

## Investigation of anti neutrophil cytoplasmic antibody presence with indirect immunofluorescence and enzyme-linked immunosorbent assay

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### Abstract

Anti-neutrophil cytoplasmic antibodies (ANCA) are a family of autoantibodies that react with proteins expressed mainly in cytoplasmic granules of polymorphonuclear neutrophil granulocytes (PMNs). The ANCA test is used to diagnose small vessel vasculitis and to monitor inflammatory activity. ANCA was initially detected using indirect immunofluorescence (IIF), which allowed differentiation of different patterns, such as p-ANCA (perinuclear) and c-ANCA (cytoplasmic). It is now common to detect antibodies by immunochemical assays using purified proteins, such as Enzyme-Linked Immunosorbent Assay (ELISA). In our study, we evaluated ANCA test results studied with IIF and ELISA methods and recorded patient diagnoses in the system. Serum samples of 4524 patients who were thought to have autoimmunity in their etiology were evaluated for ANCA presence. In accordance with the recommendation of the manufacturer (Euroimmun AG, Lübeck, Germany), serum IFA technique was evaluated for the presence of p-ANCA, c-ANCA. ELISA test (Alegria, Orgentec) was used to detect antibodies against MPO and PR3. The number of ANCA IIF positive patients was 525(11.6%). When we look at the distribution of ANCAs, 275(52.5%) formalin sensitive pANCA, 95 (18%) formalin resistant pANCA, 60 formalin sensitive cANCA(11.5%), 95(18%) formalin resistant cANCA. 18 (3.4%) of ANCA IIFA positives had PR3 antigen and 22 (4.1%) had significant antibody elevation against MPO antigen., ANCA IIF positive samples, according to the diagnosis of information registered in the operating system, consist of 424 (80.8%) autoimmune and inflammatory diseases according to disease groups, 36 (6.9%) malignancies, 18 (3.4%) infectious diseases, 47 (8.9%) are other diseases which are not included in these groups. ANCA is a determinant for many diseases, especially vasculitis. ANCA, which we found in a large number of different disease groups, was found to be an indicator that should be used in the diagnosis and follow-up of many autoimmune and inflammatory diseases.

**Keywords:** antineutrophilcytoplasmic antibodies, immunofluorescence, proteinase 3, myeloperoxidase

### 1. Introduction

Anti-neutrophil cytoplasmic antibodies (ANCA) are IgG autoantibodies directed mainly against the components of primary granules of neutrophils and monocyte lysosomes. Although several antigenic targets have been identified, those ANCA directed to proteinase 3 (PR3) or myeloperoxidase (MPO) are clinically relevant, whereas the importance of other ANCA remains unknown. Although ANCA can be found in multiple autoimmune diseases, the recognition that proteinase 3 (PR3) and myeloperoxidase (MPO) are the dominant autoantigens in small-vessel vasculitis has linked ANCA testing with the so-called ANCA-associated vasculitides (AAV; granulomatosis with polyangiitis, microscopic polyangiitis, and eosinophilic granulomatosis with polyangiitis) (1). ANCA also occur in 30-40% of patients with eosinophilic granulomatosis with polyangiitis (EGPA) and anti-GBM disease, but is uncommon in other forms of vasculitis. ANCA with different specificities have been described with varying frequencies in diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis, inflammatory bowel disease, endocarditis, chronic infections

and hematopoietic malignancies. ANCA can also develop as an adverse event during pharmacological treatment (2). ANCA was initially detected by indirect immunofluorescence (IIF) in ethanol-fixed neutrophils that appeared as two major patterns as granular cytoplasmic staining (CANCA) or perinuclear staining (P-ANCA). Today, it is common to detect antibodies by immunochemical analyses such as ELISA that use purified proteins as antigens (3,4). The minimum requirements for AAV screening with IIF on neutrophils stabilized with ethanol and, if positive, follow up with antigen-specific enzyme-linked immunosorbent assays (ELISA) enabling detection of PR3- and MPO-ANCA (5). In our study, we evaluated only the test results studied in our immunology laboratory between January 2016 and July 2018 and the patient's diagnostic records.

### 2. Materials and Methods

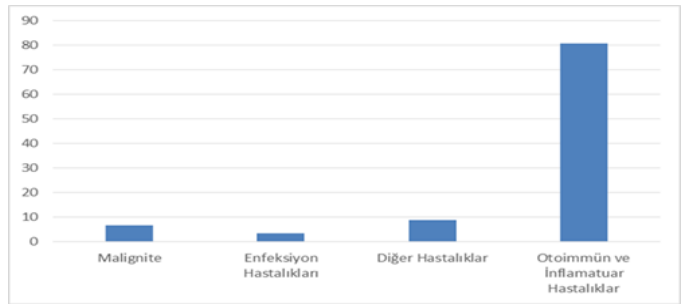
In this study, results obtained from serum samples to be studied sent to the Immunology Laboratory of Ondokuz Mayıs University Faculty of Medicine between January 2016-July 2018 and patient diagnoses recorded in the system were retrospectively examined. Serum samples of 4524 patients who

were considered to have autoimmunity in etiology were evaluated in terms of their presence. In accordance with the proposal of the manufacturer (Euromun AG, Lübeck, Germany), serums were irrigated at 1/10 for P-ANCA, c-ANCA with IFA technique. The samples were brought to room temperature before they were studied and worked in accordance with the application procedures of the manufacturer. In these slides, three different areas were evaluated for each sample by solid phase consisting of hep-2 cells, human granulocytes and formalin flaked with ethanol and human granulocytes. In addition to the ANCA, these areas allowed the study of the anti-nuclear antibody (ANA) positivity. In this way, all the fields were evaluated in terms of the separation of two antibodies from each other. Human granulocytes fixed with ethanol were used to differentiate between cANCA or pANCA. P-ANCA formaldehyde resistance status was evaluated with formaldehyde fixated neutrophils. ELISA test (Alegria, Orgentec) was used to detect antibodies against MPO and PR3. This study was found ethically appropriate by the Ondokuz Mayıs University Clinical Research Ethics Committee on 11.03.2021 with the decision number OMUKAEK 2021/68.

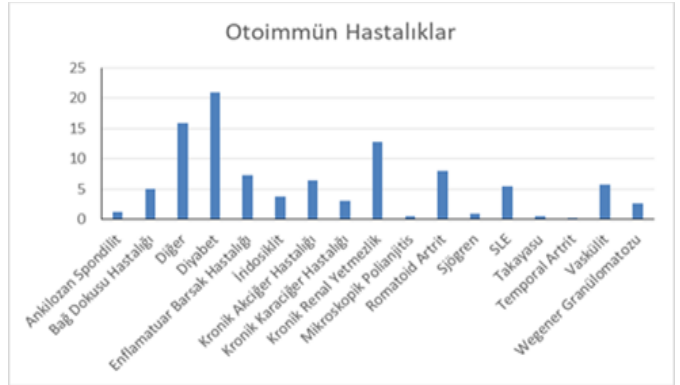
**3. Results**

In our study, the results of 4524 patients whose serum samples were tested for ANCA IFA who were admitted to the Immunology Laboratory of Ondokuz Mayıs University Hospital between January 2016 and July 2018 were evaluated retrospectively. The number of ANCA IFA positive patients without recurrence was 525 (11.6%). The distribution of ANCAs was 275 (52.5%) formalin sensitive pANCA, 95 (18%) formalin resistant pANCA, 60 (11.5%) formalin sensitive cANCA, 95 (18%) formalin resistant cANCA. In 18 (3.4%) of the ANCA IFA positive samples, significant antibody level was detected against PR3 antigen and MPO antigen in 22 (4.1%). Of the ANCA-positive patients, 222 (%42.2) were male and the average age was 47.1 and the number of female positive patients was 303 (%47.8) and the average age was recorded as 49,03. When we classified ANCA IFA positive samples according to disease groups based on information registered in the hospital information system, there were 424 (80.8%) autoimmune and inflammatory diseases, 36 (6.9%) malignancies, 18 (3.4%) infectious diseases, 47 (8.9%) are the other diseases that are not included in these groups (Fig. 1). Of the autoimmune and inflammatory diseases, 27 (6.3%) were chronic lung disease, 13 (3.4%) were chronic liver disease, 54 (12.7%) were chronic renal failure, 89 (20%, 9) were diabetes mellitus, 31 (7.1%) were inflammatory bowel disease, 21 (4.9%) were connective tissue disease, 34 (8%) were rheumatoid arthritis, 23 (5.4%) were systemic lupus erythematosus, 16 (3.7%) were iridocyclitis, 5 (1.1%) were ankylosing spondylitis, 11 (2.1%) were Wegener granulomatosis, 2 (0.4%) were microscopic polyangiitis Takayasu's arteritis, 1 (0.2%) was temporal arteritis, 4 (0.9%) were Sjogren syndrome, 24 (5.6%) were undefined vasculitis,

67 (% 15,8) had the other group of autoimmune and inflammatory diseases (Fig. 2).



**Fig. 1.** Distribution of ANCA positive samples by patient groups



**Fig. 2.** Distribution of ANCA positive autoimmune and inflammatory diseases

**4. Discussion**

Antineutrophil cytoplasmic antibodies (ANCA) have been accepted as clinically relevant autoantibodies for more than 50 years. ANCA were originally detected by indirect immunofluorescence (IIF) on ethanol fixed neutrophils revealed as two major patterns; granular cytoplasmic staining (C-ANCA) or perinuclear staining (P-ANCA). Although it can be found in multiple autoimmune diseases, proteases 3 (PR3) and myeloperoxidase (MPO) are considered dominant autoantigens in small vascular vasculitis. However, less well-defined antigen specificities, also added value in the diagnosis of inflammatory bowel diseases and autoimmune liver diseases (6). Özdemir et al. obtained 11.4% ANCA positivity in patients by IIFA method in their study. Similarly, in our study, the positivity rate of ANCA by IIFA method was determined as 11.6%. When Özdemir et al. examined the diagnosis of patients who were positive for ANCA, they found that chronic lung diseases, connective tissue diseases, arthritis and systemic diseases were the most frequent (7). In our study, the most frequent diseases in the patients were diabetes mellitus, chronic kidney failure, rheumatoid arthritis and inflammatory bowel diseases. Polymorphonuclear neutrophils and inflammatory process play an important role in the development of late diabetic vascular complications. ANCA are considered important serological markers for vasculitis (8). In our study, diabetes was diagnosed in 20.9% of ANCA positive patients. In this respect, ANCA may be a predictor of vascular complications that may develop in diabetic patients. ANCA has been reported to be present in a wide variety of inflammatory conditions. In some diseases, ANCA positivity

has been shown to correlate with some features. A chronic and systemic inflammatory disease, rheumatoid arthritis (RA) is characterized by cartilage erosion, bone damage, and a chronic synovitis that can cause fibrous ankylosis of the joints. In patients with RA, ANCA has occurred against cytoplasmic antigens of neutrophils, especially to lactoferrin (LF), and not yet fully defined some polypeptides (9). In a study performed by Bandt et al. 32% of RA patients were found to be P-ANCA or atypical ANCA positive by IFA method (10). In the study of Özdemir et al., 4 of 44 patients who were positive for ANCA were found to be RA (7). In our study, the diagnosis of RA was 8% among all cases. Systemic lupus erythematosus (SLE) is a rheumatic disease that primarily affects young women. Inflammation and organ damage occurs when the immune complexes accumulate along the walls of the arteries (11). There are a number of antibodies in SLE, and some of them have been reported to show ANCA specificity with frequency of 25%. It has been shown that the ANCA positivity in SLE is correlated with both disease activity and severity (12). In our study, 3.7% of ANCA positive patients were being followed up with the diagnosis of SLE. Besides vasculitis, ANCA has been studied in inflammatory bowel disease (IBD). In addition to some other autoantibodies, ANCAs have been shown to be more common among IBD patients than healthy controls, but their benefits as a biomarker and their possible role in the pathogenesis are discussed. In the study performed by Kılıç et al., P-ANCA was found to be positive in 65% of UC and 2.5% of healthy persons (13). The diagnosis of inflammatory bowel disease was 7.1% in our study. ANCA is a prognostic marker which should be investigated in many diseases, especially vasculitis. It is concluded that ANCA is an indicator that should be routinely monitored in both the diagnosis and the follow-up of many autoimmune diseases. On the other hand, our data support the diagnosis of 0.8 % small vessel vasculitis as a result of the ANCA tests as a supportive laboratory examination of patients with a preliminary diagnosis of small vessel vasculitis. This situation reveals the necessity of reviewing the cost-effective usage policies of ANCA tests in our hospital.

#### Conflict of interest

The authors have no conflict of interest.

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