Supplemental Information

Inhibitory Anti-Peroxidasin antibodies in Pulmonary-Renal syndromes

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Expanded Methods

Cohort Acquisition

Vanderbilt Goodpasture Disease Cohort: The Goodpasture disease cohort at the time of active disease was collected and handled exactly as previously described [1]. Samples were either serum collected within the first 72 hours of hospitalization or the first available plasmapheresis fluid irrespective of replacement colloid (either albumin or fresh frozen plasma as dicted by patient care). . Samples were de-identified after collection, used and stored according to approved uses by Vanderbilt University Medical Center's Institutional Review and use Board.

Department of Defense Serum Repository Cohort: A unique cohort was obtained from the DoDSR essentially as previously described [2]. Briefly, to perform a matched-case control study on serial samples pre-dating the onset of clinical Goodpasture's disease, patients were identified from the military database with an International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM) code for (Goodpasture's disease) anti-GBM disease (446.0) between January 1990 and October 2008. Only current or previous active duty members were included. One inpatient or two separate outpatient GP codes were required for inclusion. Additional ICD-9 codes were queried and assembled into a de-identified database as a surrogate for chart review to obtain additional clinical course insight when possible. For each case identified, three separate age, gender, race, and age of serum-matched healthy controls (as determined by lack of ICD-9- coded disease) were pulled to generate the final cohort of 6 patients and 18 matched controls with four pre-diagnosis samples. The assembled samples (~500 µL serum per time point per patient) were then shipped to Vanderbilt where all additional experimentation was performed. Researchers at Vanderbilt (A.S.M) were blinded to specimen identity for the initial screen of peroxidasin, MPO, a3, and a5 collagen IV NC1 domains. The coded data was returned to S. Olson for case-control assignment as per the stipulations if the institutional review board of Walter Reed Army Medical Center.

UNC Vasculitis Cohorts: Patients were diagnosed according to the Chapel Hill Consensus Conference.[3] ANCA serotypes were determined by indirect immunofluorescence and antigen-specific PR3 and MPO ELISA. MPO status was recomfirmed at Vanderbilt and cross-validated against results from UNC to ensure internally consistant technique and methodology. Disease activity was determined by the 2003 Birmingham Vasculitis Activity Score (BVAS) in conjunction with clinical signs of activity.[4] Patients with biopsy-proven AAV enrolled in this study gave informed, written consent and participated according to University of North Carolina Institutional Review Board guidelines. Patient demographics were similar between healthy controls and AAV patients with regard to age, sex, and race.

Preparation of Antigens- Recombinant human peroxidasin (hPXDN) was expressed in HEK293 cells and purified as described previously [5,6]. Human Myeloperoxidase was purchased from Athens Research and Technology (Athens, GA), reconstituted, aliquotted, frozen in liquid nitrogen, and stored at -80^{o}C until use. Both peroxidasin and MPO were checked for activity upon thawing by using a Tetramethylbenzidine (TMB) peroxidase assay (1µg enzyme in 100 µL TMB-ELISA Substrate Solution [Thermo-Fischer Scientific]) in the presence of 3mM NaBr and compared to pre-freezing values

for each batch to ensure internal consistency. a3, and a5 collagen IV NC1 domains were recombinately expressed in HEK293 cells and purified exactly as described [1].

Detection of Antigens by ELISA-All assays were run in 384 well format with 50 µL volumes for coating, incubation with human sera, and development. 100 µL volumes were used for blocking and wash steps. Peroxidasin (4 nM- 2.5 µg/ml), MPO (4 nM- 0.8 µg/ml), a3(IV) , and a5(IV) NC1 were applied immediately following dilution of the antigen in TBS and coated overnight at 4^{o}C on polystyrene 384 well plates (Nunc). Wash steps were then performed on a BioTek ELx50 plate washer using TBS with 0.05% v/v Tween-20 . Plates were blocked with 1% bovine serum albumin (Fraction V-RIA grade-Sigma Aldrich, St. Louis, MO) in TBS for 1hr at 37^{o}C. Human sera or plasmapheresis fluid was then diluted as defined in each experiment (1:100 for primary screen, 1:500 for most subsequent experiments) in 0.1% BSA in TBS with 0.05% v/v Tween-20. After washing, secondary antibodies (Goat Anti-Human IgG (Fc specific)−Alkaline Phosphatase conjugate)(Sigma Aldrich, St. Louis, MO) was diluted 1:2000 in in 0.1% BSA in TBS with 0.05% v/v Tween-20 and applied for 1hr at 37^{o}C. After another wash, wells were developed using p-nitrophenyl phosphate (pNPP) as the AP substrate and the absorbance at 405nm was determined (SpectraMax Plus 384 Microplate Reader, Molecular Devices, Sunnyvale, CA). All samples run in duplicate. Initial screen of the DoD cohort run on the same day in four consecutive plates with control samples repeated across plates to insure internal consistency. Blank wells which were coated in BSA and received no primary antibody or sera were used as blanks.

Competition ELISA-Antigens were coated and plates blocked as described above. Sera were diluted 1:500 in 0.1% BSA in TBS with 0.05% v/v Tween-20 with varying amounts of identical MPO or peroxidasin and allowed to pre-incubate for 12hrs at 4^{o}C before application to plates for 1hr 10min at 37^{0}C before a double wash (6 total volume changes per well). Secondary antibody and development proceeded as previously detailed.

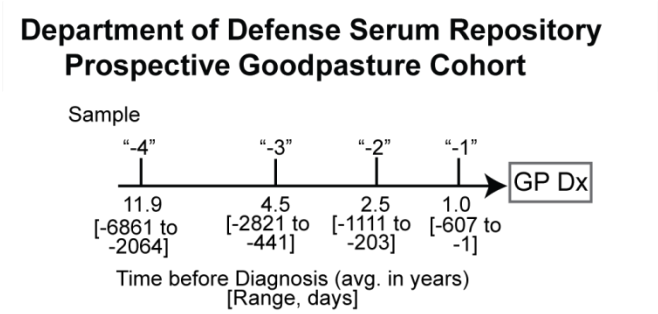
Antibody Partial Purification

Antibodies from patient sera were isolated using size exclusion chromatography. n. Frozen serum aliquots were taken from the various samples and diluted 1:1 with fresh PBS. If lipemic, they were centriguged at 14,000xg through a 0.2µ filter (Ultrafree-CL Centrifugal Filter, hydrophilic PVDF) prior to injection then re-centrifuged again to minimize any particulate. Samples were then injected in minimal volume onto a Superdex 200 10/300 GL (GE Healthcare Life Sciences) in Phosphate Buffered Saline (PBS). The IgG peak was collected and concentrations were established spectrophotometrically.

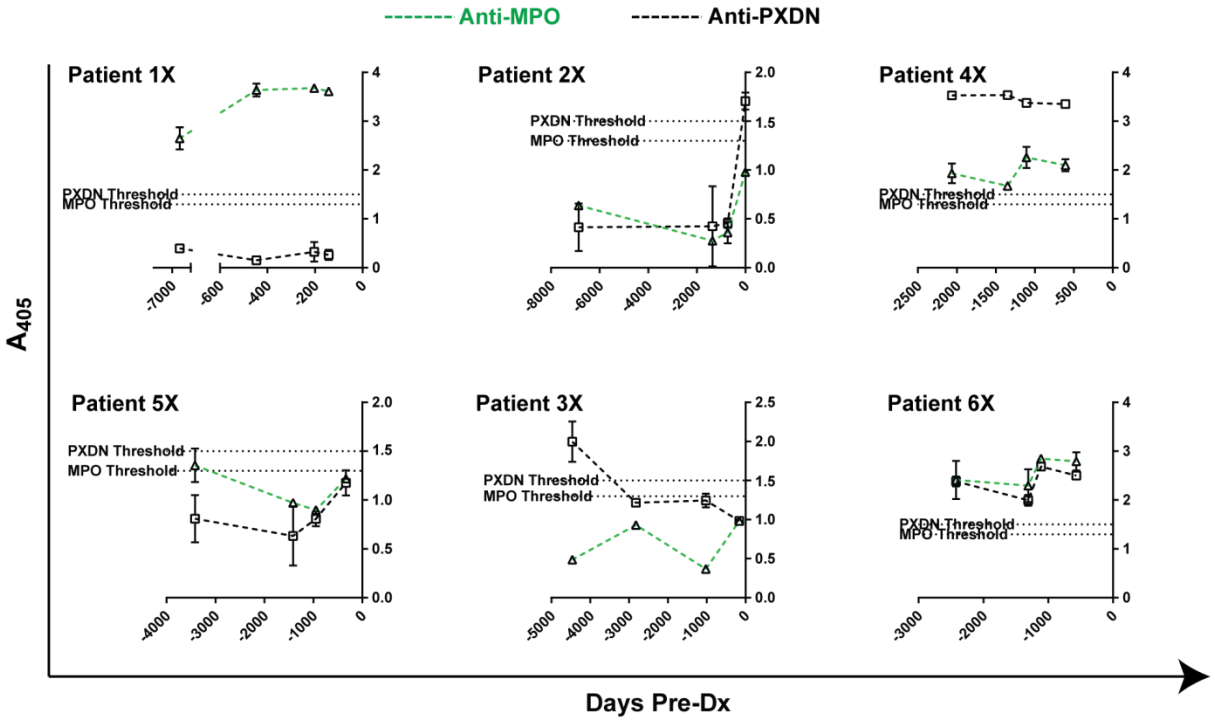
Peroxidasin inhibition kinetics- Peroxidasin was prepared as previously described [5,6]. Antibodies and peroxidasin were premixed (500 pM peroxidasin, 218 µM whole patient IgG (437.5-fold mol excess), 100µM NaBr, 140mM NaCl) and added to a 96 well black-walled plated for flourimetry. Using a GloMax® Discover fluorimeter equipped with

a sample injector (Promega Corporation), APF (aminophenyl fluorescein) (AAT Bioquest, Sunnyvale, CA) (10µM APF final concentration) was injected and an initial time point measured. Because hypohalous acids react with APF to form fluorescein, an excitation wavelength was set to 470-490nm with an emission filter set between 500-500nm. Next, initial rates were measured after the addition of 7.5 µM H_2O_2 (final concentration) using the sample injector with serial readings every 30 seconds. All patient and control samples were run on the same plate, at the same time, with the same reagent batches to ensure maximum consistency in the rate measurement.

Supplemental Figures and Tables

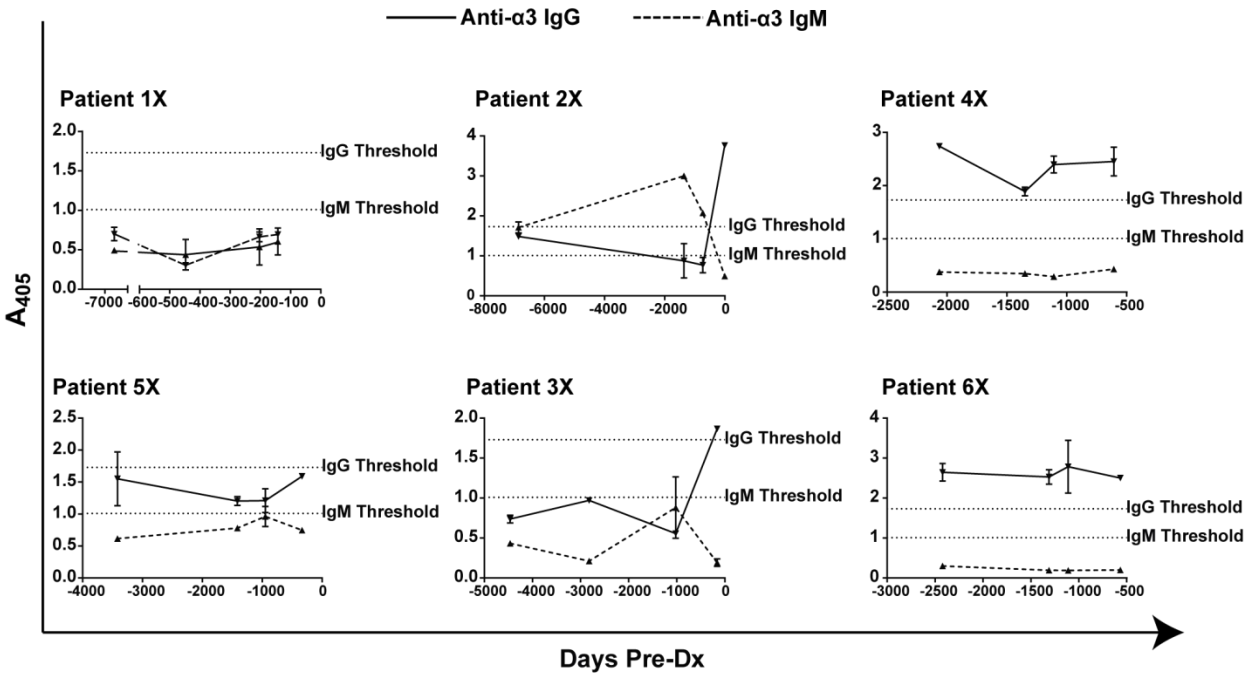


Supplemental Figure 1: Sample accrual schematic for DoDSR serum samples before GP diagnosis



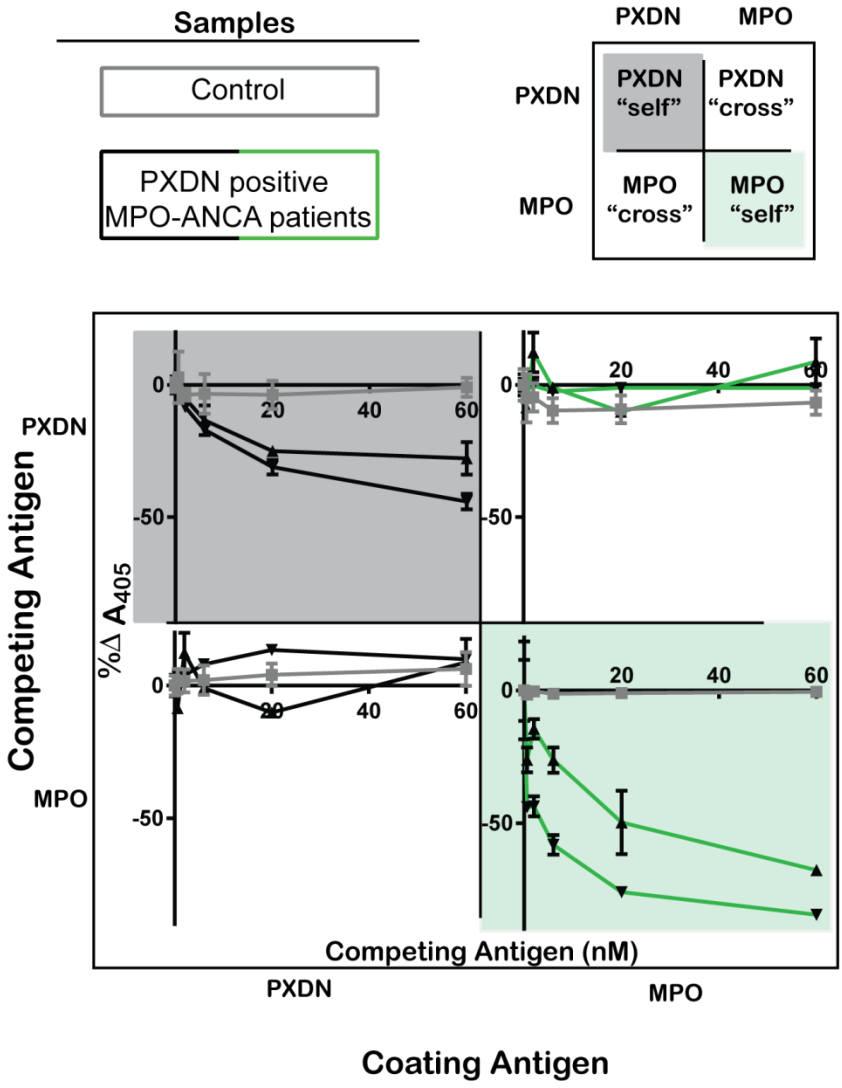
Supplemental Figure 2: Time resolved Anti-peroxidasin and Anti-MPO antibodies in pre-diagnosis GP patients by ELISA from the DoDSR.

Positivity thresholds defined as control mean (n=18) + 3 S.D.



Supplemental Figure 3: Anti-α3 IgG and IgM levels in pre-diagnosis GP patients from the DoDSR by ELISA.

Positivity thresholds defined as control mean (n=18) + 3 S.D.



Supplemental Figure 4: Anti-peroxidasin and Anti-MPO specific antibodies are present in anti-peroxidasin positive Anti-MPO AAV patients.

Antigens were coated (2.8 nM) and native, soluble peroxidasin or MPO were pre-incubated with 1:500 dilutions of patient sera for 12 hours at 4^{O}C before exposure to coated antigens . The BVAS score for the two patients was 10 and 12. These data show specific self-competition for both MPO and peroxidasin antibodies in these patients without the cross-reactivity demonstrated in the GP patients tested indicating there are both MPO and peroxidasin specific antibodies. Grey lines=controls , black lines=cases with peroxidasin as coated antigen, green lines = cases with MPO as coated antigen. Refer to key in the top right of the figure for quick reference regarding the type of competition binding being tested. "self" refers to same antigen competition, "cross" refers to the other antigen.

Supplemental Table 1. Cohort Demographics and case characteristics

Cohort	Goodpasture's disease	Prospective Goodpasture's disease	Anti-MPO AAV	Anti-PR3 AAV	Drug Induced Vasculits
Cohort Source	**Vanderbilt[†]**	**Department of Defense Serum Repository[‡]**	**UNC-Chapel Hill[€]**		
Number of samples	24	6	59	10	6
Age [range]	45 [9-66]	37 [25-42]	55 [17-89]	52 [22-70]	68 [51-87]
%Male Sex	53%	83%	51%	60%	33%
%Renal involvement (number)	100% (24/24)	66% (4/6)[‼]	96% (57/59)	ND	ND
%pulmonary involvement (number)	50% (6/12)	50% (2/4)[¥]	37% (22/59)	ND	ND
PXDN positive (%)(number)	46% (11/24)	50% (3/6)	14% (8/59)	0% (0/10)	0% (0/6)
MPO Positive (%)(number)	33% (8/24)	50% (3/6)	98% (58/59)	10% (1/10)	83% (5/6)

[†]Demographic and limited clinical information of the Vanderbilt Anti-GBM cohort. * Pulmonary involvement and final dialysis requirements based on medical records of inpatient and outpatient course where available. Demographic details of this cohort are previously published. [1]

[‡]Demographic information for the prospective Goodpasture's disease cohort from the DoDSR. Disease involvement based on ICD9 codes associated with inpatient and outpatient visits following the index hospitalization where available. Sample accrual timing detailed in Supplemental Figure 1.

[‼]Pulmonary involvement (pulmonary hemorrhage) derived from ICD9 786.3.

[¥] Acute kidney injury based on ICD9 584.9 and End Stage Renal Disease requiring dialysis ICD9 585.6 from subsequent outpatient visits used to identify ongoing dialysis. If ICD9 585.3 (Stage III CKD), the patient was deemed to not need dialysis.

[€] Demographic and limited clinical information of the UNC cohort studied. Demographic details of these samples have been previously published. [7,8] End organ involvement was assessed at the time of BVAS calculation.

Supplemental Table 2: Characteristics of Anti-peroxidasin positive patients within the Anti-MPO AAV cohort

Variable	Anti-peroxidasin status negative	positive
Number of samples	51	8
Age [range]	56 [17-89]	37 [17-85]
%Male Sex	47%(24/51)	25%(2/8)
%Renal involvement (number)	96% (49/51)	100% (8/8)‼
%pulmonary involvement (number)	37% (19/51)	38% (3/8) ‼

Demographic and limited clinical information of the UNC cohort studied. Demographic details of these samples have been previously published. [7,8] ‼ Not significantly different.

References

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