Title Page

A novel tool for non invasive diagnosis and tracking of patients with Inflammatory Bowel Disease (IBD).

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Abstract

Background

The pathogenesis of IBD involves the role of bacteria. These bacteria ferment non-starch polysaccharides in the colon producing a fermentation profile which, through altered gut permeability can be traced in urine. We propose to track the resultant volatile organic compounds (VOCs) or gases that emanate from urine using non-invasive real time tools, specifically by electronic nose and FAIMS (Field Asymmetric Ion Mobility Spectrometer) instruments.

Aim

To determine the utility of electronic nose and FAIMS instruments to detect and track the fermentation profile of patients with IBD.

Methods

62 individuals were recruited; 48 with IBD (24 with Crohn's and Ulcerative colitis respectively) and 14 controls. The disease activity was recorded and urine samples were collected. The headspace (the air above the sample) analysed using the electronic nose and FAIMS instruments.

Results

Electronic nose data analysis was by: 1. Principal Component Analysis (PCA): data are analysed together without prior categorisation. 2. Discriminant Function Analysis (DFA): samples are pre-categorised (clinical groups). FAIMS data was processed by Fisher Discriminant Analysis (FDA): pre-categorised (clinical groups). We have shown consistently using these technologies, the ability to separate those with IBD and controls with >75% accuracy; $p<0.001$. In a smaller sub-group ($n = 24$), we also demonstrated that electronic nose and FAIMS instruments can distinguish between active disease and those in remission.

Conclusions

The fermentation profile or fermentome are disparate in those with IBD compared with controls – a reflection of the bacterial diversity in health and disease. This profile also changes (and was tracked) as the disease is induced into remission. Thus the electronic nose and FAIMS offers the potential of a non-invasive, real time diagnostic tool for point of care clinical use.

1.0 Introduction

It is the accepted premise that the pathogenesis of inflammatory bowel disease (IBD) is heterogeneous involving genetic susceptibility, environmental triggers (diet, life style etc.) and influence from host bacterial colonies. Bacterial diversity has been difficult to study as less than 50% of organisms can be successfully cultured. Modern genomic techniques can circumvent this problem but are expensive, laborious and not practical for daily clinical use.

It is also apparent the shift in balance of bacteria in the host (dysbiosis) significantly influences health [1]. These colonic bacteria undergo fermentation of non-starch polysaccharides – fibre consumed by the host. The study of the resultant products of fermentation which we have termed 'the fermentome' [2,3] can be measured in urine. The latter is possible due to the altered gut permeability afforded in certain gut diseases including IBD [4,5].

The quest continues to find simple, reliable non-invasive markers to distinguish between Crohn's disease and ulcerative colitis (UC) as well as to distinguish between active disease and those in remission. In an attempt to address this important clinical problem we proposed the utilisation of real-time instruments that are able to detect volatile organic compounds and gasses that emanate from biological material. Specifically - the electronic nose (an instrument that replicates the biological olfactory system) [6,7] and also newer technology - FAIMS (Field Asymmetric Ion Mobility Spectrometry) [8].

The aim of this study was to test the potential usefulness of these techniques to differentiate between IBD subjects and controls using only urine samples. Secondly, we also sought the ability of this technique to distinguish between active IBD compared with those in clinical remission. These changes were correlated with clinical disease activity scores.

2.0 Materials and Methods

2.1 Subjects

A total of 62 patients were recruited for this study and consisted of adults aged 28 to 81 years. The study cohort included 3 groups: 24 patients with ulcerative colitis, 24 patients with Crohns disease and 14 controls. The first two groups were divided further into those with a relapse or in remission with 4 patients from each group with an acute flare of their inflammatory bowel disease. Those with an uncertain diagnosis or inconclusive radiological or histological confirmation were excluded from the study. The demographics of the subjects are shown in Table 1.

2.2 Study Design

The study had a cross sectional case control design where patients attending the inflammatory bowel disease clinics were recruited from University Hospital Coventry & Warwickshire, UK and Rotherham General Hospital, UK. Demographic data and disease activity score index was collected from the patients. Urine was then collected in a standard universal specimen container and frozen to -80C after collection for subsequent batch analysis.

2.3 Analysis

Two methods were used for the analysis of urine samples: Electronic nose and FAIMS. These methods are described below:

Urine was collected from patients and the samples were frozen in -80C after collection and stored for batch analysis. They were then left to thaw overnight to room temperature and aliquoted into appropriate sample bottles (described below). The samples were then used for analysis using the electronic nose and FAIMS experimental methods.

Electronic nose

In this study a commercial electronic nose (Fox 4000, AlphaMOS, France) was used to analyse the chemical signature of the samples. This instrument comprises of an array of 18 metal oxide gas sensors, whose resistance is modulated in the presence of a target gas/vapour and attempts to mimic the biological olfactory system. When a complex sample is presented to the chemical sensors, as each sensor is different, the response from each sensor is unique within the array. The response of all the sensors can be brought together to create a smell

'fingerprint' of that sample. When a similar sample is presented again to the instrument, it will produce the same sensor response profile and thus we are able to identify that sample. Consequently, we are able to present many different types of sample to the instrument, allow it to learn these smell fingerprints and thus characterise/identify samples.

Here 3 ml of each urine sample was aliquoted into 10 ml sample bottles with a crimp lid, fitted with a septum. The Fox 4000 electronic nose is fitted with a HS100 autosampler, which allows up to 64 samples to be run in one batch. The autosampler, moves each bottle into a preparation chamber, which heated the samples for 10 mins to 40 oC and agitates the bottle. After 10 minutes a syringe takes 2.5 ml of headspace from the sample bottle and directly injects it into the electronic nose. The change in resistance of the sensors was measured from the injection time for 120 seconds at a sample rate of 1 Hz. The instrument is flushed with clean, dry air (flow rate of 500 ml/min) for 10 minutes after each exposure to ensure that the sensors had fully recovered. Figure 1 shows a typical response of the sensor array to a urine sample.

FAIMS (Field Asymmetric Ion Mobility Spectroscopy)

For FAIMS analysis, again a commercial setup was deployed (Lonestar, Owlstone, UK). Unlike the electronic nose, this system achieves separation of chemical components on the basis of differences in ion mobilities within an electric field. FAIMS is a fairly recent technology that allows gas molecules to be separated and analysed at atmospheric pressure and room temperature. After the sample is ionised, it is composed of ions of various sizes and types. These are introduced between two metal plates and an asynchronous high voltage waveform is applied to these plates. These ionized molecules are then subjected to these high electric fields. The difference in movement of these molecules, in this high electric field, can be measured resulting in a separation of the complex mixture.

7 ml of urine was aliquoted into a standard sterilin bottle. The plastic lids (normally used) were modified with the addition of push-fit fitting (for 3 mm PTFE tubing), which allowed the bottle to be connected to the FAIMS instrument. The sterilin bottles were heated to 40 oC ± 0.1 for 30 minutes before each experiment. The FAIMS instrument was set up in a pressurised configuration with a flow rate of 2L/min. The dispersion field was stepped through 51 equal settings between 0 and 90% (the dispersion field in the ratio of the high electric field to low electric field) and for each dispersion field the compensation voltage

stepped was between +6V and -6V in 512 steps. Figure 2 shows a typical FAIMS 'plume' produced by a Crohn's disease urine sample.

2.4 Statistical Methods

Exploratory data analysis for the electronic nose was performed using PCA and DFA, and for FAIMS FDA. These techniques are extensively used for these types of experiment. Their purpose is to allow the simple interpretation of complex data to determine if differences in groups of samples can be seen. For electronic nose, analysis was undertaken using the internal AlphaMOS software (AlphaSoft v12.3)..

FAIMS data was processed in Matlab (Mathworks Inc., USA). For analysis both the positive and negative ion count matrices for each spectrum were concatenated into vectors and joined to make a single 52,224 element vector. These were then wavelet transformed using a Daubechies D4 wavelet; this mapped the data onto a basis set which, given the form of the vector, was more natural. Dimensions in the resulting vector, suitable for discrimination, were then identified. Thresholds were set for the within class scatter: $(\Sigma\sigma_i)^2$ and the between class scatter: $(\sigma_\mu)^2/(\Sigma\sigma_i)^2$, to identify dimensions for selection. (σ_i: the standard deviation of the dimension in question within the class i, and σ_μ was the standard deviation of the means of the dimension under test between classes). The exponents change the form to reflect those employed in Fisher Discriminant Analysis (FDA). FDA (a linear, pre-classified method), was applied to the dimensions identified as suitable for discrimination.

This approach gave a two dimensional input parameter space (within class scatter and between class scatter) to control the separation algorithm. This space was explored as follows. For each point in the discretised input space 50 test sets (each containing two (one in the case of the smaller flare groups) samples from each group being investigated) was re-classified. These test sets were not used in the identification of dimensions or the implementation of the FDA. This exploration identified regions in the parameter space where re-classification exceeded that which would be expected from random re-classification (number of standard deviations from the mean in each case given below), and for which the success of re-classification was robust to perturbation in the parameters while changing the number of dimensions identified. Parameter sets in this robust region was used for further analysis.

2.5 Ethics

Scientific and ethical approval was obtained from local Research & Development Department and Warwickshire Ethics Committee (ref: 09/H1211/38). Written informed consent was obtained from all patients who participated in the study.

3.0 Results

The subject characteristics of the inflammatory bowel groups and controls are described in Table 1. No statistically significant difference between the groups was noted.

Initial electronic nose analysis was undertaken using PCA, the results of which are shown in Figure 3. The results of this analysis indicate that there are differences between the smell profile of these samples. As PCA is a non-classified technique, it shows that the samples are different, but with some overlapping of classes. When the samples are re-analysed using DFA (a pre-classified technique), shown in figure 4, we are able to see the groups clearly separating. Test sets were removed and reclassified using a matrix derived using the remaining data. Each test set contained an element from each of the five groups. Accuracy of reclassification exceeded 70% (21 standard deviations from the mean, $P<0.001$ compared to uniform random reclassification). Reclassification purely according to disease group or control exceeded 88% (20 standard deviations from the mean, $P<0.001$).

The results of FDA on the FAIMS data for patients with Crohn's disease, Ulcerative Colitis and controls are shown in Figure 5. Accuracy of reclassification of the elements of the test sets exceeded 75% (10 standard deviations from the mean, $P < 0.001$, compared to random reclassification), indicating a fundamental difference in the spectra associated with disease groups. The results of FDA on the FAIMS data for Crohn's disease in remission and flare is shown in Figure 6. Accuracy of reclassification was >66% (1 standard deviation from the mean, $p<0.05$). UC in remission and flare is shown in Figure 7. Accuracy of reclassification >74%. (5 standard deviations from the mean, $p<0.001$). This indicates a difference in the spectra associated with disease state.

4.0 Discussion

Our study demonstrates that the utility of novel non-invasive and inexpensive technology to distinguish between patterns of volatile organic compounds present in the urine of patients

with Crohn's disease, ulcerative colitis and healthy individuals. This distinction was confirmed by repeating the analysis with FAIMS technology. Moreover it was also able to separate patients between those experiencing a flare of their disease and from those in a quiescent disease state. This indicates that the VOC pattern is different not just between the two groups of inflammatory bowel disease and healthy individuals but also between those who are experiencing a flare of their disease. The uniqueness of our study is the use of specific non invasive tests that to our knowledge is the first to demonstrate the use of volatile organic compounds detected by 'e' nose and FAIMS technology in urine of patients with IBD. This has enabled the generation of a characteristic chemical fingerprint. The relevance of this is given by the fact that diagnosis of inflammatory bowel disease can be very challenging and depends on a host of invasive tests that at times may not even accurately distinguish between the two groups of IBD. Our data also extend our previous studies which looked at changes of VOC profile in patients subjected to pelvic radiotherapy [7]. Using the electronic nose we were able to accurately differentiate between patients who had significant gastrointestinal side effects following pelvic radiotherapy.

The changes in VOC profile in patients with Crohns and Ulcerative Colitis compared to controls further affirm the significant contributing role of gut microbota dysbiosis in the pathophysiology of inflammatory bowel disease. VOCs are produced as a result of colonic fermentation following a complex interaction between the colonocyte, human faecal flora, mucosal integrity and invading pathogens [2]. They are emitted from bodily fluids and as a result, VOCs emitted from urine, faeces and breath may include biomarkers of use in the assessment of gastrointestinal disease. Therefore changes in the VOC profile are reflective of the variation of gut microbiota and sheds light into its causative role in pathological states [9]. It is well established that the biodiversity of gut microflora remains high in Crohns disease with enterobacteria being observed significantly more frequently in CD than in health and these changes are suggested to lead to damage to the intestinal barrier causing an inappropriate stimulation of the immune system hence promoting the inflammatory process [10-13]. Hence changes in the VOC profile in these patients indirectly represent this complex pathological process involved in the disease state.

Furthermore we demonstrated that the VOC profile is different in patients who are in disease remission and relapse states. This interesting novel finding suggests that the gut flora is altered in patients with a flare of their disease thereby adding to the evidence that changes in

gut flora are potential likely triggers of inflammation in susceptible patients with inflammatory bowel disease. It is also interesting to note that the VOC profile does not return to the profile of healthy controls in disease remission states suggesting that the baseline gut microflora diversity remains different in these patients. It is thought that this steady state of composition of gut flora is determined soon after births where the environmental conditions under which babies are born and nurtured may affect their exposure to microbes. With the use of an electronic nose we are able to demonstrate changes in VOC profile in patients with disease remission and relapse states which are reflective of these changes in gut bacteria in triggering intestinal inflammation.

Our preliminary data indicates that further validation using an electronic nose/FAIMS technology is warranted to determine its role as a clinical diagnostic tool for identifying patients with inflammatory bowel disease. This would require a specific study design that a) would involve a larger cohort of 'pre-diagnosed' patients with inflammatory bowel disease to determine predictive values against the current gold standard technique (i.e. extension of this study) and b) consequent external validation in newly recruited patients presenting with symptoms that would lead them down an inflammatory bowel disease diagnostic pathway. This would allow the electronic nose to be an inexpensive, non invasive and easy to use tool that may be used for screening thereby aiding the clinician in selecting the subset of patients who, would need further invasive investigations.

References:

1. Conte, M. P., S. Schippa, I. Zamboni, et al. 2006. Gut-associated bacterial microbiota in paediatric patients with inflammatory bowel disease. Gut 55:1760-1767
2. Arasaradnam RP, Pharaoh MW, Williams GJ et al. Colonic fermentation--more than meets the nose. Med Hypotheses. 2009 Nov;**73**(5):753-6
3. Arasaradnam R, Quraishi N, Kyrou I, et al. Insights into 'Fermentonomics': Evaluation of volatile organic compounds (VOCs) in human disease using an Electronic 'e' Nose. J Med Eng Technol. 2011 Feb;**35**(2):87-91. Epub 2011 Jan 4.
4. Arasaradnam RP, Pharaoh M, Williams G et al. Fermentonomic insights – the gut in health and disease. DDW 2010; Gastroenterology 2010; Abstract #T1267
5. Arasaradnam RP and Bardhan KD. Bioactive foods and Extracts – Cancer treatment and prevention. Taylor Francis, New York 2010
6. Gardner JW, Craven M, Dow C, Hines EL. The prediction of bacteria type and culture growth phase by an electronic nose with a multi-layer perceptron network. Meas Sci Technol 1998;**9**:120-127
7. Arasaradnam RP, Ouaret N, Joseph M et al. Radiotherapy induced gut damage: identifying 'at risk' patients using an electronic 'e' nose. Gut 2011;**60**:A108-A109 doi:10.1136/gut.2011.239301.227
8. Arasaradnam RP, Ouaret N, Thomas MG et al. Evaluation of gut bacterial populations using an Electronic 'e' Nose and FAIMS (Field Asymmetric Ion Mobility Spectrometry): Further insights into 'Fermentonomics'. J Med Eng Technol. 2012 *In press*
9. Tannock GW. The search for disease associated compositional shifts in bowel bacterial communities of humans. Trends in Microbiology 2008; **16**(10):488-495
10. Mareau P. Bacterial flora in Inflammatory bowel disease. Dig Dis 2009;**27**(1):99-103.
11. Darfeuille-Michaud A, Boudeau J, Bulois P, et al. High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. Gastroenterology 2004 Aug;**127**(2):412-21.
12. Marchesi JR, Holmes E, Khan F et al. Rapid and non-invasive metabonomic characterisation of inflammatory bowel disease. J Proteome Res 2007; **6**:546-551
13. C P Tamboli, C Neut, P Desreumaux, et al. Dysbiosis in inflammatory bowel disease Gut 2004;53:1 1-4 doi:10.1136/gut.53.1.1

Appendices

Table 1: Baseline Characteristics of Study Population

	Crohns Remission	UC Remission	Crohns Flare	UC Flare	Controls
Number	20	20	4	4	14
Mean Age (sd)	50 (11.4)	56 (15.8)	39 (10.3)	42 (16.6)	32 (8.2)
Sex: M/F	12:8	10:10	3:1	4:0	5/9
Mean BMI (sd)	25 (5.9)	28 (4.3)	22 (4.2)	25 (3.6)	
5-ASA (%)	17/20 (85%)	17/20 (85%)	2/4 (50%)	3/4 (50%)	n/a
Aza (%)	5/20 (25%)	3/20 (15%)	2/4 (50%)	1/4 (25%)	n/a
Steroids (%)	4/20 (20%)	3/20 (15%)	1/4 (25%)	2/4 (50%)	n/a
Biologics (%)	2/20 (10%)	0/20 (10%)	0	0	n/a

Figure 1: Raw electronic nose results to a Crohns disease patient.

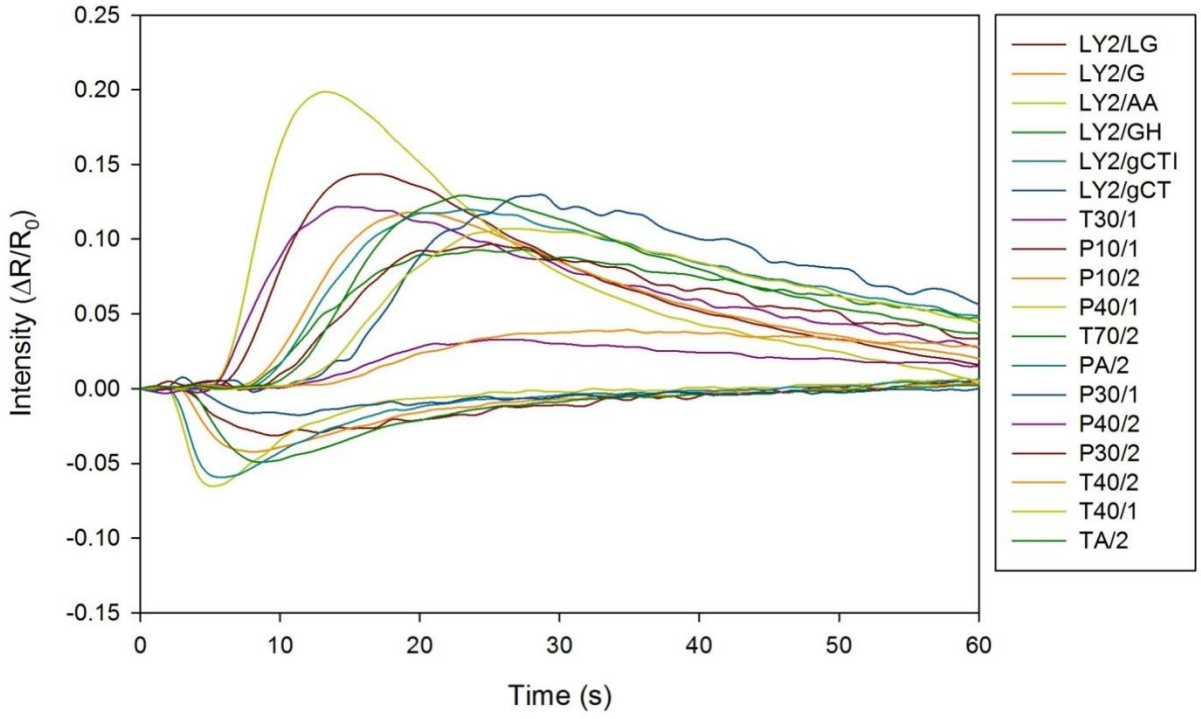


Figure 2: Raw data from the FAIMS instrument to a Crohns disease patient. Intensity is in arbitrary units of ion count.

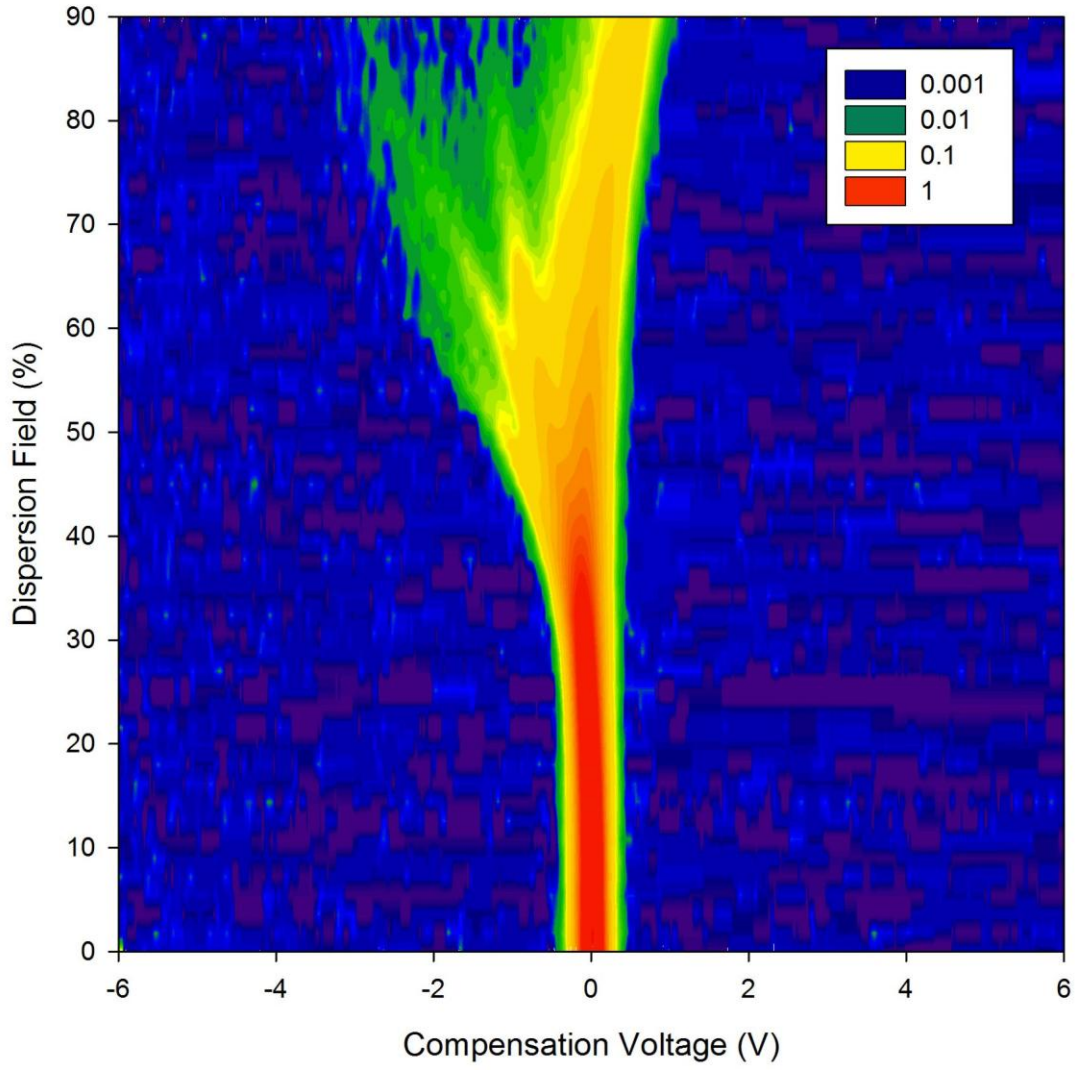


Figure 3: Principal component (PC) analysis of Fox 4000 results.

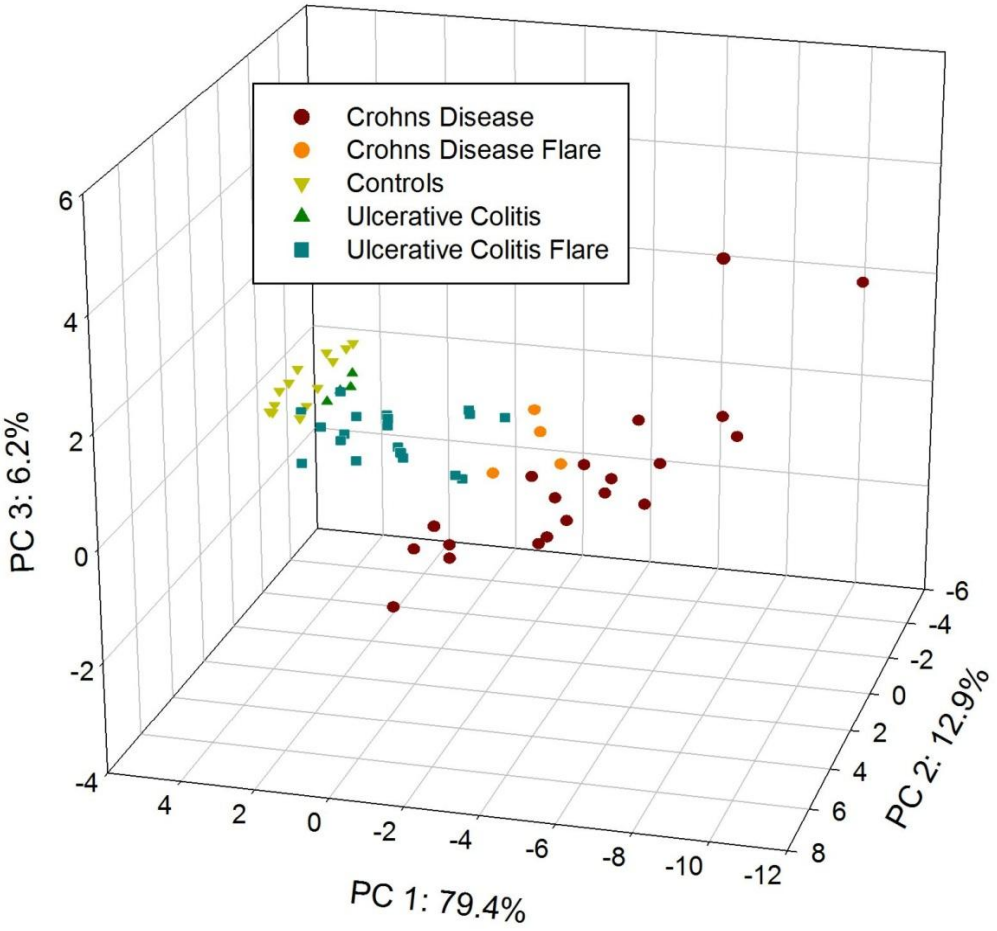


Figure 4: Discriminant Function (DF) Analysis of Fox 4000 data

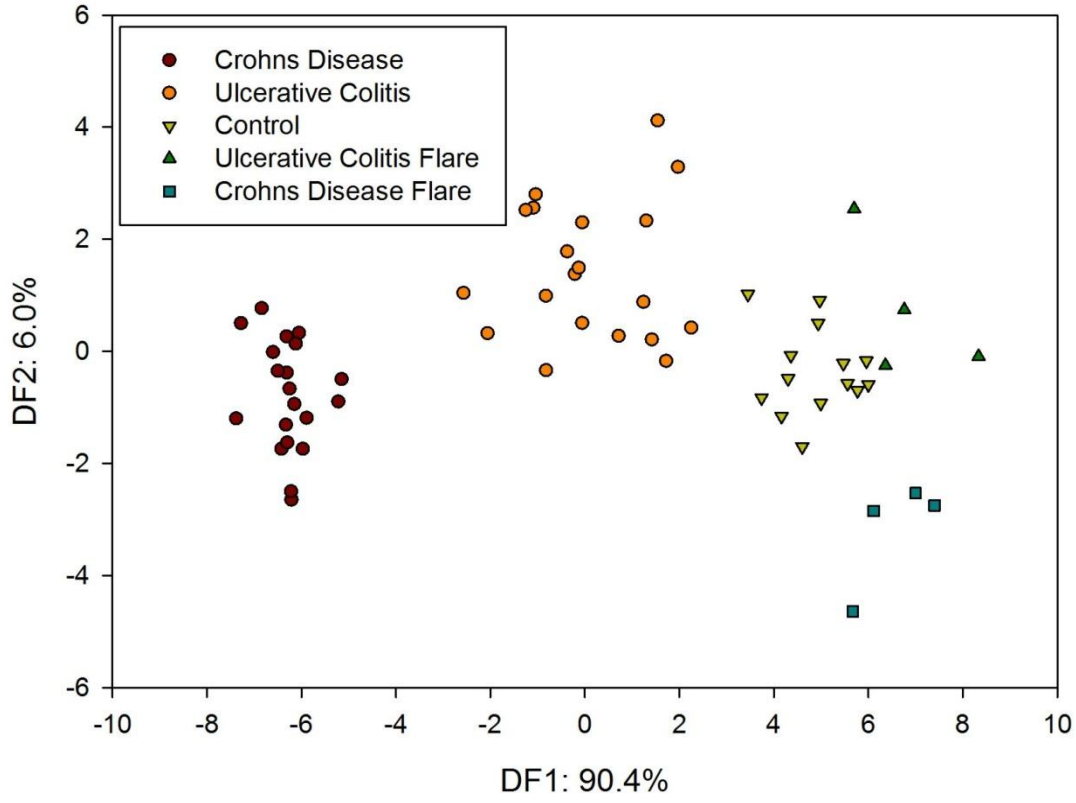


Figure 5: FDA analysis of FAIMS data by disease groups

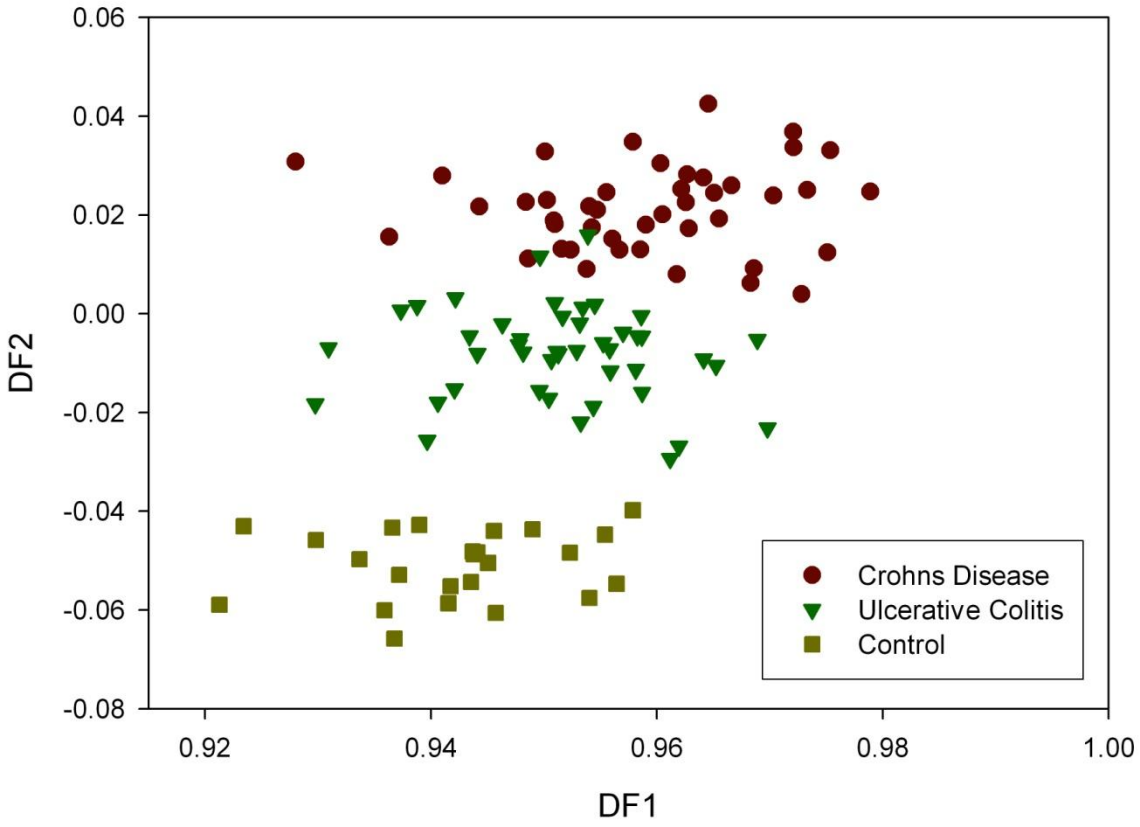


Figure 6: FDA analysis of FAIMS data for Crohn's disease in remission and flare

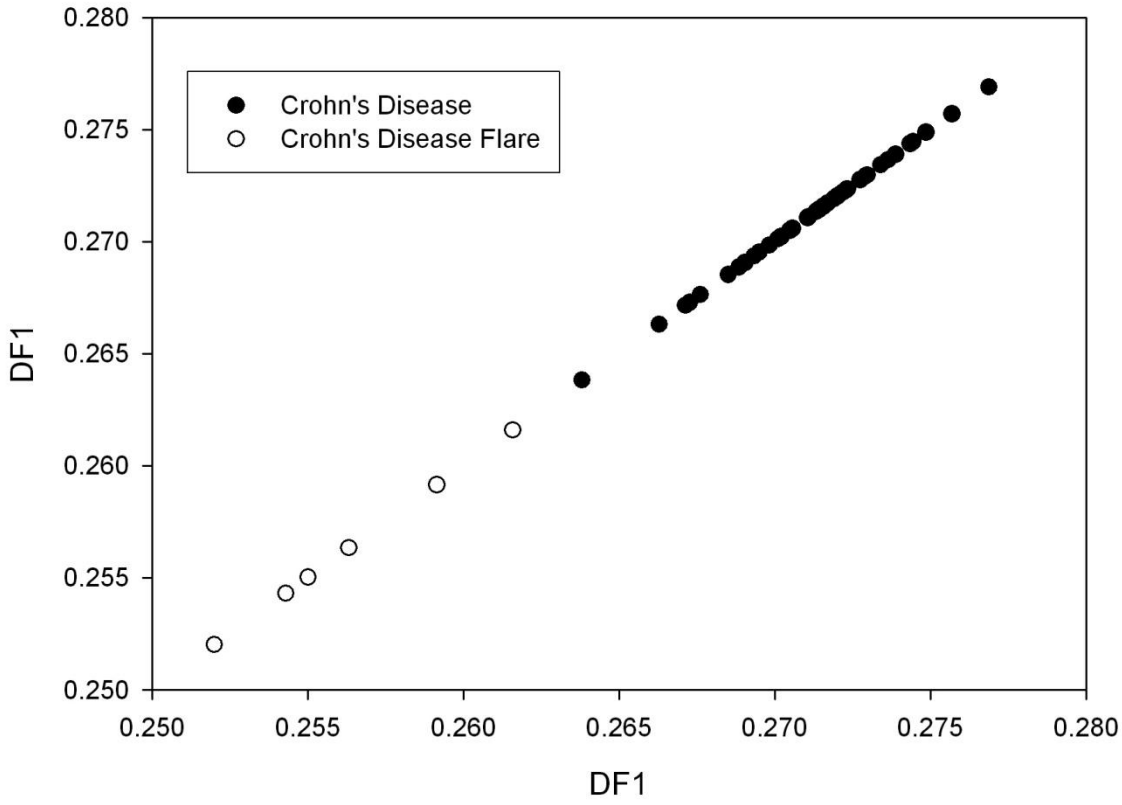
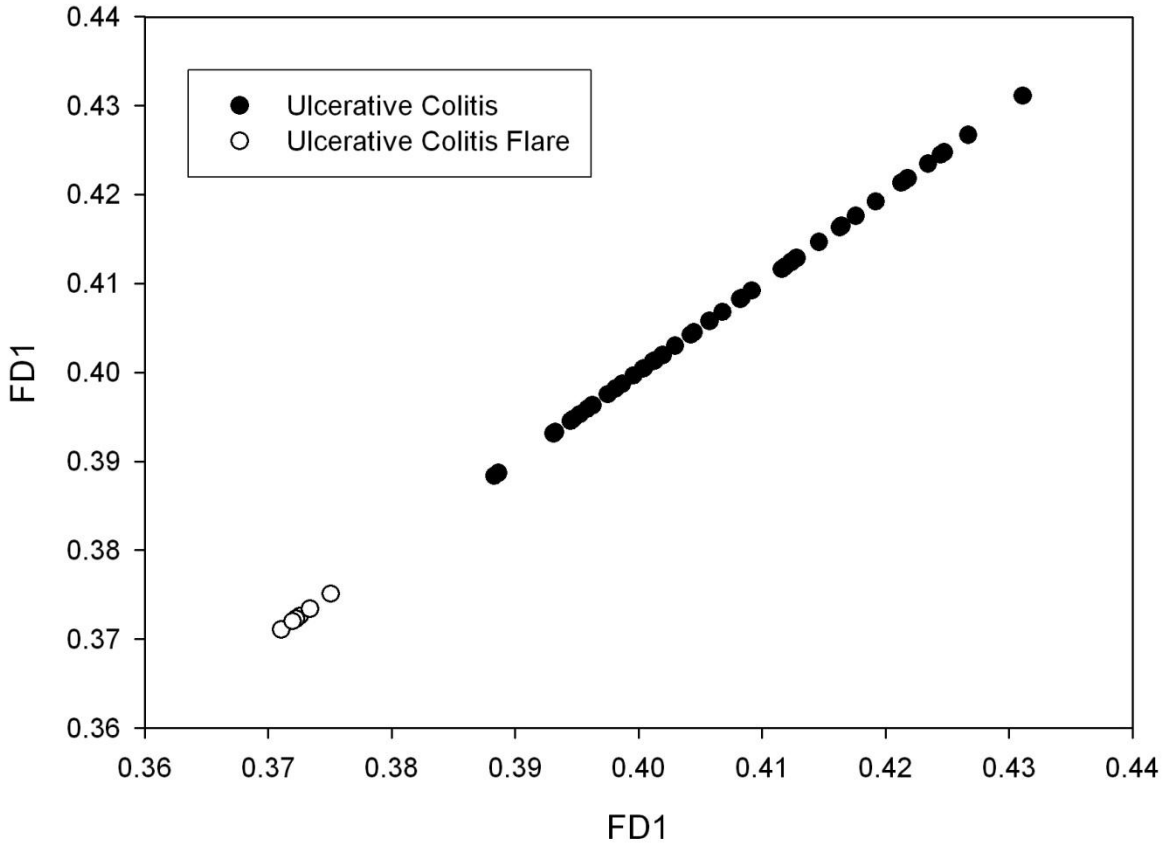


Figure 7: FDA analysis of FAIMS data for UC in remission and flare


Ulcerative Colitis
Ulcerative Colitis Flare
FD1
FD1
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