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# Diagnostic Testing and three-year data Interpretation of ANA and ANCA Test for Autoimmunity

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**Abstract:**

Out of the data was consolidated over three years, with a total of 147 ANA and 279 ANCA test results. Out of 147 patients, 67.5% showed positive results, with the majority being female. Out of 279 patients, 14 were CANCA positive, 3 were CANCA ANA positive, 7 were PANCA positive, and 4 were PANCA ANA positive.

**Keyword:** CANCA ANA, PANCA, AMA-M2, ASMA & Asp-A-NLS.

## 1. Introduction:

Autoantibodies known as ANCA are targeted towards granulocytes found in neutrophils. Serum samples from patients with Wegener's granulomatosis (WG) were the first to contain ANCA [1]. The proteinase 3 (PR3) antigen, which is a component of the neutrophil's azurophilic granules, was revealed to be the antigen identified by c-ANCA [2]. Numerous other idiopathic cases of glomerulonephritis and systemic vasculitis have also been linked to a second type of ANCA [3]. Myeloperoxidase (MPO), another component of the azurophilic granules, was discovered to be the first antigen that these p-ANCA recognized [4]. During the fixation process, MPO, a highly

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cationic protein, appears to migrate and cling to the negatively charged nuclear membrane [5]. It was discovered that ANCA also occur in various idiopathic inflammatory illnesses after its identification in systemic vasculitides [6]. ANCA have been detected in the inflammatory bowel diseases (ulcerative colitis (UC) and Crohn's disease (CD)) [7, 8], in autoimmune-mediated liver diseases [9–11], in rheumatoid arthritis (RA) [12,13], and in systemic lupus eythematosus (SLE) [14,15]. Usually, the p-ANCA type is found, but the antigen is not MPO [7]. A third type of ANCA, producing a diffuse cytoplasmic staining on ethanol-fixed neutrophils (atypical ANCA or a-ANCA), has also been described in these diseases [16].

Akaline phosphatase (ALP) is an enzyme that is present in human tissues in low but significant levels. ALP is present in high concentrations in parts of the body such as bone, liver, kidney, intestines, and the placenta [17]. Heart failure, heart attack, and serious infection can also raise the ALP level [**18**]. Each of these detection methods has its merits and limitations. Spectrometric methods are a highly accurate method of determining the composition of a species based on mass and charge. They are able to resolve elemental composition of a variety of compounds including proteins and proteases to a very high degree [19,20].

## 2. Material and Method:

### 2.1 Determination of Autoimmune liver panel antibodies:

#### i. Chemicals Used:

This kit contain slides each field 3 BIOCHIPs coated with the substrate HEp-2 cells, frozen sections of rat kidney and rat stomach, conjugate fluorescein-labeled anti-human IgG (goat), positive control – antibodies against cell nuclei (ANA), control serum with titre information, homogen, positive control - antibodies against mitochondria (AMA), negative control – autoantibody negative, buffer PBS (Phosphate buffer saline), Tween 20, glycerol embedding medium, cover glass.

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**ii. Procedure:**

10 µl of diluted sample serum applied to each reaction field of the reagent tray, BIOCHIP slides were fitted and allowed to incubate for 30 min at room temperature (+18 degree celcius to +25 degree celcius), rinsed the BIOCHIP slides with PBS-Tween for 5 min. and 10 µl of fluorescein labeled anti-human globulin added and again incubated for 30 min. at room temperature (+18 degree celcius to +25 degree celcius), washed BIOCIP slides with the PBS for 5 min. following 10 µl of embedding medium was added and slides covered with the cover slip. Slides are then observed under flourasence microscope under 20x for tissue section,infected and transfected cells and under 40x for cell substrates.

**2.2 Determination of Autoimmune liver panel profile:**

**i. Chemicals Used:**

This kit contains slides, coated with the antigens AMA M2, LKM-1, LC-1 and SLA/LP, positive control IgG human, enzyme conjugate alkaline phosphatase-labelled anti-human IgG(goat), sample buffer, wash buffer, substrate solution nitrobluetetrazoliumchloride/5-Bromo-4-chloro-3-indolylphosphate (NBT/BCIP), incubation tray.

**ii. Procedure:**

Required amount of test strips removed from the package and placed them in an empty channel. Channels of the incubation tray were filled with 1.5 ml sample buffer each. Incubated for 5 min. at room temperature on a rocking shaker. Afterwards aspirated off all the liquid. Each channel is filled with 1.5 ml diluted serum samples and incubated at room temperature for 30 min. on a rocking shaker. Aspirated off liquid from each channel and each channel is washed for 3 x 5 min. with 1.5 ml working strength wash buffer on a rocking shaker. 1.5 ml dilute enzyme conjugate

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was added in each channel and incubated for 30 min. at room temperature on a rocking shaker. Aspirated off the liquid from each channel and washed as described above. 1.5 ml substrate solution added into channels of the incubation tray and incubated for 10 min. at room temperature on a rocking shaker. Aspirated off liquid from each channel and washed each strip 3 x 1 min. with distilled water. Test strips were then placed on evaluation protocol, air dried and evaluated.

**2.3 Determination of Anti-Nutrophil Cytoplasmic antibodies:**

**i. Chemicals Used:**

This kit contain slides each containing 5 x 2 BIOCHIPs coated with the substrate granulocytes (EOH), HEp-20 cells + granulocytes (EOH) and granulocytes (HCHO), conjugate fluorescein-labeled anti-human IgG (goat), positive control with titer information: autoantibodies against granulocyte cytoplasm (cANCA), human, positive control with titer information: autoantibodies against granulocyte cytoplasm (pANCA, anti-MPO), human, negative control: autoantibody negative, human, buffer PBS (Phosphate buffer saline), Tween 20, glycerol embedding medium, cover glass.

**ii. Procedure:**

30 µl of diluted sample serum applied to each reaction field of the reagent tray, BIOCHIP slides were fitted and allowed to incubate for 30 min at room temperature (+18 degree celcius to +25 degree celcius), rinsed the BIOCHIP slides with PBS-Tween for 5 min. and 25 µl of fluorescein labeled anti-human globulin added and again incubated for 30 min. at room temperature (+18 degree celcius to +25 degree celcius), washed the BIOCIP slides with the PBS for 5 min. following 10 µl of embedding medium was added and slides covered with the cover slip. Slides are then observed under flourasence microscope under 20x for tissue section,infected and transfected cells and under 40x for cell substrates.

**2.4 IMTEC-ANA-LIA MAXX kit:**

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IMTEC-ANA-LIA Maxx is an indirect membrane-based enzyme immunoassay for the qualitative measurement of IgG class antibodies against nucleosomes, dsDNA, histones, SmD1, PCNA, ribosomal PO, SS-A/ Ro52kD, SS-B/ La, CENO-B, Scl-70, U1-snRNP, AMA M2, Jo1, PM-Scl, Mi-2 nad Ku in human serum or plasma. This assay is intended for in Virto diagnostic use only.The test is based on the principle of the line immune assay (LIA). Nuclear and associated cytosolic antigens are applied as lines on a nitrocellulose membrane. The nitrocellulose membrane is blocked to prevent unspecific reactions. During incubation of a strip with diluted patient samples autoantibodies present in the sample will bind to the antigens on the strip. For the detection of the bound antibodies a secondary horseradish peroxidase (HRO) –labelled anti- human IgG antibody is used. After addition of the substrate and stop solution the appearance of brown lines indicates the existence of (auto) antibodies against the respective antigen.

**i. Chemicals used in IMTEC-ANA-LIA MAXX kit:**

Test strip, 20x wash buffer, anti-human IgG conjugate solution, 3,3',5,5'-tetramethylbenzidin hydrogen peroxide, sulfuric acid as stop solution, incubation tray.

**2.5 Test performance:**

Test strip was placed in the tray facing its color coding upside, washed with the buffer. It was then incubated for 1 min at room temperature and washed again. Diluted sample was added to the strip and was incubated at room temperature for 30 min. then washed. Conjugate was added to the strip and incubated for 30 min. and washed. Substrate was added to the strip and incubated for 10 min. After incubation it was washed and incubated with distilled water for 1 min. After incubation with distilled water stop solution was added and incubated for 5 min. at room temperature. After incubation stop solution was removed and strip dried thoroughly. To interpret the result strip was fixed on scoring sheet and aligned it with reference line on the sheet and compared with cut-off control intensity of each strip.

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**2.5 Experimental design:**

Using immunofluorescence technique diagnosis of autoimmune disease was done. Patients prescribed for the autoantibody test their serum was collected from the blood samples. ALP, ANCA tests were performed and their respective patterns and related markers were analyzed and related autoimmune disease identified. Analyzed data of autoimmune disease test retrospectively from January 2012 to December 2013 and prospectively from January 2014 to May 2014. Total ANCA patients were 279 prescribed for test, ANA patients were 1441, anti- DNA 155, ENA 189, ALP 147. Prevalence of types of most occurring diseases their markers, patterns of the autoantibody tests were identified. Frequency of occurrence of autoimmune disease in males and females were seen. Cause of ANCA positivity and ENA, ALP positivity due to ANA positivity was identified.

**2.6 Statistical Analysis:**

Patterns of the autoantibody tests, total patients, percentage of total positive patients, percentage of the positive diseased patients were analyzed. Gender wise prevalence of auto-antibodies were analyzed and bar graph prepared.

**3. Results & Discussion:**

The screening for antibodies to extractable nuclear antigens was performed by the ALP test. In the study 67.5% out of 147 patients showed positive result and majority of them were female (27 female and 13 male). 27 patients were having ANA positive (21 female and 10 male), 2 ASMA positive (2 female and 0 male), 7 AMA-M2 positive (7 female and 0 male), 1 LKM-1 (0 female and 1 male), 4 LC-1 positive (3 female and 1 male), 1 SLA positive (0 female and 1 male), 7 AGPA positive (4 female and 3 male), 0 GBM positive, 0 Asp-A-NLS positive.

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Patterns of test were as - 12 patients were having speckled pattern (6 female and 6 male), 1 homogenous pattern (1 female and 0 male), 4 nucleolar patterns (3 female and 1 male), 7 cytoplasmic pattern (4 female and 3 male), 2 mixed pattern (2 female and 0 male) Refer table No. 01 and Graph No.01.

**Table No.01 Total year wise data of ALP antigens and patterns.**

| Test | 2012 | | | | 2013 | | | | 2014 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MALE | FEMALE | TOTAL | % | MALE | FEMALE | TOTAL | % | MALE | FEMALE | TOTAL | % |
| ALP TOTAL | 21 | 35 | 56 | **NA** | 32 | 33 | 65 | **NA** | 11 | 15 | 26 | **NA** |
| ALP POSITIVE | 2 | 9 | 11 | **19.64** | 10 | 11 | 21 | **32.31** | 1 | 7 | 8 | **30.77** |
| ANA | 1 | 4 | 5 | **45.45** | 8 | 8 | 16 | **76.19** | 1 | 5 | 6 | **75.00** |
| ASMA | 0 | 0 | 0 | **0.00** | 0 | 1 | 1 | **4.76** | 0 | 1 | 1 | **12.50** |
| AMA(M2) | 0 | 5 | 5 | **45.45** | 0 | 2 | 2 | **9.52** | 0 | 0 | 0 | **0.00** |
| LKM1 | 0 | 0 | 0 | **0.00** | 1 | 0 | 1 | **4.76** | 0 | 0 | 0 | **0.00** |
| LC-1 | 0 | 2 | 2 | **18.18** | 1 | 0 | 1 | **4.76** | 0 | 1 | 1 | **12.50** |
| SLA | 0 | 0 | 0 | **0.00** | 1 | 0 | 1 | **4.76** | 0 | 0 | 0 | **0.00** |
| AGPA | 1 | 2 | 3 | **27.27** | 2 | 2 | 4 | **19.05** | 0 | 0 | 0 | **0.00** |
| GBM | 0 | 0 | 0 | **0.00** | 0 | 0 | 0 | **0.00** | 0 | 0 | 0 | **0.00** |
| ASP-A NLS | 0 | 0 | 0 | **0.00** | 0 | 0 | 0 | **0.00** | 0 | 0 | 0 | **0.00** |
| SPECKLED | 0 | 1 | 1 | **9.09** | 6 | 3 | 9 | | 0 | 2 | 2 | **25.00** |
| HOMOGENOUS | 0 | 0 | 0 | **0.00** | 0 | 0 | 0 | **32.31** | 0 | 1 | 1 | **12.50** |
| NUCLEOLAR | 0 | 1 | 1 | **9.09** | 0 | 2 | 2 | **76.19** | 1 | 0 | 1 | **12.50** |
| CYTOPLASMIC | 1 | 3 | 4 | **36.36** | 2 | 1 | 3 | **4.76** | 0 | 0 | 0 | **0.00** |
| MIXED | 0 | 0 | 0 | **0.00** | 0 | 1 | 1 | **9.52** | 0 | 1 | 1 | **12.50** |

**Graph No.01 Year wise data of ALP patterns in total male and female**.

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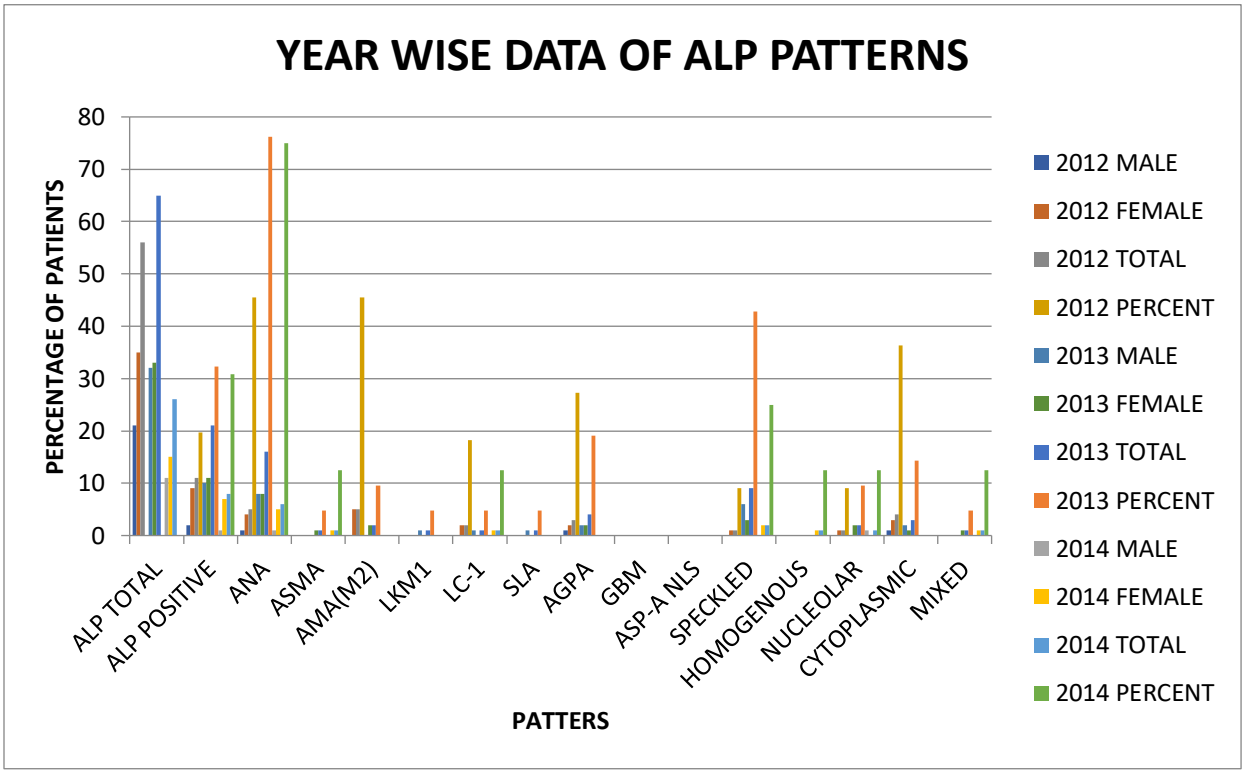


Three-year (2013,2013 and 2014) Consolidated data of total ALP test results are as –total ALP positive patients' percentage of three year is 27.21%, ANA 67.5%, ASMA 5%, AMA (M2) 17.50%, LKM-1 2.50%, LC-1 10%, SLA 2.50%, AGPA 17.50%, GBM 0%, ASP-A NLS 0%, Speckled 30%, Homogenous 2.50%, Nucleolar 10%, Cytoplasmic 17.50% and Mixed 5%. Refer Table No.02 and Graph No. 02.

**Table No. 02 Consolidated data of total ALP positive patients.**

| CONSOLIDATED TABLE OF ALP | | | | | |
|---|---|---|---|---|---|
| **TEST** | **2012** | **2013** | **2014** | **TOTAL** | **PERCENTAGE** |
| ALP TOTAL | 56 | 65 | 26 | 147 | NA |
| ALP POSITIVE | 11 | 21 | 8 | 40 | 27.21 |
| ANA | 5 | 16 | 6 | 27 | 67.50 |

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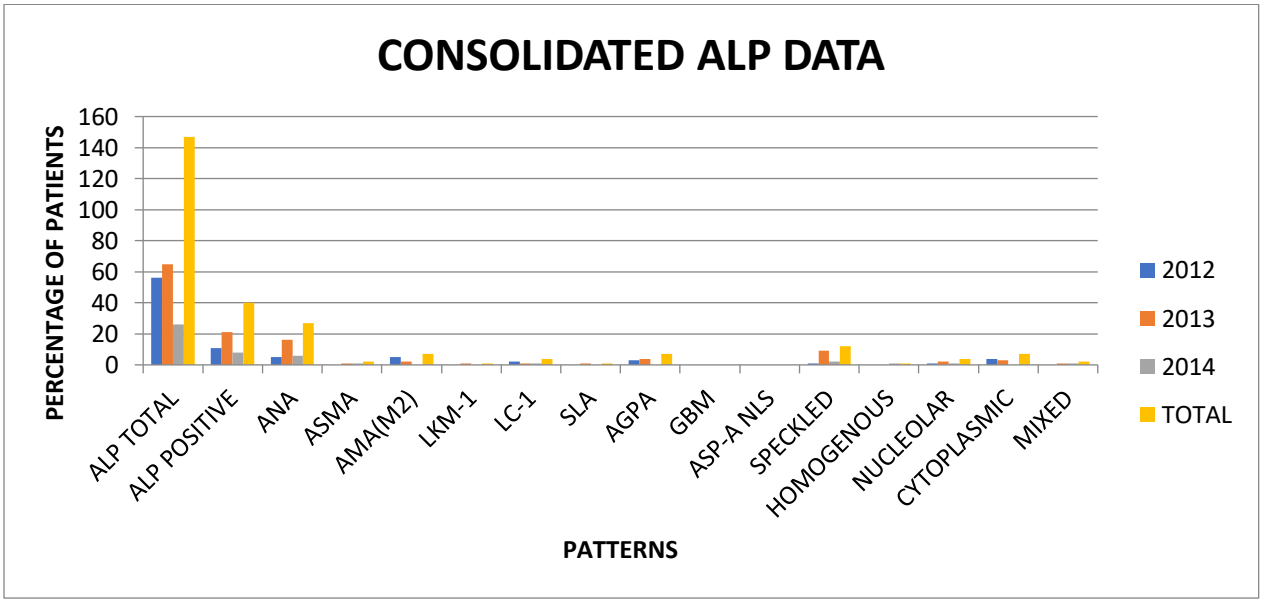
| | | | | | |
|---|---|---|---|---|---|
| ASMA | 0 | 1 | 1 | 2 | 5.00 |
| AMA(M2) | 5 | 2 | 0 | 7 | 17.50 |
| LKM-1 | 0 | 1 | 0 | 1 | 2.50 |
| LC-1 | 2 | 1 | 1 | 4 | 10.00 |
| SLA | 0 | 1 | 0 | 1 | 2.50 |
| AGPA | 3 | 4 | 0 | 7 | 17.50 |
| GBM | 0 | 0 | 0 | 0 | 0.00 |
| ASP-A NLS | 0 | 0 | 0 | 0 | 0.00 |
| SPECKLED | 1 | 9 | 2 | 12 | 30.00 |
| HOMOGENOUS | 0 | 0 | 1 | 1 | 2.50 |
| NUCLEOLAR | 1 | 2 | 1 | 4 | 10.00 |
| CYTOPLASMIC | 4 | 3 | 0 | 7 | 17.50 |
| MIXED | 0 | 1 | 1 | 2 | 5.00 |

**Graph No.02 Consolidation of year wise ALP patterns data.**




In this study, ANCA was positive in 14 cases out of 279. C-ANCA was found to be positive in 14 cases and P-ANCA was found positive 7 cases.Autoimmune hepatitis (AID) is presumed to be rare

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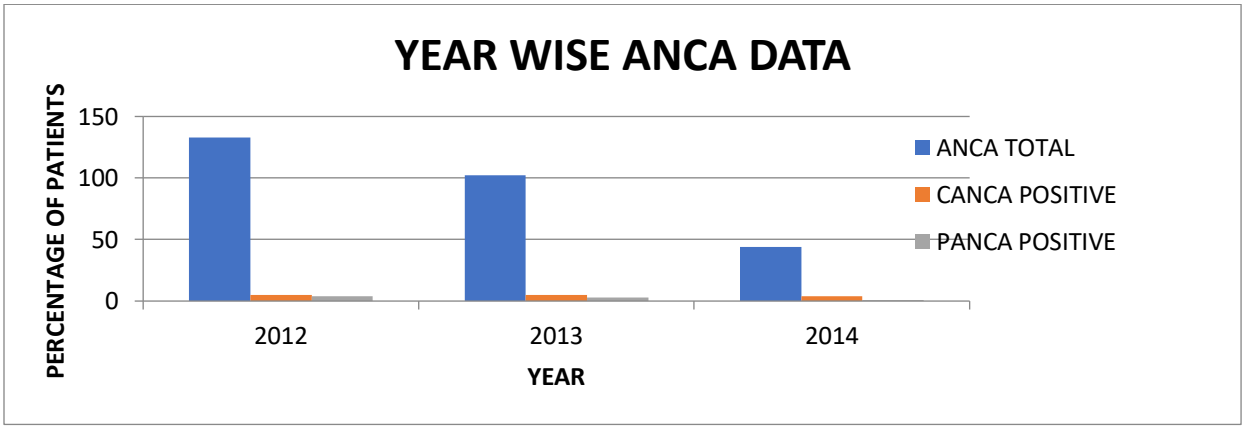
in India. Patients with chronic liver diseasesuspected to be AIH, were screened for ANA, ASMA, AMA-M2 and anti liver kidney microsomal antibodies ( anti-LKM-1) by immunofluorescence and by immunoblot method. 40 (27.21%) out of 147 total prescribed patients with chronic liver disease were diagnosed as autoimmune disease. Out of 40, 7 cases were primary biliary cirrhosis, which were positive for anti- mitochondrial antibody (AMA-M2).

**Table No. 03 Year wise ANCA data of total positive CANCA and PANCA.**

| TEST | YEAR | | |
|---|---|---|---|
| | 2012 | 2013 | 2014 |
| ANCA TOTAL | 133 | 102 | 44 |
| CANCA POSITIVE | 5 | 5 | 4 |
| PANCA POSITIVE | 4 | 3 | 1 |

**Graph No.03 Year wise ANCA data of total positive CANCA and PANCA.**




Three-year (2013,2013 and 2014) Consolidated data of total CANCA & PANCA test results are as –total Out of total 279, 14 patients were CANCA positive (5 female and 9 male) and 3 were CANCA ANA positive (2 female and 1 male), 7 patients were PANCA positive (4 female and 3 male) and 4 patients were PANCA ANA positive (2 female and 2 male). Refer Table No. 04 and Graph No.04.

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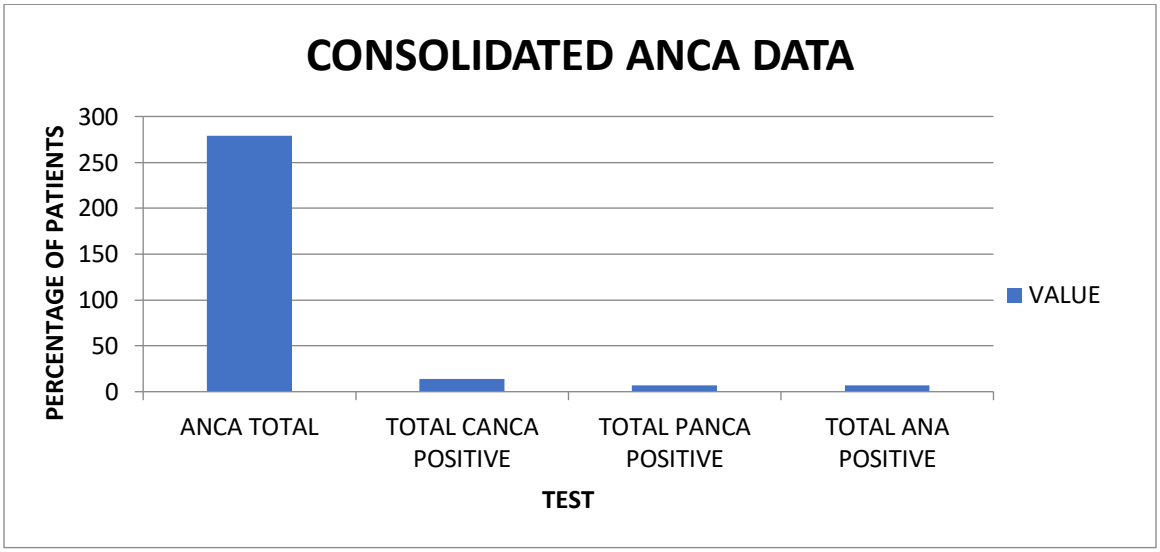
**Table No.04 Consolidate data of total ANCA patients**

| TEST | VALUE |
|---|---|
| ANCA TOTAL | 279 |
| TOTAL CANCA POSITIVE | 14 |
| TOTAL PANCA POSITIVE | 7 |
| TOTAL ANA POSITIVE | 7 |

**Graph No. 04 Total consolidated data of ANCA showing total CANCA, PANCA and ANA positive ANCA.**




**4. Summary:**

Present findings reflect that incidence of autoimmune diseases and their patterns for central India. Thus, it can be said that female is more prone to autoimmune diseases. because females show increased immune reactivity or due to different levels of gender specific hormones and reproductive functions

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