

# CHAPTER 11

## **Follow-up of avidity and titer of anti-myeloperoxidase antibodies in sera from patients with primary ANCA-associated vasculitis**

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## **Abstract**

**[Background]** Antineutrophil cytoplasmic antibodies (ANCA) is an important serologic marker for ANCA-associated vasculitis (AAV). Our previous studies in propylthiouracil (PTU)-induced AAV demonstrated that withdrawal of PTU resulted in clinical remission and significant decrease of avidity of PTU-induced anti-myeloperoxidase (MPO) antibodies. This study investigated the changes in avidity and titer of MPO-ANCA in sequential sera from some patients with primary AAV with different disease activities.

**[Methods]** Sequential sera samples of seven patients with MPO-ANCA-positive vasculitis at their initial onset, remission and relapse were collected. The avidity of MPO-ANCA was assessed by antigen-inhibition ELISA. The titer of anti-MPO antibodies was determined by a two-fold dilution of sera in MPO specific ELISA.

**[Results]** The avidity of MPO-ANCA in active phase is not significantly different from that in remission ( $724.9 \pm 828.4 \text{L/mol}$  vs.  $353.4 \pm 551.7 \text{L/mol}$ ,  $P=0.303$ ). No significant difference could be found between initial onset and remission ( $P=0.492$ ). No significant correlation could be found between aK and BVAS level, times of relapse, the number of organ involvement, serum creatinine, or CRP except for the duration of remission ( $P=0.036$ ) and ESR ( $P=0.032$ ). The titer of anti-MPO antibodies was not significantly different between initial onset and in remission.

**[Conclusion]** Avidity and titer of MPO-ANCA did not decreased significantly during remission in AAV and there is a negative correlation between avidity and the duration of remission, indicating the chronic repeated antigen stimulation was not removed, which might be the reason for recurrent relapses.

## **Introduction**

Antineutrophil cytoplasmic antibodies (ANCA) are important serological markers for certain kinds of primary systemic vasculitis, including Wegener's granulomatosis (WG), microscopic polyangiitis (MPA), Churg-Strauss syndrome (CSS) and their localized variants (renal limited vasculitis, RLV), called ANCA-associated vasculitis (AAV) [1].

The pathogenesis of AAV has not been fully elucidated, but increasing evidence suggested that ANCA play an important role [2-7]. The prognosis of untreated AAV is dismal. Since the employment of corticosteroids and cyclophosphamide, remission can be achieved in most of the patients [8]. However, up to 50% of individuals in remission will relapse within 5 years [9]. The relationship between changes of ANCA level and relapse of AAV remained controversial [10, 11]. Our previous studies on propylthiouracil (PTU)-induced vasculitis showed that once remission is achieved by withdrawal of PTU and employment of immunosuppressive therapy, relapses seldom occur [12]. Further investigation suggested that both the avidity and titer of myeloperoxidase (MPO)-ANCA decreased significantly after remission; and the avidity decreased much more quickly than the titer did [13]. Therefore, we speculate that the chronic relapses in primary AAV might be associated with the persistently high avidity of MPO-ANCA. Moreover, our previous studies suggested that in Chinese, about 80-90% of the patients with AAV are MPO-ANCA positive [14-18]. It indicated that MPO is the most important ANCA target antigen for Chinese patients with AAV. Therefore, the current study aimed to detect changes of avidity and titer of MPO-ANCA in sequential sera samples from some patients with AAV in active phase of initial onset and relapse as well as in remission.

## **Patients and methods**

### **Patients and Sera**

Patients in our hospital fulfilled the following criteria were recruited in the current study: (1) fulfilled the 1994 Chapel Hill Consensus Conference definition for AAV [19] and with positive serum perinuclear ANCA (p-ANCA) and MPO-ANCA; (2) complete clinical data on diagnosis and during follow-up; (3) sequential serum samples of initial onset and relapse before the

immunosuppressive therapy was initiated as well as remission; and (4) a relatively higher level of MPO-ANCA in all the three sera samples (above 50% of the positive control). Treatment protocols of AAV including induction and maintenance therapy were described previously [17]. All the sera were stored at -20°C until use. Informed consent was obtained for blood sampling. The research was in compliance of the Declaration of Helsinki and approved by the ethic committee of the local hospital.

### **Assessment of Disease Activity**

The vasculitic disease activity was measured by the Birmingham Vasculitis Activity Score (BVAS) [20]. “Remission” was defined as “absence of disease activity attributable to active disease qualified by the need for ongoing stable maintenance immunosuppressive therapy” (complete remission), or “50% reduction of disease activity score and absence of new manifestations” (partial remission). “Relapse” is defined as “recurrence or new onset of potentially organ- or life-threatening disease attributable to active vasculitis” (major relapse), or “recurrence or new onset of disease attributable to active vasculitis that are neither potentially organ- nor life-threatening” (minor relapse) [21].

### **Detection of Titers of MPO-ANCA**

All sera were screened for ANCA by indirect immunofluorescence using precooled ethanol-fixed normal peripheral neutrophils as substrate according to the manufacturer (Euroimmun, Lübeck, Germany) and MPO-ANCA were measured by enzyme-linked immunosorbent assay (ELISA) as described. In brief, highly purified human native MPO was coated to the wells of a Costar microtitre plate (Data Packaging Corporation, MA, USA) at 2.0mg/l in coating buffer (0.05mol/l bicarbonate buffer, pH 9.6). The volume in each well was 100µl in all steps and every sample was added in duplication, all incubations were carried out at 37°C for 1 h, and the plates were washed three times with phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBST) (Chemical reagents, Beijing, China) between stages. Sera were diluted at 1:50 with PBST and every plate contained positive, negative and blank controls (PBST). Binding was detected with a horseradish peroxidase-conjugated antihuman IgG (Gibco BRL, Carlsbad, CA, USA) at a dilution of 1:5000. The peroxidase substrate o-phenylenediamine was used at 0.4g/l in 0.1M citrate buffer (pH5.0). The results were recorded as the net OD490nm and samples were considered

positive if they exceeded mean +3 SD from 45 normal blood donors. Samples positive for MPO-ANCA in screening were subsequently tested at two fold dilutions, from 1:50 to 1:25600, to determine their titers.

### **Avidity Constant Determination**

The avidity constant (aK) was determined as the reciprocal value of the MPO molar concentration in the liquid phase resulting in 50% inhibition of MPO-ANCA binding to MPO in solid phase ELISA as previously described [13]. The higher MPO molar concentration required, the lower the avidity was. Therefore, the higher the value of aK, the higher the avidity. Briefly, the appropriate serum concentration required for a competitive assay was determined first for each patient as the serum dilution (1:50-1:3200), giving about 50%-65% of the maximum binding in the standard MPO ELISA. The competitive binding assay was performed by incubating the determined serum concentration with decreasing amounts of MPO (35mg/l - 0.068mg/l) in PBST at 4°C overnight. The mixture was then transferred to MPO-coated plates for the standard ELISA procedure.

### **Statistical Analysis**

Student's t-test was used to compare means as appropriate. Linear regression analysis was used for correlation analysis. Differences of qualitative data were compared by Chi-square test. It was considered significant difference if the P value was less than 0.05. The statistical analysis was performed in SPSS statistical software package (version 11, Chicago, Ill, USA).

## **Results**

### **Demographic Characteristics and Clinical Data**

Seven patients were recruited in the current study. Two patients were male and five were female, with an average age of 63±6.4 (range 51-69) years when the diagnosis of AAV was established. The serum creatinine at diagnosis was 346.6±236.2µmol/L (range 76-798.5µmol/L). Six patients were classified as MPA and the rest one was classified as RLV. After immunosuppressive therapy, remissions were achieved in all patients; two of which achieved partial remission and the other five achieved complete remission. The mean duration from remission to first relapse was 18.3±8.7 (range 9-33) months. The levels of BVAS were 17±3.2 (range 14-23),

1.86±3.5 (range 0-9) and 16.7±1.8 (range 15-20) at initial onset, remission and relapse, respectively. Clinical data of these patients were listed in Table 1 and Table 2.

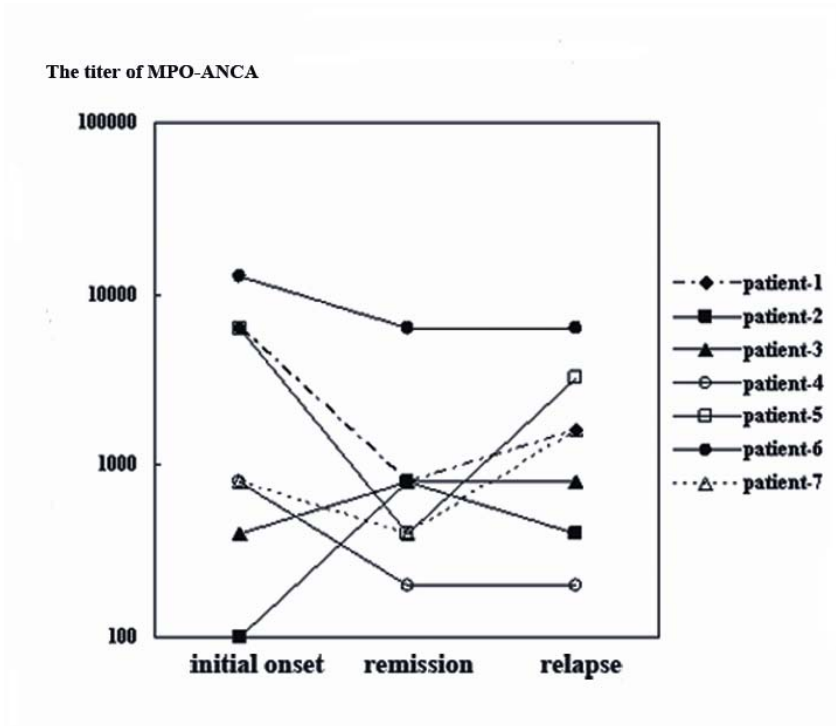


Figure 1. The titer of MPO-ANCA of patients with primary AAV in initial onset, relapse and remission

**Table 1. Clinical characteristics and titers of MPO-ANCA of patients with AAV**

No.	Age (yr)	Sex	Diagnosis	Initial Scr ( $\mu\text{mol/L}$ )	Titers of MPO-ANCA IgG		
					Initial onset	Remission	Relapse
1	58	M	MPA	138	1:6400	1:800	1:1600
2	67	F	RLV	268	1:100	1:800	1:400
3	69	F	MPA	76	1:400	1:800	1:800
4	64	M	MPA	798.5	1:800	1:200	1:200
5	51	F	MPA	333	1:6400	1:400	1:3200
6	64	F	MPA	397	1:12800	1:6400	1:6400
7	68	F	MPA	416	1:800	1:400	1:1600

[Abbreviations] MPA: microscopic polyangiitis, RLV: renal limited vasculitis

### The Titers of MPO-ANCA

As shown in Figure 1, the titers of MPO-ANCA in remission phase decreased in three patients, compared with initial onset and relapse (No. 1, 5 and 7). Two patients had their highest titers at initial onset (No. 4 and 6), and one patient had the lowest titer at initial onset (No. 3), and these three patients had equal levels for remission and relapse. For the rest one (No. 2), the titer of remission was higher than that at initial onset and relapse. No significant correlation could be found between the titers and BVAS levels ( $r=0.398$ ,  $P>0.05$ ).

### The Avidity of MPO-ANCA

**Table 2. Avidity constant (aK) of MPO-ANCA in patients with AAV**

No	Initial onset			Remission			Relapse		
	Organ involved	BVAS	aK( $\times 10^6$ , l/mol)	Duration (mon)	BVAS	aK( $\times 10^6$ , l/mol)	Organ involved	BVAS	aK( $\times 10^6$ , l/mol)
1	K,Ey	23	>2048	9	9	512	K,N	15	>2048
2	K	15	ND	16	0	256	K	15	128
3	K,L,E	18	16	11	0	96	K,L	18	32
4	K,Ep	15	192	18	0	6	K,L	16	64
5	K	15	>2048	27	0	64	K,L	16	1024
6	K,L	19	1024	14	4	1536	K,L	17	512
7	K	14	32	33	0	4	K,G,J	20	256

[Abbreviations] K: kidney, L: lung, E: ear, Ey: eye, Ep: epididymis, N: nerve, ND: not done, J: joint, G: gastrointestinal tract

The changes in the amount of MPO (mg/l) needed to give a 50% inhibition of binding in MPO-ELISA in serial sera from patients with AAV were shown in Figure 2. The higher the amount of MPO used, the lower the avidity of the MPO-ANCA detected. Compared with active phases, the avidity of four patients in remission decreased. For the other three patients, the avidity in remission was even higher than that in active phases. The avidity constant (aK) of MPO-ANCA in active phase was not significantly different from that in remission ( $724.9 \pm 828.4$  L/mol vs.  $353.4 \pm 551.7$  L/mol,  $P=0.303$ ). No significant difference of aK could be found between initial onset and remission ( $P>0.05$ ). No significant correlation could be found between aK and the BVAS level, times of relapse, the number of organ involvement, serum creatinine, or CRP, except for the duration of remission ( $P=0.036$ ) and ESR ( $P=0.032$ ) (Table 3). The duration of remission correlated with the aK of remission phase ( $P=0.036$ ), but did not correlate with that of active phase ( $P=0.788$ ).

The inhibitive amount of MPO (mg/L)

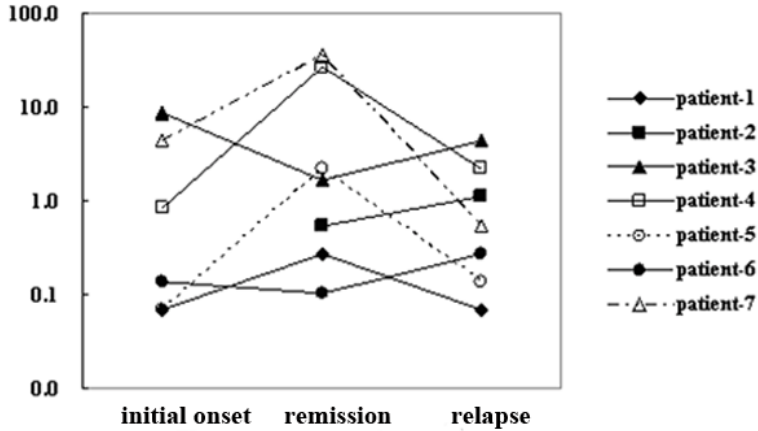


Figure 2. The changes of inhibitive amount of MPO (mg/l) needed to give a 50% inhibition of binding in MPO-ELISA from serial sera of patients with AAV in initial onset, relapse and remission.

Table 3. The correlation of MPO-ANCA avidity constant (aK) and clinical parameters

Clinical parameters	correlation with aK	
	<i>r</i>	<i>P</i> value
BVAS	0.261	>0.05
Erythrocyte sedimentation rate&	-0.595	<0.05
C-reactive protein &	-0.49	>0.05
Serum creatinine	0.219	>0.05
The number of organ involvement	0.269	>0.05
Times of relapse &	0.573	>0.05
Times of relapse §	0.478	>0.05
The duration of remission &	-0.126	>0.05
The duration of remission §	-0.786	<0.05

*r* : correlation coefficient. &: the aKs in active phase were used. §: the aKs in remission phase were used.

## Discussion

Besides the diagnostic value, the pathogenic role of ANCA has been gradually established. ANCA are presumed to reflect the disease activity. However, it is still controversial about the value of ANCA in disease activity monitoring [10, 11]. Cambridge et al. suggested that whether the autoantibody response was pathogenic would depend on many factors including the immunological characteristics of the autoantibodies, such as epitope specificity, avidity, subclass and idiotype [22]. In our previous study

on MPO-ANCA in patients with PTU-induced vasculitis, it was found that the avidity and titer of PTU-induced MPO-ANCA, especially avidity, was associated with the disease activity closely [23]. Therefore, we speculated that some of the immunological characteristics of MPO-ANCA might be associated with disease activity in primary AAV and the chronic relapses might be associated with the persistently higher avidity of ANCA even during remission.

In the current study, there was no significant correlation between titers of MPO-ANCA and disease activity. The avidity in active stage was not significantly different from that in remission stage. The main finding of our study is the failure to show the significant correlation between the avidity of MPO-ANCA and disease activity measured by BVAS.

It has been well known that the antigen-specific antibody undergoes affinity improvement after the primary response. For achieving affinity maturation, molecular modifications of the immunoglobulin genes triggered by antigen are pivotal [24]. The B-cell clones producing MPO-ANCA may take the same process. We speculate the MPO-ANCA may experience a shift from low affinity to high (affinity maturation) assisted by antigen stimulation. Therefore, the persistently high affinity of MPO-ANCA, even in clinical quiescence, may be attributed to repeated chronic antigen stimulation. It is also reasonable to suggest that high avidity of MPO-ANCA may contribute to disease activity. Therefore, the persistently high affinity of MPO-ANCA in remission phase may contribute to relapse of vasculitis.

In patients with PTU-induced vasculitis, clinical remission can be achieved through cessation of PTU and initiation of immunosuppressive therapy; a significant decrease in the avidity of PTU-induced MPO-ANCA during remission has been described previously [13]. This finding supports the speculation that repeated antigen stimulation may be the reason for persistently high avidity of MPO-ANCA in some patients with primary AAV. Our finding in the current study, no significant decrease of MPO-ANCA avidity in remission, indicates that certain unknown antigens that initiate the production of MPO-ANCA hasn't been removed. Therefore, administration of immunosuppressive therapy without removal of the chronic antigenic stimulation is not enough to prevent relapses. Meanwhile, as shown in our study, the lower the avidity of ANCA in remission was, the longer the remission maintained; which suggested the relationship between recurrent

relapses and high avidity of MPO-ANCA. Although remission could be achieved in the majority of patients with primary AAV, a high relapse rate is still a challenge, resulting in continuing morbidity and mortality, which underlines the need for uncovering the mask of the peace-breaker.

There were some limitations in the current study. Firstly, due to the method limitation, relatively higher titers are required for avidity detection. So, those patients with lower titers in either active or remission phase were excluded and the number of patients recruited in our study was limited. Secondly, all the seven patients experienced at least one relapse. They are only a subgroup of the total primary AAV patients since quite a few patients in our clinical practice never experienced relapse during follow-up. Moreover, based on the fact that most of AAV patients in Chinese were MPO-ANCA positive, we only focused on patients with MPO-ANCA and neglected PR3-ANCA-positive patients.

In conclusion, avidity and titer of MPO-ANCA did not decrease significantly during remission in some patients with AAV who experienced relapses. It indicated that the chronic repeated antigen stimulation was not removed, which may be the reason for recurrent relapses.

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