

# Relation between Asymptomatic Proteinase 3 Antibodies and Future Granulomatosis with Polyangiitis

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## Summary

**Background and objectives** The subclinical pathogenesis of granulomatosis with polyangiitis (GPA) has not been completely elucidated. Proteinase 3 (PR3) antibodies are strongly associated with GPA, but have not been evaluated before disease presentation.

**Design, setting, participants, & measurements** This was a retrospective case-control serum bank study in which PR3 antibodies and C-reactive protein (CRP) in up to three longitudinal serum samples for 27 GPA patients before diagnosis (1 day–19 years) were compared with 27 controls whose serum samples were matched for age, sex, and race. This study analyzed all patients with American College of Rheumatology criteria–confirmed disease identified in the Department of Defense electronic medical records between 1990 and 2008.

**Results** A greater percentage of GPA patients had at least one elevated PR3 antibody level ( $\geq 6$  U/ml) as well as at least one detectable PR3 antibody level ( $>1$  U/ml) before diagnosis compared with matching controls (63% [17 of 27] versus 0% [0 of 27],  $P < 0.001$ ; and 85% [23 of 27] versus 4% [1 of 27],  $P < 0.001$ , respectively). A greater percentage of GPA patients had a  $>1$  U/ml per year rate of increase in PR3 antibody level compared with matching controls (62% [21 of 26] versus 0% [0 of 26],  $P < 0.001$ ). PR3 antibody more frequently became elevated before CRP (67% [12 of 18] versus 33% [6 of 18],  $P = 0.04$ ).

**Conclusions** Subclinical PR3 antibody presence, trajectory, and temporal relationship to CRP associates with the future diagnosis of GPA. This data set further elucidates the pathogenesis of GPA.

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## Introduction

The pathophysiology of granulomatosis with polyangiitis (GPA) has become better understood over the past 3 decades (1–4). Proteinase-3 antibodies (PR3-ANCA) are associated with GPA at diagnosis. However, the exact role of PR3-ANCA in the increasingly complex pathophysiology of GPA is less clearly defined (5–7). Animal, *in vitro*, and clinical evidence support direct PR3-ANCA pathogenicity. PR3-ANCA was recently shown to induce vasculitis in a rat model (8). PR3-ANCA stimulates neutrophils *in vitro* to both release inflammatory mediators and to damage endothelial cells (1,3,9,10). Some studies showed that an increase in remission PR3-ANCA levels associates with future relapse (11). However, PR3-ANCA could simply be a passive marker or just one of multiple culprits of disease. Healthy controls with detectable PR3-ANCA, patients with “seronegative” GPA, and patients with persistent asymptomatic PR3-ANCA in post-therapeutic clinical remission have all been reported (7,11,12).

*In vivo* production of PR3-ANCA before GPA diagnosis has not been previously investigated. C-reactive protein (CRP), previously shown to be associated with GPA at diagnosis, has also not been

evaluated before disease (13,14). More importantly, the timing of PR3-ANCA production has not been compared with the timing of CRP elevation, with CRP elevation functioning as a nonspecific surrogate for early asymptomatic subclinical disease. Our objective was to describe the prediagnostic trajectory and temporal relationship of PR3-ANCA and CRP using the Department of Defense serum repository (DoDSR). We hypothesized that PR3-ANCA precedes both clinical and subclinical evidence of GPA, thus supporting the direct contribution of PR3-ANCA to GPA pathogenicity.

## Materials and Methods

### Patients

We performed a retrospective matched case-control DoDSR study of 27 patients with GPA disease. The DoDSR contains  $>50$  million military serum samples banked from biennial HIV and deployment screenings. Specimens are linked to demographic, occupational, and medical information. The index sample is banked at the time of entry into the military when recruits are cleared for service with a standardized medical examination.

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We identified 58 patients initially by International Classification of Diseases, Ninth Revision (ICD-9) code 446.40 (“Wegener’s Granulomatosis”) in the military electronic medical record and DoDSR databases between January 1990 and October 2008. Twenty-five patients had sufficient electronic medical records to confirm GPA diagnosis by meeting at least two of four American College of Rheumatology (ACR) criteria (15). One patient was excluded due to myeloperoxidase (MPO)-ANCA predominant disease. Six additional patients did not have serum in the DoDSR. A total of 18 patients with accessible electronic medical records remained. There were 33 patients identified in the DoDSR that did not have electronic medical records for review. These patients could have been diagnosed by civilian subspecialists if they were not located near a major military medical center and still had banked serum. However, it is possible that these patients were erroneously coded during an ultimately negative diagnostic evaluation. In addition to an ICD-9 code for GPA, patients were required to meet “modified” ACR criteria by having at least two additional systemic ICD-9 codes for pulmonary hemorrhage (786.3 “Hemoptysis” or “Pulmonary Hemorrhage”), renal involvement (580–589), or sinus involvement (461.9 and 473.9) to maximize patient specificity. Only 9 of 33 patients met these criteria, resulting in a combined total of 27 study participants.

The DoDSR provided a maximum of three 0.5-ml serum samples per patient to include the earliest index, the second to last, and the last samples before GPA diagnosis. In addition, the DoDSR identified one healthy control for each study participant matched for age (within 1 year), sex, race, and age of serum sample (within 90 days). Healthy controls were defined by the absence of ICD-9 codes for any chronic infectious, inflammatory, or malignant disease process in the DoDSR database.

### Laboratory Assays

The DoDSR sent the serum samples to Quest Diagnostics Nichols Institute (Chantilly, VA). PR3-ANCA assays were performed with Varelisa PR3 ANCA enzyme immunoassay kits (Phadia GmbH, Freiburg, Germany). Briefly, 100  $\mu$ l of diluted patient serum (1:101) was dispensed into wells coated with human PR3 antigen and prepared with wash buffer. After 30 minutes of incubation, the serum was removed and the wells were washed 3 times with wash buffer. This process was repeated with 100  $\mu$ l of the enzyme-labeled second antibody (conjugate) followed by 100  $\mu$ l of substrate 3,3',5,5'-tetramethylbenzidine (TMB) (incubated in the dark). After removal of the substrate TMB, 50  $\mu$ l of stop solution was added to the well. After no more than 30 minutes, absorbance (OD) was read at 450 nm with a reference wavelength of 620 nm. An individual calibration was performed for each run. The mean and median results of 432 apparently healthy participants were 0.7 U/ml and 0.6 U/ml, respectively. The average intra-assay and interassay variability were 5.5% and 5.0%, respectively (16). Clinical assays for CRP were performed with the COBA Integra C-Reactive Protein (Latex) cassette on a COBAS INTEGRA analyzer (Test CRPL2, 0-293; Roche Diagnostics, Indianapolis, IN). Quest Diagnostics was blinded as to whether the samples were from patients or controls. PR3-ANCA levels  $\geq$ 6 U/ml and CRP levels  $>$ 0.8 mg/dl were elevated.

### Statistical Analyses

The two-tailed Fisher’s exact test was used to compare all categorical values, specifically the number of study participants versus the number of control participants above various threshold PR3-ANCA values. Thresholds of  $>$ 0,  $>$ 1,  $>$ 2, and  $\geq$ 6 U/ml for PR3-ANCA were used to compare GPA patients with matched healthy controls at  $<$ 1 year, 1–5 years,  $>$ 5 years, and all time periods before diagnosis. Change in PR3-ANCA over time was analyzed using thresholds of  $>$ 0 U/ml,  $>$ 1 U/ml, and  $>$ 2 U/ml per year, as well as 0%, 20%, and 200% per year. Absolute PR3-ANCA change per year was calculated by dividing the difference between last PR3-ANCA (PR3-ANCA-L) minus the index PR3-ANCA (PR3-ANCA-I) by the difference in days (T) between the two samples (TL – TI) and multiplying the total by 365 days per year, as follows: (PR3-ANCA-L – PR3-ANCA-I)/(TL – TI)  $\times$  365. Percentage change per year was calculated by dividing PR3-ANCA-L by PR3-ANCA-I, subtracting 1, and then multiplying by 100. This value was then divided by difference in days between the two samples (TL – TI) and multiplied by 365 days per year ((PR3-ANCA-L/PR3-ANCA-I)  $\times$  100/TL – TI)  $\times$  365. Infinite odds ratio values were estimated by adding 1 to the numerator (if 0 controls were positive) or denominator (if all study participants were positive) of both the disease and control groups.

This study was approved by the Human Use Committee at Walter Reed National Military Medical Center and informed consent was waived.

## Results

### Demographics

The GPA patients consisted of predominantly Caucasian men aged  $<$ 40 years. Pulmonary and renal involvement was most common (Table 1).

### PR3 Antibody

A greater percentage of GPA patients had at least one elevated PR3-ANCA ( $\geq$ 6 U/ml) compared with matching controls at any time before diagnosis (63% [17 of 27] versus 0% [0 of 27];  $P<$ 0.001) and  $<$ 1 year before diagnosis (74% [14 of 19] versus 0% [0 of 19],  $P<$ 0.001) (Table 2).

In addition, a greater percentage of GPA patients had at least one PR3-ANCA level above the threshold of 1 U/ml versus matching controls at any time before diagnosis (85% [23 of 27] versus 4% [1 of 27],  $P<$ 0.001),  $<$ 1 year before diagnosis (79% [15 of 19] versus 0% [0 of 19],  $P<$ 0.001), and 1–5 years before diagnosis (69% [9 of 13] versus 0% [0 of 13],  $P<$ 0.001) (Table 2).

### Time Course of Antibody Development

A greater percentage of GPA patients had a rate of increase in PR3-ANCA of  $>$ 1 U/ml per year compared with matching controls (62% versus 0%,  $P<$ 0.001) (Table 3). In addition, a greater percentage of GPA patients had a rate of increase in PR3-ANCA of  $>$ 20% per year compared with matching controls (77% versus 4%,  $P<$ 0.001) (Table 3).

The mean and median number of days between the first elevation of PR3-ANCA and diagnosis of GPA were 269 and 239 days, respectively. Our results showed that 96%

| Table 1. GPA study cohort  |                                   |
|--|-----------------------------------|
| Characteristic   | Value                             |
| Age (yr)   | 33 (18–60)                        |
| Race   |                                   |
| Caucasian  | 78                                |
| African American   | 7                                 |
| Other  | 15                                |
| Meets ACR criteria for diagnosis   | 100 (27/27)                       |
| Male sex   | 89 (24/27)                        |
| Sinus involvement  | 48 (13/27)                        |
| Pulmonary involvement  | 82 (22/27)                        |
| Hemoptysis/respiratory failure   | 37 (10/27)                        |
| Renal involvement  | 85 (23/27)                        |
| ARF  | 33 (9/27)                         |
| Preceding CKD  | 0                                 |
| Hypertension (chronic)   | 41 (11/27)                        |
| Biopsy evidence of vasculitis (total)  | 66 (12/18)                        |
| Kidney   | 22 (4/18)                         |
| Lung   | 33 (6/18)                         |
| Sinus  | 11 (2/18)                         |
| Skin   | 28 (5/18)                         |
| PR3-ANCA level (U/ml) (n=9)  | 61 (10–113); 1:640 (1:160–1:1280) |
| C-reactive protein (n=7)   | 7.8 (2.1–19.4)                    |
| Therapeutic intervention (cyclophosphamide, steroids, rituximab, methotrexate, azathioprine)   | 100 (18/18)                       |
| Abnormal chest CT scan or radiograph   | 92 (11/12)                        |
| Abnormal sinus CT scan   | 80 (4/5)                          |
| Abnormal chest or sinus imaging scan   | 100 (12/12)                       |
| Concurrent anti-GBM disease  | 0                                 |
| Other antibody (ANA, dsDNA, Smith, RNP, Ro, La, SCL-70, Jo-1, GBM antibodies)  | 0 (0/8)                           |
| Data are presented as the median (range) or percentage (proportion). Comorbidity percentages may be underestimated due to incomplete background information from patients diagnosed at civilian hospitals. International Classification of Diseases, Ninth Revision codes cannot fully compensate for this limitation, because sometimes the final diagnosis is the only code listed when billing outside the military is not required. GPA, granulomatosis with polyangiitis; ACR, American College of Rheumatology; PR3, proteinase-3; CT, computed tomography; GBM, glomerular basement membrane; ANA, antinuclear antibody; dsDNA, double-stranded deoxyribonucleic acid; RNP, ribonucleoprotein; SCL-70, topoisomerase I. |                                   |

of patients with an elevated PR3-ANCA became elevated <1.5 years before diagnosis, with one outlier at 932 days.

#### Time Course of Increase in Antibody versus CRP

Of GPA patients with a clear antecedent event, 67% had an elevated PR3-ANCA before an elevated CRP (Table 4). The most extreme example was a patient with a PR3-ANCA of 148 U/ml and a CRP of 0.05 mg/dl 241 days before diagnosis. Thirty-three percent of disease patients had an elevated CRP before an elevated PR3-ANCA (Table 4). The

most extreme example was a patient with a PR3-ANCA of 1 U/ml and a CRP of 20.1 mg/dl 195 days before diagnosis.

#### Discussion

Our description of the natural history of PR3-ANCA before the diagnosis of GPA is consistent with the previously proposed complex pathophysiology of GPA (1–7). Specifically, our results support direct PR3 antibody pathogenicity in a subset of GPA patients. PR3-ANCA was elevated in 63% of disease patients predominantly <1.5 years before GPA diagnosis. Elevated prediagnostic PR3-ANCA levels with concurrent normal CRP levels in 67% of GPA patients suggest that PR3-ANCA levels cannot only elevate before overt clinical disease, but also before even subclinical serologic evidence of systemic inflammatory disease. Although there is no previous description of PR3-ANCA before GPA diagnosis, there are reports that PR3-ANCA precedes 33%–81% of postremission GPA relapses (17–20). CRP is known to be elevated before GPA relapse (21), but there has been no previous evaluation of CRP or the temporal relationship between CRP and PR3-ANCA elevation before initial diagnosis. Passive elevation of PR3-ANCA after another neutrophil activating event and before CRP elevation cannot be ruled out.

There are many explanations for why only 63% of GPA patients had elevated PR3-ANCA before diagnosis. First, the 10 study participants without an elevated PR3-ANCA could have developed elevated PR3-ANCA during the average 1038 days between the last serum sample and diagnosis. The maximum time between last sample and diagnosis was 4234 days, with three patients' last sample >6 years before diagnosis. Second, previously high PR3-ANCA could have degraded to normal levels during storage. Third, PR3-ANCA and alternate neutrophil activating mechanisms could both separately initiate GPA. Thirty-three percent of GPA patients first manifest an elevated CRP before an elevated PR3-ANCA supporting an alternate neutrophil activating mechanism. Elevated antibodies (cPR3, plasminogen, lysosomal-associated membrane protein 2, cathepsin G, lactoferrin, azurocidin, and elastase), elevated cytokines (TNF- $\alpha$ , IL-17, and IL-23), reduced regulatory T cell to effective T cell ratio, alterations in CD8+ T cell transcription, dysfunctional neutrophil apoptosis, and disrupted epigenetic silencing are all possible contributors to internal neutrophil activation (1,3,5,7,22). Environmental exposures (silica and asbestos), pathogens (*Staphylococcus aureus*, Ross virus, and *Entamoeba histolytica*), or medications (propylthiouracil and hydralazine) could all provide an external stimulus for neutrophil activation (2,3,23–28).

Our novel findings that GPA patients have higher levels of PR3-ANCA within the normal range than healthy controls are consistent with previously proposed multiple hit mechanisms of disease (1–7). The PR3-ANCA threshold of  $\geq 6$  U/ml was established based on the evaluation of patients with active clinical disease. It is possible that abnormal PR3-ANCA levels in the preclinical setting may occur in the currently accepted normal range. We previously demonstrated that a significantly larger percentage of patients with anti-glomerular basement membrane

**Table 2. Comparison of the percentage of study patients with at least one serum sample with PR3-ANCA above the thresholds >0, >1, >2, and ≥6 U/ml compared with matched healthy controls**

| PR3 Antibody, U/ml (yr) | Patients (%) | Controls (%) | Odds Ratio        | 95% Confidence Interval   | P Value (Fisher's Exact Test) |
|-------------------------|--------------|--------------|-------------------|---------------------------|-------------------------------|
| >/6                     |              |              |                   |                           |                               |
| All                     | 63 (17/27)   | 0 (0/27)     | 48.6 <sup>a</sup> | 5.7 to 413 <sup>a</sup>   | <0.001                        |
| <1                      | 74 (14/19)   | 0 (0/19)     | 57 <sup>a</sup>   | 6.0 to 73 <sup>a</sup>    | <0.001                        |
| 1-5                     | 31 (4/13)    | 0 (0/13)     | 7.2 <sup>a</sup>  | 0.72 to 72.7 <sup>a</sup> | 0.1                           |
| >5                      | 0 (0/15)     | 0 (0/15)     | 1.0 <sup>a</sup>  | 0.1 to 17.5 <sup>a</sup>  | 1.0                           |
| > 2                     |              |              |                   |                           |                               |
| All                     | 78 (21/27)   | 0 (0/27)     | 99 <sup>a</sup>   | 11.1 to 885 <sup>a</sup>  | <0.001                        |
| <1                      | 79 (15/19)   | 0 (0/19)     | 76 <sup>a</sup>   | 7.7 to 751 <sup>a</sup>   | <0.001                        |
| 1-5                     | 39 (5/13)    | 0 (0/13)     | 9.8 <sup>a</sup>  | 1.0 to 97 <sup>a</sup>    | 0.03                          |
| >5                      | 13 (2/15)    | 0 (0/13)     | 3.5 <sup>a</sup>  | 0.3 to 37.5 <sup>a</sup>  | 0.48                          |
| >1                      |              |              |                   |                           |                               |
| All                     | 85 (23/27)   | 4 (1/27)     | 150               | 15.6 to 1436              | <0.001                        |
| <1                      | 79 (15/19)   | 0 (0/19)     | 76 <sup>a</sup>   | 7.7 to 751 <sup>a</sup>   | <0.001                        |
| 1-5                     | 69 (9/13)    | 0 (0/13)     | 32.5 <sup>a</sup> | 3.1 to 338 <sup>a</sup>   | <0.001                        |
| >5                      | 13 (2/15)    | 7 (1/15)     | 2.2               | 0.2 to 26.7               | 0.5                           |
| > 0                     |              |              |                   |                           |                               |
| All                     | 96 (26/27)   | 56 (15/27)   | 20.8              | 2.5 to 176                | <0.001                        |
| <1                      | 95 (18/19)   | 37 (7/19)    | 30.9              | 3.4 to 284                | <0.001                        |
| 1-5                     | 80 (10/13)   | 46 (6/13)    | 3.9               | 0.72 to 21.1              | 0.23                          |
| >5                      | 73 (11/15)   | 40 (6/15)    | 4.1               | 0.9 to 19.3               | 0.14                          |

Not all patients had samples available for each time period. If multiple serum samples were present for a patient in a specific subgroup analysis time period, the highest antibody level dictated group assignment. For example, the one control with PR3-ANCA >1 U/ml occurred in the >5-year time period. A higher percentage of patients had an elevated PR3-ANCA <1 year before diagnosis than over all time periods because the majority of patients without a banked sample a year before diagnosis did not have an elevated level. PR3, proteinase-3.

<sup>a</sup>Estimated due to actual infinite value.

**Table 3. A comparison of the absolute and percent PR3-ANCA increase per year in GPA patients versus matching healthy controls**

| PR3 Antibody Elevation | Patients (%) | Controls (%) | Odds Ratio        | 95% Confidence Interval | P Value (Fisher's Exact Test) |
|------------------------|--------------|--------------|-------------------|-------------------------|-------------------------------|
| U/ml per year          |              |              |                   |                         |                               |
| >0                     | 81 (21/26)   | 15 (8/26)    | 23.1              | 5.5 to 98               | <0.001                        |
| >1                     | 62 (16/26)   | 0 (0/26)     | 44.2 <sup>a</sup> | 5.2 to 377 <sup>a</sup> | <0.001                        |
| >2                     | 50 (13/26)   | 0 (0/26)     | 28 <sup>a</sup>   | 3.3 to 237 <sup>a</sup> | <0.001                        |
| >10                    | 27 (7/26)    | 0 (0/26)     | 11.0 <sup>a</sup> | 1.3 to 95 <sup>a</sup>  | 0.01                          |
| % per year             |              |              |                   |                         |                               |
| >0                     | 81 (21/26)   | 15 (4/26)    | 23.1              | 5.5 to 98               | <0.001                        |
| >20                    | 77 (20/26)   | 4 (1/26)     | 83                | 9.3 to 750              | <0.001                        |
| >200                   | 50 (13/26)   | 0 (0/26)     | 28 <sup>a</sup>   | 3.3 to 237 <sup>a</sup> | <0.001                        |
| >1000                  | 23 (6/26)    | 0 (0/26)     | 9.1 <sup>a</sup>  | 1.0 to 80 <sup>a</sup>  | 0.02                          |

Of the 27 study patients, 26 had multiple serum samples available for calculation. Calculations of change in percentage per year better adjust for patient variable serum sample time intervals before diagnosis. PR3, proteinase-3; GPA, granulomatosis with polyangiitis.

<sup>a</sup>Estimated due to actual infinite value.

disease have subclinical anti-glomerular basement membrane antibody above thresholds within the normal clinical range before diagnosis compared with matching controls (29). Neutrophil plasma membrane PR3 antigen burden is one explanation for higher baseline PR3-ANCA within the detectable but normal range. GPA patients have higher membrane PR3 antigen levels likely secondary to unique gene polymorphisms (30,31). Additional insults or

hits, to include, but not limited to, Silica or pathogen exposures, may increase production of previously low-level asymptomatic PR3-ANCA to manifest clinical disease (28).

Finally, we demonstrated that PR3-ANCA levels increase over time in a statistically significant number of disease patients versus healthy controls. Previous literature describes the percentage of GPA relapses preceded by increased PR3-ANCA production as well as the potential

**Table 4. Temporal relationship between surpassed PR3-ANCA thresholds and elevated CRP levels in GPA patients**

| PR3 Antibody (U/ml) | Before CRP<br>(>0.8 mg/dl) (%) | After CRP<br>(>0.8 mg/dl) (%) | Odds Ratio | 95% Confidence<br>Interval | P Value<br>(Fisher's Exact) |
|---------------------|--------------------------------|-------------------------------|------------|----------------------------|-----------------------------|
| >/6                 | 67 (12/18)                     | 33 (6/18)                     | 4.0        | 1.0 to 16                  | 0.04                        |
| >2                  | 80 (16/20)                     | 20 (4/20)                     | 16         | 3.4 to 75                  | <0.001                      |
| >1                  | 86 (18/21)                     | 14 (3/21)                     | 36         | 6.4 to 203                 | <0.001                      |
| >0                  | 96 (23/24)                     | 4 (1/24)                      | 529        | 31.2 to 8977               | <0.001                      |

Antecedent increase in PR3-ANCA or CRP was established for patients with a serum sample that had only one biomarker above the designated threshold value. If both became elevated in the same sample or if neither was elevated in any sample, there was no antecedent elevation determined. Because subclinical PR3-ANCA levels above the threshold of 0 U/ml, 1 U/ml, and 2 U/ml within the normal clinical range were associated with future GPA, we also compared the percentage of GPA patients with an antecedent antibody above these thresholds to the percentage of patients that first had an elevated CRP. PR3, proteinase-3; GPA, granulomatosis with polyangiitis; CRP, C-reactive protein.

increased relapse risk with an ANCA four times normal, but not the rate of PR3-ANCA increase before relapse or before initial diagnosis (17–19,32).

One explanation for the data set as a whole is that GPA patients have a baseline genetic disease propensity, or first hit, reflected by persistent subclinical PR3-ANCA production. Subsequent internal or external hit(s) can either pathologically activate neutrophils or PR3-ANCA production. Activated neutrophils/monocytes increase membrane display and/or release of PR3 antigen and cytokines. PR3-ANCA and cytokines can activate neutrophils. With this evidence, it is reasonable to conclude that there could be more than one entry point into a common deleterious feedback cycle (2–4,7).

The natural history of subclinical PR3-ANCA better defines GPA pathophysiology. However, it is not realistic to screen an entire population for a disease with a low pretest probability. However, future genetic and epigenetic analysis may define a subpopulation at increased risk for disease (33,34). Polymorphisms in the PR3 promoter region, Fc- $\gamma$  IIa and IIIa receptors, CD-18, C3, C4, IL-10, and the  $\alpha$ -1 antitrypsin genes were found to be associated with GPA (3,33–35). PR3-ANCA vasculitis was specifically shown to be associated with HLA-DP, SERPINA1, and PRTN3 (33). However, at-risk genotypes do not guarantee clinical disease. Subclinical PR3-ANCA trends described in our study could provide diagnostic synergy with future high-risk genomes, epigenetic profiles, or proteomes. Evaluation of the prediagnostic serum levels of cPR3-ANCA, matrix metalloproteinases (e.g., matrix metalloproteinase-3), CD8+ T cell transcript signatures, fibrinogen, and lysosomal-associated membrane protein 2 would help to even better define the subclinical pathophysiology of GPA (1,35). In addition, further evaluation of potential IgG subclass switching of PR3-ANCA would also have potential diagnostic value (36).

Our study has unique limitations. Although we erred on having overly specific criteria for confirmation of patients diagnosed *via* ICD-9 code, we cannot completely rule out the inclusion of a patient without GPA in the study group. This, in addition to degradation of antibody in stored serum, would only reduce the statistical significance of our findings by underestimating the number of study patients with detectable or elevated antibody. Although all patients

were assigned a diagnostic code for Wegener's granulomatosis, most patients did not have confirmatory granulomas noted on histopathology even when present for review. It is possible that the study cohort included patients of microscopic polyangiitis based on strict Chapel Hill classification criteria. As used previously, small vessel vasculitis would be a reasonable alternate description of the study cohort (28). Due to de-identification requirements, serum bank results could not be linked to specific patient diagnostic data even when present, which prevents more robust secondary and subgroup analyses. We were also not able to reliably compare the timing of antibody elevation to the earliest patient symptoms. Our GPA study cohort skewed younger and more male than GPA diagnosed in the general population, most likely because it was derived from a current or recent active duty military population. Due to the retrospective evaluation of the prospectively compiled serum samples, the time from last sample to diagnosis and the intervals between samples vary between study participants. Although time-matched controls compensate for general comparisons, this makes the evaluation of specific time periods before diagnosis more challenging because of limited power in some subgroups. In addition, percentage and unit-per-milliliter rate of change per year assumes a linear rate of PR3-ANCA increase over time before diagnosis. This has not been proven. CRP is nonspecific and the results could have been confounded by undocumented infection. However, an initial CRP elevation remained elevated before GPA diagnosis in 100% of patients. In addition, 95% of all patients demonstrated an increase in CRP from their index to last sample, even within the normal range. This suggests, but does not prove, that the CRP elevations were not due to isolated acute infections. The study cohort included only PR3-ANCA GPA patients. Omission of MPO-ANCA patients was thought to be appropriate because they have genetically distinct associations compared with PR3-ANCA patients and are postulated to represent a different autoimmune syndrome (33).

The DoDSR, in conjunction with the large heterogeneous military population, provides the only known resource to analyze the subclinical trajectory of PR3-ANCA and CRP before GPA diagnosis. It is possible that our data could be incorporated with future genomic, epigenetic, and

subclinical biomarker data to formulate predictive diagnostic models for GPA that would require prospective confirmation.

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#### Disclosures

None.

#### References

- Falk RJ, Jennette JC: ANCA disease: Where is this field heading? *J Am Soc Nephrol* 21: 745–752, 2010
- Jennette JC, Xiao H, Falk RJ: Pathogenesis of vascular inflammation by anti-neutrophil cytoplasmic antibodies. *J Am Soc Nephrol* 17: 1235–1242, 2006
- Bosch X, Guilabert A, Font J: Antineutrophil cytoplasmic antibodies. *Lancet* 368: 404–418, 2006
- Kallenberg CGM: Pathogenesis of PR3-ANCA associated vasculitis. *J Autoimmun* 30: 29–36, 2008
- Savage COS: Pathogenesis of anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis. *Clin Exp Immunol* 164 [Suppl 1]: 23–26, 2011
- Kallenberg CGM: Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis: Where to go? *Clin Exp Immunol* 164 [Suppl 1]: 1–3, 2011
- Jennette JC, Falk RJ: New insight into the pathogenesis of vasculitis associated with antineutrophil cytoplasmic autoantibodies. *Curr Opin Rheumatol* 20: 55–60, 2008
- Little MA, Al-Ani B, Ren S, Al-Nuaimi H, Leite M Jr, Alpers CE, Savage CO, Duffield JS: Anti-proteinase 3 anti-neutrophil cytoplasm autoantibodies recapitulate systemic vasculitis in mice with a humanized immune system. *PLoS ONE* 7: e28626, 2012
- Falk RJ, Terrell RS, Charles LA, Jennette JC: Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals *in vitro*. *Proc Natl Acad Sci U S A* 87: 4115–4119, 1990
- Franssen CF, Huitema MG, Muller Kobold AC, Oost-Kort WW, Limburg PC, Tiebosch A, Stegeman CA, Kallenberg CG, Tervaert JW: *In vitro* neutrophil activation by antibodies to proteinase 3 and myeloperoxidase from patients with crescentic glomerulonephritis. *J Am Soc Nephrol* 10: 1506–1515, 1999
- Tomasson G, Grayson PC, Mahr AD, Lavalley M, Merkel PA: Value of ANCA measurements during remission to predict a relapse of ANCA-associated vasculitis—a meta-analysis. *Rheumatology (Oxford)* 51: 100–109, 2012
- Hagen EC, Daha MR, Hermans J, Andrassy K, Csernok E, Gaskin G, Lesavre P, Lüdemann J, Rasmussen N, Sinico RA, Wiik A, van der Woude FJ: Diagnostic value of standardized assays for anti-neutrophil cytoplasmic antibodies in idiopathic systemic vasculitis. EC/BCR Project for ANCA Assay Standardization. *Kidney Int* 53: 743–753, 1998
- Hind CR, Winearls CG, Lockwood CM, Rees AJ, Pepys MB: Objective monitoring of activity in Wegener's granulomatosis by measurement of serum C-reactive protein concentration. *Clin Nephrol* 21: 341–345, 1984
- Grönhagen-Riska C, Teppo AM, Honkanen E, Ikäheimo R: Alpha-1-antitrypsin, CRP and interleukin-6 in ANCA-positive vasculitis. *Adv Exp Med Biol* 336: 337–340, 1993
- Leavitt RY, Fauci AS, Bloch DA, Michel BA, Hunder GG, Arend WP, Calabrese LH, Fries JF, Lie JT, Lightfoot RW Jr, Masi AT, McShane DJ, Mills JA, Stevens MB, Wallace SL, Zvaifler NJ: The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis. *Arthritis Rheum* 33: 1101–1107, 1990
- Varelisa PR3 ANCA enzyme immunoassay [package insert]. Freiburg, Germany, Phadia GmbH, 2008
- Finkelmann JD, Merkel PA, Schroeder D, Hoffman GS, Spiera R, St Clair EW, Davis JC Jr, McCune WJ, Lears AK, Ytterberg SR, Hummel AM, Viss MA, Peikert T, Stone JH, Specks U; WGET Research Group: Antiproteinase 3 antineutrophil cytoplasmic antibodies and disease activity in Wegener granulomatosis. *Ann Intern Med* 147: 611–619, 2007
- Kyndt X, Reumaux D, Bridoux F, Tributou B, Bataille P, Hachulla E, Hatron PY, Duthilleul P, Vanhille P: Serial measurements of antineutrophil cytoplasmic autoantibodies in patients with systemic vasculitis. *Am J Med* 106: 527–533, 1999
- Pettersson E, Heigl Z: Antineutrophil cytoplasmic antibody (cANCA and pANCA) titers in relation to disease activity in patients with necrotizing vasculitis: A longitudinal study. *Clin Nephrol* 37: 219–228, 1992
- Boomsma MM, Stegeman CA, van der Leij MJ, Oost W, Hermans J, Kallenberg CGM, Limburg PC, Tervaert JWC: Prediction of relapses in Wegener's granulomatosis by measurement of anti-neutrophil cytoplasmic antibody levels: A prospective study. *Arthritis Rheum* 43: 2025–2033, 2000
- Kälsch AI, Csernok E, Münch D, Birck R, Yard BA, Gross W, Kälsch T, Schmitt WH: Use of highly sensitive C-reactive protein for followup of Wegener's granulomatosis. *J Rheumatol* 37: 2319–2325, 2010
- Ciavatta D, Falk RJ: Epigenetics and complementary proteins. *Clin Exp Immunol* 164 [Suppl 1]: 17–19, 2011
- Morgan MD, Day CJ, Piper KP, Khan N, Harper L, Moss PA, Savage COS: Patients with Wegener's granulomatosis demonstrate a relative deficiency and functional impairment of T-regulatory cells. *Immunology* 130: 64–73, 2010
- Stegeman CA, Tervaert JW, Sluiter WJ, Manson WL, de Jong PE, Kallenberg CG: Association of chronic nasal carriage of *Staphylococcus aureus* and higher relapse rates in Wegener granulomatosis. *Ann Intern Med* 120: 12–17, 1994
- Pendergraft WF 3rd, Preston GA, Shah RR, Tropsha A, Carter CW Jr, Jennette JC, Falk RJ: Autoimmunity is triggered by cPR-3 (105-201), a protein complementary to human autoantigen proteinase-3. *Nat Med* 10: 72–79, 2004
- Hogan SL, Satterly KK, Dooley MA, Nachman PH, Jennette JC, Falk RJ: Glomerular Disease Collaborative Network: Silica exposure in anti-neutrophil cytoplasmic autoantibody-associated glomerulonephritis and lupus nephritis. *J Am Soc Nephrol* 12: 134–142, 2001
- Beaudreuil S, Lasfargues G, Lauériere L, El Ghoul Z, Fourquet F, Longuet C, Halimi JM, Nivet H, Büchler M: Occupational exposure in ANCA-positive patients: A case-control study. *Kidney Int* 67: 1961–1966, 2005
- Hogan SL, Cooper GS, Savitz DA, Nylander-French LA, Parks CG, Chin H, Jennette CE, Lionaki S, Jennette JC, Falk RJ: Association of silica exposure with anti-neutrophil cytoplasmic autoantibody small-vessel vasculitis: A population-based, case-control study. *Clin J Am Soc Nephrol* 2: 290–299, 2007
- Olson SW, Arbogast CB, Baker TP, Owshalimpur D, Oliver DK, Abbott KC, Yuan CM: Asymptomatic autoantibodies associate with future anti-glomerular basement membrane disease. *J Am Soc Nephrol* 22: 1946–1952, 2011
- Rarok AA, Stegeman CA, Limburg PC, Kallenberg CGM: Neutrophil membrane expression of proteinase 3 (PR3) is related to relapse in PR3-ANCA-associated vasculitis. *J Am Soc Nephrol* 13: 2232–2238, 2002
- Hu N, Westra J, Kallenberg CG: Membrane-bound proteinase 3 and its receptors: Relevance for the pathogenesis of Wegener's granulomatosis. *Autoimmun Rev* 8: 510–514, 2009
- Han WK, Choi HK, Roth RM, McCluskey RT, Niles JL: Serial ANCA titers: Useful tool for prevention of relapses in ANCA-associated vasculitis. *Kidney Int* 63: 1079–1085, 2003
- Lyons PA, Rayner TF, Trivedi S, Holle JU, Watts RA, Jayne DR, Baslund B, Brechley P, Bruchfeld A, Chaudhry AN, Cohen Tervaert JW, Deloukas P, Feighery C, Gross WL, Guillevin L, Gunnarsson I, Harper L, Hrušková Z, Little MA, Martorana D, Neumann T, Ohlsson S, Padmanabhan S, Pusey CD, Salama AD, Sanders JS, Savage CO, Segelmark M, Stegeman CA, Tesaf V, Vaglio A, Wiczorek S, Wilde B, Zwerina J, Rees AJ, Clayton DG, Smith KG: Genetically distinct subsets within ANCA-associated vasculitis. *N Engl J Med* 367: 214–223, 2012
- Monach PA, Merkel PA: Genetics of vasculitis. *Curr Opin Rheumatol* 22: 157–163, 2010

35. Monach PA, Tomasson G, Specks U, Stone JH, Cuthbertson D, Krischer J, Ding L, Fervenza FC, Fessler BJ, Hoffman GS, Ikle D, Kallenberg CG, Langford CA, Mueller M, Seo P, St Clair EW, Spiera R, Tchao N, Ytterberg SR, Gu YZ, Snyder RD, Merkel PA: Circulating markers of vascular injury and angiogenesis in anti-neutrophil cytoplasmic antibody-associated vasculitis. *Arthritis Rheum* 63: 3988–3997, 2011
36. Pankhurst T, Nash G, Williams J, Colman R, Hussain A, Savage C: Immunoglobulin subclass determines ability of immunoglobulin (Ig)G to capture and activate neutrophils presented as normal human IgG or disease-associated anti-neutrophil cytoplasm antibody (ANCA)-IgG. *Clin Exp Immunol* 164[Suppl 1]: 218–226, 2011

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