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Prevalence, significance and predictive value of antiphospholipid antibodies in Crohn's disease

Sipeki N *et al.* Papp M APLA in IBD

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Abstract

BACKGROUND

AIM

To assess the prevalence and stability of different antiphospholipid antibodies (APLAs) and their association with disease phenotype and progression in inflammatory bowel diseases (IBD) patients.

METHODS

About 458 consecutive patients [Crohn's disease (CD): 271 and ulcerative colitis (UC): 187] were enrolled into a follow-up cohort study in a tertiary IBD referral center in Hungary. Detailed clinical phenotypes were determined at enrollment by reviewing the patients' medical charts. Disease activity, medical treatment and data about evolvement of complications or surgical interventions were determined prospectively during the follow-up. Disease course (developing of complicated disease phenotype and need for surgery), occurrence of thrombotic events, actual state of disease activity according to clinical, laboratory and endoscopic scores and accurate treatment regime were recorded during the follow-up, (median, 57.4 and 61.6 mo for CD and UC). Sera of IBD patients and 103 healthy controls (HC) were tested on individual anti- β 2-Glycoprotein-I (anti- β 2-GPI IgA/M/G), anti-cardiolipin (ACA IgA/M/G) and anti-phosphatidylserine/prothrombin (anti-PS/PT IgA/M/G) antibodies and also anti-Saccharomyces cerevisiae antibodies (ASCA IgA/G) by enzyme-linked immunosorbent assay (ELISA). In a subgroup of CD ($n = 198$) and UC patients ($n = 103$), obtaining consecutive samples over various arbitrary time-points during the disease course, we evaluated the intraindividual stability of the APLA status. Additionally, we provide an overview of studies, performed so far, in which significance of APLAs in IBD were assessed.

RESULTS

Patients with CD had significantly higher prevalence of both ACA (23.4%) and anti-PS/PT (20.4%) antibodies than UC (4.8%, $P < 0.0001$ and 10.2%, $P = 0.004$) and HC (2.9%, $P < 0.0001$ and 15.5%, $P = \text{NS}$). No difference was found for the prevalence of anti- β 2-GPI between different groups (7.2-9.7%). In CD, no association was found between APLA and ASCA status of the patients. Occurrence of anti- β 2-GPI, ACA and anti-PS/PT was not different between the group of patients with active versus inactive disease state according to appropriate clinical, laboratory and endoscopic scores in CD as well as in UC patients. All subtypes of anti- β 2-GPI and ACA IgM status were found to be very stable over time, in contrast ACA IgG and even more ACA IgA status showed significant intraindividual changes. Changes in antibody status were more remarkable in CD than UC (ACA IgA: 49.9% *vs* 23.3% and ACA IgG: 21.2% *vs* 5.8%). Interestingly, 59.1% and 30.1% of CD patients who received anti-TNF therapy showed significant negative to positive changes in ACA IgA and IgG antibody status respectively. APLA status was not associated with the clinical phenotype at diagnosis or during follow-up, medical therapy, or thrombotic events and it was not associated with the probability of developing complicated disease phenotype or surgery in a Kaplan-Meier analysis.

CONCLUSION

The present study demonstrated enhanced formation of APLAs in CD patients. However, presence of different APLAs were not associated with the clinical phenotype or disease course.

Key words: Crohn's disease; Ulcerative colitis; Disease progression; Antiphospholipid antibodies; Anti- β 2-Glycoprotein-I antibodies; Anti-phosphatidylserine/prothrombin; Anti-cardiolipin antibodies; Thrombosis

Core tip: Enhanced serological antibody formation is a well-known feature of inflammatory bowel diseases. Antiphospholipid antibodies (APLAs) are a

prothrombotic group of antibodies acquired in various inflammatory diseases. However their association with clinical phenotype and disease progression is still unclear in inflammatory bowel diseases (IBD). In the present study we report enhanced formation of APLAs in patients with Crohn's disease, which was not associated with clinical phenotype or disease course during follow-up in a tertiary referral IBD center from Hungary.

INTRODUCTION

Enhanced serological antibody formation is a well-known feature of inflammatory bowel diseases (IBD). A wide range of anti-microbial and autoantibodies have been reported to be associated with either Crohn's disease (CD) or ulcerative colitis (UC)^[1] as well as with complicated disease course. Anti-microbial antibodies are formed against different surface carbohydrate (anti-glycans^[2]) or protein antigens of various gut microbes^[3]. The first and still most relevant anti-microbial antibody is the ASCA (anti-*Saccharomyces cerevisiae* antibody). Autoantibodies are directed against various host proteins. Based on recent findings, their existence might also be related to enhanced microbial challenge to the gut^[4,5] due to a disturbed gut innate immune system and may trigger an exaggerated adaptive immune response. Furthermore, these serological antibodies may also be actively involved in the pathophysiology of inflammation in IBD^[6,7].

Antiphospholipid antibodies (APLAs) are a prothrombotic group of autoantibodies and established as the serological hallmark of antiphospholipid syndrome (APS)^[8]. These antibodies comprise anti-cardiolipin (ACA), anti- β 2-Glycoprotein-I (anti- β 2-GPI), and anti-phosphatidylserine/prothrombin antibodies (anti-PS/PT). APLAs, however, are also found in a variety of disorders (chronic inflammatory diseases^[9-12] or post infectious conditions^[13-16]) not necessarily exhibiting prothrombotic activity. Even if non-prothrombotic, they may have certain pathogenetic roles in several diseases as well^[17,18].

In IBD, available cross-sectional, mainly single-time point studies assessing different aspects of APLAs, came to discrepant conclusions regarding formation, prevalence, and stability of these antibodies. Their clinical significance, including association to thrombotic events in IBD is also unclear^[19-33] (Table 1). Thus a comprehensive evaluation of the primary APLAs in a large prospectively followed-up IBD cohort is required.

Current advances may add a new spark to the investigation of the role of anti- β 2-GPI antibodies in the pathomechanism of IBD. The presence of

cross-reactive epitopes on *Saccharomyces cerevisiae* and β 2-GPI^[34] has been reported in APS and raises the possibility that ASCA alone or by cross-reactivity with anti- β 2-GPI exaggerate the pathologic intestinal microvascular processes in IBD and interfering with the inhibitory effect of β 2-GPI on von Willebrand factor-dependent platelet adhesion and aggregation. Inflammation and coagulation are closely linked, interdependent processes in the microvasculature. Coagulation abnormalities at the mucosal level result in microthrombi formation, which are well-known features of CD and thought to be involved in the disease pathogenesis and progression^[35]. Theoretically, impairment in the function of β 2-GPI due to the presence of anti- β 2-GPI may deteriorate certain innate immune functions as well. A novel function of the β 2-GPI protein with important relevance to innate immunity, is its ability to bind and scavenge lipopolysaccharide (LPS) through a direct interaction between domain 5 (D5) of β 2-GPI and LPS^[36,37].

The aims of this study were to investigate in a large IBD cohort: (1) the prevalence and type of APLAs; (2) associations between the presence of APLAs and clinical phenotype of the disease or its activity; and (3) whether the presence of APLAs is associated with the disease course or development of thrombosis during prospective follow-up. Additionally, we also provide an overview of studies, performed over the last 24 years, in which significance of APLAs in IBD were assessed.

MATERIALS AND METHODS

Patient population

We performed a cohort study among adult CD and UC patients in a Hungarian tertiary IBD referral center (Gastroenterology Department of Institute of Medicine, University of Debrecen). In all, 458 well-characterized, unrelated, consecutive IBD patients with a complete clinical follow-up (CD: 271 (male/female: 120/140, age at presentation: 27.7 ± 11.6 years, disease duration: 6.0 ± 6.7 years) and UC: 187 (male/female: 86/101, age at presentation: 34.0 ± 13.2 years, disease duration: 7.4 ± 8.6 years) seen at our

outpatient clinic were included between January 1, 2005 and June 1, 2010. Serum samples were obtained at enrollment from each patient and frozen at -80C until testing. The clinical characteristics of the patients at time of inclusion and sample procurement are presented in Table 2.

Diagnosis of IBD was based on the Lennard-Jones criteria^[38]. The disease phenotype (age at onset, duration, location, and behaviour) was determined according to the Montreal Classification^[39]. Clinical disease activity was calculated according to the harvey-bradshaw index (HBI)^[40] in CD and the partial Mayo score in UC^[41]. In this study we followed the European Crohn's and Colitis Organisation guidelines^[42] and defined HBI ≤ 4 as a state of remission and ≥ 5 as a state of active disease. In case of UC, ≤ 3 was defined as a state of remission and > 4 as a state of active disease. Endoscopic activity was determined according to the Simple Endoscopic Score for Crohn's Disease (SES-CD) in CD^[43] and the endoscopic component of the Mayo score in UC^[44]. SES-CD defines endoscopic activity as ≥ 3 points and inactive disease ≤ 2 in CD, meanwhile in UC a state of active disease was defined as ≥ 1 points according to invasive partial Mayo score. Detailed clinical phenotypes were determined by thorough review of patients' medical records, which had been collected in a uniform format. Medical records that documented the presence of extraintestinal manifestations (for example, arthritis: peripheral and axial; ocular manifestations: conjunctivitis, uveitis, iridocyclitis; skin lesions: erythema nodosum, pyoderma gangrenosum; arterial (AT) and venous thrombosis (VT) or pregnancy loss; and hepatic manifestations: primary sclerosing cholangitis [PSC]), frequency of flare-ups (frequent flare-up: > 1 clinical relapse/year)^[45], medication use (e.g., steroid, immunosuppressive and/or biological use at any time), need for surgery (resection in CD and colectomy in UC), the presence of familial IBD, smoking habits, and perianal involvement were retrospectively analyzed for the period prior to the prospective follow-up.

Phenotypical characterization of IBD patients during prospective follow-up

252 of 271 CD patients and 173 of 187 UC patients were available to be enrolled into a prospective follow-up study, where the treating IBD physicians registered laboratory data, endoscopic and imaging findings, disease activity, medical treatment, date and type of complications, surgery and thrombosis during regular and extraordinary outpatient follow-up visits and inpatient stays. In Hungary, a follow-up visit is usually scheduled for every 6 months at a specialized gastroenterology center (the actual interval varies between 3–6 mo). Collected data were transferred and stored in a database for analysis. In October 1, 2013, all patients' charts and database were reviewed and updated for the data points mentioned above. Follow-up for a particular patient was terminated if there was no further record available. Median follow-up was 57.4 mo (IQR: 40.9-80.1) for CD and 61.6 mo (IQR: 46.5-81.3) for UC patients. In CD, complicated disease behavior was defined as the occurrence of stenosis or internal penetration. Perianal fistulizing disease was distinguished from internal penetrating disease and evaluated separately. Need for surgery was defined as CD-related abdominal surgery (resection). Figure 1 summarizes flow chart of the patients with CD in the cohort study. In UC, complicated disease behavior was defined as progression of the disease extent or need for colectomy.

The control group consisted of 103 age- and gender- matched healthy blood donors (male/female: 46/57, age: 35.3 ± 12.6 years old). The control subjects did not have any gastrointestinal and/or liver disease and were selected from consecutive blood donors in Debrecen.

Serological analysis

Anti- β 2-GPI, ACA and anti-PS/PT levels in serum samples were tested using the semiquantitative QUANTA Lite™ a β 2-GPI, ACAIII and aPS/PT IgA, IgG and IgM kits (INOVA Diagnostics, San Diego, California). These enzyme-linked immunosorbent assay (ELISA) kits detect IgA, IgG and IgM antibodies against β 2-GPI, cardiolipin and the PS/PT complex in human serum. Plastic microwell plate wells are coated with purified β 2-GPI, cardiolipin or PS/PT

complex. Upon incubation, serum β 2-GPI, cardiolipin and PS/PT IgA, IgG or IgM antibodies bind to β 2-GPI, cardiolipin or the PS/PT complex. Unbound protein is removed by washing, while bound antibodies are detected by human IgA, IgG or IgM horseradish peroxidase-labelled conjugate. A peroxidase substrate is then added. The presence of anti- β 2-GPI, ACA and aPS/PT antibodies is determined spectrophotometrically by measuring the signal intensity of each sample compared to a five-point calibration curve. All assays were performed according to the manufacturer's instructions and were considered positive when titers were above the manufacturer's pre-established cut-off points (for anti- β 2-GPI and ACA assays, ≥ 20 units for all the IgA, IgG and IgM, and for anti-PS/PT assays ≥ 30 units for both the IgG and IgM). In case of anti-PS/PT IgA the results are presented as OD due to lack of established calibrators. Values above the OD cut-off 0.795 were considered positive for anti-PS/PT IgA. This cut-off OD value represented the mean \pm SD values of the healthy controls. The results were documented in absolute OD values and in frequency of positivity. Of the 458 IBD samples obtained at enrollment, serologic analysis was technically successful in 451 of the 458 IBD cases.

ASCA antibody evaluation in CD patients was performed in our previous study^[46] by ELISA (QUANTA Lite™, INOVA Diagnostics, San Diego, CA) according to the manufacturers' instructions. The results are presented as arbitrary units, and values above the cut-off of 25 units were considered as positive. The results were documented in absolute values and in frequency of positivity.

All the serological assays were performed in a blinded fashion without prior knowledge of the patients' diagnosis or other clinical information.

Detection of NOD2/CARD15 SNP8, 12, 13 mutations

NOD2/CARD15 SNP8, SNP12, and SNP13 genotypes were performed previously^[46] in CD patients ($n = 235$), but not in UC patients. NOD2/CARD15 variants were detected by denaturing high-performance

liquid chromatography (dHPLC, Wave DNA Fragment Analysis System, Transgenomic Limited, United Kingdom). Sequence variation, observed in the dHPLC profile, was sequenced on both strands to confirm the alteration. Sequencing reactions were performed with the ABI BigDye Terminator Cycle Sequencing Kit v1.1 (Applied Biosystems, Foster City, CA) and samples were sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). All investigated polymorphisms were in Hardy–Weinberg equilibrium (data not shown).

Review of the literature

We performed a systematic review of studies reporting on APLAs in IBD. Papers were eligible if they presented original research in adult IBD patients and reported occurrence of APLAs and their possible association with disease activity, medication, clinical phenotype of the disease, need for surgery and thromboembolic events. Case series or case reports were not included. Studies had to have been published in peer-reviewed journals. We started searching PubMed using the following search terms: “antiphospholipid antibodies” OR “anticardiolipin antibodies” OR “anti- β 2-Glycoprotein-I antibodies” OR “phosphatidylserine-dependent anti-prothrombin antibodies” AND “inflammatory bowel disease” OR “Crohn’s disease” OR “ulcerative colitis”. Limits were human and time ranging from 1991 until 2014 (30th of November). This search revealed 15 articles. In Table 1 we summarize the prevalence and clinical significance of APLAs in IBD based on findings in relevant literature.

Ethical permission

The study protocol was approved by the regional and national committee for research ethics. Each patient was informed of the nature of the study and signed an informed consent form.

Statistical analysis

Variables were tested for normality using Shapiro Wilk's W test. Continuous variables were summarized as mean \pm SD or as medians (IQR) according to their homogeneity. To evaluate differences between IBD and healthy control group, as well as within subgroups of patients with IBD the following statistical methods were used. Categorical variables were compared with the Fisher's exact test or χ^2 -test with Yates correction, as appropriate. Continuous variables were compared with Student's t test, one-way analysis of variance (ANOVA), or Mann-Whitney's U test. Kaplan-Meier survival curves were plotted for analyzing the association between categorical clinical variables or APLAs and complicated disease outcomes during follow-up with LogRank and Breslow tests. Associations are given as odds ratio (OR) and hazard ratio (HR) with a 95%CI. A 2-sided probability value < 0.05 was considered to be significant. For statistical analysis, GraphPad Prism 6 (San Diego, CA) and SPSS 15.0 (SPSS Inc, Chicago, IL) programs were used. The statistical methods of this study were reviewed by Elek Dinya from Semmelweis University, Institute of Health Informatics, Development and Further Training.

RESULTS

Frequency of APLA Markers in IBD

The prevalence rates of anti- β 2-GPI, ACA and anti-PS/PT antibodies are presented in Table 3. ACA positivity was associated with increased risk for CD compared to the controls ($OR_{ACA} = 10.18$, 95%CI: 3.12-33.24). Of the different isotypes, ACA IgA ($OR_{ACA\ IgA} = 49.70$, 95%CI: 3.04-813.8, χ^2 -test with Yates correction) had the highest association to CD. ACA positivity was also significantly different between CD and UC. While the prevalence of anti-PS/PT was significantly different between CD and UC, there was not a significant difference between CD and the controls. No difference was found for the prevalence of anti- β 2-GPI in different groups.

Association between APLA positivity, and other serologic markers or NOD2/CARD15 genotypes in CD

In CD, no association was found between APLA and ASCA status of the patients. Neither ACA IgA nor IgG positivity differed significantly according to presence or absence of ASCA IgA and ASCA IgG. Similarly, the prevalence of ACA was not associated with the presence of major *NOD2/CARD15* mutations. *NOD2/CARD15* genotypes were available in 235 CD patients. The prevalence of any ACA was not different between patients with or without *NOD2/CARD15* mutations (16.1% and 26.1%, $P = \text{NS}$, χ^2 -test with Yates correction).

Association between APLA positivity and actual clinical, laboratory or endoscopic activity of the disease

At the time of enrollment, 26.6% of CD patients and 27.8% of UC patients had active disease according to clinical activity scores (Table 1). Occurrence of anti- β 2-GPI, ACA and anti-PS/PT was not different between the group of patients with active (2.8%, 29.2% and 18.1%) and inactive disease state (8.8%, 21.2% and 21.2%, respectively) signified by HBI ≥ 5 . Similarly, there was no correlation between the disease activity determined by partial Mayo score >4 and APLA status in UC (data not shown).

Furthermore, the prevalence of any ACA was similar between CD patients with C-reactive protein (CRP) level >10 mg/L and those with ≤ 10 mg/L (29.0% and 21.1%, $P = \text{NS}$, χ^2 -test with Yates correction).

A total of 87 CD patients had ileocolonoscopy at enrollment. The prevalence of any ACA was not different according to endoscopic disease activity denoted by a SES-CD cut-off value of ≥ 3 (inactive *vs* active: 25.6% and 29.5%). Likewise, ACA IgA and ACA IgG level was not associated with CRP level, actual HBI or SES-CD score in patients with CD applying Spearman correlation analysis (data not shown).

20.3% of the CD patients showed frequent relapse during the follow-up. The ACA prevalence was not significantly different between patients with or without frequent relapse (ACA IgA 23.5% *vs* 16.7%, ACA IgG 3.9% *vs* 10.1% and ACA IgM 2.0% *vs* 3.5%, $P = \text{NS}$ for all).

Lastly, we investigated the association between disease duration and both the presence and magnitude of serologic response in CD. The rate of any ACA positivity was the same in all four disease duration quartile groups (Q1: 26.9%, Q2: 22.7%, Q3: 22.4% and Q4: 23.0%, $P = \text{NS}$, χ^2 -test with Yates correction). The level of ACA IgA and IgG was also not associated with disease duration (Kruskal-Wallis test).

APLA Markers and disease progression in CD

A total of 154 (56.5%) CD patients had non-stricturing and non-penetrating disease (B1) according to Montreal classification at time of sampling. 143 patients were eligible for a prospective follow-up study. The median follow-up was 53.4 months (IQR: 38.0-79.3). Among these complication and surgery naïve patients, 20.3% (29/143) experienced a complication during follow-up (31.03% developed strictures, 44.83% internal penetration and 24.14% perianal perforation only). The median time to complication was 21.4 mo (IQR: 8.1-43.1). In all, 9.1% (13/143) had to undergo CD-related abdominal surgery (resection) during the follow-up period (Figure 1). Two patients had surgical intervention without previous complication due to the development of colorectal cancer. In the remaining patients the reason for surgery was the occurrence of a complication (23.1% stenosis, 46.2% internal penetration, 15.4% both). The median time to surgery was 50.0 months (IQR: 30.5-54.5).

In patients classified as B1, the progression of the disease to a first event defined as stenosis, internal and/or perianal penetration or CD-related surgery (Table 4) was not associated to presence or absence of APLA positivity. Furthermore in Kaplan-Meier analysis the likelihood for earlier progression to a disease event was similar in patients with or without APLA positivity. Of the clinical factors, disease location and smoking were those that associated with time to development of first internal penetrating and/or stricturing complication and frequent relapses to development of perianal penetrating disease (Table 5).

In UC patient group association between APLAs and clinical

phenotype or progression of the disease was not evaluated due to the lack of increased prevalence of any APLAs in UC population.

APLA Markers and thromboembolic events in IBD

In total, 5.1% (23/452) of IBD patients had at least one thromboembolic event (14 CD and 9 UC patients). In CD, 18 events of VT, 1 event of PE and 3 events of AT were diagnosed. In UC, 8 events of VT and 1 events of AT were diagnosed. 7 (1.6%) patients had a TE also prior to diagnosis of IBD. 4 (1.5%) CD patients had recurrent TE. In women, pregnancy loss occurred in 6.4% (10/156) of CD and 6.9% (7/101) of UC patients.

CD patients presenting with VT were significantly older than those without (median age: 30.0 *vs* 38.5 years, $P = 0.003$). Of the investigated clinical factors and laboratory markers, previous VT event (RR = 23.0, 95%CI: 10.6-50.1) and factor V Leiden mutation (RR = 8.3, 95%CI: 2.2-31.3) were associated with the risk of VT events. At the same time, in patients with UC, frequent relapse was associated with higher risk of VT event (RR = 6.4, 95%CI: 1.7-24.1). However, none of the APLA markers were associated with increased risk of VT events in IBD.

Table 6 summarizes the patient characteristics, prevalence of APLAs and the known genetic and serologic markers of thrombophilia according to presence and type of thrombotic events in CD patients. We further investigated the probability for thromboembolic events as a function of positivity for a certain amount of APLAs out of the whole panel. However, neither CD and nor UC patients positive for multiple APLAs showed a higher probability for the development of thromboembolic events.

Stability of APLA markers

In order to evaluate the stability in the APLA status (positive or negative for a respective antibody), we analyzed samples from same patients over various arbitrary time-points during the disease course. At least two serum samples were taken from a subgroup of CD patients ($n = 198$) and UC patients ($n =$

103). Median time between sample procurements were 13.5 mo (IQR: 6.8–22.9 mo) for CD and 12.1 mo (IQR: 5.7–20.4 mo) for UC patients. Interestingly, the anti- β 2-GPI status was very stable over time with respect to all three Ig subtypes. Only 1.5%–5.8% of either CD or UC patients had a change in their anti- β 2-GPI antibody status compared to the initial sample procurement. At the same time marked differences were found in case of ACA Ig subtypes. ACA IgM status, similar to anti- β 2-GPI, was also stable. In contrast, ACA IgG and even more ACA IgA status showed significant changes over time, mainly from negative to positive. Changes in antibody status were more remarkable in CD than UC (ACA IgA: 49.9% *vs* 23.3% and ACA IgG: 21.2% *vs* 5.8%). Stability data are not available for anti-PS/PT antibodies since measurements were only performed on the first available serum samples of the patients. APLA changes are summarized in Table 7.

After availability of tumor necrosis factor (TNF) antagonist therapy through National Health reimbursement system in 2008, 43.7% (110/252) of our patients received either infliximab or adalimumab treatment. We assessed the impact of post-enrolment anti-TNF therapy in the induction of new ACA antibody formation. 59.1% (55/93) of patients who received anti-TNF therapy and had negative findings for ACA IgA at the baseline were found to have positive results later on (negative to positive change), which was significantly higher compared to the proportion of 35.6% (36/101) found among patients who did not receive anti-TNF therapy at all ($P = 0.004$). These ratios were 30.1% *vs* 12.9% ($P = 0.033$) for ACA IgG.

DISCUSSION

To our knowledge, this is the largest study to investigate prospectively the prevalence, type, and clinical significance of multiple APLAs simultaneously in a cohort of IBD patients to date. Three different antibodies were assessed by ELISA. Contrary to routine laboratory practice, APLAs were identified by anti-IgA secondary antibody in addition to anti-IgG and anti-IgM isotypes. Moreover, in the present study we also provided an overview of relevant

APLA studies in IBD.

We demonstrated, for the first time, that enhanced ACA IgA formation is a feature of CD; the presence of ACA IgA was significantly higher as compared to UC and HC. Previously only one study^[22], involving 50 CD patients, evaluated the occurrence of ACA IgA. However, the reported prevalence rate was much lower than in the present study (6.0% vs 19.2%). At the same time, occurrence of IgM and IgG class ACA was studied more extensively in IBD, but several studies provide only total prevalence rate for these two ACA subtypes. Reported prevalence of total ACA was widely varied both in CD (0.0%-36%)^[21,22,25] and UC (1.3%-20.0%)^[30,31] which was 12.6% and 4.3%, respectively in the present study. Studies evaluating separately these two subtypes of ACA revealed that the increased ACA prevalence is mainly due to IgG subtype. Except one study^[30], occurrence of ACA IgG prevailed over ACA IgM^[20,22,25]. This is concordant with our results in CD cohort (ACA IgG: 10.2 vs ACA IgM: 3.0%).

What can be the cause of enhanced ACA formation in CD? Animal models, immunization with lipid A and lipoteichoic acid have resulted in induction of ACA and/or lupus anticoagulant (LA) formation^[47]. Moreover, appearance of ACA antibodies may be due to the sustained exposure to bacterial DNA, which is enriched in unmethylated CpG motifs. These motifs are expressed *e.g.* in *Escherichia coli* DNA^[48]. Based on these literature findings, the role of bacterial translocation (BT) in the induction of ACA, similar to anti-microbial antibodies, seems plausible^[1]. At the same time, we did not find any association between the presence of ACA and ASCA, even when assessing according to separate isotypes. This finding implies mechanisms other than BT in the formation of ACA. Moreover, in contrast to anti-microbial antibodies, APLA formation was not influenced by NOD2/CARD15 genotypes. The fact that neither the presence of ACA, nor the titers of the antibodies were affected by actual disease activity and were also not associated with the CD phenotype of frequent relapse contradict the involvement of BT in the formation mechanisms of ACA as well.

Former studies^[19-26] extensively assessed the link between disease activity and APLA formation either by clinical activity or by CRP level, but found no association. Considering that clinical, laboratory and endoscopic activity are not inevitably congruent in all cases, in the present study, we re-evaluated this relationship in a complex way, applying all the three kinds of activity parameters simultaneously, but came to same conclusion.

The prevalence of anti- β 2-GPI IgG/IgM in the present study was comparable to those reported in the study of Koutroubakis *et al*^[25] (CD: 5.0% *vs* 4.4% and UC: 7.5% *vs* 10.8%). Anti-PS/PT antibodies were not assessed previously in IBD, however we did not find enhanced antibody formation in IBD as compared to HC.

Association of certain APLAs with clinical phenotype of IBD was mainly assessed in the cross-sectional single-time point studies summarized in Table 1. In most of these studies, these antibodies were neither linked to the disease location^[19,21,22,25], behavior^[25] and need for surgery^[19] in CD patients, nor to the extent of the disease^[19,21,24,25] and colectomy^[19] in UC patients. Likewise, different APLAs were not associated with the risk of VTE events in either CD^[19,22,23,25,26] or in UC^[19,23-25]. In case of these single points cross sectional studies the serum samples were obtained at different times during the disease course. These approaches combine samples taken before, at the time of and after complications occur and only allow revealing associations, but do not have predictive capability. In the present study to enhance the potential clinical value of these markers, we applied a prospective study design, which enabled us to evaluate the potential predictive capabilities of APLAs in respect to complicated CD behavior and surgery. Predictive ability for serum markers might be beneficial at any time during the disease course but perform distinctly by chance. Thus we formed two separate groups for our CD cohort (1) complication and surgery naïve CD patients; and (2) complication and/or surgery experienced patients prior to sample procurement. Several observations suggest that the presence of perianal and internal penetrating disease signify distinct phenotypes in CD concerning all

the clinical, serologic and genetic point of view^[1,49]. Accordingly, we evaluated B1p complication separately from B3 complication. However, APLA did not proved as a predictive marker of the complicated disease in neither clinically setting of CD. A clear strength of our study was prospective follow-up design and the application of the widest panel of currently available APLAs.

Due to low frequency of different thromboembolic complications, we assessed the occurrence of these events even from the diagnosis involving the period prior to sample procurement as well. Development of VT, AT and pregnancy loss, however, did not vary according to APLA status.

At the same time, significant changes were found in ACA IgA and ACA IgG positivity over time in patients with CD. In rheumatology disorders, introduction of TNF antagonists (adalimumab, infliximab, and etanercept) was reported to induce production of various types of autoantibodies, such as APLAs^[50]. However such data are scarce in IBD. In a study by Atzeni *et al*^[20] involving 63 patients with CD, five (8%) had ACA antibodies (mainly IgM) at baseline and two additional patients developed these autoantibodies during infliximab treatment with no apparent clinical effect. Thus we investigated the possible role of the TNF antagonists therapy in our prospective CD cohort. Interestingly, the patients initially negative for ACA developed ACA IgA or IgG positivity significantly more frequently if they received anti-TNF therapy suggesting a causative association. In the non-anti-TNF treated subgroup of patients, the change of the ACA status is less well understood and needs further clarification. The pathogenic mechanism that changes the humoral response leading to development of autoimmunity during anti-TNF inhibitors therapy is unknown, but various hypothesis have been proposed^[51]. One hypothesis is that binding of anti-TNF antagonists to the transmembrane and soluble TNF, rapidly lowering TNF level and enhancing apoptotic cell death, which triggers the development of autoantibodies. Further investigations are warranted to elucidate whether the new ACA appears associated with a worse clinical outcome.

CONCLUSION

In conclusion, the present study demonstrated enhanced formation of APLAs in CD patients. The presence of the APLAs however was not associated with the clinical phenotype, disease course, or risk of venous thrombotic events during the prospective follow-up.

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Footnotes

Institutional review board statement: The study was reviewed and approved by the Hungarian National Review Board and the Institutional Review Board of the University of Debrecen.

Clinical trial registration statement: This registration policy applies to prospective, randomized, controlled trials only.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: The authors declare that they have no conflict of interest..

Data sharing statement: No additional data are available.

CONSORT 2010 statement: The authors have read the CONSORT 2010 statement, and the manuscript was prepared and revised according to the CONSORT 2010 statement.

Figure Legends

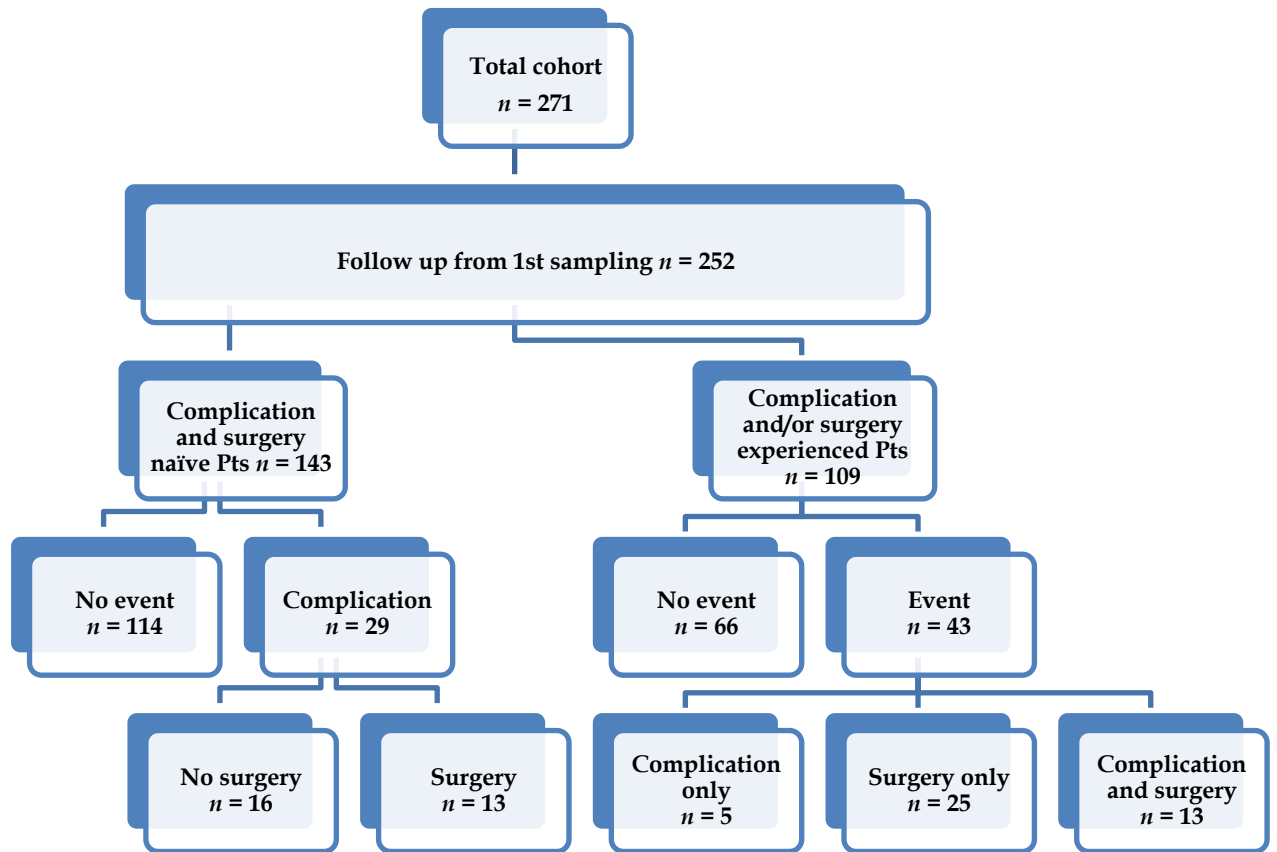


Figure 1 Flow chart of the patients with Crohn's disease in the cohort study. Event: complication and/or surgery; Complication: stricture development and/or internal penetration and/or perianal penetration; Surgery: Crohn's disease-related surgery (resection only). Pts: Patients.

Table 1 Prevalence and clinical significance of antiphospholipid antibodies in inflammatory bowel disease - review of the literature

Ref.	Study design	Group	N	ACA prevalence (%)			Anti-β2-GPI prevalence (%)			ACA		Anti-β2-GPI									
				Total	IgG	IgM	IgA	Total	IgG	IgM	IgA	Disease activity	Location	Association with disease phenotype	IS	Therapy	Disease activity	Location	Association with disease phenotype	IS	Therapy
Aichbichler et al ^[19]	HC	CD	136	4.0						No	No			No	No	No					
Atzeni et al ^[20]	AS	CD	19	5.3	5.3	0.0	0.0	0.0									No				
Caccavo et al ^[21]	CD	UC	22	36.0						No	No										
Chamouard et al ^[22]	CD	CS	50	22.0	10.0	6.0	6.0			No	No			No	No						

Chiaran	HC	40	5.0																			
tini <i>et al</i> ^[23]	CD	41	26.8						No					No								
Dalekos <i>et al</i> ^[24]	UC	19	26.3						No					No								
Koutrou bakis <i>et al</i> ^[25]	HC	100	3.0	2.0	1.0	0.0	0.0	0.0														
	CS	80	10.017.5						No	No				No No								
Lonjon <i>et al</i> ^[26]	CD	45	15.6	8.9	6.7	4.4	2.2	2.2	No	No	No					No	No	No	No	No	No	No
	UC	83	18.1	13.3	4.8	10.8	8.4	2.4	+	No				No	No	No	No					
Maher <i>et al</i> ^[27]	HC	118	2.5																			
	CD	138	11.0						No				No No									
Martino vić <i>et al</i> ^[28]	HC	40																				
	CD	7																				
	UC	19	3.9 0.0																			
Oldenb urg <i>et al</i> ^[28]	HC	102																				
	CD	24	6.6																			

<i>al</i> ^[29]		UC	21		
Saibeni <i>et al</i> ^[30]	R	HC	53	0.0	0.0
		CD	29	9.4	6.9
		UC	24		12.5
Souto <i>et al</i> ^[31]	R	HC	100		
		CD	26	0.0	0.0
		UC	75	1.3	1.3
Vecchi <i>et al</i> ^[32]	CS	IBD	20	30.0	
		HC	27	0.0	0.0
Yurekli <i>et al</i> ^[33]	CS	CD	5	7.4	3.7
		UC	22		

ACA: Anti-cardiolipin antibody; β 2-GPI: β 2-Glycoprotein-I; CS: Cross-sectional; P: Prospective; R: Retrospective; TE: Thromboembolic; HC: Healthy control; CD: Crohn's disease; UC: Ulcerative colitis; IS: Immunosuppressive.

Table 2 Clinical characteristics of inflammatory bowel disease patients at time of inclusion, *n* (%)

	CD (<i>n</i> = 271)		UC (<i>n</i> = 187)
Male/female (<i>n</i>)	115/156		86/101
Age (yr) ¹	31.0 (24.0-41.0)		40.0 (29.0-52.0)
Age at presentation (yr) ¹	25.0 (19.0-33.0)		33.0 (23.0-43.0)
Duration (yr) ¹	4.0 (1.0-9.0)		4.0 (1.0-11.0)
Familial IBD	12 (4.4)		6 (3.2)
Location			
L1	46 (17.0)	Proctitis	30 (16.0)
L2	67 (24.7)	Left-sided	104 (55.6)
L3	157 (57.9)	Extensive	53 (28.3)
L4 only	1 (0.4)		
All L4	18		
Behavior			
B1	154 (56.5)	Remitting	174 (93.0)
B2	56 (20.7)	Continuous	12 (6.4)
B3	61 (22.5)	Prolonged remission	1 (0.5)
Perianal disease	76 (27.5)		-
Arthritis	54 (19.9)		26 (13.9)
Ocular manifestations	65 (24.0)		12 (6.4)
Cutaneous manifestation	35 (12.9)		16 (8.6)
PSC	9 (3.3)		8 (4.3)
Steroid use/refractory	242 (89.3)/ 32 (13.2)		144 (77.0)/ 11 (7.6)
Azathioprine use	196 (72.3)		66 (35.3)
Surgery/multiple in CD	54 (19.6)/ 19 (7.0)		7 (3.7)
Biological use	106 (39.1)		25 (13.4)
Smoking habits			

Never	219 (80.8)		167 (89.3)
Yes	47 (17.3)		18 (9.6)
Previous	5 (1.8)		2 (1.1)
Disease activity			
Inactive HBI \leq 4	199 (73.4)	Inactive partial Mayo \leq 3	135 (72.2)
Active HBI \geq 5	72 (26.6)	Active partial Mayo $>$ 4	52 (27.8)

¹median (IQR). Location: L1: Ileal; L2: Colonic; L3: Ileocolonic; L4: Upper gastrointestinal disease; Behavior: B1: Inflammatory; B2: Stenosing; B3: Penetrating; PSC: Primary sclerosing cholangitis; Surgery: CD-related abdominal surgery and colectomy in UC; HBI: Harvey-bradshaw index; CD: Crohn's disease; UC: Ulcerative colitis.

Table 3 Anti-phospholipid antibodies in patients with Crohn's disease, ulcerative colitis, and healthy controls, *n* (%)

	CD (<i>n</i> = 265)	UC (<i>n</i> = 186)	HC (<i>n</i> = 103)
Anti-β2-GPI IgG	5 (1.9)	4 (2.2)	2 (1.9)
Anti-β2-GPI IgM	8 (3.0)	12 (6.5)	3 (2.9)
Anti-β2-GPI IgA	7 (2.6)	7 (3.8)	3 (2.9)
Any anti-β2-GPI	19 (7.2)	18 (9.7)	8 (7.8)
ACA IgG	27 (10.2) ^{b,d}	4 (2.2) ^d	2 (1.9) ^b
ACA IgM	8 (3.0)	4 (2.2)	1 (1.0)
ACA IgA	51 (19.2) ^{b,d}	1 (0.5) ^d	0 (0.0) ^b
Any ACA	62 (23.4) ^{b,d}	9 (4.8) ^d	3 (2.9) ^b
Anti-PS/PT IgG	20 (7.5)	9 (4.8)	9 (8.7)
Anti-PS/PT IgM	25 (9.4) ^{b,c}	8 (4.3) ^c	1 (1.0) ^b
Anti-PS/PT IgA	24 (9.1) ^c	7 (3.8) ^c	9 (8.7)
Any anti-PS/PT	54 (20.4) ^d	19 (10.2) ^d	16 (15.5)

^b*P* < 0.01, CD *vs* controls; ^c*P* < 0.05, ^d*P* < 0.01, CD *vs* UC. Using χ^2 -test with Yates correction. APLA: Antiphospholipid antibodies; β2-GPI: Beta2-glycoprotein-I; ACA: Anti-cardiolipin antibody; PS/PT: Phosphatidylserine/prothrombin complex; CD: Crohn's disease; UC: Ulcerative colitis.

Table 4 Antiphospholipid antibodies and clinical characteristics of complication and surgery naïve Crohn's disease patient cohort, *n* (%)

Factor	Follow-up of CD B1 at first sampling						Total Cohort	
	(n = 143)		(n = 114)		(n = 7)		(n = 13)	
	CD Total (n = 143)	B1 No Complication (n = 114)	Perianal change only (B1p) (n = 7)	Behaviour change to B2 or B3 (n = 22)	No Surgery (n = 130)	Surgery (n = 13)		
Male/female (n)	60/83	45/69	5/2	10/12	55/75	5/8		
Age at presentation (yr) ¹	25.0 (19.0-34.0)	26.0 (20.0-34.3)	17.0 (14.0-31.0)	25.0 (18.8-34.8)	25.0 (19.0-34.0)	31.0 (18.0-35.5)		
Duration (yr) ¹	3.0 (1.0-6.0)	2.0 (1.0-7.0)	3.0 (1.0-5.0)	3.0 (1.0-5.0)	2.0 (1.0-6.0)	3.0 (1.0-10.0)		
Location								
L1	30 (21.0)	24 (21.1)	0 (0.0)	6 (27.3)	26 (20.0)	4 (30.8)		
L2	51 (35.7)	44 (38.6)	3 (42.9)	4 (18.2)	48 (36.9)	3 (23.1)		
L3	62 (43.3)	46 (40.4)	4 (57.1)	12 (54.5)	56 (43.1)	6 (46.2)		
L4 only	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)		
Frequent relapse	27 (18.9)	18 (15.8) ²	4 (57.1) ²	5 (22.7)	23 (17.7)	4 (30.8)		
Smoking habit								
yes	21 (16.2)	14 (12.3) ²	2 (28.6)	8 (36.4) ²	21 (16.2)	3 (23.1)		
Follow up time from first sampling, mo ¹	53.4 (38.0-79.3)	51.7 (36.9-73.9)	79.8 (42.9-91.4)	64.7 (45.6-85.0)	50.9 ³ (37.25-74.2)	84.0 ³ (60.0-91.7)		
Positive markers								
Anti-β2-GPI IgG	1.4	1.8	0.0	0.0	1.6	0.0		
Anti-β2-GPI IgM	5.0	5.3	16.7	0.0	5.5	0.0		
Anti-β2-GPI IgA	2.8	1.8	16.7	4.5	3.1	0.0		
Any anti-β2-GPI	8.5	8.8	16.7	4.5	9.4	0.0		
ACA IgG	9.2	11.5	0.0	0.0	10.2	0.0		
ACA IgM	2.8	3.5	0.0	0.0	3.1	0.0		
ACA IgA	17.0	18.6	33.3	4.5	18.8	0.0		
Any ACA	19.9	22.1	33.3	4.5	21.9	0.0		
Anti-PS/PT IgG	8.5	8.0	0.0	13.6	7.8	15.4		
Anti-PS/PT IgM	7.8	8.8	16.7	0.0	7.8	7.7		
Anti-PS/PT IgA	9.9	9.7	16.7	9.1	10.9	0.0		

Any anti-PS/PT	20.6	19.5	33.3	22.7	20.3	23.1
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¹Median (IQR). ²Using χ^2 -test with Yates correction ($P \leq 0.01$). ³Using Mann-Whitney test ($P < 0.01$). Location: L1: Ileal; L2: Colonic; L3: Ileocolonic; L4: Upper gastrointestinal disease; Behavior: B1: Inflammatory; B2: Stenosing; B3: Penetrating. APLA: Antiphospholipid antibodies; β 2-GPI: Beta2-glycoprotein-I; ACA: Anti-cardiolipin antibody; PS/PT: Phosphatidylserine/prothrombin complex; CD: Crohn's disease.

Table 5 Univariate Kaplan Meier and Log-rank analysis of serologic and clinical factor associated with complicated disease course in B1 Crohn's disease patients (*n* = 143)

Variable	Internal and/or penetrating complication		penetrating stricturing complication		Perianal complication		penetrating CD-related abdominal surgery	
	HR (95%CI)	<i>P</i> _{LogRank-value}	HR (95%CI)	<i>P</i> _{LogRank-value}	HR (95%CI)	<i>P</i> _{LogRank-value}	HR (95%CI)	<i>P</i> _{LogRank-value}
Gender - male	1.10 (0.47-2.55)	0.83	2.69 (0.80-9.03)	0.11	0.91 (0.33-2.55)	0.86		
Smoking	3.72 (1.23-11.21) ¹	0.02 ¹	1.61 (0.36-7.19)	0.53	1.64 (0.45-5.97)	0.45		
Location (colon only)	0.41 (0.17-0.96) ¹	0.04 ¹	1.00 (0.29-3.43)	0.99	0.61 (0.22-1.74)	0.36		
Frequent relapse	1.10 (0.39-3.07)	0.85	7.29 (1.72-30.99) ¹	0.007 ¹	1.64 (0.44-6.10)	0.46		
APLA at all	1.36 (0.59-3.16)	0.47	1.30 (0.40-4.27)	0.66	1.37 (0.49-3.80)	0.55		
Anti-β2-GPI at all	0.56 (0.13-2.32)	0.42	0.99 (0.13-7.70)	0.99	0.33 (0.06-1.81)	0.20		
ACA at all	0.41 (0.13-1.26)	0.12	1.12 (0.23-5.49)	0.89	0.30 (0.07-1.27)	0.10		
Anti-PS/PT at all	1.07 (0.39-2.97)	0.89	1.74 (0.38-8.03)	0.48	1.11 (0.29-4.18)	0.88		

¹The *P*-value is < 0.05. APLA: Antiphospholipid antibodies; β2-GPI: Beta2-glycoprotein-I; ACA: Anti-cardiolipin antibody; PS/PT: Phosphatidylserine/prothrombin complex; HR: Hazard ratio; 95%CI: 95% confidence interval.

Table 6 Antiphospholipid antibodies, thrombophilia markers and clinical characteristics of total Crohn's disease according to presence and type of thrombosis, *n* (%)

Factor	Follow-up of CD Total Cohort from diagnosis (<i>n</i> = 265)				
	No thrombosis (<i>n</i> = 251)	Arterial thrombosis (<i>n</i> = 3)	Venous thrombosis (<i>n</i> = 11)	No pregnancy loss (<i>n</i> = 146)	Pregnancy loss (<i>n</i> = 10)
Male/female	104/147	1/2	7/4	146/0	10/0
Age at presentation (yr) ¹	25.0 (19.0-33.0)	40.0 (28.0-42.0)	29.5 (23.3-39.3)	26.0 (20.0-35.0)	25.5 (21.0-35.0)
Frequent relapse	48 (20.2)	0 (0.0)	4 (36.4)	33 (24.1)	2 (20.0)
Previous thrombosis	1 (0.4%) ³	0 (0.0)	4 (36.4) ³	2 (1.4)	0 (0.0)
Smoking habits					
yes	48 (19.1)	0 (0.0)	3 (27.3)	22 (15.1)	2 (20.0)
Follow up time from diagnosis, mo ¹	102.2 (63.3-172.8) ²	149.9 (130.8-219.8)	186.3 (142.0-244.2) ²	109.0 (61.8-184.6)	136.5 (95.4-180.6)
Positive markers (%)					
Anti-β2-GPI IgG and/or IgM	4.8	0.0	9.1	4.9	10.0
Anti-β2-GPI IgA	2.8	0.0	0.0	4.9	0.0
ACA IgG and/or IgM	12.1	0.0	27.3	13.9	10.0
ACA IgA	19.0	66.7	0.0	20.1	10.0
Anti-PS/PT IgG and/or IgM	14.6	50.0	36.4	18.3	40.0
Anti-PS/PT IgA	8.9	50.0	9.1	7.7	10.0
At least 1 APLA pos	48.0	66.7	54.5	52.8	60.0
At least 2 APLA pos	16.1	33.3	9.1	18.1	10.0
At least 3 APLA pos	3.2	0.0	9.1	4.9	0.0
Thrombophilia markers ³ (%)					
LA	7.5	0.0	0.0	5.1	0.0
PS deficiency (inherited and/or acquired)	8.1	0.0	25.0	12.7	0.0

ATIII (inherited acquired)	deficiency and/or	0.0	0.0	0.0	0.0	0.0
PC deficiency (inherited acquired)	and/or	3.0	0.0	0.0	4.8	0.0
FV Leiden		5.9 ⁴	0.0	42.9 ⁴	7.8	0.0
FII20210A		5.9	0.0	16.7	7.8	0.0

¹Median (IQR). ²Using Kruskal-Wallis test ($P \leq 0.01$). ³Using χ^2 -test with Yates correction ($P \leq 0.05$). ⁴Serologic and genetic markers of thrombophilia were available in 105 patients. pts: Patients; APLA: Antiphospholipid antibodies; β 2-GPI: Beta2-glycoprotein-I; ACA: Anti-cardiolipin antibody; PS/PT: Phosphatidylserine/prothrombin complex.

Table 7 Stability of antiphospholipid antibodies marker status over time in inflammatory bowel diseases patients with at least 2 samples during the disease course, *n* (%)

APLA marker	CD (<i>n</i> = 198)	UC (<i>n</i> = 103)
Anti-β2-GPI IgG		
Stable negative	194 (98.0)	97 (94.2)
Stable positive	1 (0.5)	2 (1.9)
Neg. to pos.	0 (0.0)	3 (2.9)
Pos. to neg.	3 (1.5)	1 (1.0)
Anti-β2-GPI IgM		
Stable negative	186 (94.0)	94 (91.3)
Stable positive	4 (2.0)	3 (2.9)
Neg. to pos.	6 (3.0)	5 (4.9)
Pos. to neg.	2 (1.0)	1 (0.9)
Anti-β2-GPI IgA		
Stable negative	190 (96.0)	93 (90.3)
Stable positive	1 (0.5)	4 (3.9)
Neg. to pos.	3 (1.5)	5 (4.9)
Pos. to neg.	4 (2.0)	1 (0.9)
ACA IgG		
Stable negative	139 (70.2)	95 (92.2)
Stable positive	3 (1.5)	1 (1.0)
Neg. to pos.	42 (21.2)	6 (5.8)
Pos. to neg.	14 (7.1)	1 (1.0)
ACA IgM		
Stable negative	173 (87.4)	95 (92.2)
Stable positive	3 (1.5)	1 (1.0)
Neg. to pos.	18 (9.1)	7 (6.8)
Pos. to neg.	4 (2.0)	0 (0.0)
ACA IgA		
Stable negative	73 (36.9)	78 (75.7)
Stable positive	13 (6.6)	0 (0.0)
Neg. to pos.	93 (46.9)	24 (23.3)
Pos. to neg.	19 (9.6)	1 (1.0)

Anti-PS/PT measurements were only performed on the first available serum samples of the patients. APLA: Antiphospholipid antibodies; β 2-GPI: Beta2-glycoprotein-I; ACA: Anti-cardiolipin antibody; PS/PT: Phosphatidylserine/prothrombin complex; CD: Crohn's disease; UC: Ulcerative colitis.