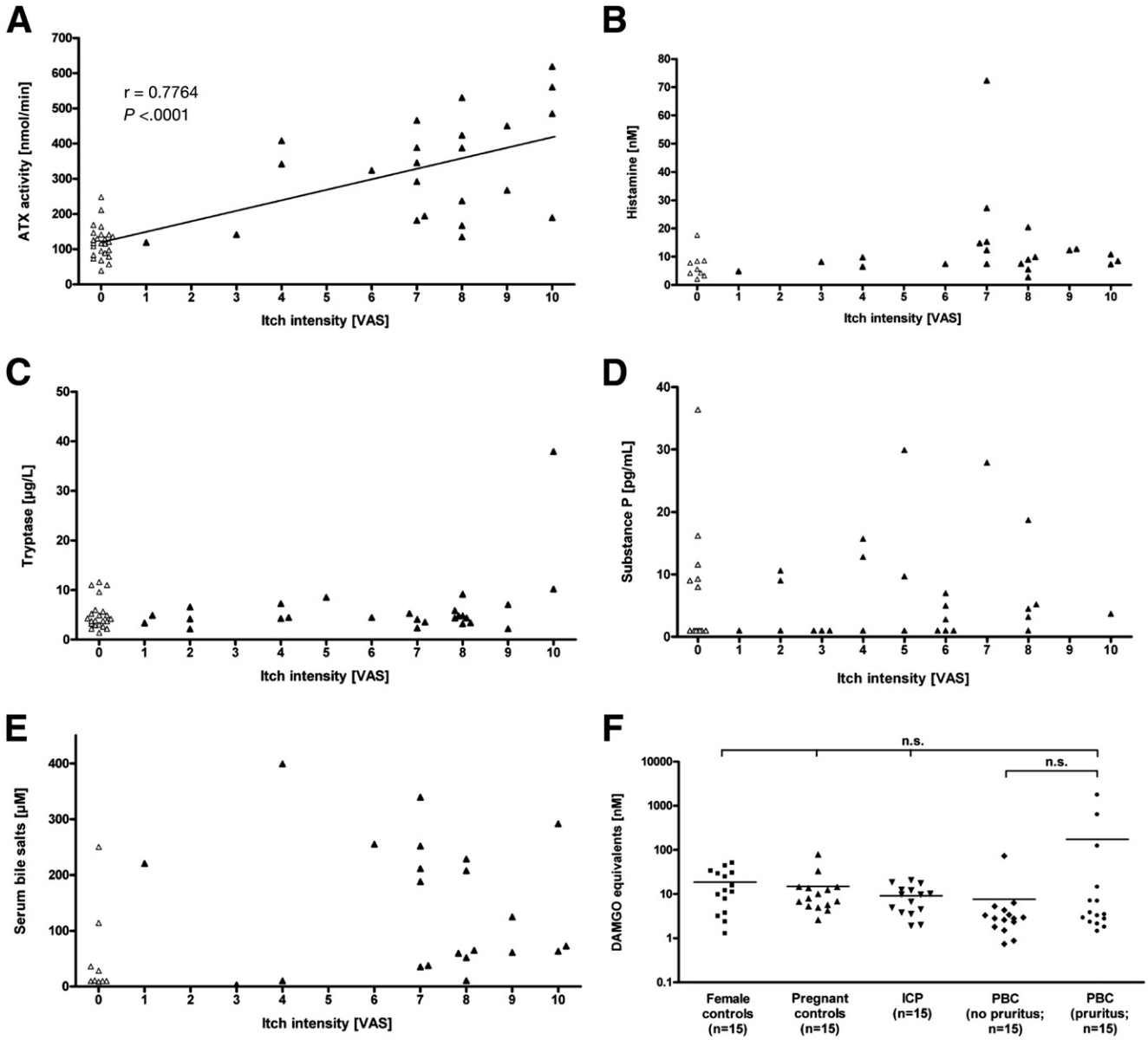

Figure 4. Only ATX activity, but not histamine, tryptase, substance P, serum bile salts, or μ-opioid activity, in serum correlated with itch intensity of patients with cholestatic itch. (*A*) ATX activity showed a significant linear correlation with the itch intensity represented on a visual analog scale (VAS) ranging from 0 (no pruritus) to 10 (most severe form of pruritus). Spearman's correlation coefficient: $r = 0.7764$, $P < .0001$. (*B–E*) No correlation between histamine levels, tryptase concentrations, substance P levels, or total serum bile salt levels and itch intensity. (*F*) Total μ-opioid activity in female and pregnant controls compared with women with ICP, PBC without pruritus, or PBC with pruritus. n.s., not significant.

mice. Scratch movements were registered as described previously.[19] Intradermal injections of LPA, but not the carrier, induced significant scratching behavior shown as number of scratch bouts per 5-minute intervals (Figure 6*A*), which was in line with a previous report.[31] Furthermore, we could demonstrate that the induction of pruritus by LPA in mice was dose dependent, showing an increased scratching behavior from 20 nmol upward (Figure 6*B*).

Discussion

Pruritus is a common and disabling symptom in cholestatic liver diseases and many other systemic disorders, including renal insufficiency; endocrine, hematologic, and metabolic diseases; various infections; and certain malignancies. The causal factors of pruritus are unknown in most of these diseases. Here we provide clinical and experimental evidence that LPA is a potential mediator of cholestatic itch.

The discovery of itch-specific sensory neurons in the skin by Schmelz et al revolutionized the research field of pruritus.[32] Thus, primary sensory neurons could be localized that only responded to the pruritogen histamine but were insensitive to mechanically induced pain stimuli. Recently, another class of itch-specific sensory neurons has been described that mediates pruritic stimuli

Table 1. Characteristics and Serum Chemistry of Cholestatic Patients With and Without Pruritus

	Patients without pruritus (n = 25)	Patients with pruritus (n = 52)	*P* value
Male/female	5/20	18/33	.15
Age (*y*)	59.9 ± 14.3	52.9 ± 13.0	.05
Disease (PBC/primary sclerosing cholangitis/other)	15/1/9	26/13/13	.76
Alkaline phosphatase (*IU/L*)	265.8 ± 219.9	313.6 ± 252.0	.45
γ-Glutamyltransferase (*IU/L*)	150.7 ± 205.6	186.2 ± 227.5	.54
Bilirubin (*mg/dL*)	3.6 ± 6.0	2.9 ± 5.0	.62
Serum bile salts (*μmol/L*)	61.4 ± 72.3	151.6 ± 125.8	.01
Albumin (*g/dL*)	4.6 ± 1.2	5.0 ± 1.3	.18
Alanine aminotransferase (*IU/L*)	69.6 ± 80.5	74.6 ± 85.0	.82
Aspartate aminotransferase (*IU/L*)	74.3 ± 105.4	70.7 ± 55.0	.85
C-reactive protein (*mg/dL*)	0.4 ± 0.5	0.5 ± 0.3	.71

NOTE. All values are expressed as mean ± SD. *P* values are for comparison between the subgroups of cholestatic patients with and without pruritus. A majority of samples were obtained from the outpatient clinic of the Academic Medical Center, Amsterdam. Additional samples were collected at the Universities of Utrecht and Rotterdam, The Netherlands; Munich Germany; and Navarra, Spain.

independent of histamine.[33] Hence, pruritoceptive nerve fibers seem to consist of different subsets of neurons, as was already known for nociceptive nerve fibers. This could explain the varying characters of itch sensations in different diseases ranging from "tickling" over "burning and painful" to agonizing pruritus.[34] We are, however, still far away from an explanation for the itch sensation that is experienced by many patients with systemic disorders.

Cholestatic disorders are frequently accompanied by itch sensation, and bile salts, and endogenous opioid peptides have, among others, been hypothesized as pruritogens.[7] However, in line with previous reports,[29,35] we have found no correlation between the severity of itch and these 2 parameters. Furthermore, although histamine is a well-established mediator of pruritus during allergic reactions, its serum levels showed no correlation with itch intensity in our patient cohort, in line with the clinical observation that antihistamines are in general ineffective in the treatment of cholestatic itch.[6] Serum tryptase is a marker of mast cell activation, and tryptase has been reported to induce pruritus via protease-activated receptor 2 (PAR-2).[36] However, in our study, tryptase concentrations were not enhanced in cholestatic patients and did not correlate with itch intensity. We observed similar negative results for substance P, which represents a pruritogen in diseases such as Sézary syndrome.[37]

In contrast, we have found strong evidence that the occurrence of itch is associated with increased systemic levels of the signaling lipid mediator LPA. Both serum LPA and ATX levels were significantly elevated in cholestatic patients with itch as compared with nonpruritic patients. ATX is a lysophospholipase D that hydrolyzes LPC into LPA and choline. Serum ATX activity, in fact, closely correlated with the extent of itch perception as objectified by the patient's scoring on a visual analog scale. Moreover, we found that relief of itch in patients with PBC by interruption of the enterohepatic cycle also significantly decreased serum ATX levels, although the molecular mechanism remains yet unclear. It is attractive to speculate that a yet undefined factor that undergoes enterohepatic circulation may increase ATX expression in cholestatic patients with pruritus. We subsequently confirmed the finding reported by others[31] that intradermal injection of LPA in mice caused short-lasting but significant scratching behavior that was dose dependent. Taken together, these findings strongly suggest that ATX and its formed product, LPA, play a causative role in the induction of itch during cholestasis.

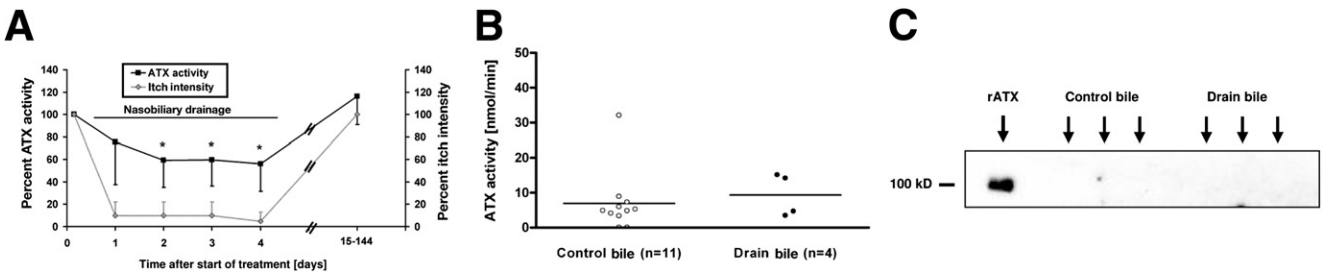

Figure 5. Serum levels of ATX respond to therapeutic interventions. (*A*) In patients with PBC undergoing nasobiliary drainage (start on day 0), ATX activity decreased with pruritus scores and increased on reappearance of pruritus several weeks later (days 15–144). Data are shown as percent changes of baseline values in 4 patients. $^{*}P < .05$. (*B*) ATX activity in bile of patients undergoing nasobiliary drainage and in control bile. (*C*) ATX protein could not be detected in bile by Western blot. Recombinant ATX (rATX) was used as positive control.

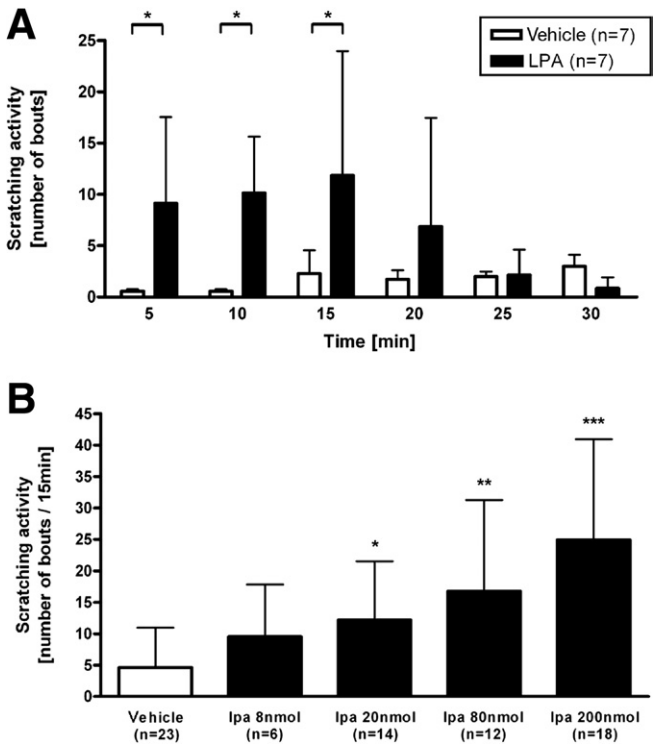

Figure 6. Dose-dependent induction of scratch responses by LPA in vivo. (A) Intradermal injections of LPA (100 nmol) led to increased scratching behavior compared with vehicle injections being significant during the first 15 minutes. Injections were performed in 7 mice. $^{*}P < .05$. (*B*) Dose-dependent scratching behavior after intradermal injections of LPA. Injections were performed in the indicated number of mice. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

Strikingly, we observed a much clearer difference between pruritic and nonpruritic patients in serum ATX levels than in serum LPA levels and concurrent serum-induced $\Delta[Ca^{2+}]_i$ in neuroblastoma cells (compare Figures 1 and 2 with Figure 3). One might argue that serum LPA levels (and the concurrent Ca^{2+} transient) should closely follow the serum ATX level, because the latter is responsible for increased LPA levels. Although we did find a clear correlation between serum ATX and LPA levels as well as Ca^{2+} transients (data not shown), the range of ATX levels was much more dynamic and levels were more consistently increased in pruritic patients. The most likely explanation for this discrepancy is that LPA is a highly unstable lipid derivative that undergoes rapid metabolism in the circulation. In addition, LPA can be formed during and after blood collection, and therefore levels depend on the procedure of processing and storage. In contrast, the enzyme ATX turns out to be highly stable in vitro and therefore represents a much more direct and reliable parameter.

The source of increased serum ATX levels remains to be determined. Enhanced levels could either be caused by increased ATX expression or by reduced clearance of the enzyme. Recently, liver sinusoidal endothelial cells were shown to play an important role in uptake and degradation of ATX.[38] This is in agreement with our observation that ATX could not be detected in bile. Although ATX has a half-life of only several minutes in circulation, its activity can easily be detected in serum, suggesting a continuous synthesis and release by peripheral cells and tissues. Among these may be endothelial cells and adipocytes,[39,40] but ATX was also reported to be expressed in the liver.[41] Interestingly, in patients with chronic hepatitis C, liver ATX messenger RNA expression was enhanced,[24] which might lead to enhanced serum ATX concentrations in these patients.[25] In our group of patients with hepatitis C virus, ATX levels were higher than in healthy controls but lower compared with cholestatic patients with pruritus.

ATX was originally described as a motility-stimulating protein secreted from melanoma cells.[8] Nowadays, the effects of ATX are believed to be mainly mediated by its enzymatic product LPA.[14] The bioactive lipid LPA is an agonist of a family of at least 6 G protein–coupled receptors that promote a great variety of biological processes, ranging from cell motility, proliferation, survival, and tumor progression to vascular development and cytokine production.[14] Furthermore, LPA has been implicated in neuronal cell functions such as brain development and neurite remodeling but also demyelination and after neurotrauma.[42] In mice, LPA initiates neuropathic pain after a single intrathecal injection.[16] Mice lacking LPA_1 receptor do not develop any signs of demyelination or neuropathic pain.[16] It was shown that LPA causes reprogramming of signal transmission through different nerve fibers. While transmission through type 3 Aδ fibers is increased, the transmission through type 1 C fibers (involving substance P) is dramatically reduced.[17] Thus, LPA may induce neuropathic pain via LPA_1 receptors on nociceptive nerve fibers and at the same time contribute to pruritus via LPA receptors on pruritoceptive neurons. Alternatively, LPA might indirectly cause pruritus by stimulating the release of a pruritogenic cytokine or lipid mediator from cells located in the skin.[43]

It is intriguing to speculate on the role of ATX and LPA in the pathogenesis of pruritus in other systemic diseases. Wound healing after injury or surgery is typically accompanied by local itch perception. LPA promotes re-epithelialization and healing of cutaneous wounds.[44] High local concentrations of LPA might thus elicit itch-specific neurons, leading to the well-known desire to scratch a wound during its healing process. Pruritus is also a common symptom in patients with lymphoma, especially in those with Hodgkin's disease.[45] Recently, Epstein–Barr virus–infected Hodgkin lymphoma cells have been shown to highly express ATX.[46] Thus, these cells might release high amounts of ATX, forming high local concentrations of LPA that not only promote tumor growth[9] but may also activate the neuronal itch pathway. Intriguingly, patients with Hodgkin's

disease with intense pruritus have a shorter survival than those without itch.[47]

Unraveling the molecular mechanisms leading to pruritus in systemic diseases will have a major impact on the development of novel treatment strategies for this agonizing symptom. At least in cholestatic disorders, ATX inhibitors and LPA receptor blockers, which are currently developed for the treatment of patients with malignancies to reduce disease progression and formation of metastasis,[48] might also represent a novel class of antipruritic drugs.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.05.009.

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Reprint requests

Address requests for reprints to: Andreas E. Kremer, MD, Tytgat Institute for Liver and Intestinal Research, Academic Medical Center, S1-164, University of Amsterdam, NL-1105 BK Amsterdam, The Netherlands. e-mail: a.e.kremer@amc.uva.nl; fax: (31) 20-566-9190.

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Conflicts of interest

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Supplementary Materials and Methods

Quantitative LPA Determination

As internal standard myristoyl-LPA (LPA 14:0) was added to serum samples reaching a final concentration of 1 μM. Lipids were subsequently extracted from 100 μL of serum by one-phase lipid extraction using 1 mL of methanol/chloroform (1:1, vol/vol). The extraction fluid was evaporated to dryness (45°C, vacuum). The residue was dissolved in 100 μl of chloroform/methanol/water (50:45:5, v/v/v) containing 0.01% NH_4OH, and 10 μL of this solution was injected into the HPLC–MS system. The HPLC system consisted of a Surveyor quaternary gradient pump, a vacuum degasser, a column temperature controller, and an autosampler (Thermo Electron, Waltham, MA, USA). The column temperature was maintained at 25°C. The lipid extract was injected onto a LiChrospher 2 × 250 mm silica-60 column, 5 μm particle diameter (Merck, Darmstadt, Germany). The phospholipids were separated from interfering compounds by a linear gradient between solution B (chloroform/methanol, 97:3, v/v) and solution A (methanol/water, 85:15, v/v). Solutions A and B contained 1 and 0.1 mL of 25% (v/v) aqueous ammonia per liter of eluent, respectively. The gradient (0.3 mL/min) was as follows: 0–10 min, 20% A–100% A; 10–12 min, 100% A; 12–12.1 min, 100% A–0% A; and 12.1–17 min, equilibration with 0% A. All gradient steps were linear, and the total analysis time, including the equilibration, was 17 min. A splitter between the HPLC column and the mass spectrometer was used, and 75 μL/min eluent was introduced into the mass spectrometer. A TSQ Quantum AM (Thermo Electron) was used in the negative electrospray ionization mode. Nitrogen was used as the nebulizing gas. Argon was used as the collision gas. The skimmer offset was set at 10 V. The spray voltage used was 3600 V, and the capillary temperature was 300°C. Selected reaction monitoring (SRM) was used to monitor precursor to product ion transition of m/z 381.2 → 227.2 for LPA(14:0) and m/z 435.25 → 281.25 for LPA(18:1). Quadrupole 1 and quadrupole 3 were maintained at 0.3 and 0.7 unit resolution (FWHM) respectively. The collision gas pressure was 0.5 mTorr and the collision energy was set at 50 V. Dwell time was 0.150 s for both the analytes and IS. All the parameters of LC and MS were controlled by Xcalibur software version 2.0.7.