



pANCA autoantibody testing by indirect immunofluorescence indicates interstitial arteritis independent of MPO-ANCA immunoassays in ANCA-associated glomerulonephritis

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The discovery of antineutrophil cytoplasmic antibodies (ANCA) in ANCA-associated vasculitis (AAV) marked a breakthrough in diagnostics [1]. Subsequently, ANCAs were found in distinct small vessel vasculitides, including microscopic polyangiitis (MPA) and eosinophilic GPA (formerly known as Churg-Strauss syndrome). The current recommended diagnostic approach for ANCA testing includes immunoassays to detect myeloperoxidase (MPO)-ANCA or proteinase 3 (PR3)-ANCA used as the primary screening method for patients suspected of having AAV without the categorical need for indirect immunofluorescence (IIF). IIF occurs in three distinct patterns: cytoplasmic (cANCA), perinuclear (pANCA), and atypical (xANCA) [2]. A cANCA staining pattern is mainly linked to specificity for PR3, whereas a pANCA staining pattern for MPO [2]. ANCA IIF using ethanol-fixed unstimulated neutrophils as a substrate can also detect ANCA autoantibody binding to neutrophil autoantigens other than MPO or PR3, and these autoantibodies cannot be detected by specific MPO-ANCA or PR3-ANCA immunoassays [3]. We here aimed to directly compare validated ANCA testing by IIF, MPO-ANCA and PR3-ANCA immunoassays with histopathological findings in a cohort of 53 kidney biopsies with confirmed ANCA GN [4]. Detailed methods are described in Supplementary Materials and Methods.

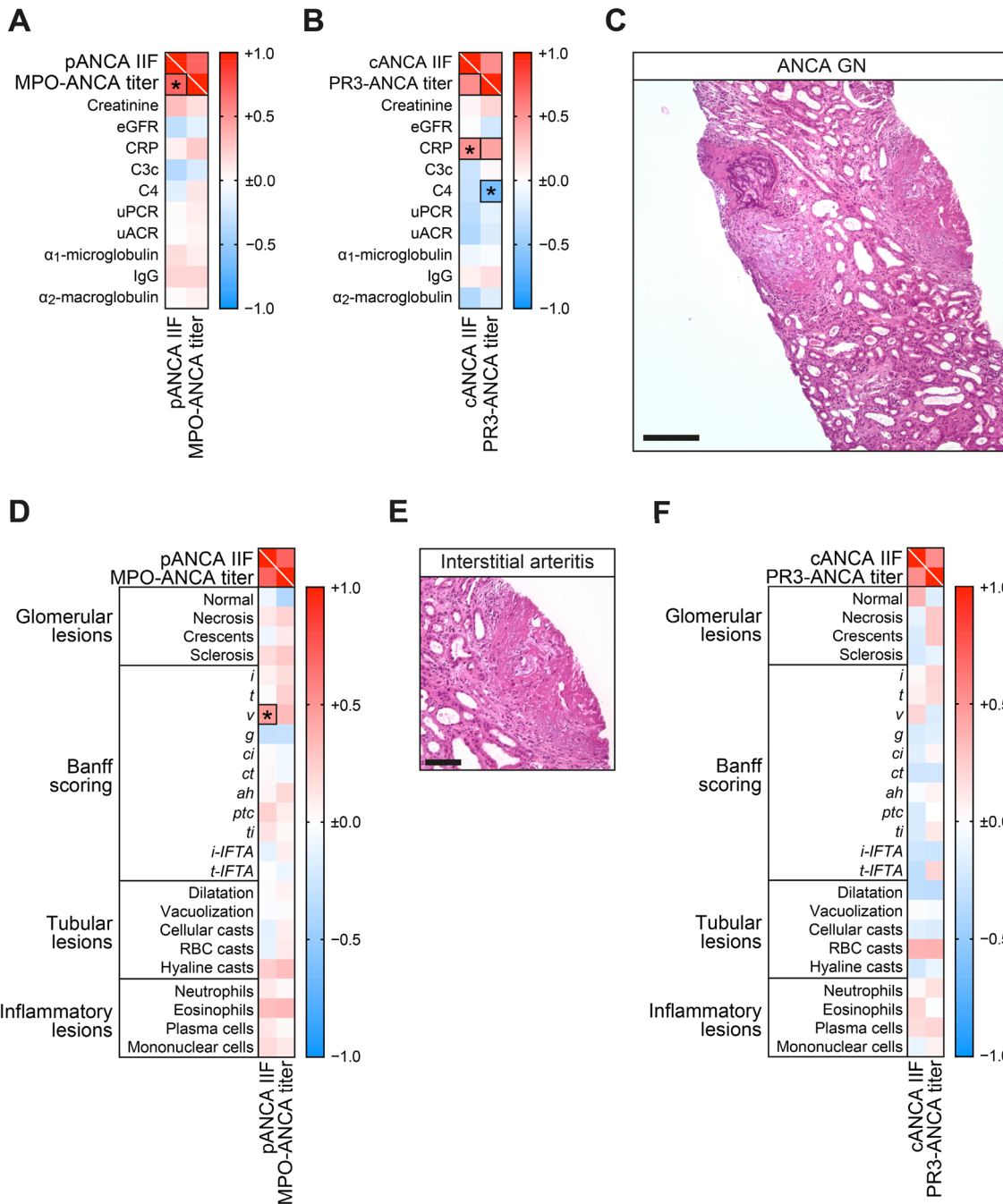
In this cohort of biopsy-proven ANCA GN, 26/53 (49.1%) were MPO-ANCA GN and 27/53 (50.9%) were PR3-ANCA GN (Supplementary Table 1). In the subgroup of MPO-ANCA GN, pANCA IIF and respective MPO-ANCA titers measured by immunoassays correlated among each other ($p < 0.0001$, Supplementary Table 2). In contrast, a less robust association was observed for cANCA IIF and PR3-ANCA titers ($p = 0.0899$, Supplementary Table 2). Among other laboratory parameters, cANCA IIF correlated with C-reactive protein (CRP) elevation in PR3-ANCA GN ($p = 0.0060$, Fig. 1A, B and Supplementary Table 2). Beyond that, PR3-ANCA titers also correlated with complement system activation reflected by low serum levels of complement factor 4 (C4, $p = 0.0107$, Fig. 1B and Supplementary Table 2). We next aimed to describe the correlation between ANCA measurements and histopathological findings in ANCA GN (Fig. 1C). While pANCA IIF and MPO-ANCA measurements did not correlate with any glomerular, tubular or inflammatory lesions, specifically pANCA IIF correlated with interstitial arteritis (v) present in 7/26 (26.9%) of patients with MPO-ANCA GN ($p = 0.0204$, Fig. 1D, E and Supplementary Table 2). In PR3-ANCA GN, cANCA IIF and PR3-ANCA measurements were not associated with any glomerular, tubular or inflammatory lesions in this subgroup (Fig. 1F). In summary, ANCA autoantibody binding to neutrophil autoantigens as confirmed by pANCA IIF correlated specifically with interstitial arteritis regardless of the respective MPO-ANCA titers in a considerable subset of patients with ANCA GN.

Besides ANCA testing for diagnostic value, its role as a disease activity marker has been the subject of frequent studies with varying outcomes. Discrepant results regarding disease activity have been a matter of discussion and can be interpreted by differences in ANCA epitopes and/or affinities

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[5]. On a mechanistic level, pathogenic ANCA autoantibodies can bind to the cell surface MPO of the proinflammatory cytokine-primed neutrophils, leading to excessive activation of neutrophils and subsequent destruction of the small vasculature [6]. Our finding that specifically pANCA IIF correlated with interstitial arteritis supports a pathomechanistic role of perinuclear targets, particularly MPO reflecting a neutrophil granule protein whose primary role in normal metabolic processes is the generation of oxygen radicals. It has previously been demonstrated that specifically MPO-ANCA induce neutrophil extracellular traps (NETs) [6].

Autoantibodies against NET components trigger neutrophils to undergo NETosis, prompting tissue damage and autoimmunity in small vessel vasculitis including AAV [7]. The ability to induce NETs directly correlated with ANCA affinity to MPO and disease activity in ANCA GN [6]. Our observation that ANCA autoantibody binding to neutrophil autoantigens as confirmed by pANCA IIF regardless of the respective MPO-ANCA titers could imply that autoantigens other than MPO might contribute to neutrophil activation and a specific contribution for AAV manifestation to distinct renal compartments with interstitial arteritis in ANCA GN.

Fig. 1 ANCA autoantibody testing by immunoassays and ANCA IIF associate with distinct clinical and laboratory parameters in ANCA GN. **A, B** Association between ANCA IIF and titers in association with laboratory parameters separated by MPO-ANCA GN and PR3-ANCA GN are shown by heatmap reflecting mean values of Spearman's ρ . Rectangle boxes indicate a Spearman's ρ more than ± 0.4 , asterisks indicate significant correlations in the linear regression analysis ($p < 0.05$). **C** Representative kidney section stained for combined elastica and H&E in ANCA GN (scale bar: 200 μm), the inset shows interstitial arteritis in ANCA GN (scale bar: 100 μm). **D** Association between pANCA IIF and MPO-ANCA titers in association with histopathological findings are shown by heatmap reflecting mean values of Spearman's ρ . The rectangle box indicates a Spearman's ρ more than ± 0.4 , asterisk indicates a significant correlation in the linear regression analysis ($p < 0.05$). **E** Representative kidney section stained for combined elastica and H&E showing interstitial arteritis in ANCA GN (scale bar: 100 μm). **F** Association between cANCA IIF and PR3-ANCA titers in association with histopathological findings are shown by heatmap reflecting mean values of Spearman's ρ . *ah* arteriolar hyalinosis, *ANCA* anti-neutrophil cytoplasmic antibody, *BVAS* Birmingham Vasculitis Activity Score, *C3c* complement factor 3 conversion product, *C4* complement factor 4, *cANCA* cytoplasmic ANCA, *ci* interstitial fibrosis, *CRP* C-reactive protein, *ct* tubular atrophy, *eGFR* estimated glomerular filtration rate (CKD-EPI), *g* glomerulitis, *GN* glomerulonephritis, *i* interstitial inflammation, *ICU* intensive care unit, *IgG* immunoglobulin G, *IIF* indirect immunofluorescence, *i-IFTA* inflammation in IFTA, *MPO* myeloperoxidase, *pANCA* perinuclear ANCA, *PR3* proteinase 3, *RBC* red blood cell, *SAPS II* Simplified Acute Physiology Score, *t* tubulitis, *ptc* peritubular capillaritis, *ti* total inflammation, *t-IFTA* tubulitis in IFTA, *uACR* urinary albumin-to-creatinine ratio, *uPCR* urinary protein-to-creatinine ratio, *v* arteritis

The main limitations of our study are its retrospective design, the small number of patients and lack of independent validation. Moreover, patients received steroids at the time of kidney biopsy that may have influenced the histopathological findings. Finally, quantification of additional ANCA autoantigens would further provide insights into a direct link between ANCA autoantibodies, neutrophil activation and AAV manifestation to distinct renal compartments (e.g., interstitial arteritis) in ANCA GN. Nevertheless, our finding that pANCA IIF specifically correlated with arteritis is especially relevant because arteritis determines renal prognosis in ANCA GN [8, 9]. Moreover, this unique association between pANCA IIF and specifically arteritis in MPO-ANCA GN regardless of glomerular or other tubulointerstitial lesions requires further investigation with regard to its pathomechanistic implications.

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Author contributors BT conceived the study, collected and analyzed data, and wrote the first draft. EB, PK and DT collected and analyzed data. SH, IAK and PS evaluated histopathological findings. PK

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Declarations

Conflict of interest The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Ethics approval The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of the University Medical Center Göttingen, Germany (no. 4/8/19). Informed written consent was obtained from all subjects involved in the study for the use of routinely collected data for research purposes as part of their regular medical care in the contract with the University Medical Center Göttingen.

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