DISCUSSION
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Localization of UC Antigens

The antigens specific for atypical pANCA have not yet been completely elucidated. Many researchers have reported proteins like lysozyme, elastase, lactoferrin, catalase, cathepsin G, BPI, α-enolase, and lamin B1 amongst many others which still remain to be characterized to be suitable candidates for atypical pANCA antigens (Barahona et.al, 2009, Nakamura et.al, 2003). Various studies have indicated that these are not cytoplasmic antigens like MPO, PR3, but they are present inside the nucleus of human neutrophils (Mallolas J, et. al, 2000, Andrea et.al, 2013). It has been reported that the High Mobility Group Proteins (HMG1 and HMG2) and few granular and non-histone chromosomal proteins may be the potential target antigens of atypical p-ANCA (Takaishi et.al, 2012). However atypical pANCA do not react with denatured double stranded DNA (Andrea et.al, 2013). This suggests that the native confirmation of the DNA is essential for the antigenic targets to maintain their antigenicity. Also, the fluorescence pattern observed on the IIF staining suggests that the antigens are localized near the chromatin rich inner periphery of the neutrophil nucleus, thus imparting the thick perinuclear staining by IIF. The antigens have a very good probability of being DNA-Protein complexes (Bossuyt, 2006). Thus in order to confirm the reported findings experimentally, a set of three experiments was designed and performed.

The first experiment of spectrophotometrically analysing the cytoplasmic and nuclear neutrophil fractions was aimed to estimate the total protein content of both the fractions. It was found that the cytoplasmic fraction contained approximately 10 times the protein load as compared to the nuclear fraction. Next experiment was aimed at determining the load of atypical pANCA antigens in both the fractions by subjecting them to a UC ELISA assay. Here the two fractions were coated onto ELISA plates as source of antigens and were made to react with UC positive sera as source of primary atypical pANCA antibody. The reaction was completed using a HRP tagged secondary antibody and TMB-H₂O₂ substrate. Although the nuclear fraction had one tenth the protein load of the cytoplasmic fraction, it showed much higher OD value than the latter. This led us to the conclusion that indeed the concentration of atypical pANCA
antigens was much higher in the nuclear fraction. The OD reading showed by the cytoplasmic fraction could be attributed to the fact that the processing steps of separation of the two fractions could have resulted in the cross over contamination of the cytoplasmic fraction with some amount of nuclear fraction. The third experiment was designed to confirm the findings of the first two experiments using Dot-Blot technique. In this experiment, MPO and PR3 purified antigen solutions, Cytoplasmic and Nuclear extracts in native and denatured (heat treated) form were blotted onto the nitrocellulose membrane and subjected to a HRP tagged secondary antibody and TMB-H$_2$O$_2$ substrate. The colour was developed and it was found that the nuclear extract in its native form showed the maximum colour intensity of the blot. There was no blot at the MPO and PR3 spotting site confirming that they were not the target antigens of atypical pANCA. Also worth noticing was the fact that the denatured nuclear fraction did not show any spot suggesting that heat treatment led to the loss of confirmation of the atypical pANCA antigens. Thus the UC antigens were localised to the inner periphery of the neutrophil nucleus.

**Autoimmune Profile Survey of UC Cases**

Studying the epidemiology of UC is an important aspect to investigating possible etiological bases for UC through genetics, race, ethnicity, or environmental exposure in any given society. While the mortality rates in UC patients are not greater, the risk increases with age, comorbidity, and severity of disease (Meenakshi et al, 2013). In the past 15 years, there has been an increase of at least 3-fold in the prevalence of UC in the developing countries (Jiang et.al, 2002). Several research groups have examined the influence of race and ethnicity on the development of UC, hence its incidence, in various geographic areas (Basson et.al, 2014). The environmental make-up of UC, or its flare-up, is quite complex and consists of external factors like childhood infections, smoking, family history as well as internal ones such as the intestinal flora (Alexandra et.al, 2013). It is of interest to note that nicotine seems to play a curative role in UC. In one study, the application of a transdermal nicotine patch as an adjunct to typical treatment regimen eased the symptoms of mild and moderate UC cases. Smoking has been positively associated with the management of symptoms of UC. The patients who are active smokers have reported experiencing milder forms of symptoms.
as compared to patients who have never smoked. (Lunney et.al, 2012). It was frequently observed that family members of IBD patients present the disease (CD or UC), or some other signs, indicative of an increased risk of developing it. Almost 40% of the immediate relatives of people suffering from IBD were reported to suffer from the disease (Yulan et.al, 2015). But controversy concerning the strength of correlation between genotype and phenotype have always existed and have been expressed in several studies. Annese et al, 2002, presented a study supporting the notion that no significant correlation exists between NOD2/CARD15 genotype and disease progression or type.

The autoimmune profile survey undertaken on demographics and environmental risk factors shed light on various aspects of UC. The data collected from the subjects was grouped under 10 variables as follows: infections occurred during childhood, vaccines administered, consumption of sugar, intake of fibres, consumption of fast food, intake of caffeine (tea and coffee), physical exercise, smoking, health and hygiene and major stressful events. These criteria were compared with the severity of disease. Also the impact of these indicators on extent of UC and the quality of living of the patients was analysed. The first occurrence of UC was seen in the age group of 30 to 45 years, which corroborates the findings of earlier studies (Weronica et.al, 2014). The loss in weight was seen to be severe in the first 10 years of the disease and plateaued out beyond this threshold. Abdominal pain was found to be the most severe symptom of UC, thus prompting the focus of therapy towards reducing the abdominal pain. Family history also was seen to play a role in the pathophysiology although a significant relation could not be established.

Tobacco in the form of cigarette was found to reduce the severity of the physical symptoms of UC. Patients consuming tobacco reported that their symptoms were better manageable. This observation supplements the finding of an earlier study relation to effect of tobacco consumption on UC wherein the researchers found that Nicotine present in tobacco helps to desensitise the nervous system, thus enabling the patients to feel the decreased severity of physical symptoms like pain (Alexandra et.al, 2013)

Physical exercise was seen to play a positive role in management of UC symptoms amongst patients. The patients undertaking regular physical exercise noted that had more manageable symptoms. Moreover, the patients who started regular
physical activity noted that it helped them to improve their quality of life in terms of acceptance of the condition, boosted self-esteem, ability to keep themselves busy with work and lead a better life. This may be attribute to the hormones being released during physical activity which help the patients to manage the disease symptoms better.

Sugar intake was seen to be positively associated with occurrence of UC. Majority of the participants used extra sugar in their food as well as beverages like tea and coffee. Fast food consumption also seemed to be positively associated with occurrence of UC since a large proportion of patients used to consume fast food more than once a week. Alcohol consumption seemed to have no effect on UC occurrence. Fruits, vegetables, bread and beverages did not seem to have any correlation with UC.

Thus the autoimmune profile survey of the UC patients and healthy controls was undertaken in order to compare the disease extent and severity, and to assess the impact of the factors on UC and the quality of living of the patients. This survey provided valuable insights into the demographic characteristics of the UC patients and the environmental risk factors associated with occurrence of UC.

Development of Diagnostic Algorithm for UC

The diagnosis of UC can be divided into two separate but related entities. The first involves the signs and symptoms as perceived by the patient and the clinician; the second involves the endoscopic, histological and serological laboratory examinations. In terms of the signs and symptoms, patients suffering from UC majorly complain of rectal blood loss, with recurrent stools and mucous release. Onset of UC is typically deceptive. In severe of the cases, pus-filled rectal flow causes pain in lower abdomen and severe dehydration, noted a lot amongst the elderly population. Blood in stools is the primary symptom in UC. Patients with UC might show symptoms such as urgency to defecate and bleeding in the rectum with passage of pus and mucus. For the clinical assessment of UC, several activity indices exist, including the Simple Clinical Colitis Activity Index and the modified Truelove-Witts severity index (DeLange et.al, 2004). By far, Endoscopic evaluation along with Histopathological evaluation of the biopsy remains the gold standard for diagnosis of UC (Nikolaus et.al, 2007). Along with the above mentioned criteria, serological markers are deployed as possible means for diagnosis. These range from antibodies and autoantibodies (Reumaux et.al, 2003), to
various products secreted by cells (Kayazawa et.al, 2002) or their metabolites (Winterkamp et.al, 2002). In IBD, a serologic response to a range of microbial and autoantigens may be developed. The utility of different antibodies in UC and CD as diagnostic or sub-clinical markers, has been a subject of vast investigation. In UC, the most significant serological marker is atypical pANCA. It has been reported to be present in more than 85% of the UC patients (Papp et.al, 2007). Along with it, other serological markers involved in diagnosis of UC are ANA, cANCA, pANCA, MPO, PR3, etc. Most of the western diagnostic companies like Mayo Laboratories and Quest Diagnostics employ a panel of assays based on these related antigens in order to enhance the predictability of the serological tests in conjunction with other clinical findings for diagnosis of UC. They have devised a set of tests which are performed sequentially which help them to narrow down on the probable diseases. This process also helps to save on the cost and time required for the diagnosis. Due to the resource limited setting in India, physicians rarely recommend a systematic panel of tests to the patients and thus end up misdiagnosing the disease. Also the patients suffer in the process due to loss of crucial time and financial resources performing various diagnostic procedures, many of which could be avoided had the doctors followed a systematic approach to diagnose UC.

Thus there was a need felt for a systematic algorithm for UC diagnosis in India due to the large amount of expenses incurred by the patients for performing various diagnostic tests, many of which could be avoided if a systematic algorithm was adopted. Also, the algorithm was necessary to save upon the precious time wasted during the diagnosis phase due to which treatment gets delayed. This diagnostic algorithm has been validated in the western countries but is not established in the Indian setting. Based upon the available literature (Papp et.al, 2007) and the results of the BioRad ANCA-IIF, ANA IIF, MPO ELISA and PR3 ELISA assays, the proposed algorithm was tested for its validity amongst the Indian population. The results confirmed the presence of atypical p-ANCA antibodies in UC as stated in earlier studies (Reumaux et.al, 2003). None of the specimens tested positive for ANA, PR3 ELISA or MPO ELISA tests.

Thus the algorithm for UC diagnosis was established and validated in Indian Population. This diagnostic algorithm will prove to be of a great help to gastroenterologists in order to follow a systematic approach for recommendation of
diagnostic tests. It will also help in reducing the cost incurred by patients to perform unnecessary diagnostic tests. This algorithm will also enable the doctors to save upon the precious time of the patient which gets wasted during the diagnosis phase and put the patients on the correct drug regimen so as to minimise the discomfort caused to them due to the symptoms of UC.

**Development of Diagnostic Assays for UC**

In UC, the blood count may reflect lessened number of RBCs due to prolonged bleeding from the rectum as a result of chronic inflammatory condition. UC patients may also show electrolyte abnormalities due to chronic diarrhoea. (Stange et al, 2006; Langan et al, 2007; Stange et al, 2008). The single most robust serological biomarker of UC is P-ANCA (Stange et al, 2008). pANCA can be found in more than 80 percent of the UC patients (Allan et.al, 2016, Nikolaus et.al, 2007). The specificity of pANCA for UC is reported at 89% (Nikolaus et.al, 2007). Most of the leading diagnostic firms around the world make use of atypical pANCA testing for serological diagnosis of UC in suspected patients. Hence atypical pANCA was employed as the biomarker of preference to develop three in-house assays as a part of this project to aid in the early diagnosis of UC. The first assay was ANCA IIF assay. The neutrophils mounted onto IIF slides using the in-house technique were observed to be bigger in size as compared to the ones on commercially available ANCA IIF slides, which made the nucleus very prominent for differentiating between typical and atypical p-ANCA staining patterns.

The dual fixative format of ethanol and formalin fixation facilitated the differentiation between typical and atypical p-ANCA which was otherwise left to the judgement of the experts observing the patterns. pANCA showed cANCA type fluorescence on formalin fixation. This observation is attributed to the fact that the pANCA antigens, present in the azurophilic granules in the cytoplasm of the neutrophils do not migrate to the negatively charged nucleus and remain localized to the cytoplasm itself, thus giving a cANCA type fluorescence. On the contrary, in case of atypical pANCA, the fluorescence was lost. This may be due to the loss of integrity in terms of the binding site of the UC antigens which are localized within the nucleus as a result of formalin treatment. Another benefit of the ANCA IIF assay was the affordability. The assay was developed at almost 1/10th the cost of the commercially
available alternatives. Cost is a huge factor responsible for low penetration of high end tests into the rural areas of India.

The second in-house developed UC assay was the UC ELISA assay. The development of this assay was based on the fact established in the earlier part of the experiments that UC antigens are located in the nucleus of the neutrophils. Hence human neutrophils were lysed to extract intact nuclei as source of UC antigens using an in-house developed and standardised protocol. These intact nuclei were used as the source of UC antigens to be coated on to the ELISA plates. These antigen-coated ELISA plates were subjected to UC serum specimens as a source of primary atypical pANCA antibodies followed by HRP conjugated secondary anti human IgG antibodies and TMB H₂O₂ substrate. The colouration observed was analysed using a spectrophotometer and a reference range was established to correlate it with concentration of atypical pANCA antibodies in patient serum. This approach has not been tried yet in the commercial diagnostic industry and hence was considered as a novel approach. Thus an in-house UC ELISA assay was successfully developed and its reference range was determined.

Going one step further from the currently available IIF based assays, we developed a novel assay which was more accurate, simplified and economical. The modified UC ANCA IIF assay made use of intact nuclei from human neutrophils coated onto IIF slides. Due to the absence of whole neutrophils, the result interpretation was simplified and could be stated as positive for fluorescence observed in the nuclei and negative for absence of fluorescence. As per the BioRad ANCA Test, the specimen results were interpreted based upon the pattern showed by them on the ethanol fixed BioRad ANCA slides. The ANCA patterns seen were: c-ANCA pattern was presented as coarse speckled granular cytoplasmic staining; p-ANCA pattern was seen as fine rimmed perinuclear staining and atypical p-ANCA pattern was observed as thick perinuclear staining along with infrequent nuclear extensions. The in-house developed modified UC ANCA IIF assay had a rather simplified interpretation of the results with positive result being fluorescence observed at the nuclear periphery and negative result being absence of fluorescence on the modified UC ANCA IIF slide. The novel assay presented minimal interference from other related autoantigens like MPO and PR3
since they were removed in the processing stages itself and hence could not be detected on the slide.

**Comparison of Performance Characteristics of In-house Assays with Commercial Gold Standard**

The role of ANCA in UC had been suggested by many researchers (James et al., 2011, Sabery et al., 2007, Reese et al., 2006, Buckland et al., 2005). In serological testing, IIF remained one of the most reliable techniques to detect ANCA in UC (Maria et al., 2009, Michele et al., 2015). However, considerable amount of expertise is required to correctly distinguish between the various patterns of ANCA. Differentiating the UC related atypical p-ANCA pattern from the non-UC autoimmune typical p-ANCA pattern on ethanol-fixed neutrophils by IIF presented various challenges. Nevertheless, given the major differences in diagnostic implications, this distinction is vital. The microscopic conditions put forward by Terjung et al., 2001, have not yet been extensively adopted in UC detection (Klebl et al., 2003). Serological specimens depicting the p-ANCA pattern on ethanol fixation and a faint or negative pattern using formalin fixation, indicated the atypical p-ANCA pattern related with UC (Maria et al., 2009). Previous study had reported that atypical IIF-ANCA samples, defined as being positive on ethanol but negative on formalin-fixed neutrophils, had a very low (< 1%) positivity rate for anti-PR3 or anti-MPO antibodies (Ming-Wei et al., 2014). In this research project, we aimed to simplify the detection of atypical p-ANCA in UC patients by employing a novel IIF based assay. Atypical p-ANCA was the predominant pattern observed amongst UC patients using the modified UC ANCA IIF assay. Similar results were found earlier (Laurent et al., 2007).

The in-house developed ANCA IIF Assay was found to be comparable with BioRad ANCA IIF Assay to distinguish between UC cases and healthy controls with a high relative specificity and PPV at a remarkably low cost. The neutrophils observed in the in-house assay were of comparatively bigger size as compared to BioRad ANCA IIF Assay, thus making the nucleus very prominent for differentiating between typical and atypical p-ANCA staining. The time required to perform the in-house assay was at least an hour lesser than the BioRad Assay. The in-house ANCA IIF Assay had a sensitivity of 71.1% and specificity of 100%. The PPV was 100% whereas the NPV
was 77.6%. The intra and inter assay variability of the In-house Assay was analysed and was found to at par with BioRad ANCA IIF. The ROC curve analysis showed equivalent curves for both in-house developed ANCA IIF Assay and BioRad ANCA IIF Assay. The AUC was also found to be same for both the assays at 0.856, which categorised the in-house developed ANCA IIF Assay as ‘good’ in terms of its accuracy and performance.

The in-house developed UC ELISA Assay was found to be comparable with BioRad ANCA IIF Assay to distinguish between UC cases and healthy controls. The BioRad ANCA IIF assay was found to be slightly more accurate as compared to the in-house UC ANCA assay. The in-house UC ELISA Assay had a sensitivity of 68.9% and a specificity of 100%. The PPV was 100% whereas the NPV was 76.2%. The intra and inter assay variability of the In-house Assay was analysed and was found to at par with BioRad ANCA IIF. The lower sensitivity could be attributed to the antigen processing steps undertaken during the development of the ELISA assay. The ROC curve analysis showed almost similar curves for both the assays with BioRad ANCA IIF Assay having the edge over in-house developed UC ANCA Assay on account of its higher sensitivity. The AUC was found to be 0.856 for BioRad ANCA IIF Assay and 0.844 for the in-house developed UC ANCA Assay, which categorised the in-house developed ANCA IIF Assay as ‘good’ in terms of its accuracy and performance.

The in-house developed Modified UC-ANCA IIF assay simplified the process of interpretation of test results by eliminating the possibility of a c-ANCA and p-ANCA type fluorescence pattern. The results were interpreted as positive for fluorescence observed in the nuclei and negative for absence of fluorescence. This test produced reasonably higher sensitivity (86.6%) as compared to that of commercial standard BioRad ANCA IIF assay (71.1%). This result can be attributed to the fact that the atypical p-ANCA specific antigens, being localized on the inner periphery of the nuclei, may have been better exposed to the ANCA antibodies in the patient sera in the modified UC ANCA IIF slides containing intact nuclei rather than the BioRad IIF slides coated with whole neutrophils. The NPV of modified UC ANCA IIF assay was also seen to be significantly higher at 88.2% than that of BioRad ANCA assay at 77.6%. Also, none of the healthy controls or the related disease Autoimmune Vasculitis control showed positivity on the modified UC ANCA IIF assay, justifying its high specificity.
The intra and inter assay variability of the In-house Assay was analysed and was found modified UC ANCA IIF assay as compared to BioRad ANCA IIF assay. The ROC curve analysis showed a better curve for both the assays with BioRad ANCA IIF Assay on account of its higher sensitivity. The AUC was also found to be 0.856 for BioRad ANCA IIF Assay and 0.933 for the in-house developed modified UC ANCA IIF assay, which categorised the latter one notch higher than BioRad ANCA IIF assay as ‘excellent’ in terms of its accuracy and performance.

Thus with a high specificity and an enhanced sensitivity the modified UC ANCA IIF assay will prove to be a better diagnostic test for UC as compared to other IIF assays available in the market. The time required to perform the in-house assay was considerably lesser than that of commercially recommended ANCA IIF Assays. c-ANCA and p-ANCA controls did not show fluorescence on the ANCA IIF test. Thus, substantiating the test results with the clinical evidence, we will be able to determine whether the patient is suffering from Ulcerative Colitis or not. The use of modified UC ANCA IIF assay as a standalone may be insufficient for diagnosis of patients suspected to have UC. However, the high sensitivity of this assay supports its use as a first line screening test for UC, which is generally not employed by gastroenterologists due to high costs involved. Also, the high specificity may also enable of modified UC ANCA IIF assay to be used as a confirmatory test for UC after corroborating the test results with physical symptoms and other clinical findings. This will be the most rapid form of diagnostic test for UC.

Amongst the three in-house assays developed, the of modified UC ANCA IIF assay showed the best performance characteristics and Receiver Operating Characteristics. The Area under ROC Curve for Modified UC ANCA IIF assay was 0.933, which is in the range of excellent diagnostic test in ROC Curve analysis. This assay was significantly superior to the other two in-house assays as well as the commercial standard BioRad ANCA IIF assay in terms of sensitivity and specificity. Hence we can conclude that Modified UC ANCA IIF assay can be employed efficiently in the Indian setting to simplify and facilitate the early diagnosis of UC.