

# Anti-annexin V antibodies in Takayasu's arteritis: prevalence and relationship with disease activity

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## SUMMARY

Annexin V has an important role in the regulation of apoptosis and antibodies directed against it have been shown to lead to apoptosis of vascular endothelial cells. To evaluate the role of anti-annexin V antibodies (AA5A) in Takayasu's arteritis (TA), we investigated these antibodies in the sera of 66 TA patients, 50 healthy controls and in the follow-up sera of 12 active TA patients by enzyme-linked immunosorbent assay. The AA5A-positive patients were analysed further for the presence of anti-endothelial cell antibodies (AECA) and anticardiolipin antibodies (ACLA) to determine the relationship of AA5A with these autoantibodies. AA5A were observed in 36% (24/66) of the patients *versus* 6% (3/50) of the controls ( $P < 0.001$ ) and in 53% (19/36) of patients with active TA *versus* 17% (5/30) of those with inactive disease ( $P < 0.01$ ). Levels of AA5A were also observed to be significantly higher in patients with TA compared to those with inactive disease ( $0.557 \pm 0.362$  *versus*  $0.259 \pm 0.069$ ;  $P < 0.0001$ ) and in patients with active disease compared to those with inactive disease ( $0.700 \pm 0.403$  *versus*  $0.385 \pm 0.205$ ;  $P < 0.0001$ ). In the follow-up study, 6/12 patients who became inactive during follow-up also showed normalization of AA5A levels. AECA and ACLA were detected in 54% (13/24) and 12% (3/24) of the AA5A-positive patients, respectively. Our results show that a significant proportion of TA patients have AA5A, which exhibit an association with AECA and because they have a correlation with disease activity thus appear to be involved in the disease pathogenesis.

**Keywords** anti-annexin V antibodies disease activity Takayasu's arteritis

## INTRODUCTION

Takayasu's arteritis (TA) is an idiopathic chronic inflammatory pan-arteritis characterized by stenosis or aneurysmal dilation of large elastic arteries, mainly the aorta and its major branches, including pulmonary and coronary arteries. It is the most common vasculitic disorder in India and the third most common vasculitis in the paediatric age group worldwide [1,2].

Immune-mediated damage of vascular endothelial cells (ECs) is the initial event in the pathogenesis of TA [3,4]. The presence of apoptotic ECs in vascular lesions and in the circulation of allied vasculopathies suggests that apoptosis may be one of the major pathways of inflammatory damage of ECs [5]. A fundamental feature of ECs and other cell types undergoing apoptosis is the externalization of plasma membrane phosphatidylserine (PS) [6]. Annexin V, which is found abundantly in ECs and has a well-characterized  $\text{Ca}^{2+}$ -dependent natural binding

affinity to anionic phospholipids such as PS, plays an important role in the regulation of proinflammatory and procoagulant activities of apoptotic cells by shielding their exposed PS [7,8]. Furthermore, it may also be important in maintaining the integrity of early apoptotic cells and in the regulation of apoptotic process itself [9–11]. A dysregulation in the expression or activity of this protein *in vivo* may therefore have an important role in vascular damage.

Recently, anti-annexin V antibodies (AA5A) have been observed to cause apoptosis of cultured ECs *in vitro* [12,13]. These antibodies are reported commonly in clinical conditions, which also contain anti-endothelial cell antibodies (AECA) and antibodies to cardiolipin (ACLA) or other phospholipids [14–17]. However, a relationship of AA5A with these autoantibodies is not yet known. Because AECA and ACLA are observed frequently in patients with TA (18), we hypothesized that AA5A, which may have an important role in vascular damage, are quite likely to be present in the disease.

We thus undertook this study to investigate the prevalence of AA5A and to determine the relationship of these antibodies with disease activity as well as with AECA and ACLA in patients with TA.

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## SUBJECTS AND METHODS

### Subjects

Sixty-six patients with TA (27 males, 39 females; mean age  $27 \pm 11$  years; range: 12–45 years) were included in the study. The diagnosis of TA was established on the basis of clinical, laboratory and angiographic findings of the disease. All patients fulfilled at least three of the American College of Rheumatology Criteria 1990 for TA [19]. These include (i) bruits over subclavian arteries or aorta, (ii) decrease or absent brachial artery pulse and (iii) systolic blood pressure difference of  $>10$  mmHg between the arms. Finally, in each case the diagnosis of TA was confirmed by angiography and all the patients were found to have angiographically proven disease. Disease activity of the patients was determined by the following criteria: (i) systemic features such as fever, arthralgias, myalgias or weight loss of unknown cause; (ii) carotidynia (painful arteries); (iii) elevated erythrocyte sedimentation rate (ESR) ( $>30$  mm/h) and (iv) elevated C-reactive protein (CRP) ( $>0.6$  mg/dL) levels. A patient was considered to be in the active stage if two or more of these criteria were present along with other features of the disease [18]. Accordingly, 36 patients had an active disease and 30 had an inactive disease. Control subjects consisted of 50 age- and sex-matched healthy individuals. Most of the controls were individuals residing in the same area as the patients, while some were paramedical staff of the Institute. After obtaining informed consent, 5 ml of venous blood was obtained from each individual and isolated serum was stored at  $-80^\circ\text{C}$  until analysis.

Patients with active disease were put on immunosuppressive therapy consisting of prednisolone and azathioprine, which were given for 2 years with tapering of the doses as the disease became less active. Twelve patients with active disease, who were positive for AA5A and visited our clinic regularly, were followed-up prospectively to determine a relationship between levels of these antibodies and remission of disease activity.

This study was approved by the Institutional Ethics Committee of Sanjay Gandhi Post-Graduate Institute of Medical Sciences (SGPGIMS), Lucknow.

### Detection of anti annexin V antibodies (AA5A)

AA5A were detected by enzyme-linked immunosorbent assay (ELISA) using recombinant human annexin V as antigen according to a validated procedure described previously, with minor modifications [20].

Briefly, gamma-irradiated 96-well flat-bottomed plates (Nunc, Roskilde, Denmark) were coated with  $0.5 \mu\text{g}/\text{well}$  of purified recombinant annexin V (BD Pharmingen, Mountain View, CA, USA) in a final volume of  $100 \mu\text{l}$  of carbonate-bicarbonate buffer ( $0.05$  M, pH 9.6) and incubated at  $4^\circ\text{C}$  for 24 h. Following this and each of the subsequent incubation steps, washing was performed with phosphate buffered saline (PBS, pH 7.4) containing 0.2% bovine serum albumin (BSA) and 0.05% Tween-20 (wash buffer). The non-specific binding sites of each well were blocked by adding  $250 \mu\text{l}$  of PBS containing 3% BSA and incubating the plate at  $37^\circ\text{C}$  for 2 h. Test and reference sera diluted 1 : 300 with wash buffer were added  $100 \mu\text{l}/\text{well}$  in duplicate, with one set of duplicate wells containing wash buffer alone (antibody free) and the plates were incubated for 2 h at  $37^\circ\text{C}$ . The bound antibodies were detected by incubating  $100 \mu\text{l}/\text{well}$  of 1 : 3000 diluted  $\gamma$  chain-specific goat antihuman IgG-alkaline phosphatase (Sigma, St Louis, MO, USA). The colour reaction was developed by adding

$100 \mu\text{l}/\text{well}$  of 1 mg/ml of p-nitrophenyl phosphate (Sigma) in diethanolamine buffer (pH 9.8) and the absorbance was read in an automated ELISA reader (Tecan Spectra, Austria) at  $405 \text{ nm}$ .

Two positive and negative sera were included as standards in each run. Each plate was read when the optical density (OD) of positive controls reached 1.0–1.2 (always within 35 min). The blank value of the plate was subtracted from the sample value to obtain specific OD for each sample. The cut-off value for determining a sample to be positive, was taken as mean  $\pm 2$  s.d. of the OD of the normal controls. The interassay coefficient of variation was 8.5%.

### Assay for AECA and ACLA

To determine a relationship of AA5A with AECA and ACLA, the patient sera, which were found to be positive for AA5A, were tested for the presence of each of these antibodies using a standardized ELISA as described previously [18].

In the AECA assay each sample was run in triplicate and results were expressed as mean optical density (OD) values. The cut-off for the positivity of these antibodies was taken as mean  $\pm$  s.d. of 25 normal healthy controls run in parallel.

All samples for the detection of ACLA were run in duplicate and results were expressed as IgG phospholipid units (GPLU) calculated as per standard samples used in the assay. A sample was considered to be ACLA positive if its GPLU was  $\geq 10$ .

### Statistical analysis

Statistical analysis was performed using *Z* statistics for parametric data and Man-Whitney *U*-test for non-parametric data. A *P*-value  $< 0.05$  was considered to be statistically significant.

## RESULTS

### Prevalence of AA5A

AA5A were observed in 36% (24/66) of patients with TA and in 6% (3/50) of controls ( $P < 0.001$ ). Their prevalence in patients with active TA and those with an inactive disease was 53% (19/36) and 17% (5/30), respectively ( $P < 0.01$ ).

The levels of AA5A were significantly higher in patients compared to controls ( $0.557 \pm 0.362$  versus  $0.259 \pm 0.069$ ;  $P < 0.0001$ ) and in patients with active disease compared to those with inactive disease ( $0.700 \pm 0.403$  versus  $0.385 \pm 0.205$ ;  $P < 0.0001$ ) (Fig. 1).

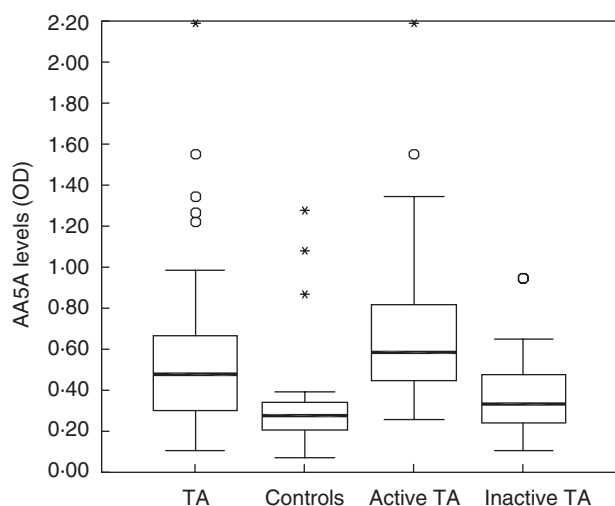
### Relationship of AA5A with disease activity

Of the 12 patient in whom a follow-up study was performed, six patients who became inactive also exhibited normalization of AA5A levels. The median follow-up period of these patients was 18 months (range: 12–24 months) (Fig. 2a). The remaining six patients who remained active during follow-up also continued to have elevated levels of AA5A, although the levels in two of these patients declined significantly but did not normalize till the last follow-up. The median follow-up period of these patients was 19 months (range: 16–24 months) (Fig. 2b).

The levels of these autoantibodies in patients in remission and in those who remained active on follow-up also correlated positively with CRP and ESR, the laboratory measures used to assess disease activity in the study. In patients in remission, however, CRP was raised in two cases and ESR had upper borderline values in another two cases, but they were found otherwise to be inactive. Similarly, two follow-up patients, who had remained

**Table 1.** Relationship of anti-annexin V antibodies (AA5A) with laboratory measures of disease activity: C-reactive protein (CRP; normal value: <0.6 mg/dl) and erythrocyte sedimentation rate (ESR; normal value: <30 mm/h) in 12 active Takayasu's arteritis (TA) patients treated with immunosuppressive therapy (the cut-off value for AA5A positivity: 0.397; calculated as mean  $\pm$  2 s.d. of controls)

Patient	Pretreatment AA5A levels (OD values)	On last follow-up				
		AA5A levels (OD values)	CRP (mg/dl)	ESR (mm/h)	Disease activity	Follow-up period (months)
1	1.596	0.301	<0.5	12	Inactive	24
2	0.951	0.282	0.9	20	Inactive	22
3	0.893	0.325	1.2	22	Inactive	12
4	0.889	0.232	<0.5	30	Inactive	20
5	0.750	0.217	<0.5	18	Inactive	16
6	0.574	0.269	<0.5	30	Inactive	12
7	1.309	0.626	5.5	16	Active	24
8	1.252	1.222	3.8	35	Active	18
9	0.955	1.022	1.5	42	Active	16
10	0.896	0.878	2.7	10	Active	20
11	0.870	1.379	1.3	55	Active	24
12	0.743	0.511	0.8	36	Active	18

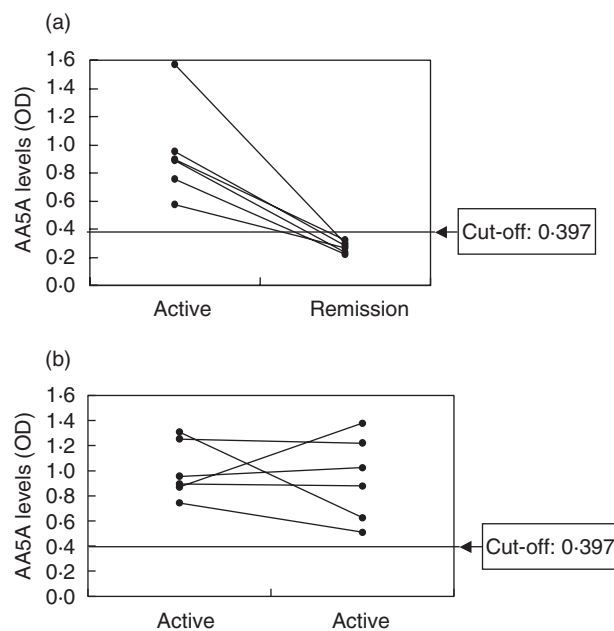


**Fig. 1.** Box plot showing levels of AA5A in Takayasu's arteritis (TA) patients ( $n=66$ ), healthy controls ( $n=50$ ), patients with active TA ( $n=36$ ) and patients with inactive TA ( $n=30$ ). The box includes observations from the 25th to the 75th percentile. The horizontal line within the box represents the median value. The upper and lower lines outside the box represent the highest and lowest value, respectively. The circles and asterisks represent outliers and extreme OD values of AA5A, respectively, in different subject groups. The levels of AA5A were significantly higher in patients with TA compared to controls ( $P < 0.0001$ ) and in patients with active TA compared to those with inactive TA ( $P < 0.0001$ ).

active on follow-up, had normal ESR but had other features of an active disease (Table 1).

#### Relationship of AA5A with AECA and ACLA

AECA and ACLA were detected in 54% (13/24) and 12% (3/24) of AA5A positive TA patients, respectively.



**Fig. 2.** Relationship of AA5A levels with disease activity in 12 prospectively followed-up active TA patients. (a) Six patients who underwent remission (median follow-up period: 18 months; range: 12–24 months) showed normalization of the levels of these antibodies. (b) The remaining six patients who continued to show disease activity (median follow-up period: 19 months; range: 16–24 months) continued to have elevated levels of these antibodies. Each line represents data of an individual patient. The straight line represents the cut-off OD value (0.397), above which a sample was taken as positive for AA5A.

## DISCUSSION

Our study shows that AA5A are present in a significant proportion of patients with TA, particularly those with an active stage of the disease. To the best of our knowledge, this is the first evidence for the presence of these antibodies in a vasculitic entity.

AA5A are newly identified autoantibodies demonstrated for the first time by Matsuda *et al.* [21] in patients with habitual fetal loss or pre-eclampsia. Since then they have been described increasingly in several systemic autoimmune disorders, including systemic lupus erythematosus (SLE) [16,17,22,23], anti-phospholipid syndrome (APS) [23–25], fetal loss [26,27], rheumatoid arthritis (RA) [20,28] and scleroderma [29]. More recently, raised levels of AA5A have been reported in two young female patients with cerebrovascular disease (CVD) and pregnancy complications, but without any clinical manifestation of a systemic autoimmune disease or major thrombotic risk factor [30]. In these diseases the prevalence of AA5A is reported to be 18–30% and a relationship of these antibodies with disease activity has been studied only in RA, with contradictory observations by two separate author groups [20,28]. Dubois *et al.* showed a strong association of AA5A levels with RA disease activity [28], while Rodriguez-Garcia *et al.* found such an association not significant [20]. We have observed an overall prevalence of AA5A in TA to be 36% and a significantly higher prevalence and levels of these antibodies in patients with active disease than in those with inactive disease showing an association of AA5A with active TA. To delineate further this relationship in the disease, we investigated AA5A in follow-up patients who initially had active disease as well as elevated levels of these antibodies. Our results, showing normalization of AA5A in patients who become inactive and their persistent elevation in those who remained active on follow-up, suggest further a relationship of these antibodies with disease activity in TA.

Almost all the AA5A associated diseases described above, including TA, frequently contain AECA and ACLA [14,18,31]. Furthermore, like AA5A, some of the AECA possess the capacity to induce apoptosis of ECs [32] and ACLA are also reported to have an association with vascular injury [33]. However, whether AA5A have a relationship with these autoantibodies has not yet been explored. In the present study, we have observed the presence of AECA in a substantial proportion (54%) of AA5A-positive patients, suggesting that annexin V might be one of the antigenic targets of these autoantibodies. In view of this co-existence of AA5A and AECA in TA, it is possible that the endothelial apoptosis-inducing capacity of AECA may be restricted to its fraction of endothelial-annexin V-specific antibodies which may have a direct pathogenic role, at least in this disease. Despite this association, AA5A *per se* do not appear to be a subset of AECA, as in addition to ECs annexin V is present in a variety of cell types and they may also recognize extracellular annexin V present in the circulation. To the best of our knowledge, this is the first study demonstrating the co-existence of AA5A and AECA in a disease. We also evaluated the relationship of these antibodies with ACLA, but in contrast to AECA we did not observe an association between AA5A and ACLA in TA. Similarly, no ACLA are found in most of the AA5A-positive patients with scleroderma [29] and neither ACLA nor other tested antiphospholipid antibodies were detected in two young patients with CVD who had persistently increased levels of AA5A [30]. AA5A present in patients with SLE have been reported to possess ACLA properties, indicating that both of these may be closely related autoantibodies [15]. However, it has been shown recently in SLE that removal of antiphospholipid activity using cardiolipin and other phospholipid liposomes had no effect on anti-annexin activity of the serum IgG fraction of SLE sera, also suggesting no association between these antibodies in this disease [13]. Our observations in

TA, together with these findings, suggest that AA5A are different from ACLA and probably from AECA as well and constitute a distinct group of autoantibodies. The existence of autoantibodies to other members of the annexin family, such as annexin I, -IV, VI and XI in different autoimmune diseases, further supports that AA5A is a separate group of autoantibodies [23,28,34,35].

The mechanism underlying the generation of AA5A is not known. It can be speculated that conditions associated with increased PS externalization in ECs, such as their persistent activation or apoptosis, are accompanied by increased release of annexin V and its binding to exposed PS. This suprathreshold surface expression of predominantly intracellular annexin V or expression of some cryptic or new antigenic epitopes in the native protein due to its binding to PS triggers the formation of these antibodies. Demonstration of activated/apoptotic ECs in lesions and/or in the circulation of different vasculopathies [5,36] and hyperexpression of annexin V in large-vessel endothelium of hypertensive pigs, and of annexin I and II in the diseased arteries of TA patients, together lend support to this hypothesis [37,38].

The pathogenic role of AA5A is not well defined. Because AA5A have been observed predominantly in thrombotic vasculopathies such as SLE and APS, most of the studies have implicated these antibodies in the induction of thrombosis [17,24,25]. However, they may have a role in inflammatory tissue damage by blocking or modulating different biological functions of annexin V, including regulation of apoptosis [10,11], PS-mediated inflammation [8,9] and calcium signalling [39]. In addition, AA5A can also play an important role in the propagation and amplification of the autoimmune response to apoptotic ECs by inhibiting or altering their physiological clearance by scavenger cells [40]. A direct pathogenic role of AA5A has been posited. Recently, these antibodies have been shown to cause apoptosis of cultured vascular ECs *in vitro* [12,13], necrosis of placental trophoblasts in a mouse model [41] and inhibition of gonadotrophin secretion from and apoptosis of human placental trophoblasts [42]. These findings, and the association of AA5A with digital ischaemia in scleroderma [29], together suggest an important role of these antibodies in vascular damage. In the present study a significantly higher association of AA5A with active disease and their normalization in patients, who underwent remission on follow-up, point towards an involvement of these antibodies in the pathogenesis of TA. However, a precise role of these antibodies in the disease is not clear at present and remains to be seen.

We thus conclude that a significant proportion of TA patients have AA5A which exhibit an association with AECA and because they have a correlation with disease activity, thus appear to have a pathogenic role in the disease. Further studies on factor(s) triggering the formation of AA5A, their pathogenic mechanism(s) and reactivity to other annexins will be important to understand the role of these antibodies in the pathogenesis of TA and other related vasculopathies.

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