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CLINICAL RESEARCH STUDY

# ANCA Are Detectable in Nearly All Patients with Active Severe Wegener's Granulomatosis

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## ABSTRACT

**BACKGROUND:** The pathogenic significance of antineutrophilic cytoplasmic antibodies (ANCA) in Wegener's granulomatosis is controversial. Their presence is influenced by the extent, severity, and activity of the disease at the time of sampling. The objective of this study was to determine the frequency of ANCA in patients with active Wegener's granulomatosis and to assess the influence of disease severity on test results.

**METHODS:** Baseline serum samples from the 180 participants in a multicentric prospective trial were tested for ANCA by indirect immunofluorescence, direct enzyme-linked immunosorbent assay (ELISA), and capture ELISA. Disease activity was measured using the Birmingham Vasculitis Activity Score for Wegener's granulomatosis. All patients had active disease at enrollment. Patients were categorized as having severe (n = 128) or limited (n = 52) Wegener's granulomatosis.

**RESULTS:** When all ANCA detection methods were combined, 166 patients (92%) were ANCA positive, including 96% with severe disease and 83% with limited disease.

**CONCLUSION:** ANCA are detectable in nearly all patients with active severe Wegener's granulomatosis, but approximately 1 of 5 patients with active limited disease are ANCA negative. Immunofluorescence and both direct and capture ELISAs are required for optimal detection, suggesting that ANCA are not recognized equally well by all testing methods. © 2007 Elsevier Inc. All rights reserved.

**KEYWORDS:** Antineutrophil cytoplasmic antibodies; Enzyme-linked immunosorbent assay; Fluorescent antibody technique; Wegener's granulomatosis

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Testing for antineutrophilic cytoplasmic antibody (ANCA) has become an essential part of the diagnostic evaluation of patients with small-vessel vasculitis, particularly Wegener's granulomatosis and microscopic polyangiitis,<sup>1,2</sup> yet the optimal means of applying these assays in the clinical setting remains unclear. In addition, whether ANCA are integral to the disease manifestations of Wegener's granulomatosis remains central to the debate about both disease nomenclature and the potential pathogenicity of ANCA.<sup>3</sup>

The reported sensitivity of ANCA for patients with Wegener's granulomatosis varies widely, ranging from 50% to 95%.<sup>1,4</sup> Many factors may contribute to this variation, including the disease extent, severity, and activity at the time of sampling;<sup>5</sup> the ANCA detection method used (immunofluorescence, direct or capture enzyme-linked immunosorbent assay [ELISA]); and whether the testing is done in a central specialized laboratory or with commercial assays known to have a high degree of interassay variability.<sup>6-8</sup>

Only a handful of large studies have evaluated ANCA in cohorts of patients with active disease and different degrees of disease activity. The largest study addressing this issue precedes the availability of antigen-specific ANCA detection methods.<sup>5</sup> Therefore, the aims of this study were to determine the frequency of ANCA in the largest cohort of patients with active Wegener's granulomatosis reported to date and to investigate the impact of disease severity on ANCA testing by using several validated assays under standardized conditions.<sup>9-12</sup>

## METHODS

### Patients and Serum Samples

Samples used in this study were obtained from the Wegener's Granulomatosis Etanercept Trial (WGET).<sup>13</sup> The protocol for WGET was approved by the institutional review board at each participating center. Informed written consent was obtained from all participants. Full details of the study design have been published elsewhere.<sup>14</sup> All patients met at least 2 of the 5 modified criteria of the American College of Rheumatology for the classification of Wegener's granulomatosis<sup>14</sup> and had active disease within 28 days before enrollment, defined by a Birmingham Vasculitis Activity Score for Wegener's granulomatosis (BVAS/WG) of at least 3. For the purpose of assigning standard therapies, patients were classified as having "severe" or "limited" Wegener's granulomatosis.<sup>14,15</sup> Severe disease denoted a condition that threatened the patient's life or the function of a vital organ (eg, rapidly progressive glomerulonephritis, alveolar hemor-

rhage, and mononeuritis multiplex, all of which are caused primarily by capillaritis). Limited disease did not pose such threats (eg, nose and sinus inflammation, lung nodules, or arthralgias/arthritis). Patients with severe disease were treated at enrollment with cyclophosphamide and glucocorticoids, and those with limited disease received methotrexate and glucocorticoids.<sup>14</sup> Disease activity was measured using the BVAS/WG, a scoring system adapted specifically for Wegener's granulomatosis and validated by the investigators before the trial.<sup>16</sup> Baseline serum samples were frozen and stored at  $-80^{\circ}\text{C}$ .

## CLINICAL SIGNIFICANCE

- Almost all patients with active severe Wegener's granulomatosis have ANCA.
- A combination of immunofluorescence, direct ELISA, and capture ELISA is required to ensure optimal detection of ANCA.

## Antineutrophilic

### Cytoplasmic Antibodies Detection Methods

Standard indirect immunofluorescence was performed using ethanol-fixed neutrophils as previously described.<sup>11</sup> Samples were categorized as C-ANCA positive if the characteristic centrally accentuated granular cytoplasmic staining pattern was detectable and as P-ANCA positive if there was a perinuclear or nuclear staining pattern. Direct ELISAs for PR3-ANCA (ANCA against proteinase-3) and MPO-ANCA (ANCA against myeloperoxidase) were performed with commercially available kits (Scimedx, Corporation, Denville, NJ) according to the manufacturer's instructions. Two different capture ELISAs developed and validated in our laboratory also were used for PR3-ANCA detection: the mature-PR3 and pro-PR3 capture ELISAs, and the recently described anti-myc capture ELISA.<sup>9,10,12</sup>

### Statistical Methods

All serum samples were tested at first thaw by all ANCA detection methods. Patients were grouped by disease severity at baseline and by subsets based on whether their baseline BVAS/WG score was in the upper or lower 50th percentile. Descriptive data were summarized as mean (standard deviation), median (interquartile range), or percentages. The Fisher exact test was used to compare categorical variables, with statistical significance taken at the level of  $P$  less than .05. Statistical analyses were performed using StatView 5.0 for Macintosh (SAS Institute, Cary, NC).

## RESULTS

A description of baseline characteristics of the 180 patients constituting the WGET cohort has been published elsewhere.<sup>15</sup> The mean (standard deviation) age was 47 (16) years, there were 108 (60%) men, and the majority

**Table 1** Antineutrophilic Cytoplasmic Antibodies Test Results in the 180 Wegener's Granulomatosis Etanercept Trial Participants Stratified by Disease Severity

	Severe WG				Limited WG			
	All Patients	BVAS/WG Percentile		P	All Patients	BVAS/WG Percentile		P
		≤50%	>50%			≤50%	>50%	
N	128	59	69		52	35	17	
BVAS/WG, median (IQR)	7 (5-10)	5 (4-6)	10 (8-12)		4 (3-5)	4 (3-4)	6 (5-8)	
C-ANCA, n (%)	100 (78)	43 (73)	57 (83)	.2041	36 (69)	24 (69)	12 (71)	1.0000
P-ANCA, n (%)	18 (14)	10 (17)	8 (12)	.4493	6 (12)	4 (11)	2 (12)	1.0000
PR3 direct ELISA, n (%)	97 (76)	38 (64)	59 (86)	<b>.0070</b>	36 (69)	22 (63)	14 (82)	.2075
MPO direct ELISA, n (%)	7 (5)	2 (3)	5 (7)	.4504	2 (4)	1 (3)	1 (6)	1.0000
Mature-PR3 capture ELISA, n (%)	100 (78)	41 (69)	59 (86)	<b>.0336</b>	36 (69)	23 (66)	13 (76)	.5316
Pro-PR3 capture ELISA, n (%)	90 (70)	37 (63)	53 (77)	.1200	32 (62)	19 (54)	13 (76)	.1432
Anti-c-myc capture ELISA, n (%)	108 (84)	45 (76)	63 (91)	<b>.0272</b>	40 (77)	26 (74)	14 (82)	.7286
All combined, n (%)	123 (96)	54 (92)*	69 (100)	<b>.0189</b>	43 (83)	29 (83)	14 (82)	1.0000

ANCA = antineutrophilic cytoplasmic antibodies; BVAS/WG = Birmingham Vasculitis Activity Score for Wegener's Granulomatosis; MPO = myeloperoxidase; PR3 = proteinase 3; ELISA = enzyme-linked immunosorbent assay; IQR = interquartile range; WG = Wegener's granulomatosis.

\*Five patients with severe Wegener's granulomatosis yielded negative results in all of the ANCA tests.

(92%) were white. Eighty patients (44%) had newly diagnosed Wegener's granulomatosis. The median (interquartile range) time since the onset of symptoms was 17 (5-50) months and since diagnosis was 5 (1-36) months. The median (interquartile range) BVAS/WG at entry was 6 (4-9).

At baseline, 160 patients (89%) were ANCA positive by immunofluorescence (136 [76%] for C-ANCA and 24 [13%] for P-ANCA). A total of 133 patients (74%) had PR3-ANCA detected by direct ELISA, and 9 patients (5%) had MPO-ANCA. With the capture ELISAs, 136 patients (76%) tested positive for PR3-ANCA reacting with mature-PR3, and 122 patients (68%) tested positive for PR3-ANCA reacting with pro-PR3. With the use of the anti-c-myc capture ELISA, 148 patients (82%) were found to have PR3-ANCA. When all these tests were combined, 166 patients (92%) had detectable ANCA.

Among the 180 patients, 128 (71%) had severe Wegener's granulomatosis and 52 (29%) had limited Wegener's granulomatosis. The results of all the ANCA detection methods are presented in Table 1. When all the tests were combined, 96% and 83% of the patients with severe and limited disease, respectively, were found to have ANCA. All patients with severe disease in the upper 50th percentile of the baseline BVAS/WG tested positive for ANCA.

Five patients with severe disease in the lower 50th percentile had negative ANCA test results by all methods applied. The clinical characteristics of these 5 patients are summarized in Table 2. Three of these patients had clinical features that are predominantly associated with necrotizing granulomatous inflammation rather than capillaritis. The other 2 patients had items of disease activity scored in the BVAS/WG that are usually attributed to capillaritis. Both of

these patients had been treated with azathioprine for more than 1 year before enrollment.

## DISCUSSION

The results of this study indicate that when a multimodality ANCA testing approach is used, nearly all patients with active severe Wegener's granulomatosis are ANCA positive. In those with active limited disease, ANCA was found in 83%. This study is unique in several respects. First, the WGET cohort is the largest cohort of patients with Wegener's granulomatosis evaluated in a protocolized fashion. Second, disease activity was recorded with a standardized, validated instrument in all patients.<sup>13,16</sup> Third, all patients had active disease at baseline with a BVAS/WG of at least 3. Fourth, the study samples were subjected to only 1 freeze-thaw cycle and were analyzed in batch form, minimizing any potential effect of the serum handling and storage on the assay results. Finally, all 3 principal ANCA detection methods were used: indirect immunofluorescence, direct ELISA, and capture ELISA.

Our findings are of particular interest with respect to the ongoing controversy about the pathogenic role of ANCA in small-vessel vasculitis, including Wegener's granulomatosis.<sup>3,17</sup> There is substantial evidence from in vitro and in vivo animal model studies supporting a pathogenic role of ANCA. The proinflammatory effects of human PR3- and MPO-ANCA are well documented,<sup>17</sup> and antibodies against MPO have been shown to cause pauci-immune small-vessel vasculitis in rodents.<sup>18,19</sup> However, the most common argument against a pathogenic role of ANCA is that a substantial proportion of patients with active Wegener's granulomatosis or other forms of "ANCA-associated vasculitis" are actually

**Table 2** Clinical Characteristics of the 5 Patients with Active Severe Wegener's Granulomatosis at Baseline and Consistently Negative Antineutrophilic Cytoplasmic Antibodies

Patients		57	69	31	40	39
Age (y)		57	69	31	40	39
Gender		Male	Male	Female	Male	Female
Diagnosis		Old	Old	Old	Old	New
Time since diagnosis (mo)		40	37	7	5	1
Creatinine (mg/dL)		1.5	0.9	0.8	-	0.5
Sedimentation rate (mm)		101	20	34	-	3
BVAS/WG items		6	5	4	6	5
		<ul style="list-style-type: none"> <li>● Arthralgias/itis</li> <li>● Fever</li> <li>● Other infiltrates secondary to WG</li> <li>● Increase in Cr &gt; 30% or decrease in Cr Cl &gt; 25%</li> </ul>	<ul style="list-style-type: none"> <li>● Arthralgias/itis</li> <li>● Bloody nasal discharge/nasal crusting/ulcer</li> <li>● Alveolar hemorrhage</li> </ul>	<ul style="list-style-type: none"> <li>● Bloody nasal discharge/nasal crusting/ulcer</li> <li>● Sinus involvement</li> <li>● Pleurisy</li> <li>● Severe anorexia and fatigue</li> </ul>	<ul style="list-style-type: none"> <li>● Arthralgias/itis</li> <li>● Fever</li> <li>● Bloody nasal discharge/nasal crusting/ulcer</li> <li>● Sinus involvement</li> <li>● Pleurisy</li> <li>● Nodules or cavities</li> </ul>	<ul style="list-style-type: none"> <li>● Arthralgias/itis</li> <li>● Retro orbital mass/proptosis</li> <li>● Bloody nasal discharge/nasal crusting/ulcer</li> <li>● Sinus involvement</li> <li>● Nodules or cavities</li> </ul>

Cr Cl = creatinine clearance; BVAS/WG = Birmingham Vasculitis Activity Score for Wegener's granulomatosis.<sup>16</sup>

ANCA negative.<sup>3</sup> Our findings indicate that ANCA can be detected in nearly all patients with active severe Wegener's granulomatosis. Although our study does not prove that ANCA causes severe Wegener's granulomatosis or any disease manifestations resulting primarily from capillaritis, the nearly complete overlap of ANCA with severe systemic manifestations of Wegener's granulomatosis is important to note.

Five patients with severe disease had no detectable ANCA at baseline. These 5 apparent exceptions are best explained by the definitions of disease severity used in the WGET. The categorization of patients as having severe or limited disease was not based on biological features, but rather on the perception of the investigator that the patient's clinical scenario required cyclophosphamide.<sup>14,16,20</sup> Thus, a patient with indolent clinical features that did not pose an immediate threat to organ function could have been classified as "severe" if the disease activity persisted or recurred on maximal remission maintenance medication (eg, methotrexate or azathioprine). For example, a patient with pulmonary nodules who did not respond satisfactorily to glucocorticoids and methotrexate could have been started on cyclophosphamide and classified as "severe," despite the absence of an immediate threat to life or vital organ function.

Our study has several limitations. A bias toward ANCA positivity cannot be excluded because the presence of PR3-ANCA was 1 of the 5 modified American College of Rheumatology criteria used to diagnose Wegener's granulomatosis for enrollment in our study. However, a positive ANCA was not an inclusion requirement, and there were only 15 patients in whom a positive ANCA was the defining inclusion criteria. Furthermore, all of the analyses were repeated excluding these patients, and the results remained unchanged (data not shown). Second, little information about MPO-ANCA in Wegener's granulomatosis can be derived from this study, primarily because of the small number of patients positive for P-ANCA or MPO-ANCA. Finally, it is important to emphasize that the extensive ANCA testing approach used in this study is rarely used in clinical practice, where indirect immunofluorescence combined or not with direct ELISA is routinely used. This may lead to an underestimation of the prevalence of ANCA, and a negative ANCA result (when only a single test is performed) should be interpreted with caution in patients with typical features of active Wegener's granulomatosis.

## CONCLUSION

Our study demonstrates that almost all patients with active severe Wegener's granulomatosis have ANCA. A combination of immunofluorescence, direct ELISA, and capture ELISA seems to be required to ensure optimal detection of these antibodies, suggesting that ANCA are a heterogeneous group of antibodies that are not recognized equally well by all testing methods.

**APPENDIX**

## The Wegener's Granulomatosis Etanercept Trial Research Group

WGET Chairman	John H. Stone, MD, MPH (The Johns Hopkins Vasculitis Center)
WGET Co-Chairman	Gary S. Hoffman, MD (The Cleveland Clinic Foundation Center for Vasculitis Research and Care)
Coordinating Center	The Johns Hopkins University Center for Clinical Trials: Janet T. Holbrook, PhD, MPH, Director Curtis L. Meinert, PhD, Associate Director John Dodge, Systems Analyst Jessica Donithan, Research Coordinator Nancy Min, PhD, Biostatistician Laurel Murrow, MSc, Trial Coordinator (former) Jacki Smith, Research Data Assistant Andrea K. Tibbs, BS, Trial Coordinator Mark Van Natta, MHS, Biostatistician
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Data and Safety	Paul L. Canner, PhD, Maryland Medical Research Institute
Monitoring Board	Doyt L. Conn, MD, Emory University (Safety Officer) Jack H. Klippel, MD, Arthritis Foundation (Chair) J. Richard Landis, PhD, University of Pennsylvania

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