Section: Microbiology

Original Research Article

SEROPREVALENCE OF ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODIES IN POST-COVID 19 PATIENTS IN A TERTIARY CARE CENTRE IN SOUTH INDIA- A CROSS-SECTIONAL STUDY

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Abstract

Background: Autoimmune diseases are classified into local and systemic based on the clinical manifestations. Anti-neutrophil cytoplasmic antibodies (ANCA) associated vasculitis (AAV) is one among the systemic autoimmune diseases. The fact that SARS-CoV-2 infection can be a 'trigger factor' for autoimmune vasculitis is an interesting topic to be explored. **Material & Methods:** This cross-sectional descriptive study was carried out for a period of 3 months from August 2021 to October 2021 at a Tertiary Care Centre in Chennai, South India. Post COVID-19 subjects who tested positive in RT-PCR test were included in the study. Sample size was 75 at a confidence level of 95 and margin of error of 10%. Blood sample was collected and Indirect immunofluorescence test was used as a screening assay for the detection of ANCA. Samples which were positive in indirect immunofluorescence were tested by Line immunoassay to confirm the presence of anti-Proteinase 3 and anti-Myeloperoxidase antibodies. Statistics: SPSS version 21 was used to find out the statistical significance of the results. **Results:** Prevalence of antineutrophil cytoplasmic antibodies by immunofluorescent assay was 9.3%. Immunofluorescent assay and Line immunoassay were compared using Chi-square test and the p value was 0. 672793. Among the ANCA positives, 42.9% were in the age group 41-50 years. **Conclusion:** Though the prevalence of anti-neutrophil cytoplasmic antibodies was 9.3% in immunofluorescent assay, none of them were positive for anti-proteinase 3 and anti-Myeloperoxidase antibodies in Line Immunoassay. Hence further studies are necessary to find out the actual prevalence of ANCA antibodies in post-COVID 19 subjects.

INTRODUCTION

Autoimmune diseases are either local involving a single organ or systemic involving more than one organ or more than one system. The basic pathogenesis is either the production of autoantibodies or the T cells becoming autoreactive. Anti-neutrophil cytoplasmic antibodies(ANCA) associated vasculitis(AAV) is one among the systemic autoimmune diseases. AAV primarily affects small- and medium-sized arteries of internal organs and skin and has similar features on kidney histology [e.g., a focal necrotizing, often crescentic, pauci-immune glomerulonephritis (GN).[1] The factors leading to the initiation of AAV remains ambiguous.

Genetic factors, infectious agents, drugs and environmental exposures are some of the triggering factors for autoimmunity.[2] It has been reported in previous studies that systemic viral and/or bacterial infections may trigger autoimmunity and cause granulomatous polyangiitis (GPA). Somer et al. reported cases of parvovirus-19 and cytomegalovirus-induced GPA.[3] Kallenberg et al. reported that the superantigens of staphylococcus aureus may be involved in first phase of GPA by activating a limited polyclonal response, possibly involving a specific autoimmune response.[4]

The Corona Virus Disease-19 (COVID-19) pandemic caused by Severe Acute Respiratory Syndrome Corona Virus- 2(SARS-CoV-2) inflicts vascular endothelial injury and occurs as a multisystem disease with protean clinical manifestations. Ultimately whether COVID-19 has any implications on AAV or not is not well understood. The fact that SARS-CoV-2 infection can be a 'trigger factor' for vasculitis is an interesting topic to be explored. SARS-CoV-2 infection may trigger autoimmunity and autoimmune diseases [5,6]

The probable mechanisms of autoimmunity following SARS-CoV-2 infection are bystander killing, molecular mimicry, viral persistence, epitope spreading, and formation of neutrophil extracellular traps (NETs).[7] High levels of NETs have been detected in circulation and renal biopsy specimens in patients with AAV.[8] The proinflammatory proteins present in the NETs are thought to play a major role in the development of vasculitis directly by causing endothelial cell injury and activating the complement system and indirectly by the production of proteinase 3(PR3) and myeloperoxidase (MPO)ANCA. Whenever there is an imbalance in the production of these proteins and clearance of NETs it can trigger autoimmunity. [9,10]

The laboratory diagnosis of AAV is by the detection of antibodies directed against neutrophils' cationic enzymes such as proteinase 3 (PR3), myeloperoxidase (MPO), elastase, lactoferrin, cathepsin G, bacterial permeability-increasing peptide, and calprotectin. They are involved in the pathogenesis of small-to-medium-sized vessel vasculitides. ANCAs can be detected by indirect immunofluorescence assay (IFA) and are named based on the staining pattern. If the cytoplasm of the neutrophil is almost evenly stained and the nucleus is not stained, it is called cytoplasmic ANCA (C-ANCA), whereas when the nucleus alone is stained without cytoplasmic staining it is named perinuclear ANCA (P-ANCA). Although not universal, PR3 is associated with the former and MPO with the latter. C- or P-ANCAs can be by-products of the aforementioned NET-formation mechanism[11] Although ANCAs are known to play a role in vasculitides in the context of autoimmune diseases, it is also produced in infections caused by Staphylococcus aureus.[12]

As SARS-CoV-2 virus causing COVID-19 has the potential to trigger autoantibody production, screening the COVID-19 patients for Antineutrophil cytoplasmic antibodies can identify the individuals at risk of developing ANCA associated vasculitis (AAV).

The aim of the study is to find out the prevalence of ANCA antibodies in post-COVID 19 subjects which enables the clinicians to make an early diagnosis of AAV in such patients and to initiate appropriate treatment thereby preventing the progression of the disease and permanent organ damage.

MATERIALS AND METHODS

Study Design and Study population

It was a cross-sectional descriptive study for a period of 3 months from August 2021 to October 2021 at a Tertiary Care Centre in Chennai, South India. The study was approved by the Institutional ethics committee vide ID.No.8.7.2021.Reg.No.ECR/1385/INST/TN/2020. Study population was COVID-19 positive subjects and the target population was COVID-19 positive subjects who have completed 3 months from the day they tested RT-PCR positive. Participants who had fulfilled the above-mentioned criteria attending post-COVID OP at the Tertiary Care Centre were recruited for the study. Sample size was 75 at a confidence level of 95%. Informed and written consent was obtained from the study participants. Age group of the study participants was 21 – 60 years. The study participants were from both the sexes in which 53 were females and 22 were males.

Diagnostic Method

Under strict aseptic precautions 5ml of blood sample was collected, centrifuged at 1500rpm/minute for 5 minutes and serum was separated. Sera were stored at -20℃. Indirect immunofluorescence test was applied as a screening method for the detection of ANCA. Human neutrophils fixed with ethanol were used to screen all samples. The platform used as confirmatory was Immunoblot (Line Immunoassay LIA) for all the samples tested positive in screening test. The LIA kits and IIF ANCA kits were procured from Medsource Ozone Biomedicals Ltd., New Delhi. Each LIA strip has a functional control, cut-off control and the antigens coated were Proteinase 3(PR 3), Myeloperoxidase (MPO) and Glomerular Basement Membrane (GBM).

Inclusion criteria

COVID-19 positive subjects who have completed 3 months from the date they tested positive in RT-PCR test were included in the study.

Exclusion criteria

COVID-19 negative subjects and subjects with pre-existing autoimmune diseases were excluded from this study.

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Statistics

The data were entered in Microsoft excel spread sheet. SPSS version 21 was used for statistical analysis of the data. Percentage of ANCA positivity in indirect immunofluorescence was calculated. Chi-square test was done to find out the significance of the methods of ANCA test at p value < 0.05.

RESULTS

The study participants were between 21 and 60 years of age and the age distribution of the participants is given in table 1.

Table 1: Age distribution of the study participants n (75)

Age in years	Number	Percentage %
21-30	13	17.33
31-40	21	28
41-50	29	38.67
51-60	12	16
Total	**75**	**100**

The highest percentage of participants were between 41 years and fifty years and the lowest percentage of participants were of age 51 to 60 years.

Sex distribution of the study participants is depicted in figure 1.

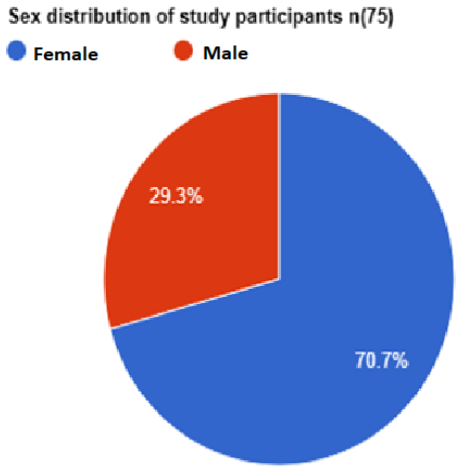

Figure 1

Table No. 2: Anti-neutrophil cytoplasmic antibodies by IIF in post-COVID 19 patients n (75)

ANCA	NUMBER	PERCENTAGE %
Positive	7	9.34
Negative	68	90.66
Total	75	100

Among the subjects recruited for the study 9.34% were ANCA positive.

By Indirect immunofluorescence 7 were ANCA positive. Age wise distribution of ANCA positivity is depicted in figure 2.

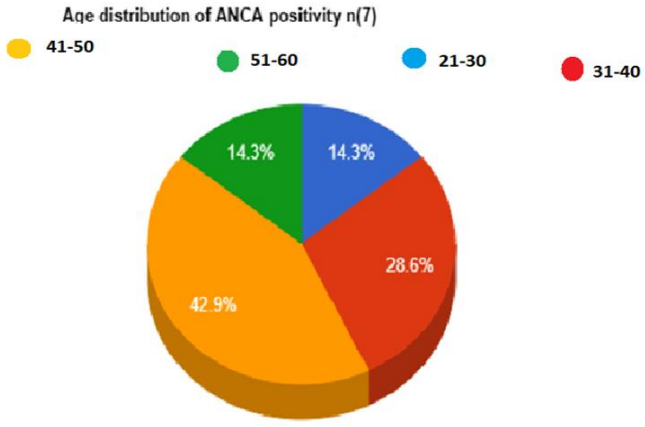

Figure 2

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The 3 different patterns of fluorescence in ANCA positives are perinuclear ANCA(P-ANCA), cytoplasmic ANCA(C-ANCA) and atypical ANCA(x-ANCA). The findings of this study are tabulated in table 3.

Table 3: Type of ANCA(IIF) among the ANCA positives n (7)

TYPE OF ANCA	POSITIVE	NEGATIVE
Perinuclear ANCA (P-ANCA)	**5**	**2**
Cytoplasmic ANCA (C-ANCA)	**2**	**5**

p value is.108809. Not significant at p < .05

P-ANCA and C-ANCA were the patterns of fluorescence observed in this study. Though statistical significance was not found between the 2 different patterns, P-ANCA was found to be more common than C-ANCA.
Immunoblot or Line immunoassay (LIA) is a confirmatory test in the detection of ANCA. All the samples which were positive in IIF were further tested by ANCA profile Line immunoassay. Table 4 shows the results of ANCA.

Table 4: Indirect Immunofluorescence vs Line Immunoassay

ANCA METHOD	POSITIVE	NEGATIVE	TOTAL
IIF n (75)	7	68	75
LIA n(7)	1	6	7

p value is .672793 Not significant at p < .05

Image 1: ANCA PROFILE IMMUNOBLOT (LINE IMMUNOASSAY) NEGATIVE

Image 2: ANTI-GLOMERULAR BASEMENT MEMBRANE (Anti-GBM) ANTIBODY POSITIVE

DISCUSSION

Viral infections predispose to inflammatory syndromes and autoimmune diseases. Genetic factors, infectious agents, certain drugs, environmental factors and other factors are attributed in the development of autoimmune diseases.[2,3,4,13] As the SARS CoV-2 virus has led to an alarming pandemic across the countries of the world and symptoms of autoimmune diseases were observed among post-COVID patients, a comprehensive study was undertaken to find out whether it has resulted in the production of Anti-neutrophil cytoplasmic antibody pathognomonic of ANCA associated vasculitis in these patients. An increase in the number of autoimmune diseases has been reported following the covid 19 pandemic.[14]
In this study maximum number of participants were of age group 41-50 years followed by 31 to 40 years. A recent study on AAV in post- covid 19 patients has stated 40.5±14.7 as the mean age of the participants and 66.6% were men.[15] Unlike the above study, number of female participants were more than the males in this study. This is due to the inclusion of the laboratory technicians as study participants from a tertiary care center in which females outnumber males.
The various methods of testing for ANCA are indirect immunofluorescence, ELISA and immunoblot. Indirect immunofluorescence method is a screening test in the detection of anti-neutrophil cytoplasmic antibodies. The "International consensus statement on testing and reporting antineutrophil cytoplasmic antibodies (ANCA)"[16] recommends IIF for screening ANCA. All those with a positive finding in IIF either peri-nuclear ANCA or cytoplasmic ANCA should be tested for the 2 ANCA specificities, proteinase 3 (PR3) and myeloperoxidase (MPO) by enzyme linked immunoassay (ELISA).[17] In this study human

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neutrophils fixed with ethanol were used as the substrate for detecting anti-neutrophil antibodies by IFA. Anti-neutrophil cytoplasmic antibodies were found in 9.3% of post-COVID 19 subjects in IFA.

The 2 major staining patterns are granular cytoplasmic staining with central accentuation, known as C-ANCA produced by anti- PR3 antibody and perinuclear staining, known as P-ANCA which is due to anti-MPO antibody and other antibodies.[18,19] Anti-PR3 (C-ANCA) antibodies are pathognomonic of Granulomatous Polyangiitis and Anti-MPO (P-ANCA) antibodies are present in microscopic polyangiitis. Apart from these, anti-MPO antibodies are also present in Inflammatory bowel diseases, primary sclerosing cholangitis, rheumatoid arthritis and other diseases. In this study,71.42% and 28.57% were P-ANCA and C-ANCA positive respectively. In a study conducted on ANCA in post-COVID 19 patients 57% were ANCA positive out of which 72% and 28% were C-ANCA and P-ANCA respectively.[20] The percentage of ANCA positivity and the percentage of the 2 different patterns of fluorescence in our study is discordant with the above-mentioned study.

As P-ANCA could be due to the presence of anti-elastase, anti-lysozyme, anti-cathepsin and anti-lactoferrin antibodies, ANCA profile line immunoassay was performed for all the 7 samples which were ANCA positive by IIF to confirm the presence of PR3 and MPO. Among those 7 one was positive for anti-GBM antibodies by Line immunoassay. However, none of them were positive for anti- PR3 and anti- MPO in Line immunoassay.

Limitations of the study

Sample size of this study is small. Though the prevalence of ANCA by IIF was 9.3% neither anti-PR3 nor anti- MPO was positive by Line Immunoassay. Hence a study with a larger sample size can detect the actual prevalence of ANCA antibodies in post -COVID 19 subjects.

CONCLUSION

The prevalence of anti-neutrophil cytoplasmic antibodies in post-COVID 19 subjects by immunofluorescent assay was 9.3%. But anti-PR3 and anti-MPO antibodies by line immunoassay were negative in all these patients. As majority (71%) of the subjects presented with P-ANCA which is quite common in diseases other than AAV further studies are necessary to elucidate the prevalence of anti-neutrophil cytoplasmic antibodies in post- COVID 19 patients. Furthermore, a regular and periodic follow up of these subjects is warranted for the early diagnosis and management of ANCA associated vasculitis (AAV).

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Conflict of Interest: Nil

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