

Phosphatidylserine-dependent anti-prothrombin antibodies (aPS/PT) in infliximab-treated patients with inflammatory bowel diseases

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Abstract

Purpose To (1) examine the occurrence and concentrations of aPS/PT and aPL in inflammatory bowel disease (IBD) patients at the beginning of and during anti-TNF- α therapy with infliximab; (2) investigate the link of the aPS/PT and aPL presence with antibodies to infliximab (ATI) formation; and (3) examine possible clinical consequences of aPS/PT and/or aPL positivity in IBD patients.

Materials and methods Thirty (30) IBD patients treated with infliximab were analyzed regarding aPS/PT, aPL, and ATI antibody serum levels by standardized ELISAs at treatment weeks 2 (W2) and 14 (W14).

Results At W2, 40 % of infliximab-treated patients had elevated aPS/PT and 16.7 % had elevated aPL serum levels. At W14, the proportion of aPS/PT-positive sera decreased to 16.6 %, whereas aPL distribution remained unchanged. Moreover, concentrations of aPS/PT have shown significant differences at W2 (16.64 [10.06; 33.06] U for IgG and 18.46 [9.18; 32.48] U for IgM) and at W14 (8.24 [2.78; 19.82] U for IgG and 8.57 [5.55; 26.82] U for IgM), $p = 0.009$ and $p = 0.003$, respectively. In ATI-positive samples, aPS/PT IgG were more frequent ($p = 0.001$ for W2 and $p = 0.003$ for W14), whereas aPS/PT IgM and aPL IgG/IgM did not show such association.

Conclusions Higher concentrations of aPS/PT IgG and IgM were found in IBD patients at the beginning of the biological treatment period compared to the maintenance treatment period. Moreover, aPS/PT IgG were more frequent in ATI-positive individuals, which was not observed in aPL. We speculate that there is a relationship between the aPS/PT and the severity of inflammation and auto-aggressive processes in IBD.

Keywords Inflammatory bowel diseases · Crohn's disease · Ulcerative colitis · Phosphatidylserine-dependent antiprothrombin antibodies · Antiphospholipid antibodies · Antibodies to infliximab

Abbreviations

ANA	Antinuclear antibodies
anti-dsDNA	Anti-double-stranded DNA antibodies
aPL	Antiphospholipid antibodies
aPS/PT	Phosphatidylserine-dependent anti-prothrombin antibodies
ATI	Antibodies to infliximab
CD	Crohn's disease
IBD	Inflammatory bowel disease
IFX	Infliximab
TNF α	Tumor-necrosis factor alpha
UC	Ulcerative colitis
W2	2nd week of the treatment
W14	14th week of the treatment

Introduction

Inflammatory bowel disease (IBD) is of a group of gastrointestinal inflammatory diseases with two major types: ulcerative colitis (UC) and Crohn's disease (CD). Tumor

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necrosis factor alpha (TNF α) plays an important role in the pathogenesis of IBD. The use of therapeutic TNF α inhibitors has brought about great progress, especially in the treatment of patients with high and persistent IBD activity, complicated disease behavior, and unresponsiveness to other immunomodulatory treatments [1]. Several therapeutic anti-TNF α monoclonal antibodies are currently used in IBD treatment; the most frequently used is infliximab, which is a chimeric monoclonal antibody against TNF α administered as an intravenous infusion.

In spite of the great advantages of infliximab in IBD treatment, the risk of the development of autoimmune processes related to infliximab administration must be mentioned, ranging from asymptomatic laboratory alterations to life-threatening systemic autoimmune diseases [2, 3]. It is known that infliximab may stimulate the production of different autoantibodies such as antinuclear antibodies (ANA), anti-double-stranded DNA antibodies (anti-dsDNA), antiphospholipid antibodies (aPL), and antibodies to infliximab (ATI). Various workgroups have shown that infliximab led to the development of ANA in 29–77 %, anti-dsDNA antibodies in 10–29 %, aPL in 21–27 %, and ATI in 7–19 % of patients treated with infliximab [4, 5].

Patients with IBD are at increased risk for thrombotic complications, which are threefold more likely in IBD patients than controls, and the relative risk exceeds 15-fold during disease flare-ups [6–9]. Generally, an inflammatory systemic state can lead to endothelial dysfunction, secondary dyslipidemia, and activation of coagulation [10, 11]. IBD patients could be at higher risk of vascular thrombosis as well through induction of thrombophilic autoantibodies, especially aPL.

We have focused on the antibodies against phosphatidylserine/prothrombin complexes (antiphosphatidylserine-dependent antiprothrombin antibodies, aPS/PT), which are a subgroup of antiphospholipid antibodies and may be associated with vascular thrombosis and positivity of lupus anticoagulant phenomena [12–15]. Since there are scanty published data concerning prothrombotic variables in active IBD patients and only a few published studies analyzing such patients treated with TNF α antagonists, we have examined (1) the incidence of aPS/PT in IBD patients with active disease; (2) the behavior of aPS/PT development during anti-inflammatory treatment with the anti-TNF α blocker infliximab; and (3) the clinical consequences of pertinent aPS/PT positivity with thrombotic events in examined IBD patients.

Materials and methods

We have conducted a study of aPS/PT, aPL, and ATI serum levels in 30 randomly selected IBD patients treated with infliximab.

Patients

The cohort of patients comprised 15 females and 15 males aged 21–63 (median 33 years) previously naïve to anti-TNF α therapy and treated with infliximab. Eighteen (18) of the participants suffered from Crohn's disease, and 12 had ulcerative colitis with moderate to severe activity.

Blood samples were collected during the induction therapy at week 2 (W2, after the first and before the second infliximab infusion) and during the maintenance treatment at week 14 (W14). Blood samples were taken from the cubital vein. Clotted blood samples were centrifuged for 10 min at ambient temperature; 1,300g and separated serum aliquots were frozen at -80°C and placed in the IBD serum bank. The frozen serum samples were thawed once on ice before analysis.

Interventions

IBD patients enrolled in the study were treated according to standard clinical practice as a scheduled strategy. Infliximab treatment using a dose of 5 mg/kg of body weight was started in the induction phase, using three intravenous infusions in weeks 0, 2, and 6. After that, if a response was achieved, maintenance therapy was continued every other month. A standard assessment of disease activity before and after the induction period was done, including clinical markers and laboratory examinations.

Immunosuppressants such as azathioprine or 6-mercaptopurine were taken by 11 of the 30 patients (37 %); additionally, corticosteroids were taken by 16 of the 30 patients (53 %) and mesalazine by 21 of the 30 patients (70 %).

Ethical aspects

The study was approved by the Institutional Ethical Committee. The purpose and procedures of the study were explained to participants, who signed informed consent forms.

Laboratory evaluation

Serum aPS/PT, aPL, ATI levels and fecal calprotectin were measured by standardized ELISAs. Serum C-reactive protein (CRP) was detected by immunonephelometry.

aPS/PT IgG and IgM were detected by QUANTA Lite[®] aPS/PT IgG and QUANTA Lite[®] aPS/PT IgM (INOVA Diagnostic Inc., San Diego, USA) by the sandwich ELISA technique. Briefly, sera were pipetted to the plastic micro-well plate wells coated with purified PS/PT complex. Upon incubation, unbound protein was removed by washing, and anti-human IgG or IgM horseradish peroxidase (HRP)

labeled conjugate was added to the wells. After further incubation and washing, a peroxidase substrate was added and the enzymatic production was stopped. The presence or absence of aPS/PT antibodies was determined spectrophotometrically at 450 nm using a MRXII (Dynatech, UK) photometer and analyzed using the software Revelation (Dynatech, UK). The reference range 0–30 Units was used per the manufacturer's recommendations.

Detection of aPL was accomplished by anti-phospholipid screen IgG/IgM (Orgentec, Mainz, Germany). Serum samples with elevated values ≥ 10 GPLU/MPLU were further investigated for IgG and IgM class autoantibodies against beta-2-glycoprotein I, cardiolipin, phosphatidylserine, phosphatidylinositol, and phosphatidic acid using standardized enzyme-linked immunosorbent assay (ThromboCombo, Orgentec, Mainz, Germany).

ATI were detected by enzyme-linked immunosorbent assay with the use of antibody to Infliximab Q-ATI ELISA Quantitative Analyses (Matriks Biotek, Ankara, Turkey). Q-ATI is a sandwich assay for the determination of antibodies against infliximab in serum and plasma samples. The reference range 0–8 ng/mL was used based on our own lab reference ranges using data from our own equipment and donors' sera.

Systemic inflammation was assessed by CRP serum levels (Dade Behring High Sensitivity CRP, Siemens Medical Solutions Diagnostics, Erlangen, Germany). Local inflammation of the intestinal mucosa was assessed with the help of fecal calprotectin measurement (EK-CAL ELISA Bühlmann, Schönenbuch, Switzerland).

Statistical analyses

Statistical analysis was performed using the software Statistica CZ 10.0 (StatSoft Inc, Tulsa, USA). Different groups were compared using the Mann–Whitney *U* test or a two-sided Kruskal–Wallis non-parametric test. The Spearman Rank Correlation Test was used to identify correlations between variables. The threshold for significance was set at $p < 0.05$.

Results

Basic descriptive characteristics of examined cohorts are presented in Table 1.

At the beginning of the infliximab treatment at W2, 38.7 % of infliximab-treated patients had elevated aPS/PT. Three (3) aPS/PT positivities were found simultaneously in IgG and IgM isotypes; nine (9) patients had solitary aPS/PT IgG.

W2 was characterized with weak to moderate aPL positivity in 16.1 % of patients. In one (1) case aPL IgG

Table 1 Descriptive characteristics of examined cohorts

Gender <i>n</i> (%)	
F	15/30 (50 %)
M	15/30 (50 %)
Age, median (IQR)	33 (29; 44)
Diagnosis <i>n</i> (%)	
CD	18/30 (60 %)
L1	8/18 (44 %)
L2	2/18 (12 %)
L3	8/18 (44 %)
B1	6/18 (33 %)
B2	7/18 (39 %)
B2 + 3	4/18 (22 %)
B3	1/18 (6 %)
UC	12/30 (40 %)
E2	4/12 (33 %)
E3	8/12 (67 %)
Concomitant treatment <i>n</i> (%)	
Immunosuppressants	11/30 (37 %)
Corticosteroids	16/30 (53 %)
Mesalazine	21/30 (70 %)
Response to the IFX treatment <i>n</i> (%)	
Responders	20/30 (67 %)
Responders with adverse events	6/30 (20 %)
Primary non-responders	1/30 (3 %)
Secondary non-responders	3/30 (10 %)

F females, *M* males, *IQR* interquartile range, *IFX* infliximab, *CD* Crohn's disease, *L1* ileal location of CD, *L2* colonic location of CD, *L3* ileo-colonic location of CD, *B1* non-stricturing non-penetrating behavior of CD, *B2* stricturing behavior of CD, *B3* penetrating behavior of CD [16], *UC* ulcerative colitis, *E2* left-sided UC, *E3* extensive UC [16]

were detected and in four (4) patients aPL IgM were found; none of these individuals has shown double IgG and IgM positivity.

At W14, proportion of aPS/PT-positive sera decreased approximately by half to 16 %, whereas aPL distribution remained unchanged, see Table 2.

Moreover, concentrations of aPS/PT have shown significant differences at W2 compared to W14 for both isotypes examined, see Fig. 1.

Interestingly, in both W2 and W14 cohorts, simultaneous aPS/PT and aPL positivity was not found.

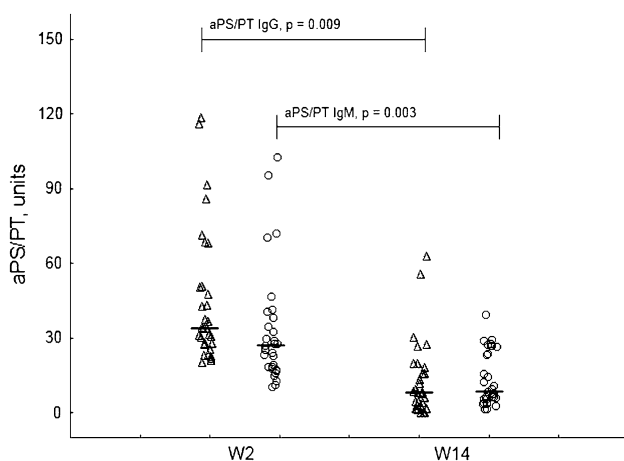
ATI were found in 2/30 (6.7 %) patients after the first infliximab infusion at W2, and in 6/30 (20 %) patients during W14 of the maintenance treatment, see Table 2.

More frequent were aPS/PT IgG and their serum levels were significantly higher in ATI-positive samples (see Fig. 2a, b), whereas aPS/PT IgM and aPL IgG/IgM did not shown such association.

Table 2 aPS/PT IgG/IgM, aPL IgG/IgM, and ATI IgG during the anti-TNF α treatment of 30 IBD patients with infliximab

	W2 n (%)	W14 n (%)
aPS/PT IgG	9 (30 %)	3 (10 %)
aPS/PT IgM	0	0
aPS/PT IgG and IgM	3 (10 %)	2 (6.7 %)
aPL IgG	1 (3.3 %)	1 (3.3 %)
aPL IgM	4 (13.3 %)	4 (13.3 %)
aPL IgG and IgM	0	0
ATI IgG	2 (6.7 %)	6 (20 %)

aPS/PT phosphatidylserine-dependent anti-prothrombin antibodies, *aPL* antiphospholipid antibodies, *ATI* antibodies to infliximab, *W2* second week of the biological treatment, *W14* 14th week of the biological treatment

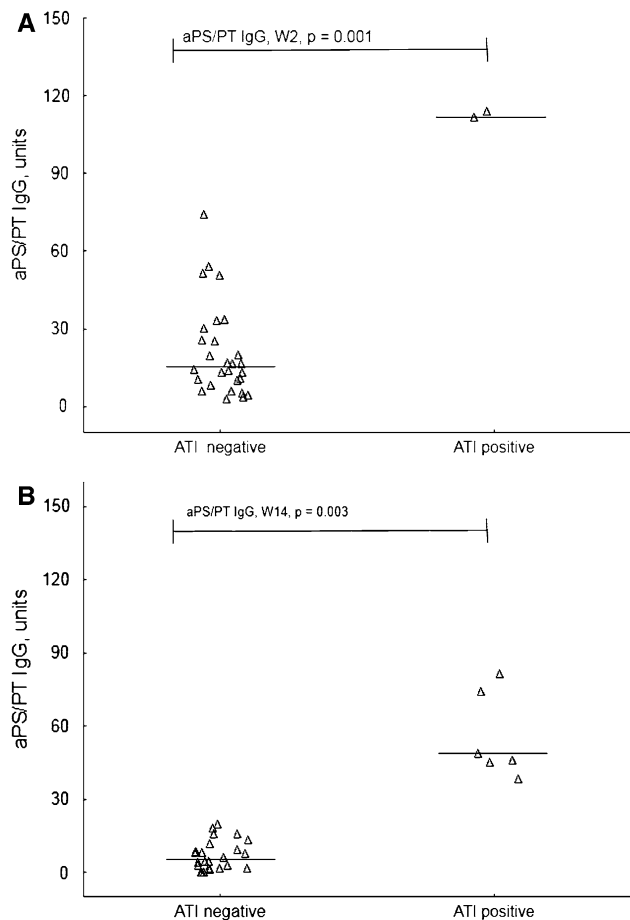
**Fig. 1** Development of aPS/PT serum concentrations during IBD treatment with infliximab. *aPS/PT* phosphatidylserine-dependent antiprothrombin antibodies, *W2* second week of the biological treatment, *W14* 14th week of the biological treatment

None of the autoantibodies monitored have shown dependence on systemic inflammation measured by serum CRP or on local intestinal inflammation measured by fecal calprotectin.

Analyses did not identify age, gender, diagnosis (CN or UC), or concomitant immunosuppressive treatment as a risk factor for developing aPS/PT.

None of the aPS/PT and/or aPL-positive IBD patients experienced clinical symptoms of antiphospholipid syndrome such as vascular thrombosis or miscarriage either before or during the biological treatment of IBD with infliximab.

In summary, aPS/PT concentrations have shown descending tendency during the biological treatment of IBD with infliximab and displayed different behavior compared to aPL and ATI; aPS/PT IgG were significantly more frequent in patients with ATI positivity.

**Fig. 2** aPS/PT antibodies in ATI negative and ATI-positive sera. **a** W2 **b** W14. *aPS/PT* phosphatidylserine-dependent antiprothrombin antibodies, *ATI* antibodies to infliximab, *W2* second week of the biological treatment, *W14* 14th week of the biological treatment

Discussion

To our knowledge, this is the first report arising from the investigation of serum aPS/PT antibodies in IBD patients during immunomodulatory treatment with anti-TNF α blocker. It shows a relatively high incidence of aPS/PT (40 %) in patients with active disease where biological treatment was needed and consequent decreasing of aPS/PT levels due to the anti-inflammatory effect of infliximab.

This behavior is different from “classic” aPL and to other organ non-specific autoantibodies observed in numerous studies [2–5, 17, 18]. It is known that anti-TNF α therapy leads to a different autoantibody formation. Occurrence and concentrations of autoantibodies increase during the biological treatment, but these autoantibodies can present without autoimmune clinical manifestations [5]. Induction of different autoantibodies by infliximab could be explained by the fact that modulation of humoral immunity may be a more general feature of infliximab

treatment, and this phenomenon is probably not disease specific.

In aPS/PT, a different tendency was found: more serious clinical condition was connected with higher and more frequent aPS/PT antibodies, whereas anti-TNF α treatment was followed by their significant decrease. Based on this observation we hypothesize that aPS/PT are independent autoantibodies different from “classic” aPL, and their roles in immunopathological cascades could be different. The mechanisms by which aPS/PT take part in inflammatory processes remain unclear. Nojima et al. [19, 20] has speculated that aPS/PT antibodies inhibit thrombin-mediated endothelial cell prostacyclin release, inhibit protein C activation, activate endothelial cells, induce pro-coagulant substances via prothrombin, and enhance platelet activation. In other words, aPS/PT can take part in the extensive cross-communication between inflammatory activity and coagulation, and their positivity could be, in this point of view, simply a consequence of sustaining inflammation.

Surprisingly, we have even more aPS/PT IgG positive samples in the ATI-positive group of patients. Immunogenicity of infliximab belongs to the emerging issues connected with the treatment with this anti-TNF α blocker. Formation of ATI can lead to limited treatment efficacy and, moreover, ATI may induce acute and/or late post-infusion reactions [21, 22]. ATI formation is another manifestation of the activity of immunopathological cascades. Predominance of aPS/PT IgG positivity in ATI-positive samples could be another evidence of assuming aPS/PT as an epiphenomenon of the inflammatory processes.

Our aPS/PT-positive patients did not experience clinically verified vascular thrombosis or other clinical signs of antiphospholipid syndrome. We realize that positivity of autoantibodies can be asymptomatic for a long time, and in definite circumstances, the clinical manifestation of the disease can occur. Our aPS/PT-positive IBD patients should be considered as at higher risk in the future, especially in the case of acute IBD flare-ups. Moreover, microvascular injury with fewer dramatic clinical signs must be considered in sustaining inflammation [23].

The current study has some limitations. Firstly, this was a retrospective study conducted on serum samples from the IBD blood bank. Therefore, we have not examined lupus anticoagulant in our cohort. It is known that aPS/PT are strongly correlated with the presence of lupus anticoagulant activity [24], and phospholipid-dependent clotting tests could be beneficial for the thrombosis risk assessment. Moreover, no data about antinuclear, anti-dsDNA and other organ non-specific autoantibodies in our cohorts are available, even though they could support or disprove proposed theory about aPS/PT as an epiphenomenon of the inflammatory processes. Future research could further test

the adequacy of this hypothesis. Thirdly, this is a pilot study, so that the sample size and the restriction of the data to one medical center only limit the generalisability of the results. Our findings are associative and cause and effect cannot be determined. However, the results have biological plausibility, and provide a theory about the differences in aPS/PT and aPL behavior during the biological treatment with anti-TNF α agents.

Conclusions

A high incidence of aPS/PT was confirmed in IBD patients with active disease, with successive decreasing of autoantibody concentrations during disease healing. Unlike aPL and other autoantibodies, aPS/PT are not induced by infliximab. Thus, aPS/PT could be considered as an epiphenomenon of inflammation with proposed links between immunopathological inflammatory cascades and coagulation.

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Conflict of interest Authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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