Journal of Translational Autoimmunity 4 (2021) 100131

Contents lists available at ScienceDirect

Journal of Translational Autoimmunity

journal homepage: www.sciencedirect.com/journal/journal-of-translational-autoimmunity

Intrinsic factor autoantibodies by luminescent immuno-precipitation system in patients with corpus atrophic gastritis

Ilaria Marzinotto [a], Ludovica Dottori [b], Francesca Baldaro [b], Emanuele Dilaghi [b], Cristina Brigatti [a], Elena Bazzigaluppi [a], Gianluca Esposito [b], Howard W. Davidson [c], Lorenzo Piemonti [a], Vito Lampasona [a,**], Edith Lahner [b,*]

[a] San Raffaele Diabetes Research Institute, IRCCS Ospedale San Raffaele, Milan, Italy
[b] Medical-Surgical Department of Clinical Sciences and Translational Medicine, Sant'Andrea Hospital, School of Medicine, University Sapienza, Rome, Italy
[c] Barbara Davis Center for Diabetes, University of Colorado Anschutz Medical Campus, Aurora, CO, USA

ARTICLE INFO

Keywords:
Atrophic gastritis
Autoimmune gastritis
Gastric autoantibodies
Intrinsic factor antibodies
Parietal cell antibodies
Luciferase immunoprecipitation system
LIPS
Pernicious anemia

ABSTRACT

Background: Corpus atrophic gastritis (CAG) may lead to intrinsic factor (IF) deficiency and vitamin B_{12} malabsorption. Intrinsic factor autoantibodies (IFA) are considered markers of pernicious anemia, but their clinical utility in CAG has not been evaluated. This study aimed to assess IFA in CAG patients and controls using a luciferase immunoprecipitation system (LIPS).

Methods: Recombinant nanoluciferase-tagged IF secreted from transfected Expi293F cells was used as antigen in an IFA-LIPS assay. IFA IgG were measured in sera from subjects undergoing gastroscopy and biopsy (updated Sydney system) mainly for anemia (57%) or dyspepsia (34%). This cohort comprised 105 patients with histologically-proven-CAG (cases: median age 64 years, 68% females) and 110 subjects with suspected CAG that were histologically negative (controls: median age 67 years, 54% females). Cut-off values were selected by Q-Q-plot analysis (negative: <2.5 arbitrary units).

Results: IFA levels were higher in cases than in controls (Mann-Whitney:$p < 10^{-5}$). The ROC-AUC was 0.67 (95% CI 0.60–0.73, $p < 0.0001$). The IFA LIPS sensitivity and specificity for CAG were 32% (95% CI 24–42) and 95% (95% CI 90–99). This diagnostic performance remained similar after stratification for the presence/absence of anemia, dyspepsia or vitamin B_{12} deficiency. IFA levels were higher in females compared with males ($p = 0.0127$). In females aged <65 years, IFA-positives were more prevalent than in males (43.5% vs 6.6%, $p = 0.011$).

Conclusions: The IFA-LIPS assay discriminated between CAG patients and controls showing a good specificity (95%) at the cost of sensitivity (32%). IFA-positivity occurred independently from anemia and vitamin B_{12} deficiency, but was more frequent in younger females. IFA testing should be considered in patients at high clinical suspicion of CAG.

1. Introduction

Intrinsic factor is a glycoprotein secreted by gastric parietal cells located in the corpus oxyntic mucosa that plays a key role in the transport and absorption of vitamin B_{12} by the terminal ileum. Vitamin B_{12}, also called cobalamin, is a vital micronutrient, and deficiency of intrinsic factor and vitamin B_{12} can lead to important consequences, with effects including severe hematological or neurological disorders, and sometimes, life-threatening cardiovascular disease [1,2].

Intrinsic factor deficiency may occur as a result of gastric parietal cell loss due to gastric oxyntic mucosa atrophy in the context of autoimmune corpus atrophic gastritis [2,3], but also, less commonly, due to

Abbreviations: Hp, *Helicobacter pylori*; PA, pernicious anemia; CAG, corpus atrophic gastritis; IFA, intrinsic factor autoantibodies; PCA, antibodies against gastric parietal cells; LIPS, luminescent immuno-precipitation system.

* Corresponding author. Gastroenterology, Sapienza University of Rome; Medical-Surgical Department of Clinical Sciences and Translational Medicine; Via Grottarossa 1035, 00189, Rome, Italy.
** Corresponding author. San Raffaele Diabetes Research Institute, IRCCS Ospedale San Raffaele, via Olgettina 60, 20132, Milan, Italy.
E-mail addresses: lampasona.vito@hsr.it (V. Lampasona), edith.lahner@uniroma1.it (E. Lahner).

https://doi.org/10.1016/j.jtauto.2021.100131
Received 6 August 2021; Received in revised form 28 October 2021; Accepted 29 October 2021
Available online 1 November 2021

multifocal atrophic gastritis with or without *Helicobacter pylori* (Hp) infection [4,5]. These conditions may clinically manifest with anemia or dyspepsia, but can remain asymptomatic for a long time [3,4]. In Western countries, the most frequent condition linked to vitamin B_{12} deficiency is pernicious anemia (PA), a megaloblastic anemia arising from vitamin B_{12} malabsorption due to intrinsic factor deficiency as consequence of corpus atrophic gastritis (CAG) [2]. Besides PA, vitamin B_{12} deficiency may give rise to a wide range of neurological symptoms from sensory-motor neuropathy leading to ataxia, to cognitive decline, and psychiatric disorders, thus strengthening the importance of its timely diagnosis [2].

Intrinsic factor autoantibodies (IFA) are considered the most reliable pre-endoscopic markers of PA, gaining renewed importance after the disuse of the Schilling test [2,3,6–8]. Antibodies against gastric parietal cells (PCA) are commonly used as serological markers of autoimmune CAG [3,4,7], but the ability of IFA to diagnose CAG in the absence of PA has not been clarified so far. According to 2014 British guidelines for the diagnosis and treatment of cobalamin and folate disorders [8], "all patients with anemia, neuropathy or glossitis, and suspected of having PA, should be tested for IFA regardless of cobalamin levels (Grade 1A)", and "patients found to have a low serum cobalamin level in the absence of anemia and who do not have food malabsorption or other causes of deficiency, should be tested for IFA to clarify whether they have an early/latent presentation of PA (Grade 2A)", while "testing of PCA for PA diagnosis is not recommended (Grade 1A)" [9]. In contrast, other experts suggest that PCA is an appropriate screening test for PA, recommending that IFA be reserved for confirmatory testing or in patients with positivity towards other autoantibodies that confound the use of PCA [7,10]. Still other reports suggest the usefulness to screen for both PCA and IFA in the setting of CAG [11,12].

Recent data on the clinical usefulness and diagnostic performance of IFA in PA or CAG are scanty. Currently, there is no gold standard method for measuring IFA. The performance of the available diagnostic methods (IIF, RIA, ELISA, etc.) appears variable [12–15], although a recent study reported that automated ELISA based assays had the highest relative sensitivity and specificity [16]. A previous study showed an overall IFA-positivity by ELISA in 27% of patients with CAG, and the combination of PCA and IFA increased diagnostic performance, particularly in CAG with PA [11]. An innovative liquid-phase method called LIPS (Luciferase Immuno-Precipitation System) has been used recently to determine circulating autoantibodies in a wide range of diseases including CAG [17–19]; for this condition, the diagnostic performance of LIPS-assayed PCA confirmed them as reliable markers of oxyntic mucosa damage [19,20]. Given this precedent, the current study aimed to assess the presence and diagnostic performance of IFA determined by LIPS in patients with CAG and controls.

2. Materials and methods

This paper was drafted according to STARD 2015 guidelines to ensure the quality of reporting [21].

2.1. Study population and design

In this cross-sectional study, we used archived sera from a prospective case-finding study of 215 consecutive adults presenting at our centre (May 2017–April 2018) with a clinical suspicion of CAG who matched the inclusion criteria [20]. Informed consent was provided by all participants and the study conducted with the approval of the local ethical committee (No.5390/2019). The clinical suspicion of CAG mainly arose due to the presence of anemia (61.9%) or dyspepsia (31.2%), or less frequently for other reasons such as a family history of autoimmune disease or gastric malignancy (6.9%). Adult (>18 years) subjects with one of the following criteria underwent esophagogastroduodenoscopy with three biopsy samples taken from antrum and three from the corpus mucosa, following a standardized protocol for histopathological assessment (updated Sydney System) [11,20,22]. Diagnosis and grading of gastritis were based on the presence of focal or complete oxyntic glands loss and/or their replacement with intestinal or pseudo-pyloric glands [11,20,22]. The exclusion criteria were: 1) age<18 years, 2) a previous diagnosis of CAG, 3) previous inclusion in an endoscopic surveillance program for gastric malignant conditions. According to the histopathological assessment, 105 (48.8%) subjects were defined as cases with a diagnosis of CAG, and 110 (51.2%) as controls without evidence of CAG. The main characteristics of the included cases and controls are shown in Supplemental Table 1. All subjects were Caucasians, with no statistically significant difference in age or sex between cases (median age 64 years, range 18–88; 67.6% female) and controls (median age: 67 years, range 23–90; 53.6% female). Sera from these subjects were used previously in a study focusing on serological markers of CAG, including PCA against subunits A and B of the gastric parietal cell proton pump ATP4 [20]. In the current study these sera, preserved at −20 °C, were re-analyzed for IFA using the LIPS assay. Data from the previously reported LIPS ATP4B PCA assays [20], were used to compute the diagnostic performance when combining IFA with ATP4B PCA.

2.2. Helicobacter pylori status

Both patients and controls were investigated by histology and serology (GAPtestIgG, Bio-Rad, Milan, Italy) for the presence of *Helicobacter pylori* (Hp). Serological positivity was not different between cases and controls (47.8% vs 55.9%, $p = 0.3617$), while histology found less Hp positives in cases than in controls (6% vs 19.4%, $p = 0.0051$).

2.3. Definition of vitamin B_{12} deficiency and pernicious anemia

For the current study, vitamin B_{12} deficiency was defined as serum levels below a threshold of 200 ng/l. PA was defined as the presence of CAG and anemia (hemoglobin levels <13 g/dl for men and <12 g/dl for women) in association with vitamin B_{12} deficiency [3,13].

2.4. Assessment of intrinsic factor autoantibodies (IFA) by LIPS assay

All serum samples included in this study were previously tested for the presence of ATP4A and ATP4B antibodies by LIPS [18,19]. For the IFA LIPS, the human gastric intrinsic factor (IF) was expressed as a fusion protein (IF-Nluc) comprising full length IF joined by a 9 amino acid linker to a modified Nanoluc reporter (Promega) [23] (Fig. 1A). Expi293F cells (Invitrogen) were transfected with the IF-Nluc expression plasmid and grown as suspension cultures for 48 h at 37 °C in serum-free medium with constant agitation. The supernatant containing IF-Nluc was then harvested and clarified by centrifugation. The luciferase activity of the recombinant antigen was then quantified using a Berthold Centro xS960 luminometer (Berthold, Germany) following addition of Nanoglo substrate (Promega), and expressed as light units (LU) measured over a 2 s interval [23]. For LIPS, 1 μl of each test serum was pipetted in duplicate into the wells of a 96 Deep-well plate (Beckman, USA) followed by the addition of 25 μl of Tris buffered saline pH7.4, 0.1% v/v Tween 20 (TBST) containing 4×10^6 LU equivalents of IF-Nluc antigen. The plate was then incubated for 2 h at room temperature. To recover the immune-complexes, 50 μl of a TBST solution containing 6 μl of a 50% slurry of rProtein A 4 Fast Flow sepharose (GE Healthcare, Little Chalfon, UK) was added to each well. After incubation for 1 h at 4 °C with constant shaking, the plate was washed by centrifugation, aspiration of the supernatant and dispensing of cold TBST (5 cycles, 750 μl/well of TBST at 4 °C). After the last wash, the supernatant was removed and the resin pellet transferred to an Optiplate (PerkinElmer, USA), followed by the addition of 40 μl of NanoGlo substrate and readout in the luminometer. The measured LU were converted into arbitrary units (AU) using a standard curve consisting of serial dilutions of a strongly IFA-positive sample in normal serum and a liner regression

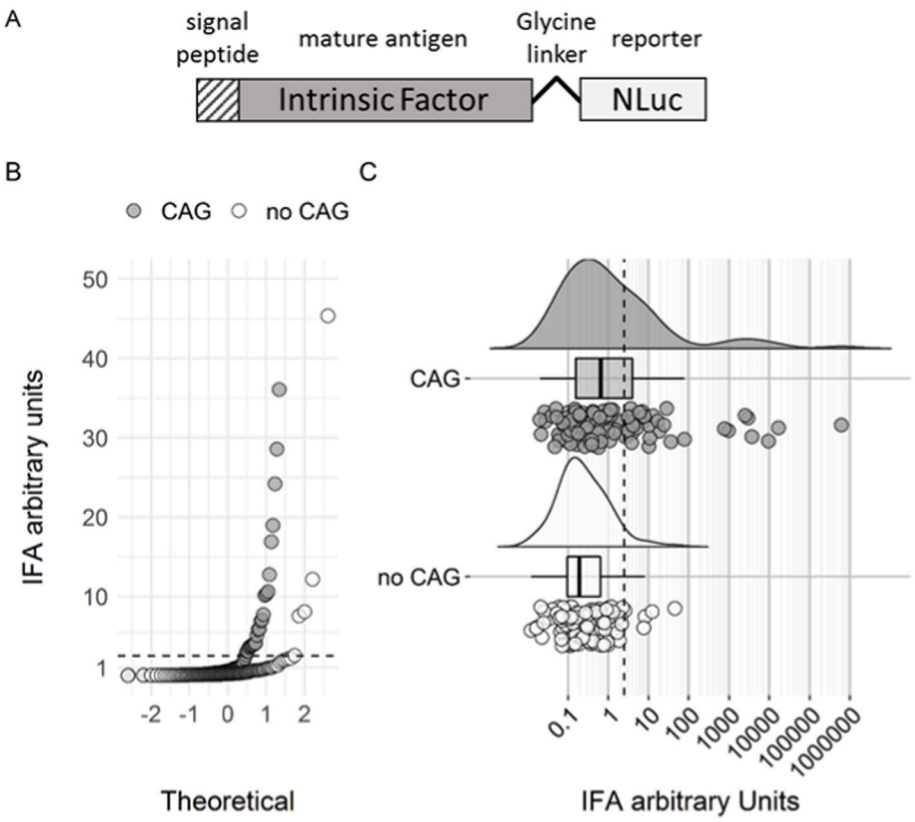

Fig. 1. Distribution of IFA levels measured by LIPS in the study subjects. **A.** *IF-Nluc design*: The full-length human IF coding sequence minus the stop codon (dark grey box) with its natural signal peptide (shaded) was linked via a flexible linker of 9 amino acids to a modified NanoLuc® protein (light grey box). **B.** *IFA assay threshold selection.* Quantile-quantile (Q–Q) plot showing the distribution of IFA measured by LIPS (AU) and their theoretical ranking in subjects with (CAG, grey dots) or without (no CAG, white dots) histologically confirmed corpus atrophy. The horizontal dashed line at the distribution inflection point indicates the calculated threshold for positivity (2.5 AU). **C.** *IFA levels measured by LIPS are significantly higher in CAG cases compared with controls.* Rain cloud plot showing the distribution of IFA AU in cases (CAG) and controls (no CAG). Shown are levels measured in each sample (circles), their probability density estimate (half violin plots, up-scaled to maximum width for better visualization), and box-plots showing the median, IQR, and whiskers extending to 1.96 times the median. The dashed vertical line indicates the assay threshold for positivity.

algorithm using log transformed values. Sera that showed LU at saturation of binding in the assay were serially diluted, retested and the measured AU were corrected according to the dilution factor. In light of the not normal distribution of measured AU in control we selected a cut-off for positivity (≥2.5 AU) based on a non-parametric approach using a quantile-quantile (Q-Q) plot analysis. Repeated measurements (n = 5) of the standard curve showed a Coefficient of Variation (CV) of the IFA LIPS ranging from 9 to 19% for serial dilutions from 1:100 to 1:12,800 and of 30–43.2% for dilutions 1:25,600 to 409,600.

All LIPS analyses were performed at the San Raffaele Diabetes Research Institute of the IRCCS Ospedale San Raffaele, 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.

2.5. Comparison of IFA measured by LIPS vs ELISA

As a reference method for the measurement of IFA, we used the commercial Anti-Intrinsic Factor ELISA (IgG) kit (EUROIMMUN, Lübeck, Germany), approved for diagnostic use. Following the manufacturer's instruction, we tested a subset of 45 serum samples from our cohort, comprising 33 patients with confirmed CAG and 12 subjects without CAG. Moreover, we tested the serial dilutions of two strongly IFA-positive samples in normal serum, of which one is used as standard curve in the LIPS assay. The absorbance of samples and the background were read at 450 nm and 655 nm, respectively, in an ELISA 680 Microplate reader (Bio-Rad). IFA levels were calculated by interpolation of the absorbance (after subtraction of the background) using a standard curve consisting of three calibrators provided by the kit (corresponding to 200, 20 and 2 relative units/ml (RU/ml)). The ELISA threshold for positivity was set at ≥20 RU/ml, according to the manufacturer's instructions.

2.6. Statistical evaluation

Data are expressed as median (IQR or 95% CI), and/or number/total (percentage, %). Differences between cases and controls, and between subgroups selected on the presence or absence of anemia and vitamin B_{12} deficiency, were analyzed using Fisher exact tests and/or Mann-Whitney tests, as appropriate. Two-tailed p values < 0.05 were considered statistically significant. To define a threshold level for IFA-positivity a Q-Q plot analysis was performed and a threshold was selected around the inflection point of IFA units at which observed values deviated from the theoretical straight line of normally distributed quantiles. To determine the percent agreement of assigned scores in LIPS and ELISA (corrected by pure chance), the Gwet's first Agreement Coefficient (AC1) was calculated [24]. A Receiver-operating characteristics (ROC) curve analysis was performed to compare the diagnostic performance of IFA in different subgroups of subjects. The diagnostic performance of IFA was defined in terms of sensitivity, specificity, positive likelihood ratio (LR+) and negative likelihood ratio (LR-). Statistical analyses were conducted in R [25], and figures were produced using the package ggplot2 [26].

3. Results

3.1. Diagnostic performance of IFA in CAG by LIPS

IFA levels measured by LIPS were significantly higher in cases with histologically confirmed CAG compared with controls without evidence of corpus atrophy (median CAG AU 0.643 [IQR 0.155–3.910] vs no-CAG AU 0.192 [IQR 0.097–0.634], $p < 0.0001$). Using an assay threshold for positivity of 2.5 AU based on a Q-Q plot analysis (Fig. 1B), the IFA LIPS showed a sensitivity for CAG of 32% (95% CI 24–42%) and a specificity of 95% (95% CI 90–99%) (Fig. 1C), a LR+ of 7.19 (95% CI 2.92–17.68) and a LR-of 0.71 (95% CI 0.62–0.81).

The area under the ROC curve (AUC), a threshold independent

analysis of diagnostic performance, was 0.672 (95% CI 0.605–0.734, $p < 0.0001$). We then calculated the partial ROC-AUC after imposing a specificity ≥90% ($pAUC_{90}$) as a more relevant proxy of assay performance consistent with commonly adopted thresholds for positivity for related assays: the $pAUC_{90}$ was 0.027 (Fig. 2A).

The diagnostic performance for CAG was similar after stratification of the subjects according to the presence or absence of either anemia or dyspepsia or vitamin B12 deficiency (Table 1 and Fig. 2B–D).

3.2. Comparison of IFA LIPS results with a commercial ELISA test

We compared the performance of our IFA-LIPS with that of a commercial ELISA kit for the measurement of IFA, using a subset of serum samples from 33 CAG cases (of which 22 positive and 11 negative in LIPS) and 12 no CAG controls (of which 4 positive and 8 negative in LIPS). In this subset, both LIPS and ELISA showed higher IFA levels in CAG patients compared to controls without gastric atrophy, with a significant difference observed in LIPS only (LIPS median CAG AU = 8.94 [IQR 0.86–347.2] vs no-CAG AU = 0.23 [IQR 0.06–8.08], p = 0.012; ELISA median CAG AU = 2.048 [IQR 0.11–37.1] vs no-CAG AU = 0.20 [IQR 0.12–0.33], p = 0.067). IFA levels measured by ELISA were only partially correlated with those obtained by LIPS in both CAG and in no-CAG subjects ($R^2 = 0.36$ and 0.33, respectively) (Supplemental Fig. 1A). Using the cut-off indicated by the manufacturer, the commercial ELISA assay identified as positive 39.3% (13/33) of CAG cases, compared to 66.7% (22/33) in LIPS. Conversely, the IFA ELISA identified as positive 0% (0/12) of controls without gastric atrophy compared to the 33.3% (4/12) in LIPS (Supplemental Fig. 1B). Overall, the concordance of assigned scores was also partial, with a percent agreement of 64.4% and an Agreement Coefficient 1 (AC1) of 30.6% (95% CI: 1–60.1, p-value = 0.043).

We compared the analytical sensitivity of the assays by testing serial dilutions in normal serum of two strongly IFA-positive sera, one of which was used as reference serum in the LIPS assay. In the case of our LIPS reference serum (sample #15173), the IFA-LIPS was able to reliably detect above the threshold of positivity the serial dilutions from 1:100 down to the 1:3200. None of these dilutions were above the threshold in ELISA (Supplemental Fig. 2). Similarly, using serial dilutions of a different serum (sample #SA450) which tested strongly IFA-positive in both assays (LIPS AU: 610,668 and ELISA RU/ml: 185.6), the LIPS confirmed its ability to detect as IFA positive the dilutions from 1:100 down to the 1:51,200, while none of the dilutions were above the assay threshold in ELISA (Supplemental Fig. 3).

3.3. IFA levels and demographic-clinical characteristics of CAG cases

IFA levels were similar in CAG cases stratified by characteristics such as the presence of anemia, vitamin B_{12} deficiency or corpus-restricted atrophy. Regarding sex, IFA showed higher levels in females compared with males (p = 0.0127) (Table 2A).

Within our cohort IFA prevalence was similar after stratification by age above or below the median (65 years), vitamin B_{12} deficiency, and presence of corpus-restricted atrophy, the typical histological feature of autoimmune atrophic gastritis. Regarding sex, we observed a trend towards an increased IFA prevalence in females (p = 0.0804) (Table 2B). Despite the low global sensitivity for CAG of the IFA assay alone, the

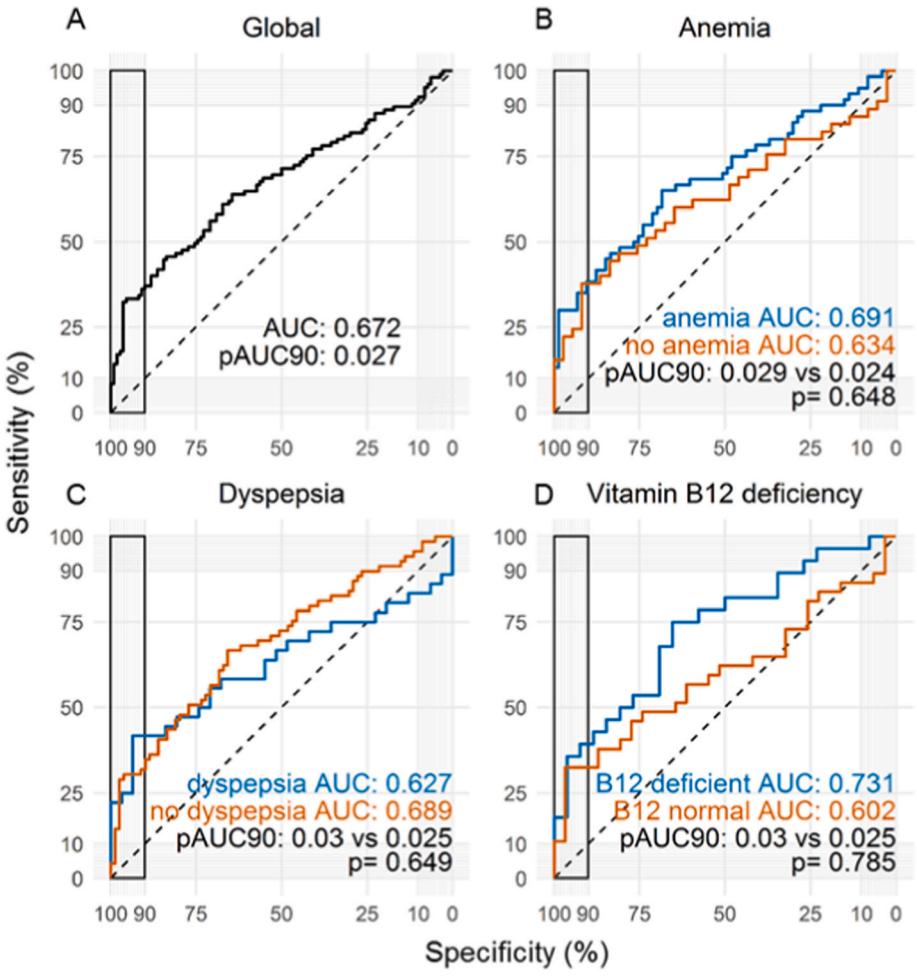

Fig. 2. *Receiver-operating characteristics (ROC) curves of the IFA LIPS assay in subjects with (cases) or without CAG (controls), stratified by different clinical characteristics.* Shown are the total area under the curve (AUC) and the partial AUC for 90–100% specificity ($pAUC_{90}$, grey rectangle delimited) together with the p-value of the difference between $pAUC_{90}$. **A.** IFA ROC curve (black line) in CAG cases (n = 105) and controls (n = 110). **B** IFA ROC curves in CAG cases and controls presenting with (blue line, n = 60 and n = 73) or without (orange line, n = 45 and n = 37) anemia **C.** IFA ROC curves in CAG cases and controls presenting with (blue line, n = 36 and n = 31) or without (orange line, n = 69 and n = 79) dyspepsia. **D.** IFA ROC curves in CAG cases and controls presenting with (blue line, n = 28 and n = 26) or without (orange line, n = 37 and n = 31) vitamin B_{12} deficiency. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Sensitivity, specificity, and positive and negative likelihood ratio (LR) of positive IFA LIPS in CAG cases and controls stratified by clinical characteristics.

	CAG - no CAG (N)	Sensitivity % (95 CI)	Specificity % (95 CI)	LR+% (95 CI)	LR-% (95 CI)	AUC	$pAUC_{90}$
Global	105–110	32 (24–42)	95 (90–99)	7.2 (2.9–17.7)	0.71 (0.62–0.81)	0.674	0.027
Anemia	60–73	28 (17–41)	99 (93–100)	20.7 (2.8–150.9)	0.73 (0.62–0.85)	0.691	0.029
no anemia	45–37	38 (24–53)	89 (75–97)	3.6 (1.3–9.8)	0.70 (0.54–0.90)	0.642	0.024
Dyspepsia	36–31	39 (23–57)	94 (79–99)	6.2 (1.5–25.3)	0.65 (0.49–0.86)	0.635	0.030
no dyspepsia	69–79	29 (19–41)	96 (89–99)	7.6 (2.4–24.6)	0.74 (0.63–0.86)	0.689	0.025
B_{12} deficiency	28–26	32 (16–52)	96 (80–100)	8.4 (1.1–61.5)	0.71 (0.54–0.92)	0.731	0.030
B_{12} normal	37–31	32 (18–50)	97 (83–100)	10.1 (1.4–73.1)	0.70 (0.55–0.88)	0.602	0.025

Table 2
IFA levels and clinical characteristics of patients with CAG.

A. IFA arbitrary units expressed as median and interquartile range (IQR) stratified for the main clinical characteristics of patients with CAG.

	N	Median	IQR	*p*
Females	71	0.45	0.122–1.679	0.0127
Males	34	0.19	0.099–0.745	
Age ≥65 years	51	0.43	0.142–3.973	0.346
Age <65 years	54	0.83	0.208–3.871	
Anemia as clinical presentation yes	60	0.33	0.099–0.997	0.0617
no	45	0.30	0.155–2.574	
Vitamin B_{12} deficiency[a] yes	28	0.32	0.133–1.018	0.3922
no	37	0.53	0.130–1.548	
Corpus-restricted atrophy* yes	81	0.67	0.145–4.084	0.8986
no	19	0.65	0.222–2.687	

B. Main clinical characteristics of patients with CAG and either positive or negative for IFA

	IFA-negatives (n = 71) N (%)	IFA-positives (n = 34) N (%)	*p* value
Females	44 (61.9)	27 (79.4)	0.0804
Age ≥65 years	35 (49.2)	16 (47.1)	0.8383
Anemia as clinical presentation	43 (60.5)	17 (50.0)	0.3996
Vitamin B_{12} deficiency[a]	19 (43.2)	9 (42.8)	1.0000
Corpus-restricted atrophy*	53 (79.1)	28 (84.8)	0.5941

[a] Data on vitamin B_{12} deficiency available from 65 CAG patients (44 IFA-negative and 21 IFA-positive). *As typically present in autoimmune atrophic gastritis. Corpus-restricted atrophy data was available for 100 CAG patients (67 IFA-negative and 33 IFA-positive).

simultaneous presence of anemia and a positive IFA test was linked to a high LR + for CAG of 20.68 (95% CI 2.83–150.96) (Table 1).

After stratification of CAG cases by sex and age (above or below the median), a greater prevalence of IFA-positives was observed in females aged below 65 years (43.5%,17/39) compared to males (6.6%,1/15) (Fisher exact test,$p = 0.011$) while no differences were present in cases aged>65 years (female 31.2%,10/32, males 31.5%,6/19. Fisher exact test,p = ns) (Supplemental Fig. 4).

A modest negative Spearman's correlation between IFA arbitrary units and age was present in CAG cases (rho −0.21, $p = 0.0332$) but not in controls (Supplemental Fig. 5), which however lost statistical significance when adjusted for the number of comparisons (p = 0.0664).

3.4. IFA assay combined with ATP4A + ATP4B assay

We next analyzed the diagnostic performance for CAG when IFA were combined with ATP4A and ATP4B antibodies, two other autoantibody biomarkers of CAG (20). We tested several algorithms based on alternative combinations of IFA AND/OR ATP4A AND/OR ATP4B antibodies (Table 3A).

A stringent algorithm in which IFA+ AND ATP4A+ AND ATP4B+ were used yielded a sensitivity for CAG of 29.0% (95%CI 20–38), a specificity of 98% (95%CI 94–100), a LR+ of 15.7 (95%CI 3.9–64.1) and a LR-of 0.7 (95%CI 0.6–0.8). A relaxed algorithm in which IFA+ AND/OR ATP4A+ AND/OR ATP4B+ were used yielded the greatest sensitivity for CAG of 83.0% (95% CI 74–90), but the lowest specificity of 85% (95%CI 76–91), a LR+ of 5.4 (95%CI 3.4–8.4) and a LR-of 0.2 (95%CI 0.1–0.3).

Table 3
Diagnostic performance for CAG of autoantibody combinations and with regard to clinical presentation.

A. Diagnostic performance for CAG of autoantibody combinations.

CAG vs no CAG	Sensitivity	Specificity	LR+	LR-
IFA+	32 (24–42)	95 (90–99)	7.2 (2.9–17.7)	0.7 (0.6–0.8)
ATP4A+ and ATP4B+	75 (66–83)	89 (82–94)	6.9 (4.0–12.0)	0.3 (0.2–0.4)
ATP4A+ and/or ATP4B+	79 (70–86)	87 (80–93)	6.2 (3.8–10.2)	0.2 (0.2–0.4)
IFA+ and/or ATP4A+ and ATP4B+	79 (70–86)	86 (79–92)	5.9 (3.6–9.5)	0.2 (0.2–0.4)
IFA+ and/or ATP4A+ and/or ATP4B+	83 (74–90)	85 (76–91)	5.4 (3.4–8.4)	0.2 (0.1–0.3)
IFA+ and ATP4A+ and ATP4B+	29 (20–38)	98 (94–100)	15.7 (3.9–64.1)	0.7 (0.6–0.8)

B. Diagnostic performance for CAG of the IFA+ and/or ATP4A+ and/or ATP4B + algorithm.

CAG vs no CAG	Sensitivity % (95 CI)	Specificity % (95 CI)	LR+ % (95 CI)	LR-% (95 CI)
Anemia	85 (73–93)	88 (78–94)	6.9 (3.7–12.8)	0.2 (0.1–0.3)
Dyspepsia	81 (64–92)	84 (66–95)	4.9 (2.2–11.3)	0.2 (0.1–0.5)
B_{12} deficiency	86 (67–96)	88 (70–98)	7.4 (2.5–21.8)	0.2 (0.1–0.4)

C. Diagnostic performance for CAG of the IFA+ and ATP4A+ and ATP4B + algorithm.

CAG vs no CAG	Sensitivity % (95 CI)	Specificity % (95 CI)	LR+ % (95 CI)	LR-% (95 CI)
Anemia	25 (15–38)	100 (95–100)	250*	0.8 (0.7–0.9)
Dyspepsia	33 (19–51)	100 (89–100)	330*	0.7 (0.5–0.8)
B12 deficiency	32 (16–52)	100 (87–100)	320*	0.7 (0.5–0.9)

D. ROC-AUC for CAG using combined IFA - ATP4A - ATP4B testing

	CAG vs no CAG	AUC	$pAUC_{90}$
ATP4A- ATP4B combined	*Global*	0.808	0.0535
	Anemia	0.877	0.0692
	dyspepsia	0.696	0.0505
	B_{12} deficiency	0.879	0.0821
IFA-ATP4A-ATP4B combined	*global*	0.840	0.0540
	anemia	0.902	0.0708
	dyspepsia	0.710	0.0460
	B_{12} deficiency	0.949	0.0851

Sensitivity and specificity are expressed as % (95% CI).
*calculated assigning a specificity of 99.9%.

The algorithms were further combined with the clinical features of anemia, dyspepsia and vitamin B_{12} deficiency. The addition of clinical features led to markedly improved specificity and LR + for CAG when using the stringent antibody algorithm, while the performance of the relaxed algorithm was more modestly affected (Table 3B–C).

Similarly, the ROC curve analysis of different antibody combinations confirmed the previous findings suggesting that the addition of IFA testing might improve diagnostic performance for CAG particularly when high specificity thresholds are imposed (Fig. 3).

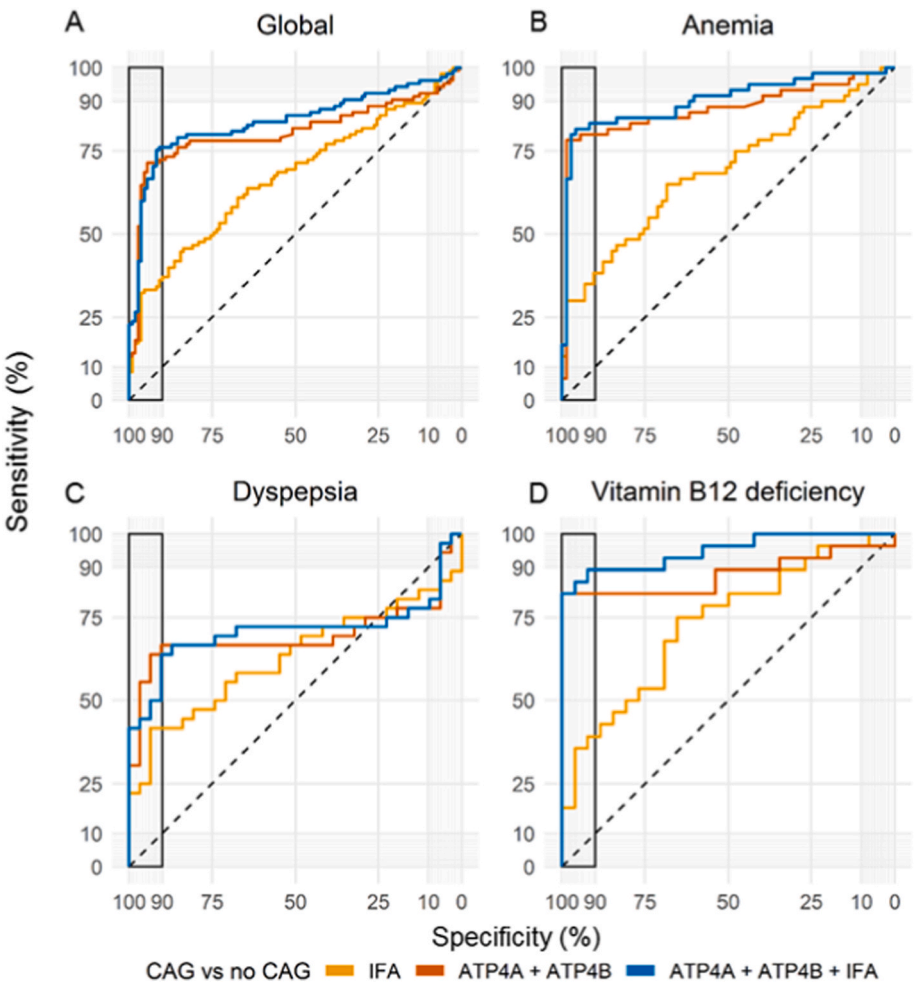

Fig. 3. *Receiver-operating characteristics (ROC) curves of combined IFA, ATP4A and ATP4B LIPS assays in subjects with (cases) or without CAG (controls), stratified by different clinical characteristics.* Shown are the ROC curves for the following antibody combinations: IFA alone (yellow line), ATP4A AND/OR ATP4B (orange line), IFA AND/OR ATP4A AND/OR ATP4B (blue line) **A**. ROC curves in CAG cases (n = 105) and controls (n = 110). **B** ROC curves in CAG cases and controls presenting with anemia (n = 60 and n = 73) **C**. ROC curves in CAG cases and controls presenting with dyspepsia (n = 36 and n = 31). **D.** ROC curves in CAG cases and controls presenting vitamin B_{12} deficiency (n = 28 and n = 26). . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

IFA are still considered the most reliable serological markers of PA [2,3,6–8], but their potential in CAG without PA has not been defined so far. Results from the current study showed that IFA-positivity was detectable in 34 of 105 histologically diagnosed CAG cases and in 5 out of 110 controls without bioptic evidence of corpus mucosa atrophy, thus yielding a sensitivity of 32% at a specificity 95%. According to earlier studies, IFA were positive in 40–60% of cases of PA [27]. Therefore, the overall frequency of IFA observed in the current study was similar, notwithstanding the fact that our study population contained both CAG patients with overt PA, and those with either latent PA or without any anemia. This is an innovative finding strengthening the potential clinical utility of IFA in the whole spectrum of patients with CAG. The study population for the current study was prospectively recruited based on a high suspicion of CAG following clinical presentation with long-standing and unexplained dyspepsia or anemia. It might have been expected that positivity towards IFA would have been less frequent in subjects presenting with dyspepsia than in those with any type of anemia, or more specifically with macrocytic anemia and/or vitamin B_{12} deficiency that are highly suggestive of PA. While the presence of IFA in subjects presenting with anemia was associated with the best positive likelihood ratio for CAG, the findings of the current study did not support this hypothesis, as they showed that the diagnostic performance of IFA in subjects presenting with anemia (28% sensitivity and 99% specificity) or at high clinical suspicion of PA (vitamin B_{12} deficiency) (32% sensitivity and 96% specificity) was not strikingly different from that of subjects presenting with dyspepsia (39% sensitivity and 94% specificity) and was similar to that of the whole study population.

Overall, these data show that IFA may be found in subjects with CAG independently from the presence of vitamin B_{12} deficiency and/or PA. Older studies found IFA-positivity exclusively [28] or almost exclusively [29] associated with PA. A study published 50 years ago described IFA-positivity in CAG patients without PA [30] showing that all but one of the IFA-positive CAG patients continued to absorb vitamin B_{12} normally without progression to PA over an observation period from three to seven years [30]. PA shares with CAG the presence of corpus oxyntic mucosa atrophy [3,4], ultimately, resulting in the loss of intrinsic factor producing cells together with the (eventual) production of neutralizing IFA leading to vitamin B_{12} malabsorption [1,2], but the nature and extent of the gastric mucosal damage might not be the same in all patients. CAG cobalamin depletion may take place over a long time [31,32], and the onset of overt PA may be preceded by prolonged vitamin B_{12} deficiency [2,29]. The amount of vitamin B_{12} absorbed by patients with CAG likely depends upon the amount of the available intrinsic factor, and this in turn is related to the number of remaining gastric parietal cells and to the presence of factors interfering with the action of intrinsic factor, such as neutralizing IFA [30]; a very low or un-detectable intrinsic factor secretion together with the presence of neutralizing IFA are probably necessary to prevent sufficient vitamin B_{12} absorption. Thus, serious consideration should be given to the inclusion of IFA assessment in the clinical evaluation of patients with suspected CAG and when positivity towards IFA is found, gastroscopy with corpus and antral mucosa biopsies should be performed to confirm the presence of CAG, irrespective of the pattern of anemia or the presence of vitamin B_{12} deficiency.

A previous ELISA-based study showed an overall IFA-positivity of 27% in CAG patients, but when CAG was associated with overt PA (macrocytic anemia and vitamin B_{12} deficiency) the positivity rate reached 37% compared to 19% in subjects with latent PA (vitamin B_{12} deficiency without macrocytic anemia) and 15% in those with normal hemoglobin and vitamin B_{12} levels [11]. In the current study, we used a LIPS assay based on human recombinant antigens tagged with a highly active luciferase reporter and antigen-autoantibody binding in liquid-phase, which provides an optimal platform for the detection of both conformational and linear epitopes. In contrast, solid-phase assays like ELISA often show a narrower dynamic range and a suboptimal detection of conformational epitopes, in particular after optimization for background noise [18,33,34].

The current study also showed that younger female CAG patients had higher IFA prevalence, possibly as a result of the more vigorous humoral immune reaction linked to female sex hormones [35]; these findings were consistent with the observed modest negative correlation between IFA levels and age. This finding seems to be in contrast with previous reports showing that IFA-positivity rates increased with age and disease duration [8,36]. Recently, a similar trend of age was observed for PCA in patients with autoimmune CAG [19,37]. This may be explained by a process of progressive mucosal destruction and subsequent antigen loss, as also shown by others [12], a phenomenon perhaps more frequent in elderly patients with a higher probability of having a longer natural history of the disease. In contrast, at a mean follow-up of 6 years PCA were shown to disappear over time, while IFA tended to increase [36]. The current study had a cross-sectional design and cannot provide answers to the time trend of IFA production. Well-designed longitudinal studies are necessary to test the hypothesis that IFA titers may decline due to depletion of the antigen source resulting from progression of oxyntic mucosa atrophy, as reported in other autoimmune conditions [38].

Finally, the current study showed that combining IFA with antibodies to ATP4A and/or ATP4B, which in combination were the best performing screening strategy for CAG in our previous study (20), provided a significant increase in sensitivity (83%), albeit at the cost of decreased specificity (85%). The clinical value of IFA is far from being definitively established: according to 2014 British guidelines regarding the diagnosis of cobalamin/folate disorders, IFA testing should be performed in all patients with anemia, neuropathy or glossitis, and suspected of having PA, regardless of cobalamin levels, and in patients with low serum cobalamin levels in the absence of anemia and who do not have other causes of deficiency, to rule out an early/latent presentation of PA [8]. The same guidelines do not recommend PCA testing for PA diagnosis [8], but PCA has been reported to represent an appropriate screening test for PA, with IFA reserved for confirmatory testing [7,10]. Other studies suggest the usefulness to screen for both PCA and IFA in the setting of suspected autoimmune gastritis [11,12]. The current study provide data to support this concept; seven PCA-negative CAG patients were IFA-positive, thus raising the level of clinical suspicion of autoimmune atrophic gastritis that might not have been suspected with PCA testing alone.

The current study has some limitations. First, it was a *post-hoc* study, as the sera used were prospectively collected for a previous serological study [20]; when the previous study was designed, the IFA LIPS assay had not been developed. Nevertheless, our study assessed IFA in a substantial sample of well-characterized subjects with and without CAG and allowed for the comparison of IFA results with those of the ATP4A and ATP4B tests. A second limitation was that pre-treatment vitamin B_{12} levels were not available for all the study subjects. Finally, not all the controls included in this study had a healthy stomach as 19% of controls had active Hp gastritis, and perhaps the IFA LIPS performance might have been improved if healthy subjects had been used as control; on the other hand, the fact that controls were composed of subjects without corpus atrophy irrespective of the presence of a healthy gastric mucosa or a superficial gastritis provides a more stringent context to test the diagnostic performance of our IFA LIPS assay. It should also be noted that although biopsy sampling was performed according to the updated Sydney system, the possibility that some of the controls might have had small focal areas of CAG at the time of gastroscopy which was not sampled by the biopsies cannot be eliminated, which if true, might explain positivity toward gastric autoantibodies in some controls.

5. Conclusion

In conclusion, this newly developed LIPS assay for IFA was able to discriminate between CAG patients and controls showing a high specificity at the cost of sensitivity (95% and 32%), similar to an ELISA assay for PA. IFA-positivity was more frequent in younger females but independent from anemia and vitamin B_{12} deficiency, suggesting that positivity to IFA has a complex etiology. Thus, IFA testing should be considered in the clinical evaluation of patients at high clinical suspicion of CAG in addition to PCA, and positivity requires histological confirmation by gastroscopy plus gastric mucosa biopsies.

CRediT author statement

Ilaria Marzinotto: Investigation and formal analysis, Validation, writing, review & editing. Ludovica Dottori: Investigation and data curation. Francesca Baldaro: Data curation. Emanuele Dilaghi: Data curation, endoscopic investigations. Cristina Brigatti: Methodology. Elena Bazzigaluppi: Methodology. Gianluca Esposito: Endoscopic investigations, Data curation. Howard W Davidson: Methodology, Review & Editing, Supervision. Lorenzo Piemonti: Supervision.Vito Lampasona: Conceptualization, Methodology, Validation, Investigation, Formal analysis, writing, review & editing. Edith Lahner: Conceptualization, Investigation, Formal analysis, writing, review & editing.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtauto.2021.100131.

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