Original Research Article

Comparative evaluation of Inflammatory cells and Interleukins in Irritable Bowel Syndrome subtypes

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Abstract

Background: Irritable Bowel syndrome is a gastrointestinal disorder with a high prevalence but its etiopathogenesis is not very clear. In recent years role of immunogenic activation as one of the causative factors has gained acceptance. This study was undertaken to analyse the difference between patients presenting predominantly with diarrhoea [IBS-D] and those with predominant constipation [IBS-C] with respect to immune cells [CD3 , CD8 Intraepithelial lymphocytes (IELs) and Mast cells] and the cytokine profile assay as well as to assess the correlation between the immune cells and the cytokine levels.**Methods:** Fifty one clinically diagnosed IBS patients and twenty three healthy controls were included. IBS patients were further subgrouped into IBS-D and IBS-C based on predominant stool pattern. Biopsies from descending colon were taken after detailed clinical history and thorough full length colonoscopy. IHC for CD3, CD8 IELs and Mast cells was done and counts were given. Simultaneously Interleukins levels were assayed by ELISA. **Results:**Mean (SD) values of both CD 3 and CD8 +ve IELs and Mast cell counts were higher in IBS-D subgroup compared to IBS-C subgroup. However the difference between these two groups was found to be statistically significant for mast cells only.Level of IL-2, IL-6 and IL-8 were significantly high in both IBS- D and IBS C subgroups as compared to controls. On comparing the two subgroups, only IL8 showed a significant difference between the two. The increase in mast cells correlated positively with CD3 lymphocytes and IL-8. **Conclusion**: Inflammation has a significant role in the causation of IBS along with psychological disorders. We also advocate different treatment strategies for IBS-D and IBS-C patients as they show different levels of expression of immune cells and inflammatory cytokines.

Keywords: CD3 and CD8 lymphocytes,Mast cells, Cytokines ,IBS

Introduction

Irritable bowel syndrome (IBS) is a common condition affecting the digestive system, with a mixed group of recurrent symptoms that include abdominal pain, bloating and altered pattern of defecation- diarrhea/ constipation/or both.[1] In India, the prevalence reported varies from 4% to 7%. [2]

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The highest incidence have been seen in the second to fourth decades with some authors having reported IBS in pediatric age group as well.[3] IBS has been reported to be more common in women than men in many Western countries,[4] but studies from Asia have not reported any such predilection. [5,6] IBS has no specific diagnostic markers till date. Thus its diagnosis is based solely on clinical presentation, Rome IV criteria being the most recent one used worldwide. [7] It states that IBS is to be diagnosed clinically when there has been recurrent abdominal

pain on average at least 1 day a week in the last 3 months associated with two or more of the following:

1. Related to defecation
2. Associated with a change in the frequency of stool
3. Associated with a change in form (consistency) of stool

Traditional theories regarding its pathophysiology visualized it as a three part complex of altered Gastrointestinal (GI) motility, visceral hyperalgesia and psychopathology. However, with time and ongoing research, various factors like environmental impact, psychosocial stressors, gut flora alterations, gut epithelial barriers abnormalities, infections and even genetic factors are said to contribute to its pathophysiology. [8,9]

Despite lack of overt morphological inflammation, the importance of immune factors has gained significant importance in the last decade and a half. [10] Increased numbers of mucosal immune cell infiltrates, especially Intraepithelial lymphocytes (IELs) and Mast cells, have been reproducibly demonstrated in IBS population.[11] However, Antigen-presenting cells have been little investigated. [12] IBS patients are said to have altered blood cytokine profile due to disturbance between proinflammatory and anti-inflammatory cytokines. Increased level of proinflammatory cytokines especially IL-2, IL-6, IL-8 and TNF-α have been documented in many studies.

However status of anti-inflammatory cytokines, especially IL-10, is controversial. [13-15] In Asia , the studies done in IBS patients are few and comparative studies between IBS- D and IBS –C even less.

Hence present study was done with emphasis on comparison between diarrhoea predominant (IBS-D) and constipation predominant (IBS-C) subgroup with respect to CD3;CD8 IELs and mast cells and different interleukins (IL-2, IL-6, IL-8 and IL-10) as well as correlation of individual Interleukins with these immune cells.

Material and method

The study was conducted over a period of one year with fifty five patients and thirty healthy controls after taking approval of the institutional ethical committee. The patients were chosen on the basis of Rome IV clinical criteria. The controls were the age and sex matched attendants of these patients with apparently no health issues whatsoever. All the patients and controls were enrolled in study after thorough counseling and proper consent. After proper bowel preparation, they underwent colonoscopy using model number GIF-V70 endoscope (Olympus Corporation, Tokyo) during which the entire lower gastrointestinal tract was thoroughly examined to rule out any obvious abnormality.This was followed by descending colon biopsy for histopathological evaluation and immune histochemistry. Biopsies taken were fixed in 10% (v/v) formal saline and processed routinely. 2-3 micron thick sections were taken and stained with Hematoxylin and Eosin for morphological details.

Immunohistochemistry for CD3 ,CD8 IELs and Mast cell Tryptase was done. The primary monoclonal antibodies used were from Biogenex, Fremont, California, USA. The morphometric counts were done by three observers [MB,AS,VK] in the areas showing maximal density for the cell of interest. Lymphocyte count was noted in the surface epithelial lining as well as the crypts lining using pinhole method and were reported per hundred epithelial cells. Only those lymphocytes which showed positive membranous staining were counted. For Mast cells, counting in the lamina propria in ten high power fields (hpf) was done and their average was given as number of mast cells/hpf. Only those cells which showed diffuse granular cytoplasmic positivity were counted. 2 ml of blood sample was collected from controls and patients in plain vaccutainer for analysis of cytokines IL-2, IL-6, IL-8 and IL-10.Human ELISA kit [“Ray BiotechR’’ Norcross, GA, U.S.A.] was used for measuring their serum levels.

Inclusion criteria: All patients irrespective of their age and sex who clinically fulfilled the ROME IV criteria were included in the study.

Exclusion criteria: Patients who fulfilled the ROME IV criteria but had abnormal findings on colonoscopy or had histopathological findings consistent with a diagnosis other than IBS, including Lymphocytic colitis(with intraepithelial lymphocytes>20/ intraepithelial cells)/Collagenous colitis (with subepithelial collagen thickness upto 10 micron and Intraepithelial lymphocytes <20/ 100 intraepithelial cells) and Mastocytic Enterocolitis (with mast cells > 20/100 hpf) were excluded from study.

Statistical analysis: Graph Pad Prism for Windows (Version 4) (SanDiego, California) was used for statistical analysis. Student “t” test , Chi square test with/without Yates’ correction and Pearson’s correlation test were used as and where required. *P* values at ≤ 0.05 were taken as critical level of significance uniformly.

Results

The study comprised of 51 patients and 23 controls finally as 4 cases and 7 controls were excluded because

of abnormal colonoscopic findings. Out of the 51 patients, 38 patients were assigned to IBS-D group and 13 patients were assigned to IBS-C group based on their predominant clinical presentations as defined in the Rome IV criteria.

Controls: The mean age of presentation was 31.26 years (31.26±7.71). Male to female ratio was 8.69:1 On histopathology all of them showed normal mucosal finding**.**The mast cell counts ranged from 3 to 4 cells/ hpf . CD3+ lymphocytes ranged from 3 to 4 and CD8+ lymphocytes ranged from 4 to 5 per 100 intraepithelial cells.Both CD3+ and CD8+ lymphocytes were more commonly seen in epithelial lining of crypts as compared to the surface epithelium.

Patients: The mean age of presentation was 35.98 years (35.98±15.24). Male to female ratio was 10:1. The most common presentation in patients was found to be abdominal pain , which was related with the act of defecation(100%) followed by abdominal distension (70.90%), feeling of incomplete evacuation (60.00%), diarrhoea (61.82%), constipation (23.64 %) and both diarrhea and constipation mixed (14.54%). Stress was associated in 63.65 % patients. On histopathology all of them showed mild to moderate increase in lymphocytes, plasma cells, eosinophils and mast cells (Fig 1a,2a). The mast cell counts ranged from 5 to 14 cells/hpf (Figure 2a,2b). CD3+ lymphocytes ranged from was 8 to 14 per 100 epithelial cells (Figure 1c,2c) and CD8+ lymphocytes ranged from 5 to 9 per 100 epithelial cells (Figure 1d,2d). Both CD3 + and CD8 + lymphocytes were higher in the surface epithelium as compared to epithelial lining of crypts. When these values for IBS patients were compared to controls, the difference was statistically significant for both mast cells and IELs. Even their values in individual IBS subgroups were also significantly high (P = <0.0001 for all).However, significant difference amongst the three parameters could be observed only with mast cells when the two groups were compared among themselves. [Table 1]

On correlating numbers of mast cells with IELs' , only CD3+ lymphocytes showed significant positive correlation(p=<0.005); no correlation was found with CD8 + lymphocytes. [Table 2]

Serum Interleukin Assay- This was done in all twenty three controls but in fifty patients only as one patient's sample was rendered insufficient for assessment.

The mean value of IL-2, IL-6 and IL8 were significantly higher in IBS patients as compared to controls. However, the IL-10 value was lower in these patients as compared to controls, though not statistically significant (Fig 3).

On comparing mean value of each of the four Interleukins between IBS-D and IBS-C subgroups, all three proinflammatory cytokines –IL 2,6 and 8 were higher in IBS –D subgroup compared to IBS –C subgroup. [Table 3] However, only IL 8 was found to have statistically significant difference between the two. (Fig 2)

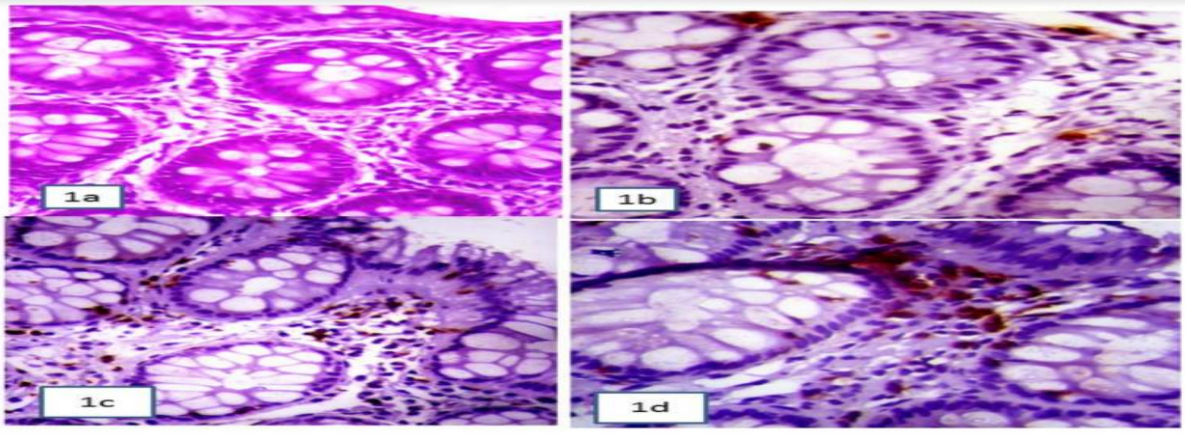

Fig 1a: Biopsy from IBS- C patient showing Colonic mucosa with occasional Inflammatory Cells (H & E X 400)

Fig 1b: Biopsy from IBS- C patient showing few TryptasePostive Mast cells in lamina propria (IHC X 400)

Fig 1c: Biopsy from IBS-C patient showing few CD3+ Lymphocytes in surface and crypt epithelium (IHC X 400)

Fig 1d: Biopsy from IBS- C patient showing few CD8+ Lymphocytes in surface and crypt epithelium (IHC X 400)

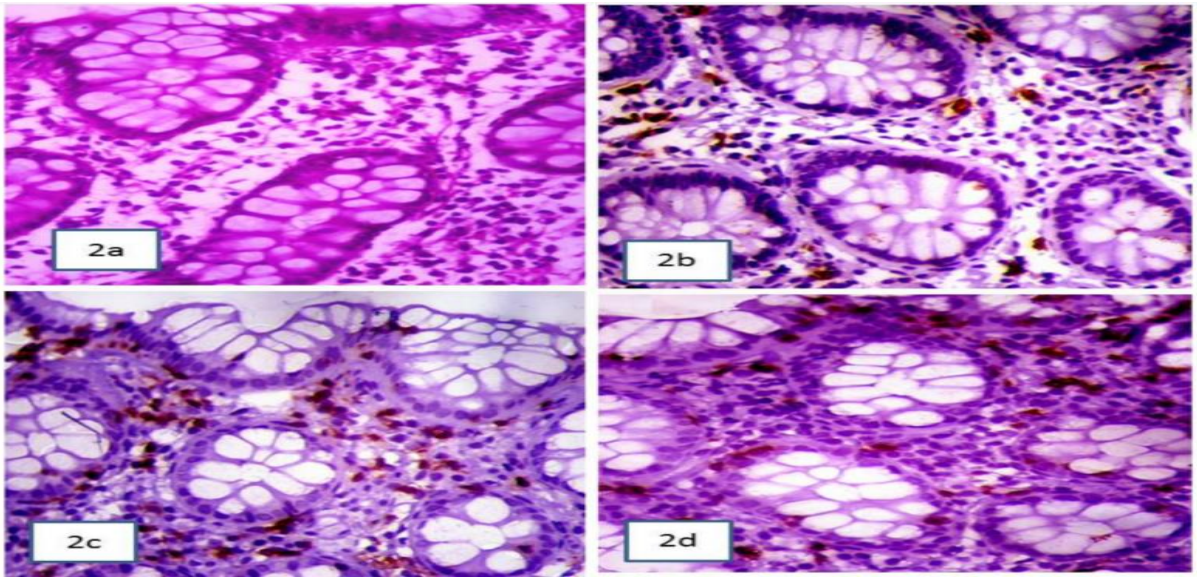

Fig 2a: Biopsy from IBS –D patient showing raised inflammatory cells in surface and Crypt Epithelium as well as in Lamina Propria (H & E X 400)

Fig 2b: Biopsy from IBS –D patient showing increased Tryptase positive Mast cells in lamina propria (IHC X 400)

Fig 2c: Biopsy from IBS –D patient showing increased CD3+ lymphocytes in surface and crypt epithelium (IHC X 400)

Fig 2d: Biopsy from IBS –D patient showing increased CD8+ lymphocytes in surface and crypt epithelium (IHC X 400)

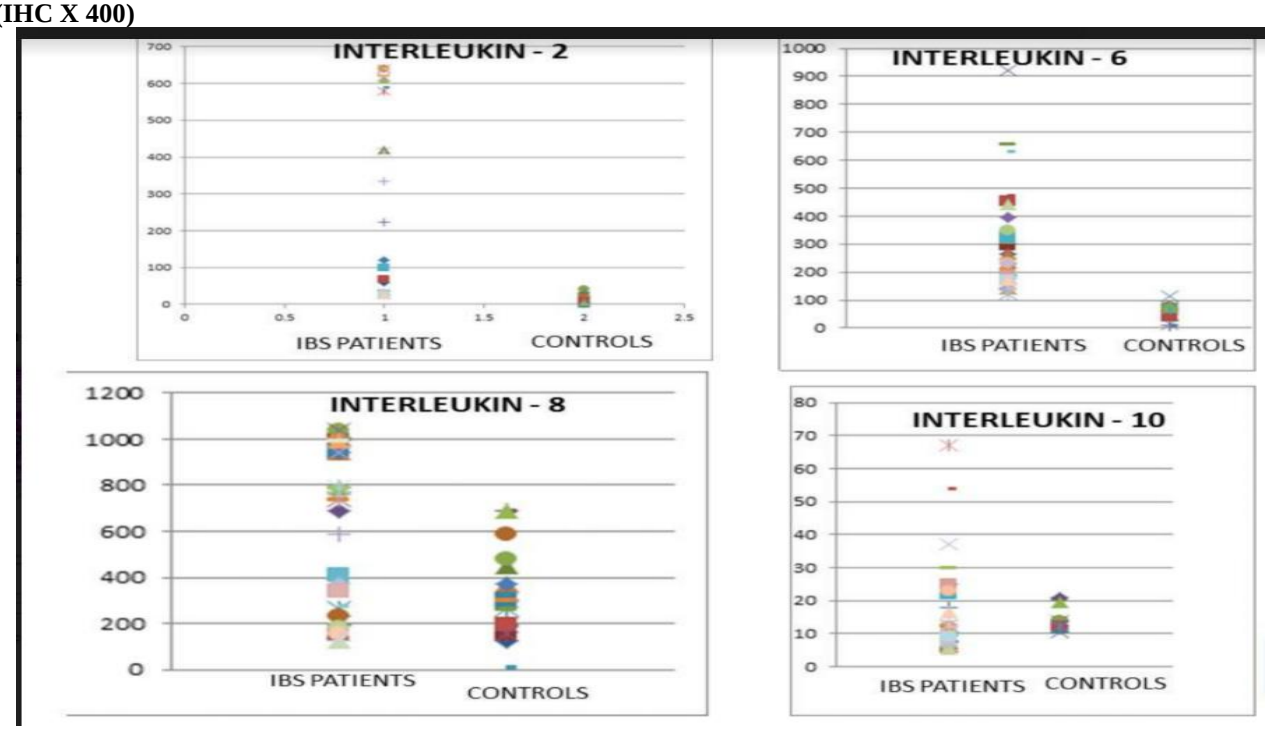

Fig 3: Scatter diagram showing simultaneous comparison of individual Interleukin levels in IBS patients and controls

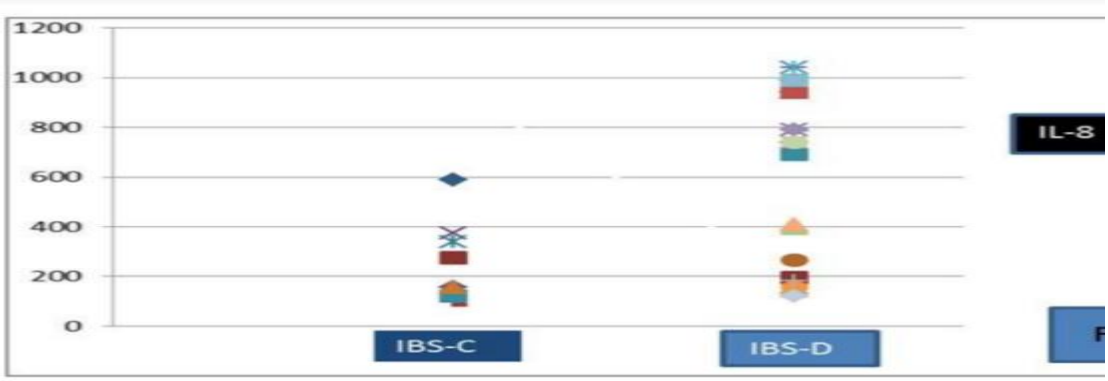

Fig 4: Scatter diagram showing simultaneous comparison of Interleukin-8 level in IBS-C and IBS-D subgroup , which showed a significant difference.

Table 1 :Comparison of intraepithelial lymphocytes (CD3, CD8) and Mast cells in controls, IBS-D and IBS-c subgroups

Type Of Cell	Control (A)	IBS- (B)	IBS-C (C)	*p* value		
				A vs B	A vs C	B vs C
CD 3^+ IEL'S	3.3 ± 0.64	11.84±2.90	10.44±2.46	<0.0001	<0.0001	(NS)*
$CD8^+$ IEL'S	4.1±0.70	7.60±2.17	7.46±1.59	<0.0001	<0.0001	(NS)*
Mast Cells	3.8±0.87	11.73±2.39	8.06±2.26	<0.0001	<0.0001	<.0001

* (NS)=not significant

Table 2:Correlation of intraepithelial lymphocytes and mast cells in IBS patients

TYPE OF IEL	r	*P* VALUE
CD 3^+	0.401	<0.005
CD 8^+	0.210	(NS)*

*NS= NOT SIGNIFICANT

Table 3:Comparison of values of Interleukins 2,6 and 8 between controls, IBS-D and IBS-C subgroups

IL	Control (A)	IBS-D (B)	IBS-C (C)	*P* value		
				A VS B	A VS C	B VS C
IL-2	51.82±27.19	285.56±179.06	196.0±78.76	<0.0001	<0.0001	NS*
IL-6	12.78±10.31	245.72±265.15	148.1±227.54	<0.0001	0.006	NS*
IL-8	315.82±198	899.40±303.46	559.0±239.68	<0.0001	.0024	.0006

*(NS)=not significant

Table 4: Correlation of Interleukins 2,6 and 8 with Mast cells and Intraepithelial lymphocytes

Interleukins	r(mast cell)	*P* value
IL-2	0.233	(NS)#
IL-6	0.12	(NS)#
IL-8	0.584	(S)*

#NS= NOT SIGNIFICANT ; *S= SIGNIFICANT

Correlation between Interleukin levels and inflammatory cells: On correlating individual interleukins with both $CD3^+$ and $CD8^+$ IELs and mast cells, a positive correlation was seen between mast cells and IL-8 only (P=<0.005). Rest of the interleukins did not show significant correlation with either mast cells or IELs. **[Table 4]**

Discussion

IBS is amongst one of the common conditions encountered in gastroenterology clinical practice. Throughout the world, about 10-20% of adults and adolescents are stated to suffer from IBS. Even after extensive research being done in this field, there is no specific laboratory test or biomarker available which can help in diagnosing this condition till date. [1] Though routinely IBS patients are not advised for colonoscopic examination and biopsy, when done, it is mainly to rule out either other entities with similar clinical presentation or for research purposes. In most of such studies done , biopsies from various segment of intestine (both small and large) have shown only low grade inflammation by routine methods. But, with use of advanced techniques, morphologic changes mainly in the form of increased intraepithelial lymphocytes, mast cells, Antigen Presenting Cells and less consistently plasma cells, neutrophils, entero chromaffin cells and B lymphocytes have also been revealed. [10] In this study, all the twenty three controls showed normal colonic histopathology. All the fifty one cases showed mild increase in inflammatory cells in the epithelial lining as well as the lamina propria. This was similar to observations made by Patel *et al* in their study. [14] We observed that compared to controls, IBS patients showed significantly increased IELs (both CD3+ and CD8+). Both of these were more raised in IBS-D subgroup than in IBS-C subgroup, though, statistically the difference was insignificant for either. Similar observations have been made in few other studies-Cremon *et al* in colonic biopsies of patients with IBS showed that counts of CD3+ cells, CD8+ cells, and T lymphocytes were increased. [15] A review article by Bashashati *et al* reported the CD3+ T cells to be increased in the rectosigmoid and the descending colon of the IBS patients which was possibly in relation to higher CD4+ T cells.[16] Martin *et al* too in their review reported increased CD3+ T lymphocytes though the findings were not consistent across all studies included in their review. [17] In contrast, some studies have shown no change in the number of CD3+ and CD8+ cells in patients with IBS. [18,19] In a study by Ilias *et al*, CD3+ and CD4+ cell counts in rectal and terminal ileal biopsy specimens were lower in the IBS group than in the controls. However, when the IBS-D and IBS-C groups were compared, IEL counts of the IBS-D group was higher than the IBS-C, as seen in our study also.[20] On assessing mast cells , a significantly high mast cell count was seen in both types of IBS patients as compared to controls which was in accordance with the findings of many studies done in recent years as reported in the review articles by Wouters *et al* and Lazaridis *et al*.[21,22] However, there are a few studies which have not found any such differences in the mast cell counts. [23-25] Even decreased counts in IBS have been reported in a study by Braak *et al*. [19] Coeffier *et al* and Liang *et al* have reported that only IBS –D patients show raised mast cells. [26,27] In contrast, Balestra *et al* showed them to be raised only in IBS-C, not IBS-D. [28] In our study, we found a significant difference in mast cell counts between IBS-C and IBS-D.We saw a significant positive correlation between CD3+ lymphocytes and mast cells.

Ilias *et al* too in their study have documented positive correlation between serotonin positive cells and CD 3, 4, 8 positive IELs in the IBS- D subgroup of their cases. [20]

The levels of all the three proinflammatory cytokines i.e. IL-2, IL-6 and IL-8 were significantly raised in the IBS-D and IBS-C subgroups compared to controls but only IL-8 level showed significant difference between the two.

Somewhat similar findings have been reported in many other studies as shown in the review papers of Lazaridis *et al* and Bashashati *et al*. These articles do also mention a few studies where no such difference has been reported in IBS cases versus controls.[23,29] To the best of our efforts, we could not find any study that has analysed the difference between the cytokine profiles of IBS-C and IBS-D patients.The levels of anti- inflammatory cytokine IL-10 in present study was found to be decreased in IBS patients compared to controls, moreso in IBS-D subgroup but the difference was insignificant. Unlike proinflammatory interleukins, the status of IL-10 in IBS is quite controversial, with most of the studies not observing any significant differences; a few documenting their lower levels [14,23,27] and Vara *et al* even reporting their higher levels in the IBS patients.[30] On correlating Interleukins with mast cells and IELs , significant positive correlation was seen only between IL-8 and mast cells. Here too, we could not find any such correlation study(ies) done previously between interleukins and inflammatory cells. But our

observation is supported by the paper of Patrix *et al* who have stated that IBS patients have increased serum concentration of IL-8 which is the main cytokine responsible for attraction of mast cells and granulocytes. [10] To the best of our knowledge, this is the first study from India that has attempted to evaluate the differences between IBS- D and IBS-C patients at the cellular level. From this study it can be concluded that apart from pschycosomatic causes, inflammation has a potential role in causation of IBS as proven by the altered number of immune cells and cytokine profile. Besides that, the role of inflammation is more pronounced in IBS-D patients as compared to IBS-C patients, hence, this subgroup of patients may require a different treatment strategy than the rest.The main drawback of present study was the smaller sample size in our IBS- C subgroup compared to the IBS- D subgroup; also the number of females were quite less compared to males. Hence, the inference drawn may not be reproducible and universally applicable. That is why we recommend that future studies on a similar assessment pattern on larger sample sizes with comparable number of both sexes and both types of IBS [IBS-D and IBS-C] may be of great help in accurately analyzing whether these two groups of patients behave differently at the cellular level and whether they require different modes of treatment or not?

Abbreviations

IBS: Irritable Bowel Syndrome
IBS-C: Constipation predominant Irritable Bowel Syndrome
IBS-D :Diarrhoea predominant Irritable Bowel Syndrome
IELs : Intra Epithelial Lymphocytes
IL : Interleukins

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