

Rivaroxaban limits complement activation compared to warfarin in antiphospholipid syndrome patients with venous thromboembolism

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Essentials

Complement activation has a pathogenic role in thrombotic antiphospholipid syndrome (APS)

Coagulation proteases such as factor Xa can activate complement proteins

Complement activation markers were elevated in anticoagulated thrombotic APS patients

Complement activation decreased in APS patients switching from warfarin to rivaroxaban

Abstract

Background

Complement activation may play a major role in the pathogenesis of thrombotic antiphospholipid syndrome (APS). Coagulation proteases such as factor Xa can activate complement proteins.

Aims

To establish whether rivaroxaban, a direct factor Xa inhibitor, limits complement activation compared to warfarin in APS patients with previous venous thromboembolism (VTE).

Methods

111 APS patients with previous VTE, on warfarin target INR 2.5, had blood samples taken at baseline and at day 42 after randomisation in the RAPS (Rivaroxaban in Antiphospholipid Syndrome) trial. Fifty-six patients remained on warfarin and 55 switched to rivaroxaban. Fifty-five normal controls (NC) were also studied. Markers of complement activation (C3a, C5a, terminal complement complex (SC5b-9) and Bb fragment), were assessed.

Results

APS patients had significantly higher complement activation markers compared to NC at both time points irrespective of the anticoagulant. There were no differences between the two patient groups at baseline, or patients remaining on warfarin at day 42. In 55 patients randomised to rivaroxaban, C3a, C5a and SC5b-9 were significantly lower at day 42; median (ng/mL) [confidence interval] 64 [29-125] vs 83 [35-147], 9 [2-15] vs 12 [4 -18] and 171 [56-

245] vs 201 [66-350] respectively, but levels of Bb were unchanged. There were no correlations between rivaroxaban levels and complement activation markers.

Conclusions

APS patients with previous VTE on warfarin exhibit significantly increased complement activation, which is likely to occur via the classical pathway, and is decreased by rivaroxaban administration. Rivaroxaban may therefore potentially provide benefit additional to its anticoagulant effect in this patient group by limiting complement activation.

Introduction

The complement system is an important part of the innate immune system, forming an important link with adaptive immune responses [1], and interacts directly with the haemostatic system [2;3]. It is activated via the classical, alternative and lectin pathways (CP, AP and LP respectively) [4], which converge at the level of C3. The CP is triggered by antigen-antibody complexes, and the LP by sugars recognised primarily by mannose binding lectin. Following activation of several proteases, a C3 convertase proteolyzes C3 into C3a and C3b, the latter forming a C5 convertase. In the AP, C3 is hydrolysed spontaneously and then binds factor B (FB), which is cleaved by factor D to produce fragment Bb and a C3 convertase, with the liberation of fragment C3a [5]. The AP can also be potentiated by plasma kallikrein, which cleaves FB. C3b acts as the central effector molecule in C5 convertase, with cleavage of C5, producing C5a and C5b. The terminal complement complex (TCC, SC5b-9) is generated by the assembly of C5b through C9 and forms the membrane attack complex, mediating the irreversible target cell membrane damage associated with complement activation. The anaphylatoxin C3a has a half-life of several hours, but in serum is cleaved rapidly to the more stable C3a-desArg, which provides a marker of complement activation [6]. C5a and SC5b-9 levels are markers of terminal complement pathway activation [7-9]. FB is unique to the alternative complement pathway, hence Bb fragment is a marker of AP activation [10].

Disorders of complement activation or dysregulation can result in excessive complement generation and thrombosis, such as in paroxysmal nocturnal haemoglobinuria (PNH) [11], atypical haemolytic uremic syndrome (aHUS) [12], thrombotic thrombocytopenic purpura (TTP) [13] and antiphospholipid syndrome (APS) [14;15].

Clinical studies and murine models suggest involvement of the complement system in thrombotic APS. APS patients exhibit low serum complement C3 and C4 levels, with C3a, C3a-desArg and C4a significantly higher than in healthy controls, suggesting that APS-associated hypocomplementemia is due to complement activation rather than complement deficiency [14;16]. The potent inflammatory mediator C5a is implicated in APS, and complement inhibition may ameliorate thrombosis induced by antiphospholipid antibodies (aPL) [17;18]. Mice treated with IgG from APS patients with high levels of aPL, subjected to a femoral vein pinch model of thrombosis, developed larger thrombi and higher TF activity than controls [19]. The co-administration of rEV576 (coversin), a recombinant protein inhibitor of C5 activation, resulted in significantly smaller thrombi and reduced tissue factor (TF) activity [19]. Furthermore, complement activation is a central mechanism in murine aPL-induced pregnancy loss and intrauterine fetal growth restriction [20;21]. Widespread complement activation may also play a role in catastrophic APS (CAPS) (22-24) and anecdotal case reports suggest possible efficacy of eculizumab (Soliris), a humanised monoclonal antibody that inhibits C5 [25;26].

Limited *in vitro* data suggest that thrombin, as well as coagulation factors (F) XIa, Xa, IXa, and plasmin, can activate C5 to C5a in the absence of C3 [2]. Complement activation can amplify coagulation and inhibit fibrinolysis, through C5a, which induces expression of TF and plasminogen activator inhibitor 1 (PAI-1) [2]. The anti-complement activation effect of heparin, first described in 1929 by Ecker and Gross [27], may be through its inhibitory effects on thrombin and FXa, which are known to cause activation of C3 and C5 producing C3a, C5a and subsequently SC5b-9.

We hypothesised that rivaroxaban, as a direct FXa inhibitor has the potential to inhibit FXa induced complement activation of C5 to C5a. The aim of this study was to establish, in

patients with APS and previous venous thromboembolism (VTE), whether rivaroxaban administration limits complement activation compared to warfarin, the current mainstay of anticoagulation in this patient group.

Patients and methods

Patients, normal controls and blood sampling

Venous blood samples were collected, as part of translational research, from patients recruited to the RAPS (Rivaroxaban in Antiphospholipid Syndrome; [<http://isrctn.org/ISRCTN68222801>] prospective randomised controlled trial (RCT) of rivaroxaban versus warfarin in patients with thrombotic APS, with or without systemic lupus erythematosus (SLE) [28]. The patients comprised those with previous venous thromboembolism (VTE), on long-term anticoagulation with warfarin, target INR 2.5 (range 2.0-3.0), who were eligible for RAPS and fulfilled the revised international consensus criteria for definite APS [29]. All patients were on warfarin for at least 3 months following a VTE prior to entry into the study and had no new thrombotic events during the trial treatment period. The current study was approved by the Research Ethics Committee and the Research and Development Office at University College London Hospitals (UCLH) NHS Trust (Reference number: 13/EM/0150). All patients gave informed, written consent for the translational research (Reference number: 12/SC/0566). Patients were randomised to remain on warfarin target INR 2.5 or to switch to rivaroxaban 20 mg once daily.

Venous samples were taken into 4mL Vacutainer K2E tubes containing 7.2mg EDTA (Vacutainer K2E; Becton Dickinson, Oxford), or 0.105M citrate (Vacutainer 9NC), using a 21 gauge butterfly needle with minimal stasis, at baseline (day 0, on warfarin) and day 42

(range 30-56 days after randomisation). In patients randomised to receive rivaroxaban, blood samples were taken at 2-4 hours following the last dose of rivaroxaban. Samples were also collected from 55 normal controls (NC), who were members of staff, previously shown to be aPL negative. Individuals who were pregnant or receiving oestrogen-containing preparations were excluded from the study as there can be increased levels or activation of complement in these situations [30;31]. EDTA (complement activation markers) and citrate (all haemostasis tests) plasmas were prepared within 1 hour of collection by double centrifugation at 2000g for 15 minutes and stored at -80°C. Immediately prior to analysis, the samples were thawed at 37°C. Of the total 116 patients (57 rivaroxaban and 59 warfarin), including 22 patients with SLE, recruited to the RAPS trial and available for study, four patients were excluded due to plasma haemolysis and one because there was no day 42 sample leaving 111 patients (55 on rivaroxaban and 56 on warfarin) with 11 SLE patients in each group.

Laboratory assays

Antiphospholipid antibodies

All aPL assays were performed at baseline. Lupus anticoagulant (LA) was assessed by Dilute Russell's viper venom time (DRVVT) using LA1/LA2 screen/confirm reagents (Siemens Healthcare) in equal volume mixtures with pooled normal plasma, to correct for warfarin-induced coagulation factor deficiencies, in accordance with international and national guidelines [32;33]; and the Taipan venom time/Ecarin clotting time ratio (TVT/ECT), Diagnostic Reagents Ltd, Thame, UK), which shows little impact of warfarin.

IgG and IgM anticardiolipin (aCL) and anti- β 2 glycoprotein I (a β 2GPI) antibodies were assessed by ELISA (Quanta Lite, Inova, Werfen). Moderate/high aCL positivity was defined as >20 GPLU/MPLU, and a β 2GPI positivity as >20 SGU/SMU.

Complement assays

Complement activation markers were measured as C3a-desArg (C3a), C5a-des-Arg (C5a), SC5b-9 and Bb fragment (Bb) using commercial enzyme-linked immunosorbent assay (ELISA) (MicroVue kits, Quidel Corp, from Pathway Diagnostics Ltd, Dorking, UK). Samples from each patient were tested on the same ELISA plate. The intra- and inter-assay coefficient of variation (CV) was as follows: C3a 1.8% and 3.4%; C5a 1.9% and 2.4%; SC5b-9 2.0% and 2.6%; Bb 1.9% and 2.7%, respectively.

International Normalised Ratio and rivaroxaban levels

International Normalised Ratio (INR) values were measured using PT Fib HS Plus on a TOP500 (Werfen, Warrington, UK), using an analyser specific ISI in the two groups of patients on warfarin. Rivaroxaban levels were measured in 55 patients receiving rivaroxaban on day 42 using an amidolytic anti-Xa assay (Biophen DiXal; Hyphen BioMed, Neuville-Sur-Oise, France).

Statistical analysis

There were no suitable data on which to base power calculations; this was a pilot study based on the samples available from the RAPS trial. Data analysis was performed using MiniTab 16 (Minitab Ltd, Coventry, UK) and GraphPad Prism[®] version 6 (GraphPad Software, Inc. La Jolla, USA). Results were reported as median or mean based on the distribution of

results with 95% confidence interval (CI). Multiple group comparisons were performed using Kruskal-Wallis ANOVA and paired comparisons using Mann-Whitney 'U' test after adjusting the significant p-values by Bonferroni correction. Examination of correlations was performed using the Spearman rank correlation. Normal ranges of complement activation markers were derived as mean \pm 2SD, after log transformation of data where necessary. A p value of <0.05 was considered significant.

Results

INR and rivaroxaban levels

INR at baseline: (median [CI]) 2.7 [2.5 to 3.0] and 2.6 [2.4-2.9] for the two groups and 2.8 [2.4 to 2.9] in those remaining on warfarin at day 42. In patients randomised to rivaroxaban, the rivaroxaban levels at day 42 varied between <50 and 461ng/mL: median [CI] 155 [139-184].

Antiphospholipid antibodies

Table 1 shows the baseline characteristics of the study subjects. 14/55 (25%) and 19/56 (34%) patients were triple aPL positive (presence of LA, aCL and a β 2GPI antibodies) in the rivaroxaban treated group and warfarin treated group, respectively. LA was present in 43/55 (78%) in the rivaroxaban treated group and 40/56 (71%) of the patients who remained on warfarin.

Complement activation markers

The normal reference ranges, as measured in the 55 NC, for C3a, C5a, SC5b-9 and Bb fragment were: 27-96 ng/mL, 0.8-13.0 ng/mL, 60-164 ng/mL and 0.7-1.7 µg/mL, respectively. APS patients had significantly higher median complement activation markers compared with NC at both baseline and day 42, irrespective of the anticoagulant ($p < 0.0001$ for C3a, C5a, SC5b-9 and Bb fragment; Fig. 1-4). At baseline (when all patients were on warfarin), there were no differences between the two patient groups for these markers and there were no differences at day 42 in those who remained on warfarin. C3a, C5a, SC5b-9 and Bb were elevated in 42 (38%), 44 (40%), 80 (72%) and 33 (30%) of the 111 patients, respectively.

Increased C3a, C5a, SC5b-9 and Bb fragment levels were not associated with the presence of triple positivity for aPL, or with the presence of LA. Furthermore, no significant differences were observed in the complement activation markers between patients with primary APS and those with SLE-associated APS on warfarin or rivaroxaban. Increased complement activation markers were not associated with higher or lower INR values. The 55 patients randomised to receive rivaroxaban displayed significantly lower median C3a, C5a and SC5b-9 on day 42 (levels on day 42, compared to baseline levels on warfarin (Fig. 1B-3B); median [CI] for C3a, C5a and SC5b-9 for rivaroxaban (day 42) versus warfarin (day 0): C3a (ng/mL) 64 (29-125) vs 83 (35-147), ($p=0.004$); C5a (ng/mL) 9 (2 - 15) vs 12.0 (4 -18), $p=0.02$; and SC5b-9 (ng/mL) 171 (56-245) vs 201 (66-350), ($p=0.002$). However, there were no significant differences in median levels of Bb fragment, between baseline and day 42 on rivaroxaban (Fig. 4B). Of the 55 patients randomised to receive rivaroxaban, 22 (40%), 25 (45%), 38 (69%) and 19 (35%) had elevated C3a, C5a, SC5b-9 and Bb, respectively at baseline. Of

these, all patients showed a decrease in C3a (mean decrease 24%) and C5a (mean decrease 32%), 35/38 in SC5b-9 (mean decrease 25%) and 14/19 in Bb (mean decrease 11%), at day 42 after switching to rivaroxaban (Fig. 5). Compared to the group remaining on warfarin, the decreases in values associated with rivaroxaban were significant for C3a ($p < 0.009$, OR 54.5, CI 2.9-1022.0), C5a ($p < 0.005$, OR 24.6, CI 1.3-470.0), and SC5b-9 ($p < 0.005$, OR 9.6, CI 2.6-36.3).

There were no significant correlations between rivaroxaban levels and C3a, C5a, SC5b-9, or Bb fragment levels and there were no significant associations between higher or lower rivaroxaban concentrations (top and bottom tertiles 190-461 and 50-138ng/ml, respectively) and elevated complement markers.

Discussion

This study demonstrates, in the context of the RAPS RCT, that APS patients with previous VTE treated with warfarin exhibit significantly increased complement activation compared with NC, in agreement with previous studies [14;16]. The mechanism of this and which pathway is involved is unclear (activation could occur through the classical, lectin, or alternative pathways), since C3a, C5a, SC5b-9 and Bb fragment were all elevated. The novel observation in this study is that this complement activation is significantly decreased by administration of rivaroxaban, a direct FXa inhibitor. Our data indicate that the limiting effect of rivaroxaban on complement activation is likely to occur via the classical pathway as patients treated with rivaroxaban had significantly lower anaphylatoxins (C3a and C5a) and terminal complement component (SC5b-9), with Bb fragment (a marker of alternative pathway activation) unchanged. We did not measure specific markers of the lectin pathway; however, preliminary evidence from Breen et al suggests a lack of association of the lectin

pathway and complement activation in patients with aPL [34]. There were no correlations between rivaroxaban levels and C3a, C5a, SC5b-9 and Bb fragment levels.

In this study, increased C3a, C5a, SC5b-9 and Bb fragment levels were not associated with the presence of triple positivity for aPL or LA and no significant differences in complement activation marker levels were observed between patients with primary APS and those with SLE-associated APS. Other studies have also reported significantly increased levels of complement activation markers in APS patients compared to NC [14;16] in APS patients. In a study of 186 patients with aPL (95 with previous thrombotic complications, 52 with obstetric APS and 39 with asymptomatic aPL), Breen et al [16] found significantly increased levels of fragment Bb and C3a compared to NC. Oku et al [14], studied 36 primary APS patients, 42 non-SLE connective tissue diseases, and 36 NC, and found that primary APS patients with low serum C3 or C4 had significantly higher levels of C3a or C4a than the control groups. Among patients with primary APS, hypocomplementemia was significantly more frequent in patients with LA, although no relationship was found with the clinical manifestations of APS. Devreese et al [35] also found increased levels of C3a in patients with aPL, but used citrate rather than EDTA plasma samples, which may result in higher levels of C3a and other markers due to *in vitro* complement activation. Overall, the clinical studies, including our observations and animal data support the hypothesis that complement activation may play a major role in the pathogenesis of APS [14-26].

The mechanism of rivaroxaban-induced inhibition of complement activation is unknown but may be related to its FXa inhibitory effect; either by direct inhibition of FXa proteolysis and activation of complement proteins, or indirectly by inhibition of pro-inflammatory effects of FXa with downstream complement activation. FXa mediated activation of the complement

system *in vitro* is characterised by physiological (2µg/mL) and supraphysiological FXa levels (20-1002µg/mL), whereas thrombin induced cleavage is observed mainly at supraphysiological thrombin levels [2]. However, these observations were made in the absence of a phospholipid surface and may not apply *in vivo*. FXa also activates protease activated receptor-1 (PAR-1) and PAR-2 receptors on the vascular endothelium, leading to upregulation of intracellular processes and inflammatory reactions [36-38]. Heparin which inhibits FXa and thrombin, has inhibitory effects on the production of proinflammatory cytokines from LPS-stimulated monocytes [39] and an anti-inflammatory effect during venous thrombosis [40], which could contribute to its inhibitory action on complement activation [41;42]. In a mouse model of sickle cell disease, dabigatran, a direct thrombin inhibitor, and rivaroxaban have been demonstrated to reduce both systemic and local inflammatory responses through inhibition of thrombin and FXa respectively [43]. However, translation of the results from mouse models to humans is limited because of differences in the coagulation and complement systems and also their regulation [44].

The differences in C3a, C5a and SC5b-9 levels, but not Bb fragment, between APS patients treated with rivaroxaban or warfarin could be due to variation in the degree of activation of the AP and CP. Although patients treated with rivaroxaban had significantly lower complement activation markers compared to patients treated with warfarin, both patient groups had complement activation marker levels significantly above those of the NC.

The lack of correlation observed between complement activation markers and rivaroxaban levels, or INR (in warfarin-treated patients), suggests that the effects on complement activation were not related to the intensity of anticoagulation.

In this study, complement activation was tested when peak rivaroxaban concentrations were expected, with maximal inhibition of factor Xa, however some patients had rivaroxaban levels lower than expected (<160 ng/mL) at 2-4 hours after the last rivaroxaban dose (based on population pharmacokinetics studies) [45]. Patients with lower rivaroxaban levels did not have significant differences in C3a, C5a and SC5b-9 levels compared to those with expected therapeutic (or higher) rivaroxaban levels, suggesting that patients treated with rivaroxaban have stable inhibition of complement activation. Furthermore, complement activation markers have short half lives (C5a ~1min, C3a a few hours, SC5b-9 50-60min) [12;46;47] therefore, complement activation is likely to involve a chronic, continuous mechanism. Since warfarin affects the post-translational maturation of FX, reducing plasma FX levels and decreasing its ability to bind phospholipid surfaces, it may not be able to abolish complement activation, whereas rivaroxaban being a small, high affinity, direct FXa inhibitor is able to be more effective.

Rivaroxaban, as well as being efficacious, safe and convenient, is fixed dose with predictable effect (so that routine anticoagulant monitoring is not required), and has fewer drug interactions than warfarin. Results of the RAPS trial (completed) and other clinical studies (ongoing) [48-50] will provide more robust information on the role of rivaroxaban and other DOACs in patients with thrombotic APS, and put into context anecdotal case reports and small case series of recurrent thrombosis after switching APS patients from warfarin to a DOAC [51-54]. Rivaroxaban significantly reduced complement activation compared to warfarin, with an absolute mean reduction in C5a of 32% and in SC5b-9 of 25%. Although complement activation was not restored to normal levels, it is biologically plausible that an inhibitory effect of rivaroxaban on complement activation may potentially provide benefit

additional or adjunctive to its anticoagulant effect in patients with APS. Such an effect of rivaroxaban in preventing complement activation might also have wider applicability in other thrombotic states associated with complement activation, such as PNH, where VTE is the main cause of mortality, accounting for 40–67% of PNH-related deaths [11]. When such patients require long-term anticoagulation, the use of rivaroxaban may be valuable in restricting overall complement activation in addition to its anticoagulant effect. The limitations of this study are that: complement activation markers were only assessed at a single time point in each patient and the degree of activation may vary with time; and we did not use assays that discriminate between classical and lectin pathway activation. Future, *in vitro* studies are required to confirm the route of complement activation in APS, the role of FXa and the mechanism of reduction of complement activation by FXa inhibitors.

In conclusion, we describe the novel observation that rivaroxaban limits complement activation in APS patients with previous VTE compared with warfarin. This action of rivaroxaban is likely to occur via the classical pathway as patients treated with rivaroxaban had significantly lower anaphylatoxins (C3a and C5a) and terminal complement component (SC5b-9), but Bb fragment (a marker of alternative pathway activation) was unchanged. The observations in rivaroxaban-treated patients may reflect inhibition of factor Xa cleavage of complement proteins, or inhibition of its pro-inflammatory effects (and consequent complement activation). Rivaroxaban may therefore potentially provide benefit additional to its anticoagulant effect in this patient group by limiting complement activation.

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Authorship

DRJ Arachchillage was involved in study concept and design, performance of assays, analysis and interpretation of data, and prepared the first draft of the manuscript. M Efthymiou and A Chitolie performed assays and revised the manuscript. IJ Mackie and SJ Machin were involved in interpretation of data and revised the manuscript. BJ Hunt, DA Isenberg and M Khamashta revised the manuscript. H Cohen was involved in study concept and design, obtaining funding, interpretation of data, and revision of the manuscript. All authors approved the final manuscript submitted.

Conflict of interest

H Cohen has received institutional research support from Bayer Healthcare Pharmaceuticals (Newbury, UK), with honoraria for participation in meetings and an Advisory Board diverted to a local charity. DRJ Arachchillage has received honoraria from Bayer Healthcare Pharmaceuticals for participation in an international meeting. Other authors have no relevant conflict of interest to declare.

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Table 1. Baseline characteristics and aPL status of study participants

Characteristics	Normal controls (N=55)	Rivaroxaban (N=55)	Warfarin (N=56)
Age (years) mean (SD)	41 (12)	47 (17)	50 (14)
Sex			
Male	19 (35)	15 (26)	17 (29)
Female	39 (65)	42 (72)	42 (71)
Number of SLE patients (%)	n/a	11 (19)	11 (19)
CrCL (mL/min) Mean (CI)	Not done	92.1 (85.2 to 99.6)	95.4 (87.8 to 103.7)
ALT (IU/L)	Not done	21.2 (18.6 to 24.1)	19.6 (17.4 to 22.2)
Median INR (CI)			
Baseline	n/a	2.6 (2.3-2.9)	2.7 (2.4-3.0)
Day 42	n/a	n/a	2.6 (2.3-2.8)
Rivaroxaban level (ng/mL) Median (CI)	n/a	155 (139-184)	n/a
APS- defining aPL (Miyakis categories):			
Category I	n/a	23/55	32/56
Category II :IIa,		29/55	21/56
IIb		2/55	3/56
IIC		1/55	0/56

SD = standard deviation; CI = confidence interval; SLE = systemic lupus erythematosus; INR = international normalized ratio; n/a = not applicable; aPL = antiphospholipid antibodies.

The Miyakis (29) antiphospholipid syndrome categories were as follows: I: the presence of more than one aPL in any combination; IIa: presence of lupus anticoagulant alone; IIb: anticardiolipin antibody present alone; IIc: anti- β_2 glycoprotein-I antibody present alone.

Figure 1

Comparison of C3a levels in patients randomised to remain on warfarin (A) or to switch to rivaroxaban (B), at day 42 compared to their baseline values and with normal controls.

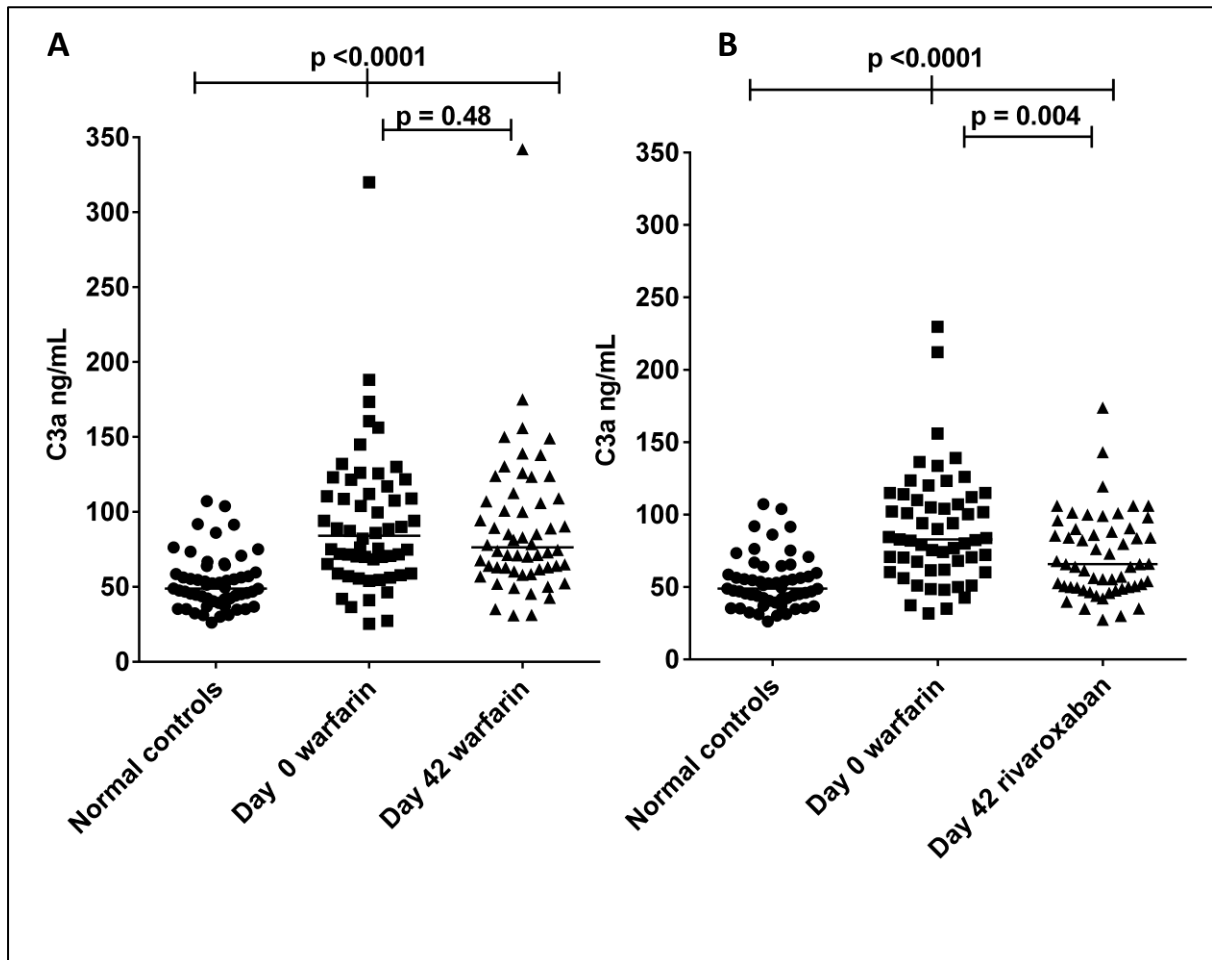


Figure 2

Comparison of C5a levels in patients randomised to remain on warfarin (A) or to switch to rivaroxaban (B), at day 42 compared to their baseline values and with normal controls.

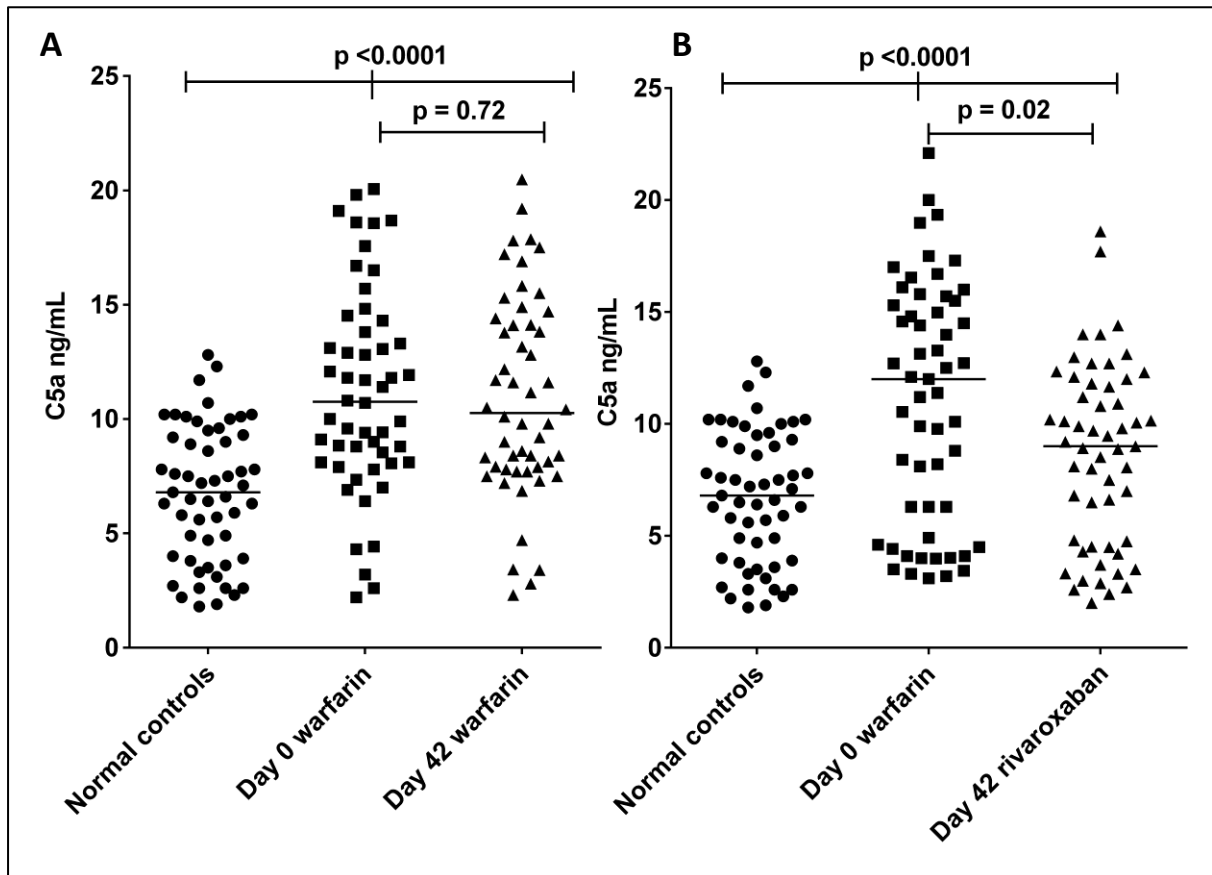


Figure 3

Comparison of SC5b-9 levels in patients randomised to remain on warfarin (A) or to switch to rivaroxaban (B), at day 42 compared to their baseline values and with normal controls.

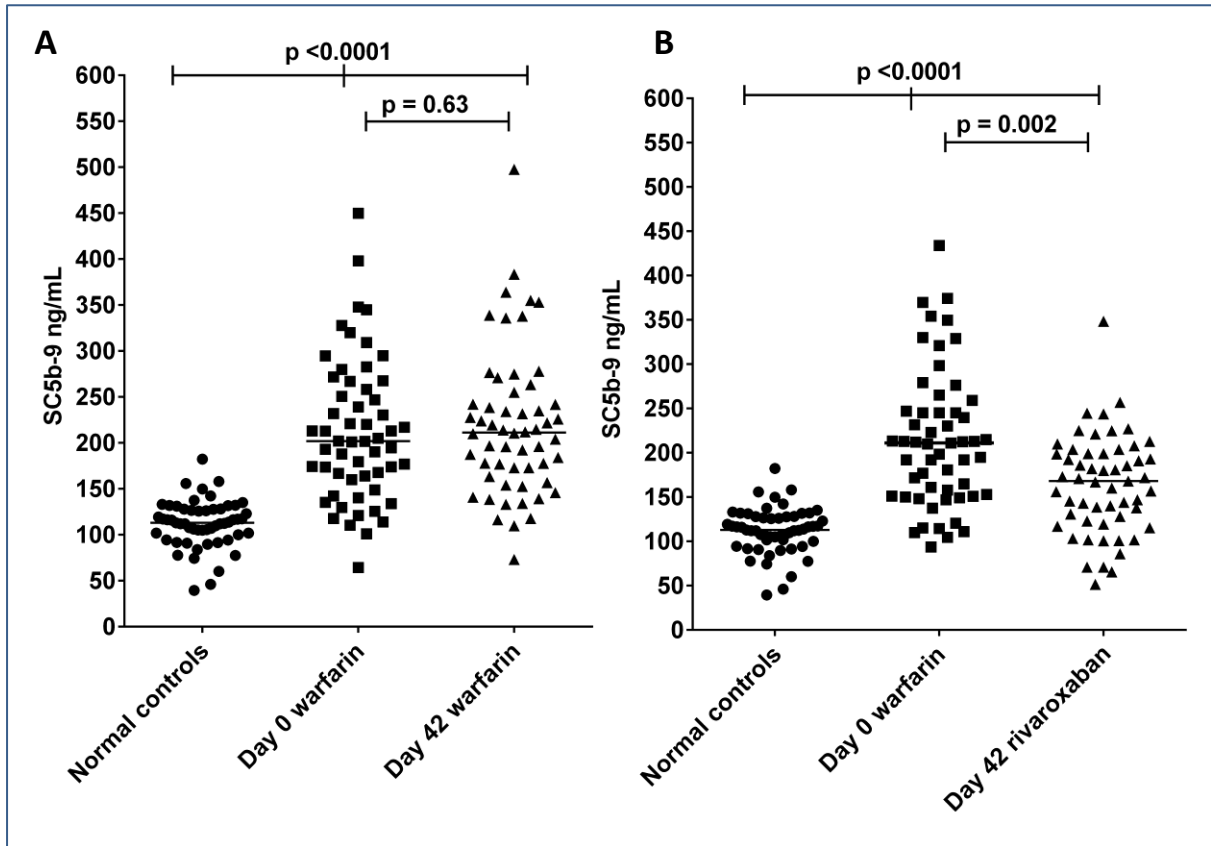


Figure 4

Comparison of Bb fragment levels in patients randomised to remain on warfarin (A) or to switch to rivaroxaban (B), at day 42 compared to their baseline values and with normal controls.

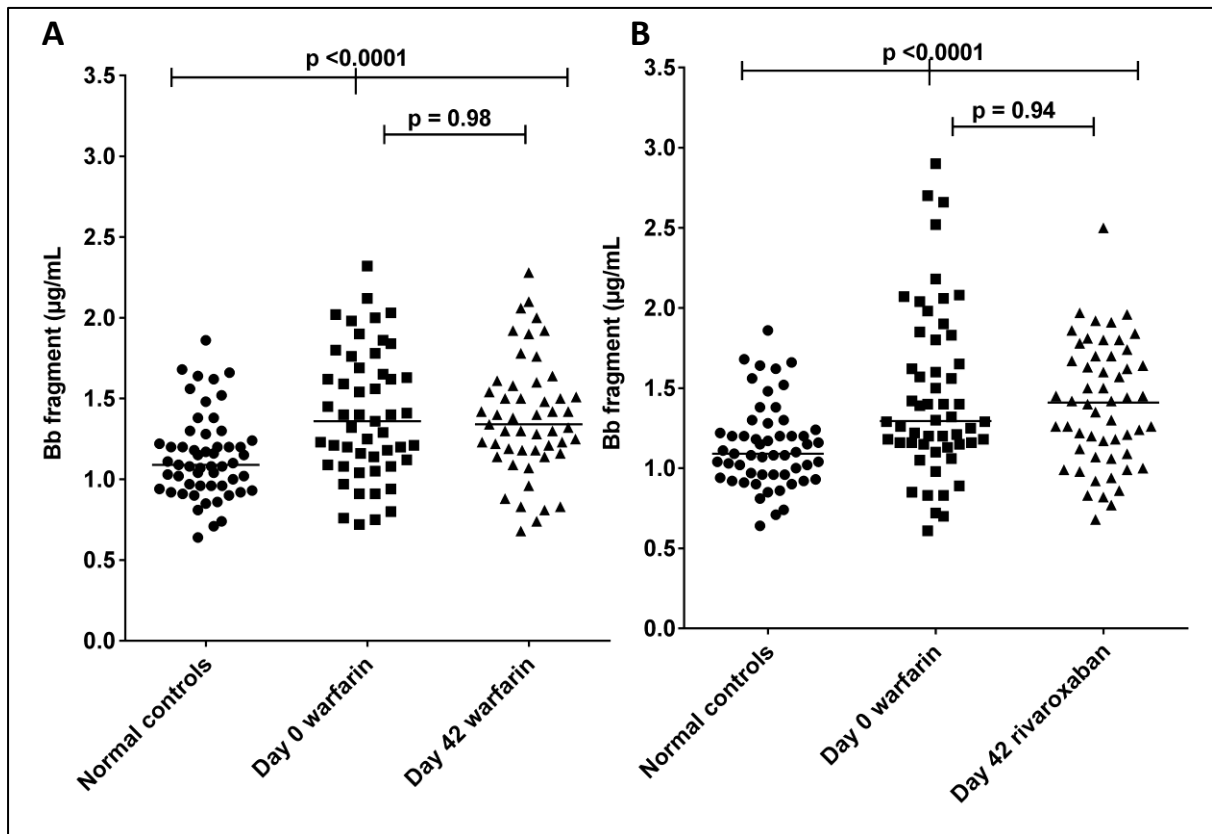
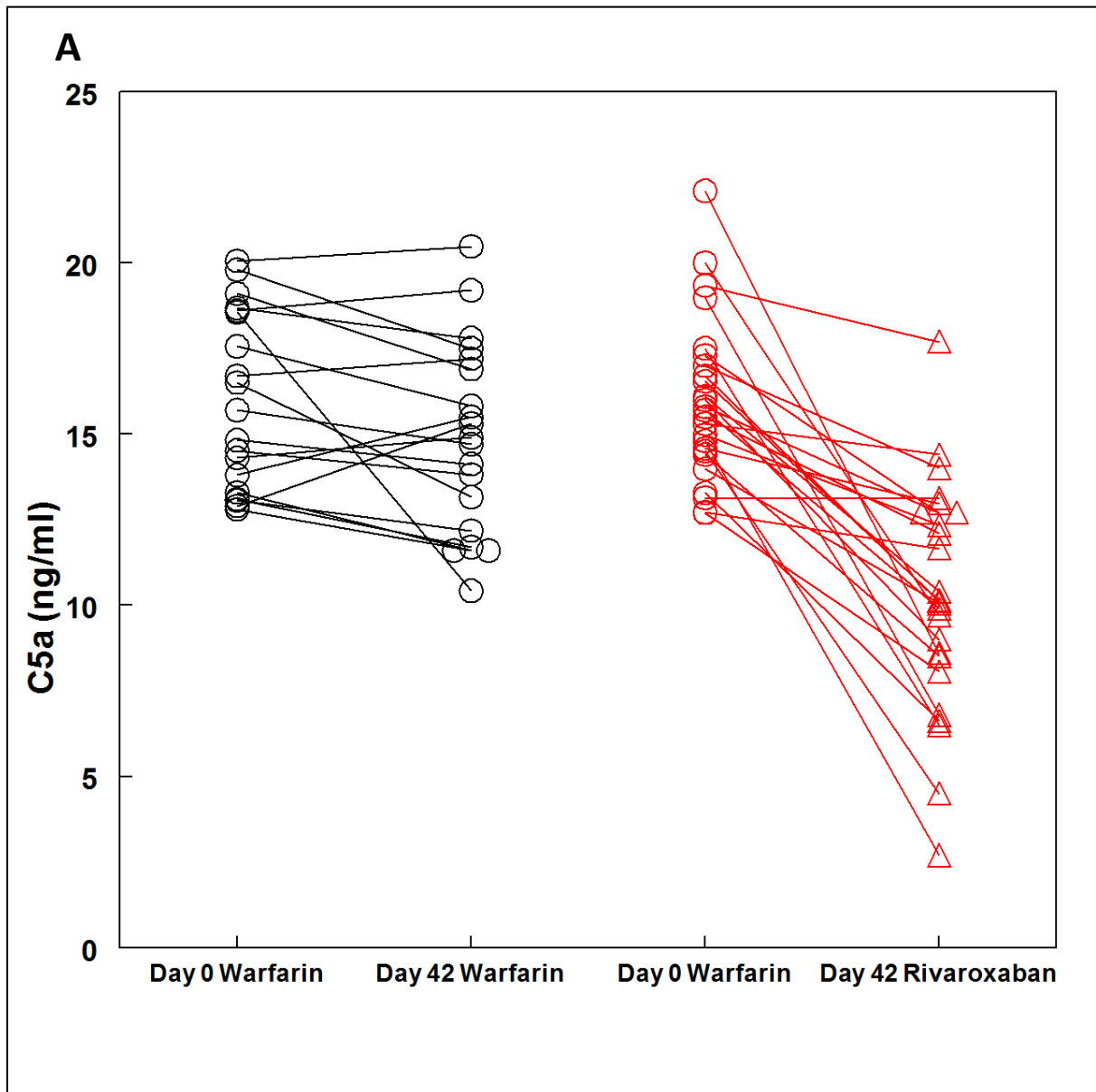


Figure 5

Patients with baseline elevation of C5a (A) or SC5b-9 (B) –effect of rivaroxaban



B

