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Complement activation prior to symptom onset in myeloperoxidase ANCA-associated vasculitis but not proteinase 3 ANCA associated vasculitis - A Swedish biobank study

L Johansson[1], E Berglin[1], O Eriksson[2], AJ Mohammad[3,4], J Dahlqvist[5], S Rantapää-Dahlqvist[1]

[1]Department of Public Health and Medicine/Rheumatology, Umeå University, Umeå, Sweden
[2]Department of Immunology, Genetics & Pathology, Uppsala University, Uppsala, Sweden
[3]Department of Clinical Sciences/Rheumatology, Lund University, Lund, Sweden
[4]Department of Medicine, University of Cambridge, Cambridge, UK
[5]Department of Medical Sciences, and Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden

Objective: Increased soluble levels of complement effectors have been demonstrated in active anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), but the timing of complement activation in the autoimmune inflammation remains elusive. This study investigated whether the complement system is activated before onset of symptoms in AAV.

Method: The Swedish National Patient Register and Cause of Death register were linked to registers of five biobanks to identify individuals sampled before AAV symptom onset. Diagnosis of AAV and time-point for symptom onset were confirmed by reviewing medical records. We identified 64 presymptomatic individuals with serum samples > 1 month < 10 years from AAV symptom onset and 122 matched controls. Complement factors (C2, C5) and activation markers (C5a, C4b) were measured using Luminex technology.

Results: Presymptomatic individuals had higher levels of C5 up to 6.5 years before symptom onset, compared with controls [median (IQR) 80.7 (131.9) vs 46.6 (63.4) μg/mL, p = 0.05]. Levels of C5a increased significantly during the pre-dating time (p = 0.033) until symptom onset. The complement levels were significantly higher in presymptomatic myeloperoxidase (MPO)-ANCA$^+$ individuals versus MPO-ANCA$^-$ and proteinase-3-ANCA$^+$ individuals. C5 was significantly increased in cases with renal involvement at diagnosis versus controls (p = 0.022), whereas levels of both C5 and C5a were significantly increased in presymptomatic individuals diagnosed with microscopic polyangiitis after onset compared with controls (C5: p = 0.027; C5a: p = 0.027).

Conclusion: Activation of the complement system is an early event in the pathogenesis of AAV and is mainly associated with MPO-ANCA$^+$ AAV and with microscopic polyangiitis.

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a group of potentially life-threatening autoimmune diseases characterized by necrotizing inflammation of small blood vessels and the presence of ANCAs (1). ANCAs are directed against neutrophil cytoplasmic constituents, e.g. proteinase-3 (PR3) and myeloperoxidase (MPO), and have the potential to activate neutrophils (2), which may trigger activation of the complement system (3).

Studies in mouse models of anti-MPO immunoglobulin G-mediated glomerulonephritis suggest that complement is activated in AAV, in particular the alternative pathway, and deficiencies in complement components C5 and factor B seem to provide protection from disease development (3). In subsequent human studies, activation of the complement system was confirmed in kidneys of AAV patients (4). Analyses of renal biopsies have shown C3c, C4d, and mannose-binding lectin depositions, suggesting that both the classical and lectin pathways, besides the alternative pathway, are involved (5). There are several studies on circulating levels of complement components in AAV, where an increase in factor B levels and activation fragments C5a and C3a in both PR3- and MPO-positive (+) AAV have been detected, mainly during active disease (6, 7). The receptor of C5a, a common effector of all three pathways, has been launched as a target in recent therapeutic development for AAV (2, 8).

The aim of this study was to investigate whether complement activation is an early process in the pathogenesis of AAV, pre-dating symptom onset, by analysing levels of C2, C5, C4b, and C5a in serum samples from presymptomatic individuals.

Solbritt Rantapää-Dahlqvist, Department of Public Health and Clinical Medicine, Rheumatology, University Hospital, SE-901 85 Umeå, Sweden. E-mail: solbritt.rantapaa.dahlqvist@umu.se

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Method

This study was performed using serum samples collected from individuals before the onset of symptoms of AAV. The

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identification of individuals and samples has been presented in detail in a previous publication (9), while the procedure will be summarized here. The Swedish National Inpatient Register and the Cause of Death Register were used to identify individuals diagnosed with AAV as first diagnosis in the discharge summary and/or cause of death between 1987 and 2011. The personal identity numbers of the individuals with AAV were linked to the registers of five biobanks in Sweden (age ≥ 18 years and samples > 1 month and < 10 years before date of symptom onset). Medical records were reviewed to identify the time-point for symptom onset and to confirm the diagnosis of AAV using the European Medicines Agency (EMA) algorithm (10). Information about ANCA positivity at diagnosis and clinical manifestations at symptom onset and at diagnosis was collected from the medical records.

The study approved by the locally appointed ethics committee (Dnr 2016-419-32M) and complies with the Declaration of Helsinki.

Medical records were available for 504 individuals, although 206 and 184, respectively, were excluded as they did not fulfil the time limit or the algorithm for AAV diagnosis, and 27 samples could not be located (9). Of the remaining presymptomatic individuals, 64 had available serum samples at a median [interquartile range (IQR)] of 4.4 (5.4) years pre-dating onset (Table 1). Two controls without an AAV diagnosis per case, except for one per case in six, were matched for age, gender, and sampling date from the same biobanks (n = 122) (mean ± sd age 52.5 ± 18.1 years). MPO-ANCA was positive in two of the controls (1.7%) and PR3-ANCA in one of the controls (0.8%).

All samples were stored at –80°C, for a mean ± sd storage time of 21.8 ± 8.0 years, and thawed rapidly only once, in connection with the analysis, to avoid complement activation. Serum levels of complement factors (C2 and C5) and activation markers (C4b and C5a) were measured using EMD Millipore MILLIPLEX Human Complement Panel-1, Luminex technology (Merck, Darmstadt, Germany). All

Table 1. Demographic and clinical characteristics of AAV pre-symptomatic individuals before symptom onset, and at symptom onset and at diagnosis

Characteristics	All Cases (n=64)	Pre-symptomatic PR3-ANCA$^-$N=45	Pre-symptomatic PR3-ANCA$^+$ N=19	Pre-symptomatic MPO-ANCA$^-$ n=55	Pre-symptomatic MPO-ANCA$^+$ N=9
At sampling					
Age mean (SD), years	52.7± 18.1[1]	56.2±16.0	44.3±20.6*	50.7±17.8	65±15.6*
Predating time before symptom onset, median (IQR), years	4.4 (5.4)	4.6 (5.08)	3.8 (7.4)	5.5 (6.02)	2.0 (2.6)*
Storage time, mean (SD)	21.8±7.9	22.7±7.9	19.7±7.6	21.9±10.6	21.2±10.6
At symptom onset					
Age mean (SD), years	56.5 (18.3)	60.5 (16.1)	47.1 (20.1)*	54.8 (18.2)	67.0 (15.6)*
Involvement of kidney, lung or peripheral nervous system, n (%)	27/59 (45.8)	21/41 (51.2)	6/18 (33.3)	20/50 (40.0)	7/9 (77.8)*
At diagnosis					
Positive ANCA$^-$ frequency at diagnosis, n (%)	60/63[2] (95.2)	41/44 (93.2)	19/19 (100)	51/54 (94.4)	9/9 (100)
Age mean (SD), years	57.1 (18.1)	61.1 (15.8)*	47.6 (20.1)	55.3 (18.0)	67.6 (15.5)*
Pulmonary and/or kidney manifestations, n (%)	50/64 (78.1)	33/45 (73.3)	17/19 (89.5)	41/55 (74.5)	9/9 (100)
Ear-nose-throat manifestations, n (%)	38/64 (59.4)	26/45 (57.8)	12/19/ (63.2)	36/55 (65.5)*	2/9 (22.2)
EMA algorithm[3]					
EGPA	3/64 (4.7)	3/45 (6.7)	-	3/55 (5.5)	-
GPA	47/64 (73.4)	32/45 (71.1)	15/19 (78.9)	43/55 (78.2)	4/9 (44.4)
2a	38/64 (59.4)	25/45 (55.1)	13/19 (68.4)	34/55 (61.8)	4/9 (44.9)
2b	2/64 (3.1)	2/45 (4.4)	-	2/55 (3.6)	-
2c	1/64 (1.6)	1/45 (2.2)	-	1/55 (1.8)	-
2d	6/64 (9.4)	4/45 (8.9)	2/19 (10.5)	6/55 (10.9)	-
MPA	14/64 (21.9)	10/45 (22.2)	4/19 821.1)	9/55 (16.4)	5/9 (55.6)
3a	11/64 (17.2)	7/45 (15.6)	4/19 (21.1)	6/55 (10.9)	5/9 (55.6)
3b	3/64 (4.7)	3/45 (6.7)	-	3/55 (5.5)	-

[1] PR3- and MPO-ANCA were analysed as previously described (9), [2]ANCA data from medical records (p-ANCA and MPO-ANCA, c-ANCA and PR3-ANCA), [3] EMA algorithm for stratifications of vasculitis; eosinophilic granulomatosis polyangiitis (EGPA), granulomatosis polyangiitis (GPA; subgroups 2a-2d) and microscopic polyangiitis (MPA; subgroups 3a-b)(10)
*<0.05 comparisons between MPO- and PR-3-ANCA positivity vs. negativity, respectively

assays were performed according to the manufacturers' instructions using a 2 h protocol.

Statistics

Continuous data were compared using non-parametric tests, the Kruskal–Wallis test or Mann–Whitney U test when appropriate, e.g. for comparison of the complement concentrations between controls and presymptomatic individuals or antibody-positive or antibody-negative individuals. The Joncheere–Terpsta test was used for trend analysis. Logistic regression analyses were performed to adjust for pre-dating time. p-Values ≤ 0.05 (two-sided) were considered significant. All statistical analyses were performed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA).

Results

Complement levels in presymptomatic individuals and controls, and development over time

No significant differences in concentrations of the complement biomarkers, C2, C5, C5a, and C4b, were found between AAV presymptomatic individuals as one group and controls (Figure 1A–D). During the pre-dating time, the levels of C5a increased significantly in the presymptomatic individuals (p = 0.05). Compared with controls, the levels of C5 in all presymptomatic individuals were significantly increased closer to symptom onset, i.e. from ≤ 6.5 years until onset [median (IQR) 80.7 (131.9) vs 46.6 (63.4) µg/mL, p = 0.05] and for C5a from ≤ 5.0 years until onset [70.9 (108.2) vs 43.3 (63.6) ng/mL, p = 0.05] (Figure 1A, B). The complement levels were unrelated to sample storage time (cases: p = 0.091–0.287; controls: p = 0.084–0.244).

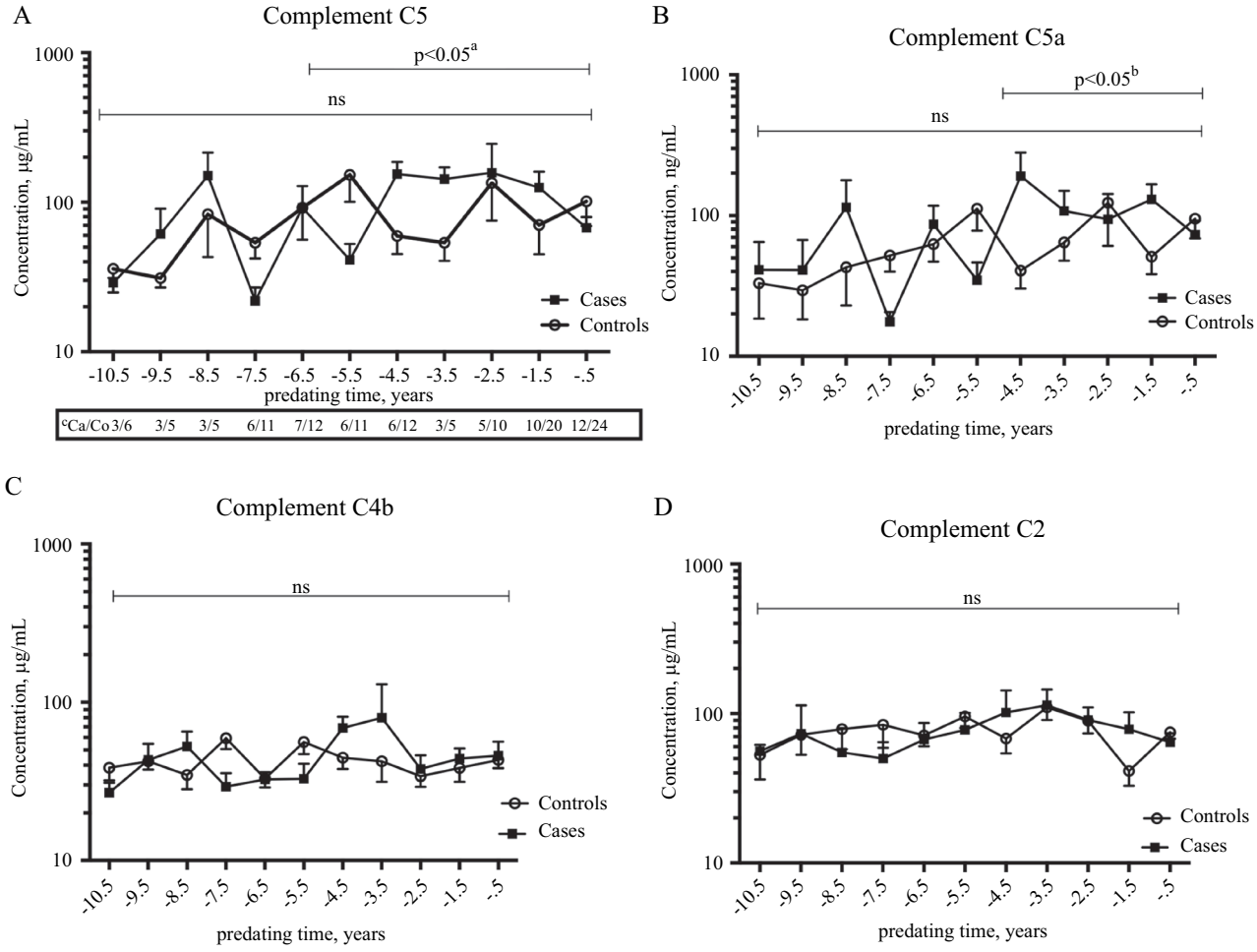

Figure 1. Concentration of complement factor presented as the mean for each time-point for (A) C5, (B) C5a, (C) C4b, and (D) C2, before symptom onset in presymptomatic individuals and in controls. Error bars represent standard error of the mean. The y-axis has a logarithmic scale. The significance values refer to comparisons between cases and controls. The number of cases and controls during each time-point is presented under the graphs. [a] Statistical calculation on samples from −6.5 years until symptom onset based on 45/64 cases and 85/121 controls; [b]statistical calculation on samples from −5 years until symptom onset based on 36/64 cases and 69/121 controls; [c]Ca/Co, number of cases/controls analysed for each time-point. The numbers of cases and controls for each time-point were identical for complement factors C5 and C2 and activation fragments C4b and C5a.

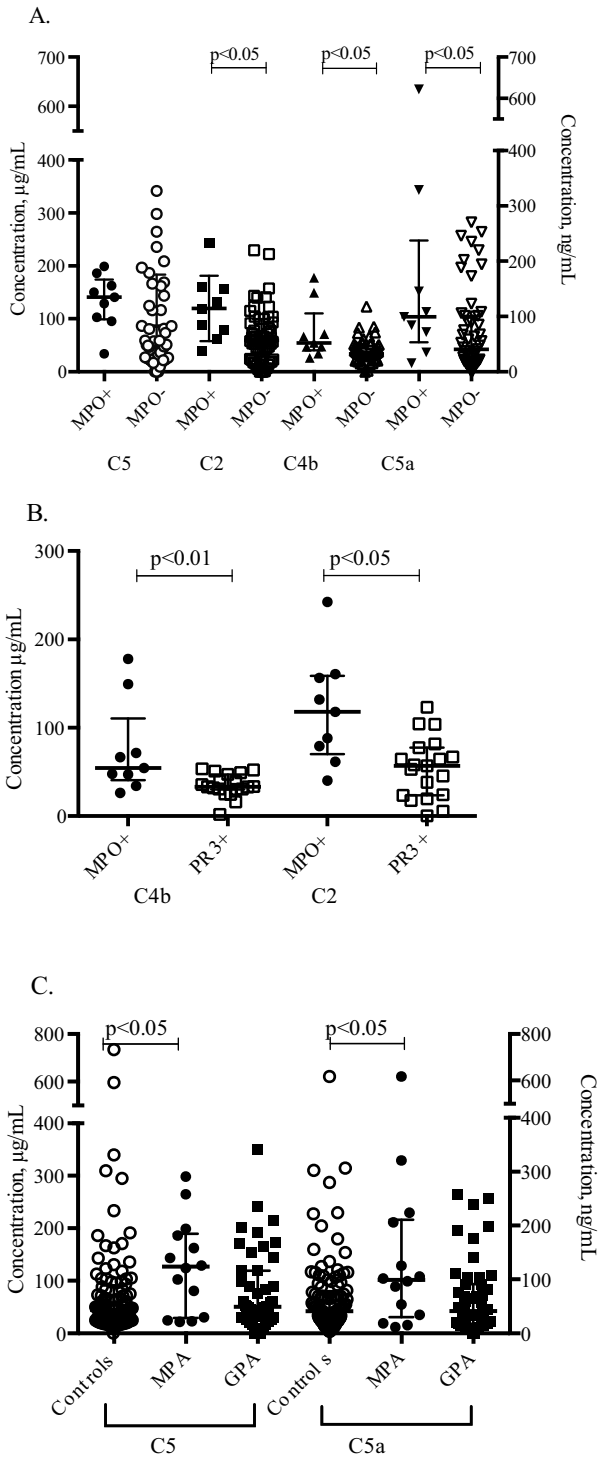

Figure 2. Serum levels of complement factors [C2 and C5 (µg/mL)] and activation fragments [C4b (µg/mL) and C5a (ng/mL)], presented as median values and interquartile range. Samples from presymptomatic individuals with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) are stratified for myeloperoxidase (MPO)-ANCA positivity vs negativity for C2, C5, and C4b (µg/mL) (left y-axis) and C5a (ng/mL) (right y-axis). The p-values refer to comparisons between (A) positivity and negativity, (B) levels of C4b (µg/mL) and C2 (µg/mL) in MPO- and proteinase-3 (PR3)-ANCA positive presymptomatic AAV cases, and (C) levels of C5 (µg/mL) (left y-axis) and C5a (ng/mL) (right y-axis) in presymptomatic samples of the cases stratified for the diagnosis after disease onset [microscopic polyangiitis (MPA) and granulomatosis polyangiitis (GPA) AAV] and in controls.

Complement levels stratified for PR3-ANCA and MPO-ANCA

The levels of C2, C5a, and C4b were significantly higher in presymptomatic MPO-ANCA$^+$ individuals than in MPO-ANCA negative (–) cases, irrespective of time before symptom onset (Figure 2A). No significant differences in the levels of any of the complement factors were found between PR3-ANCA$^+$ and PR3-ANCA$^-$ individuals. The levels of three of the factors were significantly increased in MPO-ANCA$^+$ cases compared with PR3-ANCA$^+$ (C2: $p < 0.01$; C5: $p < 0.05$; and C4b: $p < 0.01$). After adjustments for pre-dating time, the levels remained significantly increased for C2 and C4b ($p < 0.01$ and 0.05, respectively) (Figure 2B). Adjustments for renal involvement as the presenting symptom, in 33% of MPO-ANCA$^+$ and 28% of PR3-ANCA$^+$ cases, did not affect the differences for C2 and C4b (data not shown).

Complement levels in relation to symptoms at onset and at diagnosis

There were no significant differences in complement levels between cases with and those without manifestations from lungs, kidneys, nerves, or ear, nose, and throat as a debut symptom (data not shown). Likewise, there was no significant difference in levels of complement components between individuals who had renal and/or lung manifestation at diagnosis of the disease and those without. However, individuals with renal involvement as the first symptom or at diagnosis had a higher concentration of C5 compared with controls [median (IQR) 102.8 (113.9) vs 44.3 (62.9) µg/mL, $p = 0.055$; and 77.2 (65.7) vs 43.8 (63.9) µg/mL, $p = 0.022$, respectively].

Levels of C5 and C5a were significantly increased in presymptomatic individuals who were subsequently diagnosed with microscopic polyangiitis (MPA) after onset compared with controls [median (IQR) C5: 126.7 (160.4) vs 44.3 (63.9) µg/mL, $p = 0.027$; and C5a: 101.8 (184.9) vs 42.1 (58.7) ng/mL, $p = 0.027$, respectively]. There were no significant differences in the concentrations of pre-dating samples of C2, C5, C5a, or C4b between cases later diagnosed with granulomatosis polyangiitis (GPA) and controls (Figure 2C).

Discussion

In this study, using serum samples from individuals who subsequently developed AAV, we found increased levels of C5 collected up to 6.5 years before symptom onset and a gradual increase in C5a towards symptom onset. We have previously reported that the presence of both MPO- and PR3-ANCAs pre-date the onset of symptoms of AAV by up to 9 years (9). The present study further supports the hypothesis of an inflammatory pathogenic process starting years before symptom onset in AAV and suggests that activation of the complement system is an early event in AAV pathogenesis.

In our study, specifically MPO-ANCA$^+$ presymptomatic individuals showed increased levels in three complement biomarkers compared with MPO-ANCA$^-$ individuals. These results are in line with previous findings of a crucial role of the complement system in necrotizing glomerulonephritis with MPO-ANCA (4), and, in contrast to studies in patients with already developed AAV (7), suggest that the contribution of complement activation to the inflammatory cascade in presymptomatic individuals is larger in MPO-ANCA$^+$ vasculitis than in PR3-ANCA$^+$ vasculitis. These findings support differential pathogenic mechanisms behind MPO- and PR3-ANCA$^+$ vasculitis (2).

We selected four complement biomarkers for analysis: C2, C5, C5a, and C4b. Whereas the production of the enzymically inactive C5 and C2 may increase during inflammation as part of the acute-phase response (11), C4b and C5a are active effectors (12), specifically induced by complement activation (13). As PR3-ANCA$^+$ AAV has previously been associated with higher levels of acute-phase proteins than MPO-ANCA$^+$ AAV (14), we propose that the increase in complement components in MPO-ANCA$^+$ AAV identified in this study represents a specific activation of the complement system, rather than a general inflammatory reaction. The generation of C4b occurs upstream of C5a and is the result of C4 activation by either the classical or lectin pathway. When comparing C4b and C5a, we observed, foremost, increased C5a levels in pre-dating samples, suggesting an activation of the alternative, rather than the classical or lectin pathway in MPO-ANCA$^+$ vasculitis.

The limitations of the present study are the small sample size and the use of serum, rather than plasma, both resulting from the difficulties in obtaining pre-onset patient samples for rare disorders. Owing to the lack of chelating agents in serum, the complement system may be activated postsampling, during handling (13). To account for this, samples from presymptomatic individuals were compared to those of controls selected from the same biobanks as the cases, ensuring identical handling of samples. We have focused on complement biomarkers, although additional factors within the innate immune system can activate complement; for example, pentraxin-3, a soluble pattern recognition receptor, has been described as a biomarker in AAV (15).

Despite the difficulties mentioned, with 64 cases included, this comprises the largest study so far on complement levels during the presymptomatic phase of AAV. The major strengths of this study are the use of pre-onset biobank samples and the detailed ascertainment of the AAV diagnosis. Together, these advantages allowed us to perform unique comparative analyses of pre-onset patient AAV samples.

Conclusion

Activation of the complement system is an early event in the pathogenesis of AAV and is mainly associated with MPO-ANCA$^+$ AAV and with microscopic polyangiitis.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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