Journal of Clinical and Nursing Research, 2024, Volume 8, Issue 6
http://ojs.bbwpublisher.com/index.php/JCNR
Online ISSN: 2208-3693
Print ISSN: 2208-3685

Antibody Pattern: Correlation of Age and Gender in ANA and Anti-ds-DNA Prevalence

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Abstract: The study was designed to find the prevalence of ANA antibodies and anti-dsDNA antibodies in samples tested at AFIP Rawalpindi and their correlation with age and gender and to find positive and negative predictive values of ANA antibodies. For this purpose, twelve thousand nine hundred sixty-seven (12,967) patients were analyzed for ANA with four hundred sixty-eight (468) healthy samples tested as control and four thousand seven hundred three (4,703) patients tested for ds-DNA antibodies. Retrospective data of all samples tested by indirect immunofluorescence (IIF) for ANA antibodies and dsDNA antibodies was collected. To address positive and negative predictive values another control group (autoimmunity not suspected) of serum samples was taken from the healthy population. For the first group, age, gender, ANA antibodies and ds-DNA antibodies results (both tests performed by IIF) data was collected from a computer record cell; for the second control group, ANA antibodies were performed by IIF. 12,967 and 4,703 samples (Group 1) were tested for ANA antibodies and dsDNA antibodies, respectively, during this period. 1,119 (9%) and 99 (2%) were found positive for ANA antibodies and ds DNA antibodies. Among these positive samples, 850 (76%) and 73 (74%) were females respectively. Gender predisposition towards autoimmunity (ANA) was found significant with a *P* value of ($P = 0.001$). Relation of age was also found significant with anti-ANA antibodies with a *P* value of ($P = 0.001$). This study shows a negative correlation between age ($P = 0.025$) and gender ($P = 0.001$) with anti-dsDNA which is also significant. High prevalence was found below the mean age of 38 years (SD ± 16.635) for ANA antibodies and the mean age of 35 years (SD ± 15.066) for ds-DNA antibodies. The age of ANA antibodies and dsDNA antibodies positive patients ranged from 1 year old to 98 years old and 2 years old to 95 years old respectively. In the second (autoimmunity-free) control group, a total of 468 samples were tested for ANA antibodies and 9 (2%) were found positive. Positive predictive value (PPV) was 8.6% and negative predictive value (NPV) was 98%. ANA is a sensitive test for autoimmunity and it is significantly related to female gender and increasing age. The low prevalence of ANA antibodies among clinically suspected cases suggests that rationalization of test prescriptions is needed. Anti-ds-DNA is also a sensitive test for diagnosis of SLE and it is significantly related to female gender and increasing age.

Keywords: ANA; Anti ds-DNA; Autoimmunity; SLE; Immune fluorescence

Online publication: July 18, 2024

1. Introduction

Autoimmune diseases are those conditions in which the body's immune system starts attacking its parts such as cells, tissues and organs. The normal immune system malfunctions in such situations and starts targeting normal body parts ultimately leading to inflammation and destruction of tissues. These autoimmune conditions are categorized into two types including organ-specific disorders and non-organ-specific kinds. In the former category, organs and tissues change the functions of endocrine glands including the adrenal, pancreas, and thyroids. In the latter type of disease, effects spread all over the body like connective tissue diseases (CTDs), Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA), and Dermatomyositis [1]. This condition is strongly linked with some other diseases like connective tissue diseases, particularly with specific autoantibodies (blood proteins). These proteins can facilitate in diagnosis process. The most commonly tested antibodies are antinuclear antibodies (ANA) and anti-double standard deoxyribose nucleic acid antibodies (anti-dsDNA) [2].

One particular kind of autoantibodies called ANA antibodies is capable of attaching to and eliminating particular cell nucleus features. Since ANA antibodies are uncommon in normal people, concentrations of > 1:160 are usually regarded as remarkable. Only the fine-speckled arrangement appears in healthy people despite the fact that patients with CTDs exhibit fine-speckled patterns more frequently [3]. The functionally precise term for ANA is "autoantibodies to cellular antigens." ANA is linked to a number of autoimmune conditions, including mixed connective tissue disease (MCTD), RA, systemic sclerosis, Sjogren's syndrome, systemic lupus erythematosus (SLE), primary vasculitis, and idiopathic inflammatory myopathies [4]. Extensive inflammatory processes represent the multi-system tissue-related illness known as systemic lupus erythematosus, or SLE or Lupus, which is a common consequence of systemic lupus. Every system in the individual body could be affected by SLE including the central nervous system. Malar rash, cutaneous lupus lesions, butterfly rash, and mouth ulcer gangrene are signs of systemic lupus flare-ups [5].

An inflammatory condition known as Sjögren's syndrome is linked to the activation of the lacrimal glands in the eyes and the salivary glands in the mouth. There may not be a deeper connective tissue illness for Sjogren's syndrome to be an initial disorder. Dry eyes or "grittiness", dry mouth, joint pain, and exhaustion are typical symptoms [6]. A class of illnesses known as primary vasculitis results in inflammation of the blood vessel linings. Vasculitis is linked to CTDs impacting small arteries, such as leukocytoclastic vasculitis, RA, SLE, and Henoch-Schoenlein purpura. The ANA test uncovers autoantibodies in a person's serum. Immunofluorescence (IF) is the conventional technique used to conduct an ANA test. For the identification of antinuclear autoantibodies, indirect immunofluorescence on human epithelial (HEp-2) cells is regarded as the most effective diagnostic technique. The enzyme-linked immunosorbent assay (ELISA) is another widely used ANA testing technique [7].

Because of its extremely high productivity, multiplex bead technology-based semi-quantitative solid-phase tests have gained FDA approval and gained widespread use lately. Over the past few decades, studies have shown a consistent increase in autoimmune illness in Westernized countries. The autoimmune diseases related to rheumatoid arthritis, endocrinology, gastrointestinal disorders, and neurological disorders showed yearly percentage rises of 7.1%, 6.3%, 6.2%, and 3.7%, respectively. Autoimmune diseases are common in Pakistan, Finland, Denmark, the USA, the UK, the Netherlands, Italy, Spain, and New Zealand [8]. There is a broad spectrum in the proportion of positive autoantibody tests across distinct autoimmune rheumatic diseases: 90–100% in SLE, 60–80% in systemic sclerosis (SSc), 40–70% in Sjogren's syndrome, 30–80% in polymyositis/dermatomyositis, and 30–50% in RA. It has also been demonstrated that there are racial differences in the frequency of autoantibodies [9].

Anti-dsDNA antibodies belong to a class of ANA that specifically target double-stranded DNA antibodies as their intended antigen. They have a role in the pathophysiology of lupus nephritis and are diagnostic for SLE. It is among the standards used by the American College of Rheumatology (ACR) to categorize SLE patients.

The majority of antibodies are standard antibodies for illness detection and belong to the IgG class against dsDNA [10]. IgM and IgA isotype anti-dsDNA antibodies, on the other hand, appear to be less selective for SLE. In SLE, the amount of circulating anti-dsDNA antibodies varies according to the severity of the illness. A rise in inflammation may occur concurrently with or even ahead of an increase in antibody concentrations. Physicians, therefore, track levels and evaluate illness development at regular intervals. Concentrations are examined frequently at durations of 1–3 months and 6–12 months, respectively, in instances of more aggressive lupus compared to less active lupus [11]. Over time, numerous techniques for measuring antibodies to double-stranded (ds-DNA), a crucial diagnostic indicator of SLE, have been established. The *Crithidia luciliae* indirect immunofluorescence test (CLIFT), flow cytometry, multiplex assay, ELISA, Farr assay, and other solid phase immunoassays are some of these techniques. International findings have been made on the prevalence of anti-dsDNA antibodies in SLE patients. North America recorded the largest prevalence, whereas Africa observed the smallest incidence [12]. Asia, Australia, and the United States had greater SLE incidences than European nations. With a female-to-male ratio ranging from 1.2:1 to 15:1, females exhibited the greatest incidence of anti-dsDNA antibodies across all trials. Anti-dsDNA and ANA are extensively applied in the qualitative and quantitative detection of autoimmune disorders, according to research conducted nationwide and in various regions [13].

Since ANA and ds-DNA antibodies are crucial for the identification of autoimmune diseases, they are included in this investigation to determine the frequency of these antibodies in Pakistani patients with clinically suspected autoimmune disorders. Finding the prevalence of ANA and anti-dsDNA antibodies, as well as their correlation with age and gender, was the main goal of the current study. Other objectives included determining positive and negative prognostic indicators of ANA. The present research aims to investigate the prevalence of ANA and anti-dsDNA antibodies and their correlation with age and gender in patients who were referred to AFIP for ANA and dsDNA antibody testing. Research on the frequency of anti-dsDNA and ANA antibodies could support medical professionals in diagnosing autoimmune diseases in patients who may be at risk and in creating strategies to treat them.

2. Methodology

The current study was a cross-sectional study of our chosen samples. All samples referred to AFIP Rawalpindi for ANA & Anti-dsDNA antibodies testing are included in our study. Lipaemic & Hemolyzed samples are excluded from the study. Patients with suspected autoimmune disorders screened for ANA and dsDNA antibodies. Most of the samples are taken from AFIP, CMH Rawalpindi and some are referred from all the remaining CMHs of Pakistan. According to all the previous research, the prevalence of ANA and ds-DNA antibodies was approximately 2–3%. According to this prevalence, my sample size is 374 for ANA and 356 for ds-DNA antibodies. However, 12,967 samples for ANA are included in this study with 468 healthy control samples and 4703 samples for dsDNA antibodies. Non-probable consecutive sampling technique was used to collect the data. Data was analyzed using descriptive and cross-sectional statistics such as prevalence, frequency and percentages of all the variables. The study finds the *P* values by using the Pearson Chi-Squared Test. To check the correlation of my results with age and gender, this study used Pearson Correlation Analysis. To find the positive and negative predictive value by using this formula:

$$PPV = A/ (A+B)$$

$$NPV = D/(C+D)$$

A 3 mL blood sample was collected in a gel tube after the proper consent of the patient. Age, gender and lab IDs of all patients were recorded for all those samples, which were collected for analysis. Samples were centrifuged, and serum was separated. The IF technique was used for testing. Indirect immunofluorescence is a very sensitive and specific method for the detection of ANA and anti-dsDNA antibodies. Frozen sections of rat

kidney/liver are used as a substrate for ANA and *Crithidia luciliae* for anti-dsDNA antibody detection. Patients' serum containing ANA and anti-dsDNA antibodies is applied on the substrate in a predetermined solution. It binds the antigen at the Fab portion. An antihuman IgG antibody conjugated with a fluorescent dye (FITC) in predetermined dilution is added which binds to the Fc portion of the serum antibody. The presence of ANA and anti-dsDNA antibodies is detected by observing under a fluorescence microscope.

ANA and ds-DNA antibodies positive samples were used as positive control and positive QC samples from the UK were used the same way. The pooled normal human serum was used as a negative control. The reagents used were fluorescent antibody conjugate (antihuman IgG FITC) and PBS buffer and the materials were fluorescence microscope, micropipettes and tips, test tubes, multi-well slides, cryostat, glass jar, moist boxes and discarder. A worksheet of test sera was prepared. The controls and test sera were inactivated at 56 °C for 30 minutes. A 1:10 dilution of the sera in PBS (serum 10 µL + PBS 90 µL) was formulated. Slides were taken out from the freezer (Rat kidney substrate for ANA and *Crithidia luciliae* for ds-DNA) and kept at room temperature for 15 minutes. 10 µL of test/control sera was dispensed on respective wells. These were incubated at room temperature for 20 minutes in a moist box and then rinsed with PBS buffer. Slides kept in PBS for 20 minutes. Slides were taken out from the moist box and blotted the excess fluid. The fluorescent antibody conjugate was diluted in 1/70 dilution. Dispensed 10 µL in each well. Incubated in a moist chamber at room temperature for 20 minutes. Rinsed in the PBS.

Kept in a PBS jar at room temperature for 20 minutes. Removed excess fluid and do not dry wells. 1–2 drops of the mounting medium were placed (90 mL glycerine + 10 mL PBS) on the slides. Coverslips were put up and observed under the microscope in the dark room. Observed the different patterns of ANA antibodies and ds-DNA antibodies.

3. Results

The average mean of our study was 38.21 ± 16.635 with a minimum age of 2 years old and a maximum age of 98 years old. All graphs and pie charts show the analysis of the data of the prevalence of ANA tested at AFIP with clinically suspected cases of connective tissue disease and their correlation with age and gender. Group A represents the patient sample while Group B represents the control samples of healthy persons. In our present study, 91% (11,848) of samples were negative and 9% (1,119) were positive for ANA. In the present cohort, 76% of patients were female while 24% were male for ANA conditions. **Figure 1** illustrates the distribution of healthy controls in our present cohort. The correlation of age and gender with abnormal ANA results were demonstrated in **Table 1** and **Figure 2**. On the other hand, **Table 2** shows positive and negative predictive values.

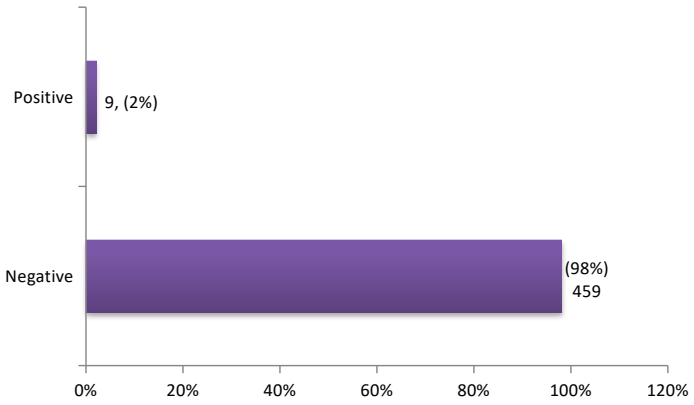

Figure 1. Distribution of healthy controls.

Table 1. Correlation of age and gender with abnormal results of ANA

No.	Variables	Pearson Correlation Coefficient (*r*)	*P* value
1.	Age	0.004	0.001
2.	Gender	-0.125	0.001

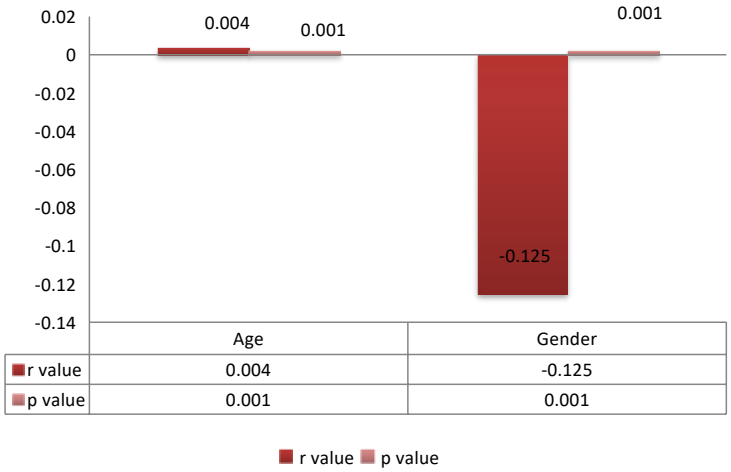

Figure 2. Association of age and gender with abnormal ANA results

Table 2. Positive and negative predictive value (PPV & NPV)

	Positive	Negative	Total
Patients sample	1119 (a)	11848 (c)	12967
Control sample	9 (b)	459 (d)	468
Total	1128	12307	13435

*True positive = 1,119
True negative = 459
Total patients sample = 12,967
All negative = 468
Total = 12,967
PPV = 1,119 / 12,967 = 8.6%
NPV = 459 / 468 = 98%
Accuracy = (1,119 + 459) / 12,967 = 12%

3.1. Anti-double-stranded DNA antibodies

Table 3 shows the mean and standard deviation of demographic variables, with 35.41 ± 15.066 and 2 years old and 95 years old for minimum age and maximum age, respectively. The distribution of positive ds-DNA patients of gender and age is shown in **Figure 5** and **Figure 6**, respectively, while the association of these demographic variables is shown in **Table 4** and **Figure 7**.

Table 3. Table mean and standard deviation (SD) of demographic variables for ds-DNA

Demographic variables	Mean ± SD	Minimum age	Maximum age
Age	35.41 ± 15.066	2 years	95 years

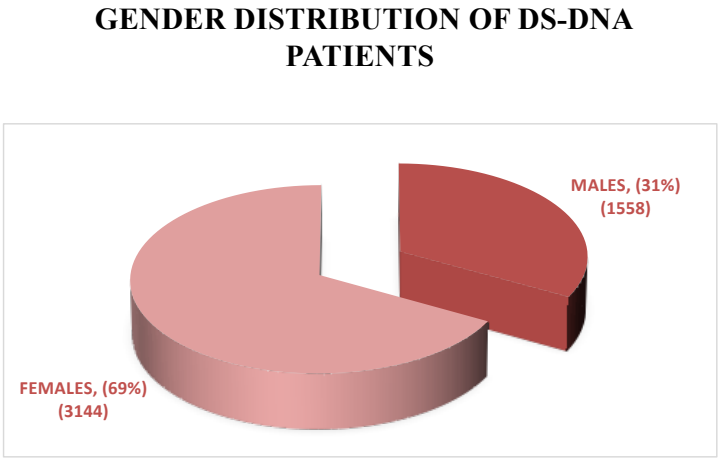

Figure 3. Gender distribution of ds-DNA patients.

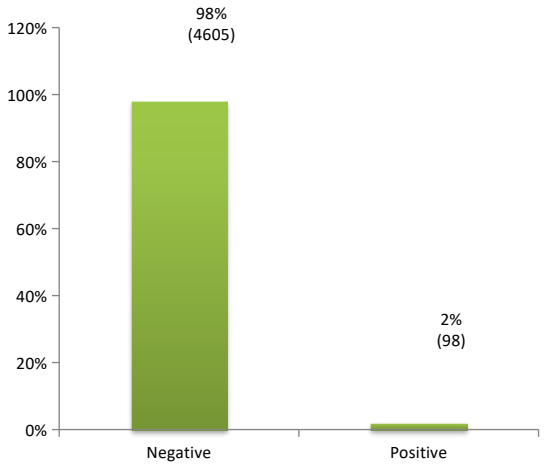

Figure 4. Distribution of positive and negative ds-DNA patients.

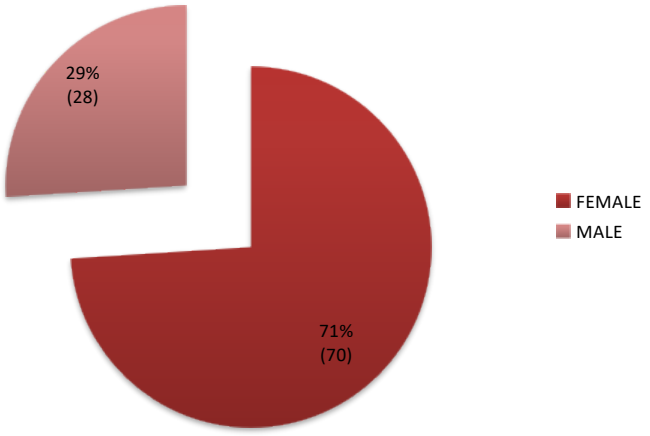

Figure 5. Gender distribution of positive ds-DNA patients.

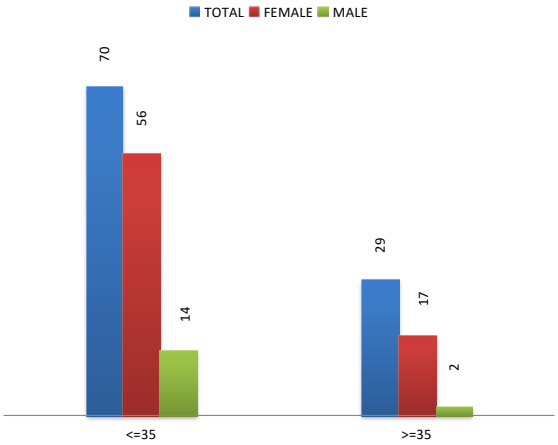

Figure 6. Age distribution of positive ds-DNA patients.

Table 4. Correlation of age and gender with abnormal results of ds-DNA

No.	Variables	Pearson Correlation coefficient (*r*)	*P* value
1.	Age	-0.054	0.001
2.	Gender	-0.025	0.001

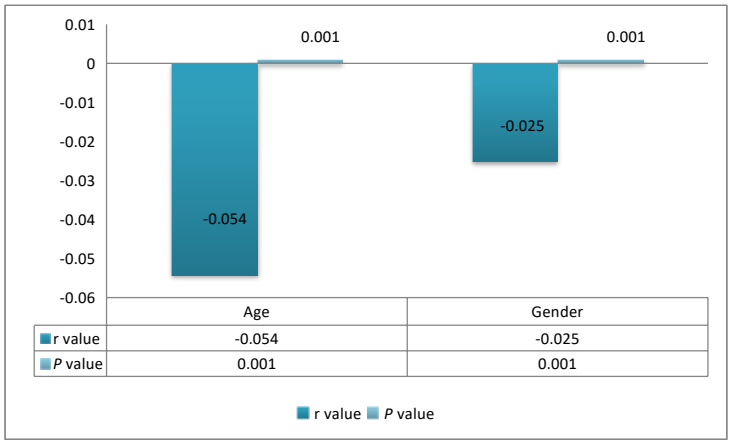

Figure 7. Association of age and gender with abnormal ds-DNA results.

4. Discussion and conclusion

When autoimmune illness is recognized at an early stage, problems from the condition may be addressed or mitigated. To avoid difficulties in detecting such a common but avoidable condition, awareness programs that are the cause of the spike in newly identified patients must continue, as should lowering the barrier for testing high-risk individuals through an affordable, non-invasive, and dependable antibody diagnostic [14]. The diagnostic assay for ANA is one of the most well-known autoimmune assays and is frequently used in clinical. Immunofluorescence is the standard technique for doing an ANA test and is considered the most common approach. The fluorescence test for antinuclear antibodies, or FANA, is referred to as such at certain facilities [15].

The typical analytical result in the majority of CTDs, ANA, has significant diagnostic implications. In the present research, investigators found that the prevalence of ANA was 2%, compared to 3% in their previous analysis of healthy participants. A year before the illness is diagnosed, some individuals with antibodies belonging to the ANA family may exhibit symptoms [16]. 70% of patients with SLE have antibodies against double-stranded DNA, which are particular to the disease. Conversely, < 0.5% of healthy people might possess these antibodies. The overall prevalence of ANA in the Chinese population was 5.92%, and it showed a positive correlation with age. Every age category showed notable variations, except those above 80 years old [17]. ANA shows positive in the 20- and 40-years old ranges in the female group. According to data from the National Health and Nutrition Examination Survey conducted between 1999 and 2004, the prevalence of ANA among individuals aged 12 years old and older in the United States is 13.8%, and over 32 million people have tested positive for the disease. According to other research, the incidence in Japanese people is 9.5% at a 1:100 cutoff level dilution, whereas it is 12.3% in Indian people [18].

In Pakistan, an alarming number of people indulge in oral addictive habits e.g. smoking, snuff and tobacco chewing, gutka, and sheesha. A recent study from Karachi, Pakistan reported that the majority of males and females practice their addictive habits in public [19]. In Southeast Asia, tobacco is used in diverse forms, including cigarettes, sheesha and smokeless tobacco (Naswar, Paan, and Gutka). These are all the risk factors of autoimmunity. There is plenty of evidence that environmental factors are noteworthy in the development of autoimmune diseases [20]. The concordance rates for autoimmune diseases in monozygotic twins are well below 100% which indicates the interaction of environmental factors with genetics in determining disease susceptibility. A lot of the autoimmune diseases run in families. A large number of diseases affect specific ethnic populations. Genomics has revealed specific genetic mutations common to people with different autoimmune diseases. According to the present study, the risk factors of autoimmunity are age, gender, environmental factors, genetic factors, family history, obesity and smoking. Quite smoking, taking care of your gums, keeping a healthy weight and avoiding environmental factors helps to reduce the chances of autoimmune disorders [21].

This study showed that ANA positivity in females (76%) was higher than in males (24%). This indicated that females have a higher ANA titer than males even at a relatively immature stage. This is mainly because of hormones such as estrogen due to an unknown cause. Because ANA-IIF is a primary screening test for adolescent SLE, this method may be used routinely to screen for ANA-positivity in younger people. Anti-ds DNA antibodies are also most prevalent in females (74%) as compared to males (26%) and are highly diagnostic markers and specific for SLE. There was a very low prevalence of ANA in healthy populations (2%) which indicates that rationalization of prescription is needed. Gender predisposition towards autoimmunity (ANA) was found 100% significant with a P value of (P = 0.001). Relation of age was also found significant with anti-ANA antibodies with a P value of (P = 0.001). This study shows a negative correlation between age (P = 0.001) and gender (P = 0.001) with anti-ds-DNA which is also significant. High prevalence was found below the mean age (38 years) of ANA and mean age (35 years) of ds-DNA.

ANA and ds-DNA detection by indirect immunofluorescence is a sensitive test for autoimmunity and it is significantly related to female gender and increasing age. The low prevalence of ANA among clinically suspected cases suggests that rationalization of test prescriptions is needed.

Authors contribution

Conceptualization: Usama Ahmed, Muhammad Zubair
Investigation: Hijazi Mallick, Amber Khan, Muhammad Zubair, Usama Ahmed
Analysis: Usama Ahmed
Writing: Usama Ahmed, Hijazi Mallick, Amber Khan, Muhammad Zubair

Disclosure statement

The author declares that they have no conflict of interest.

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