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Luminescent Immunoprecipitation System (LIPS) for Detection of Autoantibodies Against ATP4A and ATP4B Subunits of Gastric Proton Pump H+,K+-ATPase in Atrophic Body Gastritis Patients

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OBJECTIVES: Circulating autoantibodies targeting the H+/K+-ATPase proton pump of gastric parietal cells are considered markers of autoimmune gastritis, whose diagnostic accuracy in atrophic body gastritis, the pathological lesion of autoimmune gastritis, remains unknown. This study aimed to assess autoantibodies against ATP4A and ATP4B subunits of parietal cells H+, K+-ATPase in atrophic body gastritis patients and controls.

METHODS: One-hundred and four cases with atrophic body gastritis and 205 controls were assessed for serological autoantibodies specific for ATP4A or ATP4B subunits using luminescent immunoprecipitation system (LIPS). Recombinant luciferase-reporter-fused-antigens were expressed by *in vitro* transcription-translation (ATP4A) or after transfection in Expi293F cells (ATP4B), incubated with test sera, and immune complexes recovered using protein-A-sepharose. LIPS assays were compared with a commercial enzyme immunoassay (EIA) for parietal cell autoantibodies.

RESULTS: ATP4A and ATP4B autoantibody titers were higher in cases compared to controls ($P < 0.0001$). The area under the receiver-operating characteristic curve was 0.98 (95% CI 0.965–0.996) for ATP4A, and 0.99 (95% CI 0.979–1.000) for ATP4B, both higher as compared with that of EIA: 0.86 (95% CI 0.809–0.896), $P < 0.0001$. Sensitivity-specificity were 100–89% for ATP4A and 100–90% for ATP4B assay. Compared with LIPS, EIA for parietal cell autoantibodies showed a lower sensitivity (72%, $P < 0.0001$) at a similar specificity (92%, $P = 0.558$).

CONCLUSIONS: Positivity to both, ATP4A and ATP4B autoantibodies is closely associated with atrophic body gastritis. Both assays had the highest sensitivity, at the cost of diagnostic accuracy (89 and 90% specificity), outperforming traditional EIA. Once validated, these LIPS assays should be valuable screening tools for detecting biomarkers of damaged atrophic oxyntic mucosa.

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INTRODUCTION

The H+, K+-ATPase, present in the parietal cells of the gastric oxyntic mucosa, is a gastric proton pump that functions to maintain an acidic environment within the stomach by transporting H+ and K+ ions against their concentration gradients using energy derived from the hydrolysis of ATP. H+, K+-ATPases are P-type ATPases existing as heterodimers: the alpha-subunit, coded by the ATP4A gene on human chromosome 19q13.1, contains the catalytic site and mediates ion transport; the beta-subunit, coded by the ATP4B gene on human chromosome 13q34, acts to stabilize the alpha-subunit and is essential for enzyme function.[1–3]

Circulating autoantibodies targeting this H+/K+-ATPase proton pump of the gastric parietal cell are considered diagnostic markers in patients with autoimmune gastritis and pernicious anemia,[4–7] and currently, the diagnosis of autoimmune gastritis relies on the demonstration of these autoantibodies.[8] Autoimmune gastritis is a chronic disorder with a prevalence of ~2% in the general population and up to 10% in patients with other autoimmune diseases, such as type I diabetes or autoimmune thyroid disease.[9–14]

Atrophic body gastritis (AGB) is the pathological lesion of autoimmune gastritis, characterized by the disappearance of oxyntic mucosa leading to reduced or absent secretion of gastric acid.[15–19] From a pathogenetic point of view, atrophic body gastritis is a highly complex condition, which may be considered as consisting of three groups: (i) classical autoimmune *H. pylori*-negative atrophic body gastritis with a

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spared antrum; (ii) multifocal atrophic body gastritis involving the antral mucosa with active *H. pylori* infection and often negative for parietal cell autoantibodies, and (iii) a third group with overlapping features: body atrophy with a normal or inflamed antrum, active or past *H. pylori* infection and positivity to parietal cell autoantibodies.[8] *H. pylori*-related atrophic body gastritis and autoimmune gastritis are linked to increased risk for gastric cancer and type 1 gastric carcinoids[8,20] and the need of endoscopic surveillance in these patients has been argued both in Europe and in the USA,[21,22] thus making screening and timely diagnosis of this condition an important issue.

Autoantibodies against parietal cells are currently used as a serological marker to screen patients with other autoimmune disorders for autoimmune gastritis.[7,8] In patients with type I diabetes, autoimmune thyroid disease, and vitiligo, positivity to parietal cells autoantibodies have been reported in up to 25%,[10,11] 32%,[13,14,23] and 8%[24] of patients, respectively.

Despite their current use as serological markers, the diagnostic utility of these circulating autoantibodies remains largely unknown and studies on their diagnostic accuracy in patients with atrophic body gastritis are lacking. Positivity to autoantibodies against parietal cells has been shown in 7.8–19.5% of the general population.[25,26] Previous studies mainly employed enzyme immunoassays (EIA) or indirect immunofluorescence to detect autoantibodies against parietal cells that are reactive to either ATPase subunit (4A and/or 4B) of the gastric pump.[7,27] Some studies have argued that the H+, K+-ATPase 4A subunit (ATP4A) may be the major antigen,[28,29] but only few studies focusing on antibodies directed towards the H+, K+-ATPase 4B subunit have been performed so far.[30] The aim of this study was thus to assess the presence of autoantibodies against ATP4A and ATP4B subunits of parietal cells H+, K+-ATPase in patients with atrophic body gastritis and controls.

METHODS

Patients and study design. Following a case–control study design, serum samples of 104 cases with histologically proven atrophic body gastritis and 205 controls without atrophic body gastritis were assessed for autoantibodies (IgG class) specific for either the ATP4A or ATP4B subunits using the luminescent immunoprecipitation system (LIPS).

Cases. In this study, 104 patients consecutively diagnosed over a 3-year period with histologically proven atrophic body gastritis (female 71.4%, median age 56 years, range 21–83 years) were included in the study. Pernicious anemia was present in 43 (41.3%) of patients. Atrophic body gastritis was diagnosed at our institution in patients who presented with long-standing dyspepsia or who were referred from the Hematological Department for anemia or the Endocrinology Department for autoimmune thyroid disease.[16] Inclusion criteria were: (i) presence of atrophic body gastritis; (ii) complete clinical questionnaire at the time of diagnosis of atrophic body gastritis comprising demographical, life style, comorbidity and family history items; (iii) availability of a serum sample drawn at the time of atrophic body gastritis diagnosis and preserved at −20 °C for the assessment of autoantibodies against ATP4A and ATP4B subunits of the parietal cell H+, K+-ATPase. **Table 1** gives details regarding the main features of the included patients.

Table 1 Main features of the 104 cases with atrophic body gastritis

Clinical features	
Female gender	74 (71.1)
Age, years, median (range)	56 (21–83)
Fasting gastrin (pg/ml)	410 (25–2,700)
Pepsinogen I (ng/ml)	10 (0–330)
Pernicious anemia	43 (41.3)
Iron deficiency anemia	39 (37.5)
Autoimmune thyroid disease	30 (28.8)
Histological features	
Severe body atrophy	44 (42.3)
Presence of body intestinal metaplasia	62 (59.6)
Severe body intestinal metaplasia	11 (10.6)
Antrum spared	46 (44.2)
H. pylori *status*	
Histology positive	22 (21.1)
Serology positive (IgG anti-*H. pylori* antibodies)	53 (51)
Negative at histology and serology	29 (27.9)

Data expressed as number (%) when not otherwise indicated.

The presence of atrophic body gastritis was defined on the basis of histological confirmation of gastric corporal mucosal atrophy, fasting hypergastrinemia, and low pepsinogen I levels.[5,12] All patients underwent gastroscopy with standardized biopsy sampling from the antrum ($n=3$) and body ($n=3$) mucosa for conventional histopathological examination. The degree of gastritis was assessed according to the updated Sydney System.[31] Atrophy of the gastric body mucosa was defined as focal or complete oxyntic gland loss and/or their replacement by metaplastic pyloric or intestinal glands.[5,12] *H. pylori* immunoglobulin G antibodies were determined using a commercial ELISA kit (GAP test IgG, Bio-Rad, Milan, Italy). ABG patients were defined as having active *H. pylori* infection when both histology and ELISA serology were positive.[5,12,32] The presence of pernicious anemia was defined as the presence of a macrocytic anemia (hemoglobin concentration <14 g/dl for males and <12 g/dl for females, mean corpuscolar volume ≥ 100 fl) associated with low levels of serum cobalamin (normal values 190–950 pg/ml) and response to vitamin B_{12} treatment.[33]

Controls. To investigate the diagnostic accuracy of autoantibodies against ATP4A and ATP4B, these autoantibodies were also assessed in a control group without evidence of atrophic body gastritis. Sera from 205 controls (female 76.6%, median age 49 years, range 22–85 years) were included in the study. Controls were similar to cases with regard to gender ($P=0.37$), but were slightly younger than cases ($P<0.05$). The control subjects were consecutively recruited over a 3-year period in our Gastroenterology Department on the basis of the following inclusion criteria: (i) normal hemoglobin and mean corpuscolar volume values (absence of anemia and/or macrocytosis); (ii) normal values of pepsinogen I, a well-accepted noninvasive surrogate biomarker for gastric body atrophy when gastric biopsies for pathological evaluation are not available;[34] (iii) consensus to participate in the study and availability of a serum sample preserved at −20 °C for the assessment of autoantibodies

against ATP4A and ATP4B subunits of parietal cells H+, K+-ATPase.

All cases and controls were of Caucasian origin. None of the patients or controls included in the study were on treatment with anti-secretory drugs (proton pump inhibitors and/or H_2 antagonists). The study was performed in a blinded fashion, so that the case–control status of samples as well as patients' characteristics were unknown to the investigators performing the serological analyses. The study was approved by the local Ethical Committees, and patients and controls provided signed informed consent.

H. pylori status of cases and controls. Of the 104 cases, 22 (21.1%) were positive by histology (active infection) and 53 (51%) by serology (IgG anti-*H. pylori* antibodies) for *H. pylori*; the remaining 29 (27.9%) patients were *H. pylori*-negative based on both tests. Among the 205 controls, positivity to IgG anti-*H. pylori* antibodies was present in 65 (31.7%).

Assessment of autoantibodies against ATP4A and ATP4B subunits of parietal cells H+, K+-ATPase. Serum samples of cases and controls were assessed for autoantibodies (IgG class) specific for either the ATP4A or ATP4B subunits by LIPS.

Cloning of ATP4A and ATP4B coding sequences into luciferase expression vectors. The coding sequences for the ATP4A and ATP4B antigens were cloned after PCR amplification into a modified pCMV-TnT plasmid (Promega, Madison, WI, USA) expression vectors as in frame fusion proteins joined via a short glycine linker to a luciferase reporter. For ATP4A, a fragment of the coding sequences (aa 394–607), predicted to lie on the cytosolic face of the membrane,[28,35] was subcloned into pCMV-TnT-Rluc fused to a modified Renilla luciferase reporter (Promega).[36] For ATP4B, the entire coding sequences of the antigen was cloned into pCMV-TnT-Nluc fused to a modified Nanoluc luciferase reporter (Promega).[37]

Expression of chimeric luciferase-antigens. Rluc-ATP4A was expressed *in vitro* using the TnT SP6 Quick Coupled Transcription/Translation kit (Promega), based on transcription by the SP6 phage RNA polymerase and translation by a rabbit reticulocyte lysate cell-free expression system. Nluc-ATP4B was expressed in eukaryotic cells, using the Expi293 expression system (Thermo Fisher Scientific, Waltham, MA, USA). In the Expi293 expression system, recombinant protein expression is achieved by high efficiency transfection of Expi293F, a derivative of HEK293 cells, adapted to growth in suspension in a defined composition, serum free medium. After 48 h of growth with agitation, transfected Expi293F cells were pelleted and lysed with passive lysis buffer (Thermo Fisher Scientific). Expression of recombinant antigens was assessed by quantification of luciferase activity in the lysates after the addition of Renilla luciferase assay system substrate or NanoGlow substrate (Promega), reconstituted according to the manufacturer instructions, for ATP4A and ATP4B, as appropriate. Luciferase activity was measured using a Berthold Centro xS960 luminometer (Berthold, Germany) and expressed in light units (LU) emitted over a time interval of 2 s. Recombinant antigen preparations were aliquoted and stored frozen at − 80 °C.

Detection of autoantibodies to ATP4A or ATP4B by LIPS. Recombinant luciferase-antigens were used in LIPS[38] immunoassays for detection of autoantibodies to either ATP4A or ATP4B. In brief, 1 µl of human serum from each test sample was seeded into the wells of a Multiscreen HTS 96 GV filter plate (Merck Millipore Biosciences, Daran, MA, USA). Recombinant luciferase antigens corresponding to an activity of 4×10^6 LUs in a total volume of 25 µl of Buffer A (PBS pH7.4 containing 0.1% v/v Tween20) was then added to each well and the plate incubated for 2 h at room temperature. To recover immune complexes, 6 µl of a 50% slurry of rProtein A 4 Fast Flow sepharose (GE Healthcare, Little Chalfon, UK) diluted in 50 µl of Buffer A was added to each well, and incubated for 1 h at 4 °C with shaking. Plates were then washed by filtration 12 times with 150 µl/well of Buffer A at room temperature. After the last wash, the plates were air-dried, then 25 µl of Buffer A followed by 40 µl of either Renilla luciferase or NanoGlow assay substrates was added to each well, and the plate immediately read in the luminometer for 2 s. Results were converted to arbitrary units (AU) using a reference ATP4A or ATP4B autoantibody positive index serum and a negative control serum according to the formula: (LU test serum – LU Negative Control serum)/(LU Positive index serum – LU Negative Control serum). Sera that showed binding equal or higher than the appropriate index serum were serially diluted and re-tested to titrate the amount of autoantibodies, measured AU were then multiplied according to the first dilution factor that yielded linearly decreasing results.

All LIPS analyses were performed at the IRCCS San Raffaele Scientific Institute Division for Genetics and Cellular Biology, Milan, Italy, and the researchers who performed the LIPS measurements (VL, CB and IM) were blinded to the clinical data and to the diagnoses of cases and controls.

In 83 (79.8%) cases and 185 (90.2) controls, autoantibodies against parietal cells were assessed by using a commercial EIA kit (QUANTA LiteTM GPA, INOVA Diagnostics, San Diego, USA, cut-off <20 units).

Statistical evaluation. Data were expressed as median (range) and/or number/total (percentage, %). Differences between cases and controls, as well as differences between ATP4A and ATP4B autoantibody titers within subgroups of patients (pernicious anemia, gender, autoimmune thyroid disease, absence of active *H. pylori* infection) were analyzed by χ^2 or Mann–Whitney tests, as appropriate. Two-tailed P values <0.05 were considered statistically significant. A threshold for positivity for the ATP4A and the ATP4B autoantibody assays was selected using receiver-operating characteristic (ROC) curve analyses. A pairwise comparison of LIPS assays and EIA ROC curves was performed.[39,40] The diagnostic performance of ATP4A and ATP4B autoantibody testing as well as EIA in cases with respect to controls was expressed in terms of sensitivity, specificity, and positive predictive value expressed in percentages. Statistics were performed using a dedicated software package (MedCalc Software, Mariakerke, Belgium, version 12.7.8).

RESULTS

As shown in **Figure 1a,b**, the ATP4A and ATP4B autoantibody titers were significantly higher in cases (ATP4A: median 3003 AU, range 54–47,429 AU; ATP4B: median 2,762 AU, range

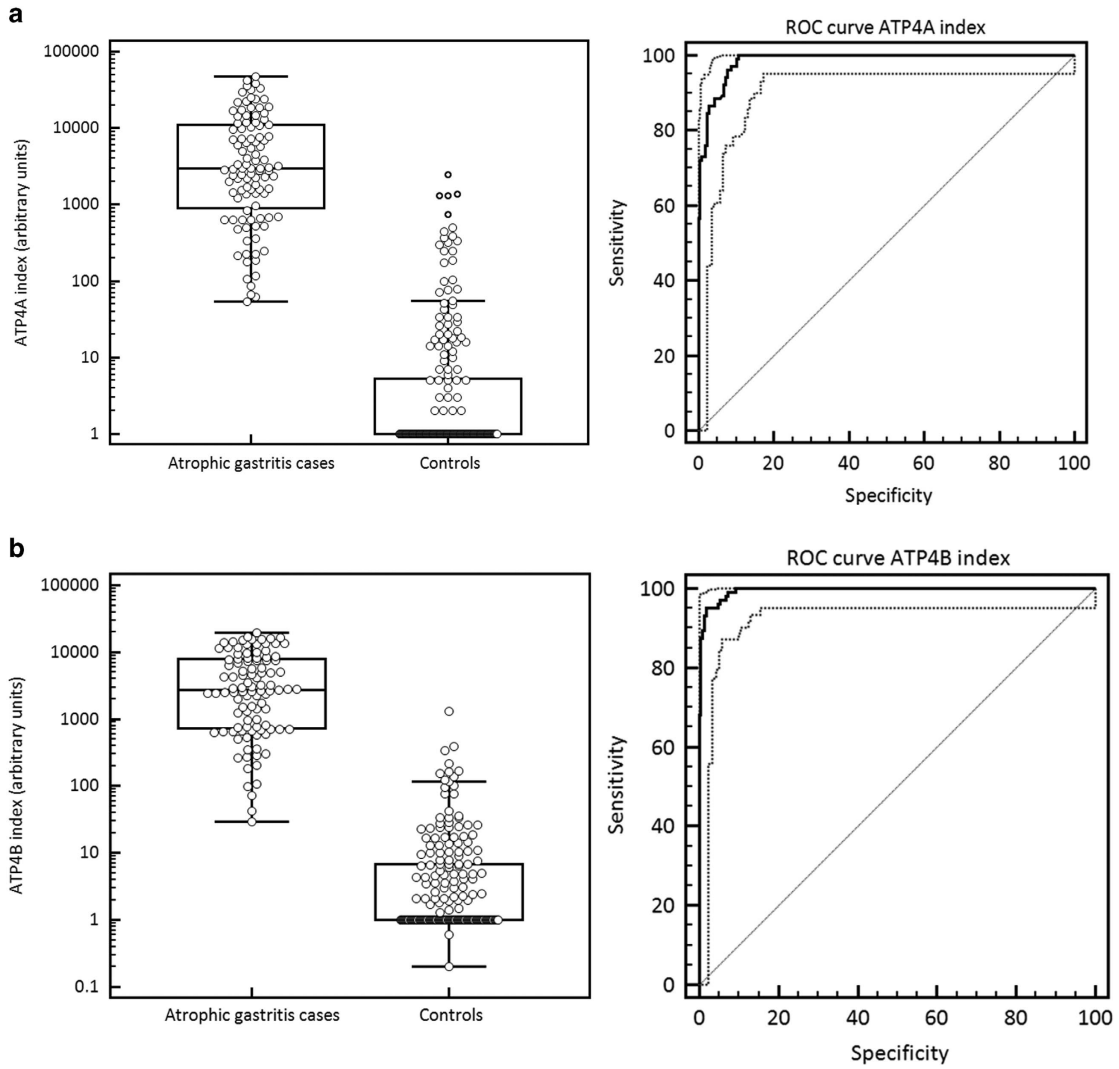

Figure 1 Index values and receiver-operating characteristic (ROC) curves of ATP4A and ATBP4B assays in cases and controls. (**a**) Left panel: ATP4A indices for each case ($n=104$) and control ($n=205$) are shown. The median, 95% CI for the median (box) and range (whiskers) are indicated. Right panel: ROC curve of the ATP4A assay. Dashed lines indicate the 95% confidence interval. A cut-off value of >51 arbitrary units (AU) gives 100% sensitivity (95% confidence interval (CI) 96–100) at 89% specificity (95% CI 84–93). (**b**). Left panel: ATP4B indices for each case ($n=104$) and control ($n=205$) are shown. The median 95% CI for the median (box) and range (whiskers) are indicated. Right panel: ROC curve of the ATP4B assay. Dashed lines indicate the 95% confidence interval. A cut-off value of >28 AU gives 100% sensitivity (95% CI 96–100) at 90% specificity (95% CI 86–94).

29–19,796 AU) compared with controls (ATP4A: median 1 AU, range 1–2,440 AU; ATP4B: median 2 AU, range 0.2–1,301 AU; $P<0.0001$ for both assays). For the ATP4A assay, the area under the ROC curve was 0.98 (95% CI 0.965–0.996) with a significance level (area = 0.5) of $P<0.0001$. For the ATP4B assay, the area under the ROC curve was 0.99 (95% CI 0.979–1.000) with a significance level (area = 0.5) of $P<0001$. As shown in the scatter diagram of correlation between ATP4A and ATP4B autoantibody titers (**Figure 2**), a statistically significant correlation between the two assays was shown (Spearman's coefficient of rank correlation (rho) = 0.895. 95% CI 0.870–0.915, $P<0.0001$) indicating a parallel reactivity against both, the ATP4A and the ATPB antigens.

Based on ROC curve analysis, a threshold for positivity for each assay was selected corresponding to the highest value at which maximal sensitivity was achieved. For the ATP4A assay, this corresponded to 52 AU and yielded a sensitivity of 100% (104/104) and a specificity of 89% (23/205). For the ATP4B assay, this corresponded to 28 AU and yielded a sensitivity of 100% (104/104) and a specificity of 90% (20/205). We then adopted as a classifier for atrophic gastritis or control status the simultaneous positivity in both assays. Based on this decision algorithm, the sensitivity in the atrophic gastritis patients was stable at 100% (104/104) while specificity in control increased to 95% (10/205; **Table 2**).

Comparison between performance of ATP4A and ATP4B LIPS assays and parietal cell antibodies EIA. In 268 subjects (83 cases and 185 controls), EIA for parietal cell autoantibodies were available. The area under the ROC

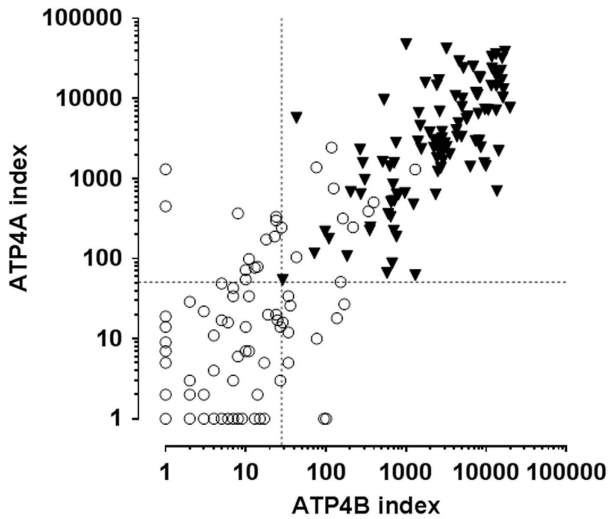

Figure 2 Correlation between the ATP4A and ATP4B assays. The ATP4A and ATP4B indices for each case (Black triangles) and control (white circles) are shown. Cut-off values for each assay are indicated (solid lines). Spearman's coefficient of rank correlation (rho) = 0.895 (95% CI 0.870–0.915; $P < 0.0001$).

Table 2 Summary of performance of ATP4A and ATP4B autoantibodies detected by LIPS and parietal cell autoantibodies detected by EIA in atrophic body gastritis

		Sensitivity (%)	Specificity (%)	PPV%
LIPS	ATP4A	100	89	82
	ATP4B	100	90	84
	Combined ATP4A +ATP4B	100	95	91
EIA	Parietal cell autoantibodies	72[a]	92	81

EIA, enzyme immunoassays; LIPS, luminescent immunoprecipitation system; PPV, positive predictive value.
[a]$P < 0.0001$ by χ^2 test.

curve of EIA was 0.86 (95% CI 0.809–0.896), being significantly lower as compared with the area under the ROC curve for ATP4A and ATP4B assays ($P < 0.0001$).

As shown in **Table 2**, the specificity and positive predictive values of EIA, 92 and 81%, respectively, were similar to those of ATP4A and ATP4B LIPS assays, while the 72% sensitivity was significantly lower compared with the 100% sensitivity values of the LIPS assays ($P < 0.0001$).

Role of *H. pylori* status in controls who tested positive for ATP4 and/or ATP4B autoantibodies. Positivity to anti-parietal cell antibodies has been reported in *H. pylori* positive subjects who did not have atrophic body gastritis, therefore we investigated the *H. pylori* status of controls who tested positive for the ATP4B and/or ATP4A assay. Of the 205 control subjects, 23 (11.2%) tested positive for ATP4A autoantibodies and 20 (9.7%) tested positive for ATP4B autoantibodies. Ten controls tested simultaneously positive for ATP4A and ATP4B (**Figure 2**; **Table 3**).

Ten (43.5%) of the 23 controls with positivity to ATP4A and eight (40%) of the 20 controls with positivity to ATP4B had a positive anti-*H. pylori* IgG test, consistent with a potential role of *H. pylori* infection in triggering reactivity against ATP4A and/or ATP4B in a subgroup of control subjects. The ATP4A and ATP4B autoantibody titers of the "false positive" controls were significantly lower as compared with the "true positive" atrophic body gastritis patients (ATP4A: median 299 AU (range 51–2,440 AU) vs. median 2,949 UA (range 54–47,428 AU), $P < 0.0001$; ATP4B: median 367 UA (range 218–1,301 AU) vs. median 2,761 (range 29–19,796 AU), $P < 0.0001$).

Table 3 *H. pylori* status of the 23 controls who tested positive for ATP4A autoantibodies and of the 20 controls who tested positive for ATP4B autoantibodies

Sample	ATP4A index, AU	*H. pylori* status	ATP4B index, AU	Sample
#1	**2,440**	Positive	**118**	#1
#2	**1,373**	Positive	**76**	#2
#3	**1,305**	Negative	1	–
#4	**1,302**	Positive	**1,301**	#3
#5	**750**	Positive	**125**	#4
#6	**504**	Negative	**395**	#5
#7	**449**	Positive	0.6	–
#8	**390**	Positive	**340**	#6
#9	**366**	Positive	8	–
#10	**332**	Negative	24	–
#11	**317**	Positive	**163**	#7
#12	**299**	Negative	23	–
#13	**246**	Negative	**218**	#8
#14	**245**	Negative	28	–
#15	**188**	Negative	23	–
#16	**173**	Positive	18	–
#17	**104**	Negative	**43**	#9
#18	**99**	Negative	11	–
#19	**79**	Negative	14	–
#20	**77**	Negative	13	–
#21	**72**	Negative	10	–
#22	**55**	Positive	10	–
#23	**51.4**	Negative	**153**	#10
	18	Negative	**137**	#11
	27	Negative	**170**	#12
	1	Negative	**101**	#13
	1	Negative	**95**	#14
	10	Positive	**77**	#15
	26	Negative	**36**	#16
	12	Negative	**34**	#17
	5	Positive	**34**	#18
	34	Negative	**34**	#19
	16	Negative	**29**	#20

AU, arbitrary units.
Positive index values are in bold: positivity to Ab ATP4A cut-off >51 AU; positivity to Ab ATP4B cut-off >28 AU.

Relationship between ATP4A and ATP4B autoantibody and clinical features of atrophic body gastritis. Atrophic body gastritis may manifest in the context of classical autoimmune gastritis with a spared antrum, as *H. pylori*-related multifocal atrophic body gastritis, and as a mixed form with overlapping features; thus, a potential relationship between ATP4A and ATP4B autoantibodies and clinical features was also investigated. As all of the atrophic body gastritis patients tested positive for ATP4A autoantibodies and 95.2% were positive for ATP4B, a comparison of clinical features between patients with positive and negative auto-antibodies could not be performed; therefore, the autoantibody titers between patients with and without specific clinical features were compared to investigate possible relationships.

As shown in **Figure 3** and **Table 4**, patients with atrophic body gastritis having a spared antral mucosa (the typical

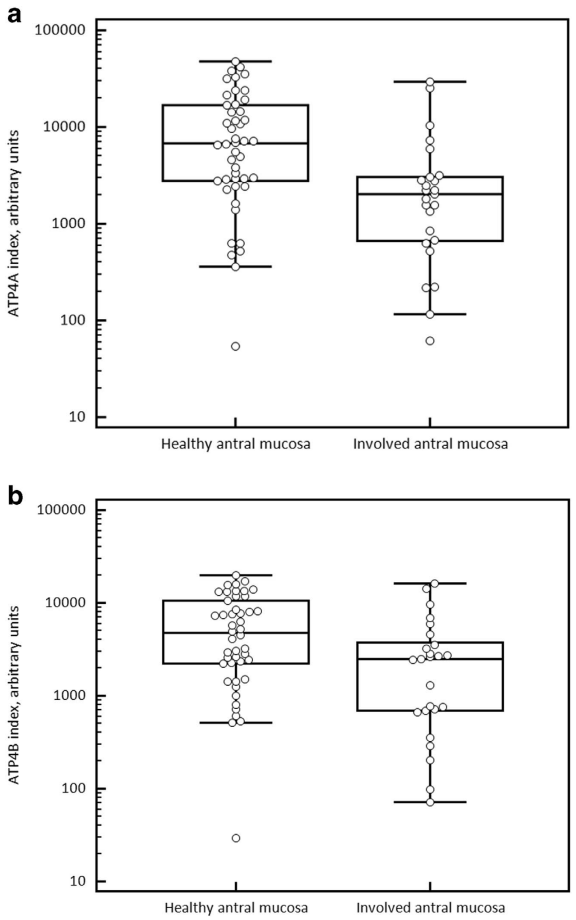

Figure 3 Comparison between ATP4A and ATP4B autoantibody titers in atrophic body gastritis patients with a healthy antral mucosa and those with an atrophic or inflamed antral mucosa. (**a**) ATP4A indices for each case with a healthy antral mucosa ($n=45$) and with an atrophic or inflamed antral mucosa ($n=25$) are shown. The median 95% CI for the median (box) and range (whiskers) are indicated. The ATP4A titer is significantly higher in the atrophic body gastritis patients with a spared antrum (median 6,750AU; range 46–47,429) compared with those with an involved antral mucosa (median 2,014 AU; range 25–29,224; $P=0.0009$). (**b**) ATP4B indices for each case with a healthy antral mucosa ($n=45$) and with an atrophic or inflamed antral mucosa ($n=25$) are shown. The median 95% CI for the median (box) and range (whiskers) are indicated. The ATP4B titer is significantly higher in the atrophic body gastritis patients with a spared antrum (median 4,721 AU; range 29–19,795) compared with those with an involved antral mucosa (median 2,473 AU; range 72–16,095; $P=0.0136$).

histological feature of autoimmune gastritis) had a significantly higher ATP4A and ATP4B autoantibody titer compared to those having an inflamed or atrophic antral mucosa (more typically linked to *H. pylori*-correlated atrophic gastritis). The ATP4B autoantibody titer, but not the ATP4A autoantibody titer, was also significantly higher in patients with concomitant autoimmune thyroid disease and in those without active *H. pylori* infection (**Table 4**), suggesting a closer relationship of this autoantibody to gastric autoimmunity. This is supported by the analysis of the 5 atrophic body gastritis patients who were negative for ATP4B autoantibodies: they were aged between 21 and 56 years and all were female, but only one had a spared antrum, none had severe atrophy, intestinal metaplasia or pernicious anemia, only two had associated autoimmune thyroid disease and all five were *H. pylori* positive (4 with active infection at histology and 1 with positive serology).

DISCUSSION

The LIPS assays for the parietal cell K+/H+ ATPase described herein show a high diagnostic performance for atrophic body gastritis, with 100% sensitivity for both, the ATP4A and the ATP4B assays. According to our results, IgG autoantibodies against the ATP4A and ATP4B antigens are virtually always present in patients with atrophic body gastritis and thus they seem to represent reliable serological biomarkers of oxyntic mucosa damage *tout court* suggesting a close relationship of oxyntic mucosa atrophy and the expression of these autoantibodies.

Biomarker tests have been defined as clinically useful when they result in improved measurable clinical outcomes of value to clinical decision-making compared with routine management without use of the test.[41] Autoantibodies against parietal cells are often used to screen for autoimmune gastritis in patients with other autoimmune diseases:[7,8] in patients with type I diabetes, autoimmune thyroid disease, and vitiligo, positivity to parietal cells autoantibodies up to 25%,[10,11] 32%[13,14,23] and 8%,[24] respectively, has been reported. Atrophic body gastritis is a condition linked to an increased risk of gastric neoplasia.[8,19,42,43] To better address invasive and cost-intensive gastroscopy (currently essential for diagnosis of atrophic body gastritis), noninvasive screening tools such as serum pepsinogen I and gastrin have been proposed.[34,44] According to a recent meta-analysis, screening for atrophic gastritis by serum pepsinogens has 69%

Table 4 Comparison of ATP4A and ATP4B autoantibodies titers of atrophic body gastritis patients with respect to clinical features

	ATP4A autoantibodies titer			ATP4B autoantibodies titer		
	Presence of clinical feature	**Absence of clinical feature**	***P-value*[a]**	**Presence of clinical feature**	**Absence of clinical feature**	***P*-value[a]**
Female gender	2,970 (54–41,996)	2,777 (87–47,428)	0.6155	2,736 (29–19,796)	2,762 (511–16,364)	0.7252
Antrum spared[b]	**6,750 (54–47,429)**	**2,014 (62–29,224)**	**0.0009**	**4,721 (29–19,796)**	**2,473 (72–16,095)**	**0.0136**
Pernicious anemia	2,833 (62–47,429)	5,709 (54–41,996)	0.2494	2,663 (183–16,095)	3,193 (29–19,796)	0.6417
Autoimmune thyroid disease	5,846 (54–38,353)	2,490 (10–47,429)	0.0645	**5,805 (29–19,796)**	**2,567 (72–16,364)**	**0.0174**
Severe corporal atrophy	3,248 (62–35,537)	2,930 (54–47,429)	0.3927	3,365 (531–16,095)	1,857 (29–19,796)	0.0629
Mild corporal atrophy	655.5 (54–41,997)	3.583 (62–47,429)	0.1142	786 (29–17,217)	3.127 (72–19,796)	0.0634
No active *H. pylori* infection	2,312 (54–47,429)	3004 (62–38,393)	0.4354	**2,918 (72–19,796)**	**905 (29–13,377)**	**0.0264**

[a]By Mann–Whitney rank sum test.
[b]Typical histological feature of autoimmune gastritis.
Data are expressed as median (range) of arbitrary units.Bold values are significant at $P<0.05$.

sensitivity at 88% specificity and an 88% AUC.[31] Another screening tool which combines gastrin 17, pepsinogens I and II and *H. pylori* antibody levels showed 50% sensitivity at 80% specificity for atrophic gastritis.[45] According to the results of the present study, the assessment of IgG autoantibodies against ATP4A and/or ATP4B may be proposed as biomarker not only for autoimmune gastritis, but also for other forms of atrophic body gastritis, and positive patients should be advised to undergo gastroscopy with biopsies to establish diagnosis of atrophic body gastritis and to rule out neoplastic complications of this condition.

This is the first study assessing the diagnostic potential of autoantibodies to ATP4A and ATP4B both separately and in combination using LIPS in subjects with atrophic body gastritis, thus no previous data for comparison of our results are available. However it should be noted that Burbelo *et al*.[30] reported that 2/90 (2.2%) of control subjects in the 2010 Diabetes Autoantibody Standardization Program showed ATP4B positivity using a related LIPS assay, yielding a higher specificity compared to the 10% "false positive" rate of the current study. Most previous studies employed EIA or indirect immunofluorescence to detect autoantibodies against parietal cells, which do not discriminate reactivity to specific ATPase subunits of the gastric pump.[7,27] Typically EIA is the preferred method, as it shows high concordance among laboratories and is ~30% more sensitive than immunofluorescence.[7,27] Recently, a novel radioimmunoprecipitation assay was reported for the ATP4A subunit,[28] which shows similar performance characteristics to the LIPS assay described above (VL, HWD & JMW; data presented at the 13th Meeting of the Immunology of Diabetes Society, Lorne, Australia, December 2013). Compared with EIA, radioimmunoprecipitation assays are usually a more accurate and precise method for autoantibody assessment, but have the disadvantage of requiring special laboratory conditions and instrumentation.[7]

The high diagnostic accuracy for atrophic gastritis of ATP4A and ATP4B autoantibodies observed in this study might be explained by at least two reasons. First, sensitive LIPS assays were employed for assessment of these autoantibodies. LIPS, is a technique that offers several advantages over current alternatives. For example, solid phase assays such as Western blotting, EIA and protein arrays, often have a narrow dynamic range for measuring autoantibodies and may show sub-optimal detection of conformational epitopes, even after optimization for background noise. In contrast, liquid phase assays such as radioimmunoprecipitation and LIPS readily detect autoantibodies directed against both conformational and linear epitopes, offering high sensitivity and specificity for many autoimmune diseases. In addition, since protein targets are genetically fused to luciferase, there is no need to purify the antigens, and no use of radioactivity. Together, these advantages suggest that LIPS is an ideal platform for the assessment of autoantibodies in a wide range of diseases including atrophic body gastritis.[38,46,47] The results in the present study showed that LIPS assay outperformed traditional EIA yielding a significantly higher sensitivity and resetting to zero the proportion of ("false") negative atrophic gastritis patients to autoantibodies against H+,K+-ATPase antigens.

Second, all of the included cases with atrophic body gastritis had a histologically proven presence of gastric mucosa atrophy which was severe in 42.3%, with associated intestinal metaplasia in 59.6%, and spared antrum (completely healthy antral mucosa) in 44.2%. Thus, the LIPS assays against ATP4A and ATP4B tested a well characterized specific target population with histological diagnosis of atrophic damage and a high likelihood of positivity to parietal cell autoantibodies. Therefore, the diagnostic accuracy of this assay as a screening tool needs to be validated in a population of subjects undergoing biopsy with suspected (but not confirmed) disease.

The LIPS assays described above show high specificity; only 11.2 and 9.7% controls tested positive for ATP4A and ATP4B, respectively, a figure which was similar to that observed with commercial EIA (7.6%). *H. pylori* infection has been associated with anti-parietal cell autoantibodies, with positivity reported in up to 20.7% of *H. pylori* positive patients.[25,26] In our study, ~40% of the ATP4A or ATP4B positive controls had a positive *H. pylori* serology, possibly explaining the reactivity against the ATPase as a consequence of the infection in this subgroup, as previously shown and linked to antigen mimicry.[48,49] In the remaining ATP4A or ATP4B positive controls who tested negative for *H. pylori*, the "false positivity" may be explained by an unspecific autoreactivity as described for anti-mitochondrial, anti-nuclear, and anti-smooth muscle autoantibodies.[50,51] In a cross-sectional study performed in Spain, besides *H. pylori* infection, other factors associated with autoantibodies against parietal cells were female gender, insulin resistance, menopause, and high serum levels of soluble CD40 ligand.[26] A recent study assessing the presence of ATP4A autoantibodies in diabetic children also failed to show an association between *H. pylori* serology and positivity to ATP4A autoantibodies,[35] suggesting that factors other than *H. pylori* may also lead to autoreactivity against the parietal cellsATPase.

In our study, female gender between cases and controls was not different, but controls were slightly younger than cases (49 vs. 56 years, $P<0.05$). It has been reported that positivity to anti-parietal cell antibodies increases with age.[27] Thus, we cannot exclude the possibility that the specificity observed for ATP4A and ATP4B autoantibodies in this study might overestimate that in the general population.

Some differences emerged between the autoreactivity against the ATP4A and ATP4B subunits. A spared antrum, the classical histological feature linked to autoimmune gastritis, was associated with higher autoantibody titers against ATP4A and ATP4B, compared with multifocal atrophic gastritis. Other features more closely linked to autoimmune gastritis, such as an absence of active *H. pylori* infection and concomitant autoimmune thyroid disease, were associated with higher autoantibody titers against ATP4B only. The ATP4A subunit is believed to be the major antigen,[28,29] while the ATP4B subunit is supposed to stabilize the alpha-subunit and to perform essential enzyme function.[1–3] These results suggest that the reactivity against the different ATPase subunits might differ according to different clinical features of atrophic body gastritis patients, possibly offering some insights regarding the pathogenetic role of these autoantibodies to be clarified in future studies.

In conclusion, positivity to autoantibodies against both the ATP4A and ATP4B subunits, is closely associated with atrophic body gastritis. Both, the ATP4A and the ATP4B assay had the

highest sensitivity, at the cost of diagnostic accuracy (89 and 90% specificity), outperforming traditional EIA. Once validated, these LIPS assays should be valuable screening tools for detecting biomarkers of damaged atrophic oxyntic mucosa.

CONFLICT OF INTEREST

Guarantor of the article: Bruno Annibale, MD.
Specific author contributions: study design: Edith Lahner, Bruno Annibale, and Vito Lampasona; development of LIPS assays: Cristina Brigatti, Ilaria Marzinotto, Emanuele Bosi, Lorenzo Piemonti, Howard W Davidson, Janet M Wenzlau, and Vito Lampasona; LIPS assays: Ilaria Marzinotto and Vito Lampasona; data analysis: Edith Lahner, Giulia Scalese, Marilia Carabotti, and Bruno Annibale; first draft of the manuscript: Edith Lahner; and all authors contributed to its completion. All authors approved the final draft submitted.
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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Circulating autoantibodies targeting the H+/K+-ATPase proton pump of the gastric parietal cell are considered diagnostic markers in patients with autoimmune gastritis.
- ✓ Studies on their diagnostic accuracy in patients with atrophic body gastritis, the pathological lesion of autoimmune gastritis, are lacking.
- ✓ Previous studies employed enzyme immunoassays (EIA) or indirect immunofluorescence to detect autoantibodies against parietal cells that are reactive to either ATPase subunit (4A and/or 4B) of the gastric pump.

WHAT IS NEW HERE

- ✓ This is the first study assessing the diagnostic potential of autoantibodies to ATP4A and ATP4B both separately and in combination using luminescent immunoprecipitation system (LIPS) in subjects with atrophic body gastritis.
- ✓ The LIPS assays for the parietal cell K+/H+ ATPase described herein show a high diagnostic performance for atrophic body gastritis, with 100% sensitivity and a 89–90% specificity for the ATP4A and ATP4B assays, respectively, outperforming commercial EIA.
- ✓ IgG autoantibodies against the ATP4A and ATP4B antigens seem to represent reliable serological biomarkers of atrophic oxyntic mucosa.

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