**Analysis and hierarchical clustering of blood results prior to diagnosis in paediatric inflammatory bowel disease**

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**Contributors’ Statement**

Dr Ashton conceptualised and designed the study, analysed the data and wrote the manuscript with help from all authors.

Professor Beattie and Professor Ennis conceptualised and designed the study and helped to write the manuscript

Dr Mossotto helped with data analysis, commented on and reviewed the manuscript.

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Abstract

Background-Paediatric inflammatory bowel disease (PIBD) is associated with a diagnostic delay. Blood tests are a routine part of the work-up in children with chronic abdominal symptoms (pain, diarrhoea).Normal tests cannot exclude PIBD. We analysed blood results at diagnosis over a 5-year period.

Methods-Patients diagnosed from 2013-2017 were identified from the Southampton-PIBD database. Results were obtained up to 100 days prior to diagnostic endoscopy. Erythrocyte sedimentation-rate (ESR), C-reactive protein (CRP), albumin, haemoglobin, platelets, packed-cell-volume (PCV), white-cell-count (WCC) and alanine transferase (ALT) were analysed. Hierarchical clustering (HC) was applied to normalised results.

Results-Two-hundred-and-fifty-six patients were included, Crohn’s disease (CD)-151, ulcerative colitis (UC)-95 and IBD-unclassified-10. Median age 13.48 years, 36.7% female.

HC of patients revealed novel groupings enriched for CD and UC, characterised by specific patterns of results.

In PIBD, 9% presented with all normal bloods, 21.9% with normal CRP and ESR. Abnormal results were seen in all tests- ESR (56.4% of patients), CRP (53.4%), albumin (28%), haemoglobin (61.9%), platelets (55.6%), PCV (64.6%), WCC (22.7%) and ALT (7.2%).

Normal inflammatory markers were more common in UC compared to CD (UC=34%, CD=15.6%, p=0.0035). UC (14.4% normal) presented with all normal results more frequently than CD (CD=5.3%), p=0.02). CRP, ESR and platelets were significantly higher in CD compared to UC. Albumin and haemoglobin were significantly lower.

Conclusions-Most cases of PIBD present with >1 abnormal blood result, although 1/11 patients present with normal bloods and 1/5 present with normal inflammatory markers. HC offers the potential to produce novel groupings to inform disease categorisation and best management.

Key words: Paediatric; Inflammatory Bowel Disease; Diagnostics; Crohn’s disease; Ulcerative Colitis

Introduction

Paediatric inflammatory bowel disease (PIBD) is a chronic, relapsing and remitting condition presenting with symptoms such as abdominal pain, diarrhoea and blood in the stool (1). Consisting of Crohn’s disease (CD), ulcerative colitis (UC) and IBD unclassified (IBDU), the incidence of PIBD is rising, with an increased emphasis on providing a timely and accurate diagnosis (2,3). Despite this there is frequently a diagnostic delay, with a recent Canadian study reporting symptoms for several years in some cases prior to referral and diagnosis (4). Strategies to assist general practitioners and general paediatricians in identifying children with possible IBD are therefore important.

Blood tests are a standard, well established and accessible part of the diagnostic work up in children with chronic abdominal symptoms (5,6). Patients with normal tests are often not referred onto specialist care, and specifically normal inflammatory markers have been considered reassuring (7). Whilst normal blood tests may be reassuring in children presenting with common, chronic abdominal symptoms, a proportion of patients diagnosed with IBD will present with some or all normal laboratory values (8). Mack *et al* (2007) described up to 9% of CD patients and 19% of UC patients presenting with normal haemoglobin, erythrocyte sedimentation rate (ESR), platelet count and albumin (9).

Faecal calprotectin (FCp) represents a potentially useful test, although it is not always reliable (especially in young children). It should only be used in those with clinical suspicion and there is not yet a definitive consensus for an abnormal cut-off value (10,11). The specificity of FCp ranges from 0.59 at >100μg/g to 0.95 at >800μg/g, with sensitivity at corresponding values displaying an inverse trend, 0.97 at >100μg/g to 0.73 at >800μg/g (12). The use of laboratory markers in addition to symptoms for diagnosis of PIBD has been analysed, with FCp proving the most useful and blood tests providing only some additional benefit in a single study (13). Despite this, blood results remain a vital part of the work-up of children with possible IBD.

Providing an update of blood results at presentation within a contemporary PIBD cohort is important. In this study we report and analyse baseline blood results of a cohort of patients with a subsequent diagnosis of PIBD, including the prevalence of normal bloods in CD, UC and IBDU. We hypothesise that identification of different strata based on patterns of blood results alongside observation of enrichment for specific abnormal tests may lead to novel patient subgrouping. We apply hierarchical clustering techniques to patients in order to identify i) novel subgroupings of children beyond the traditional CD/UC classification ii) blood test results associated with subsequent diagnosis (CD vs UC vs IBDU).

Methods

All patients (n=275) diagnosed with PIBD from January 1st 2013 to December 31st 2017 at Southampton Children’s hospital were eligible for inclusion in the study. Patients and dates of diagnosis were identified from the prospectively maintained Southampton PIBD database. All patients included were diagnosed in line with the modified Porto criteria (14).

Results for erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), albumin, haemoglobin, platelets, packed cell volume (PCV), white cell count (WCC) and alanine transferase (ALT) were automatically retrieved from the University Hospital Southampton (UHS) laboratory results system by the data science team at UHS/Southampton Biomedical Research Centre. Where results were not available from UHS the referring hospital records were searched. Results were obtained with age and gender specific normal reference ranges as provided by the UHS clinical pathology service or by the referring hospital laboratory. There were no differences in gender reference values for the age groups studied (<17 years).

Imaging and faecal calprotectin results were excluded from the analysis as they have not been routinely available to primary or secondary care physicians across the region over the study period.

Blood results were retrieved from 0 to 100 days prior to the day of diagnostic endoscopy (supplementary table 1). Where multiple blood tests per patient were available the blood results from initial presentation to the paediatric gastroenterology service were used. Where no results were accessible during the allocated time (n=19, 6.9%), patients were excluded from further analysis. All excluded patients were referred directly for endoscopy (from regional clinics run by the Southampton team).

Results were grouped and analysed by test (CRP, ESR etc.) and patient diagnosis. Blood results were then pooled and the proportion of patients with normal blood results were analysed (calculated as a percentage of patients with a result for that blood test). Statistical analysis was performed using Fisher’s exact test, Mann-Whitney U-test and simple linear regression was performed to analyse age at diagnosis (SPSS v24 IBM). Sensitivity was calculated for all blood tests (true positives/(true positives + false negatives).

In order to identify novel groups and enrichment of patients, data were normalised to a mean value of zero using the standardise function in excel (Microsoft) (based on calculations of standard deviation and mean). These data were then used for production of heatmap and box-whisker plots. The heatmap was grouped using an agglomerative approach based on average linkage and Euclidean distance to identify the most similar groups of patients as determined by blood test results using the Morpheus software (Broad Institute)(15). Briefly, all normalised data were entered into the Morpheus online application-

1. Similarity (or dissimilarity) between every pair of objects (patients) was calculating based on the ‘distance’ between objects. Here we employed the default Euclidean distance measure. All entered variables (blood results) were employed to characterise an object prior to clustering.
2. Objects are clustered into a hierarchical tree. Pairs of objects that are close in proximity based on the linkage function (average linkage), based on distances generated in step one, are placed into binary clusters, these clusters are then grouped into larger clusters until a hierarchical tree (dendrogram) is formed. The distance (similarity) between objects is represented by data merges (internal nodes), with higher nodes representing more dissimilar objects.
3. Clusters are then determined by the level at which the hierarchical tree is ‘cut’ into the groups.

Cases were labelled post-hoc by patient diagnosis (CD vs UC vs IBDU). These novel groups were interrogated for enrichment of patient diagnosis and underlying drivers of similarity (blood results). Statistical analysis for enrichment was with Fisher’s exact test.This study was registered as a service evaluation with UHS information governance department.

Results

Two-hundred and fifty-six (256) patients were included in the analysis, 151 with CD, 95 with UC and 10 with IBDU. Median age at diagnosis was 13.48 years, 36.7% (n=94) were female. Not all blood tests results were available for all patients, the mean number of tests per patients was 7.5 (range 2-8). As expected the CD group was enriched for male patients compared to the UC group (p=0.0092). There was no difference in median age of diagnosis between CD and UC (13.46 years vs 13.61 years). The median time from date of blood result to diagnosis was 8 days (range 0-99 days).

*Normal blood tests*

When considering all patients with PIBD, 9% presented with all normal blood tests (Table 1), 21.9% of patients presented with normal inflammatory markers (ESR, CRP) and 19.1% of patients presented with a normal full blood count (FBC, consisting of haemoglobin, platelets, WCC, PCV).

*Abnormal blood tests*

For individual results the most likely tests to be abnormal in PIBD were haemoglobin (61.9%), PCV (64.6%), ESR (56.4%) and platelets (55.6%). CRP was high in 53.4% of patients. Albumin was low in 28% of patients and ALT was high in 7.2% of patients.

The proportion of patients presenting with abnormal blood tests for PIBD, CD and UC can be seen in figure 1.

*Comparison of Crohn’s disease vs Ulcerative colitis*

Patients diagnosed with CD were significantly more likely to have abnormal inflammatory markers when compared to UC (UC= 34% normal, CD= 15.8% normal, p= 0.0035). When considering FBC CD were significantly more likely to be abnormal when compared to UC (UC= 30.9% normal, CD= 12.5% normal, p= 0.0005). Considering all blood tests UC was significantly more likely to present with all normal results than CD (UC= 14.4% normal, CD= 5.3% normal, p= 0.02). See table 1.

*Blood test median values*

Median values were calculated for all tests, and for both CD and UC. Inflammatory markers (CRP and ESR) were significantly higher in CD rather than UC (CRP-median value CD= 24.5mg/L, UC= 4mg/L, p=0.00001, ESR--median value CD= 27mg/L, UC= 12mg/L, p=0.0001).

Platelets were significantly higher in CD compared to UC (median value- CD= 445mg/L, UC= 381.5mg/L, p=0.0001). Albumin was significantly lower in CD (median value- CD= 32mg/L, UC= 38mg/L, p=0.00001. ALT was significantly higher in UC compared to CD (median value- CD= 12U/L, UC= 16U/L, p=0.00001). There was no significant difference for haemoglobin, WCC or PCV. See table 2.

*Normalised data analysis and hierarchical clustering*

Data for all blood tests was normalised and used to construct a heatmap and box-whisker plot. Hierarchical clustering was applied to group patients by similarity (average linkage, Euclidean distance), see figure 2. Figure 3 shows normalised data for all results displayed as a box-whisker plot. The clusters and cluster statistics (cluster stability, dissolution and recovery metrics) for 12 distinct clusters are included as a supplementary dataset (supplementary table 2).

Through hierarchical clustering patients were automatically grouped by the overall similarity of their blood results. The distance between patients reflects similarity between patients (shorter distance = more similar blood results). These data were split into 12 groups, assigned as clusters A-K from left to right (bottom to top) on the heatmap.

Patients were labelled by their diagnosis after endoscopy (post-hoc). IBDU occurs throughout the heatmap and does not cluster together. CD typically clustered together in the presence of low albumin or high inflammatory markers, with UC less likely to form distinctly clusters.

Further analysis identified distinct outlying groups at the top (clusters J + K, cluster stability 0.71 and 0.47 respectively) (highlighted in yellow, characterised by high CRP and low albumin) and bottom (cluster E, cluster stability 0.68) of figure 2 (highlighted in green, characterised by normal albumin and low haemoglobin) of the heatmap. These groups were enriched for Crohn’s disease (85.7%, 12/14 cases, p=0.049) and ulcerative colitis (66.7%, 12/18 cases, p=0.01) respectively.

Interestingly an additional novel group of nine patients (cluster H, cluster stability 0.63) was observed (highlighted in pink) comprised of 4 Crohn’s disease, 3 ulcerative colitis and 2 IBDU patients. This group was characterised by an isolated increase in white blood cell count, representing a novel grouping.

*Sensitivity of blood results*

Sensitivity as the proportion of patients presenting with abnormal tests was calculated for all blood tests. It can be inferred that in children presenting with chronic abdominal symptoms (of PIBD) individual blood tests have limited utility in the diagnostic work up (sensitivity ranging from 22.71-64.6%). However the pooled results have a sensitivity of 91%, meaning that 1/11 patients with a subsequent diagnosis of PIBD will present with all normal results, equating to 23 patients in this cohort, over 4 per year.

*Age at diagnosis and gender*

In all PIBD cases neither CRP nor ESR were significantly correlated with age at diagnosis (CRP- R2=0.011, p=0.070, ESR- R2=0.001, p=0.686). For Crohn’s disease age at diagnosis was significantly correlated with CRP value (R2 = 0.031, p = 0.022), older children presented with higher CRP values. This was not seen with ESR. Neither CRP nor ESR significantly correlated with age at diagnosis in UC.

Platelet count was not correlated with age at diagnosis in either PIBD, CD or UC.

Inflammatory markers were compared between males and females, neither CRP nor ESR were significantly different between groups (CRP- male 14mg/L, female = 9mg/L, p=0.055, ESR- male 20.5mm/hr, female 22mm/hr, p=0.72).

Blood results with known variation by age or gender (normal values are different for different ages or gender), including haemoglobin, PCV, WCC and albumin were not investigated for association with age at diagnosis or gender.

Discussion

The majority of patients over a five year period with a subsequent diagnosis of PIBD will present with at least one abnormal blood test (91%). However 1/5 will present with normal inflammatory markers and 1/11 will present with all normal blood results. Patients with a subsequent diagnosis of CD are significantly more likely to have abnormal results compared to UC. Normal blood tests (especially normal inflammatory markers) should not preclude from further investigation and referral (faecal calprotectin, diagnostic endoscopy) in children with significant chronic symptoms or a high index of suspicion (1,3).

Hierarchical clustering of normalised data revealed novel groups separate from the main patient data. The main outlying groups at the top (characterised by high CRP and low albumin) and bottom (characterised by normal albumin and low haemoglobin) of the heatmap are significantly enriched for Crohn’s disease and ulcerative colitis respectively. An additional group of nine patients clustered based on an isolated increase in white cells, this group contained CD, UC and IBDU and represented a novel cluster, distinct from traditional diagnostic subtypes. Most patients do not cluster into large distinctive subgroups and occur throughout the heatmap. IBDU patients appear throughout the heatmap and do not cluster together. These data provide an additional framework to help with early differentiation between CD and UC based on both number/pattern of abnormal blood results, the degree of abnormality and enrichment of CD/UC in novel grouping of patients through hierarchical clustering.

This study provides a large cohort of patient blood results at diagnosis, with an extensive number of tests analysed. Sabery *et al* (2007) focused on fewer tests. The proportion of patients with abnormal haemoglobin or ESR was described in up to 83% of patients with a subsequent diagnosis of PIBD (8). Mack *et al* (2007) detailed blood results at diagnosis of PIBD (ESR, haemoglobin, platelets and albumin) and demonstrated all normal results in 9% of CD and 19% of UC patients, with individual tests, ESR, haemoglobin, platelets and albumin, being normal for all IBD in 18%, 24%, 43% and 50% respectively (9). Our data show a higher proportion of patients presenting with normal values (seen in 44%, 38%, 54% and 72% of patients) for the respective blood tests. In 1995 Beattie *et al* in a cohort of 91 children referred with chronic gut symptoms to paediatric gastroenterology described no CD patients presenting with all normal blood tests and 100% of patients presenting with a raised CRP (16). In our study, a comparable 5% of CD patients had normal results however 29% had normal CRP and over 1/7 had a normal CRP and ESR. Recent data from Day *et al* detailed blood results at diagnosis (ESR, CRP, platelet count and albumin), reporting all normal tests in 13% of patients with CD and 41% of those with UC, which is very comparable to our data (15.8% and 34% respectively) (17). This apparent change in presenting phenotype, with more patients with normal results, may reflect a change in disease type. However it is more likely to reflect the significant increase in incidence and improved identification of disease at an earlier stage (4).

The patient groupings determined by hierarchical clustering in this study present a potential clinical application for these data. Patients with high CRP and low albumin were heavily enriched for Crohn’s disease, whilst those with normal albumin and low haemoglobin were enriched for ulcerative colitis. This allows clinicians to discuss the probable subtype of disease, including treatment implications with families prior to endoscopy, based on blood results alone. It would be interesting to scrutinise patients with these specific patterns of blood markers at diagnosis in a novel independent group of paediatric patients.

Data from 2003 comparing CD with UC showed a similar effect to seen in our data, ESR and platelets were significantly higher whilst haemoglobin and albumin where significantly lower in CD compared to UC (18). Our data demonstrated that CRP is significantly higher in CD, but haemoglobin was similar in CD and UC. It is well established that CD is more likely to have a systemic inflammation, which in turn is likely to be associated with more severe blood abnormalities. Younger children presenting with CD were more likely to have a normal/lower CRP value (positive age, CRP correlation), this may increase the difficulty in making a prompt diagnosis in early-onset PIBD (19).

Significant advances have been made in the diagnosis and treatment of PIBD over the last 20 years. However accessible diagnostic models to classify patient risk have lagged behind. The use of machine learning, multi-omics and artificial intelligence to aid physician diagnosis and stratification of patients is now beginning to occur but only in a research environment (20,21). Despite this progress the eventual utility to a general paediatrician or primary care physician may be limited, with simple and accessible strategies required to stratify patients for referral. A recent systematic review and meta-analysis detailing the utility of laboratory tests demonstrated that by adding FCp value to symptoms of PIBD led to improvement of diagnostic accuracy (area under the curve (AUC) by 0.26). In comparison the best blood marker (ESR) was less useful increasing the AUC by only 0.16 (13). There is clearly a place for blood results in the diagnostic work up of children with chronic gut symptoms however multiple normal blood tests and a history consistent with IBD should not be ignored (especially in the presence of features such as a family history of IBD), triggering the use of additional investigations (such as faecal calprotectin or ultrasound). In children, the differentiation between common abdominal pain or a transient diarrhoeal illness and IBD can be difficult and requires clinical expertise in conjunction with appropriate investigations (22).

This study has several limitations; we were unable to include patients who were referred with a possible diagnosis of PIBD whose endoscopy and histology were then normal. The study would benefit from these patients as a control group, however they cannot reliably be identified from the patient record leading to significant concerns over introducing bias. The decision was made to exclude them. We were therefore unable to calculate specificity in addition to sensitivity. In addition patients without bloods at UHS prior to diagnosis were excluded, reducing the sample size and therefore statistical power. However these patients were referred from across the same region and followed the same diagnostic pathway as included patients. This study benefits from prospective inclusion of all patients onto the Southampton PIBD database, capturing a real-life cohort, utilises standardised automated blood result data collection and uses age and gender specific normal ranges (obtained with each blood result) to interpret the data.

Conclusions

There may be a trend towards patients presenting with more normal blood results, perhaps driven by earlier identification of disease. It is important for general practitioners, general paediatricians and specialist services to keep a diagnosis of IBD in mind even if blood tests (especially inflammatory markers) are normal. Use of hierarchical clustering identified groups enriched for Crohn’s disease and ulcerative colitis characterised by specific blood results. Clinicians can use this model to help identify the sub-diagnosis (CD vs UC) in patients with PIBD. Future development of a simple risk stratification model, based on symptoms and accessible tests (bloods, FCp etc.) could provide a framework to reduce the diagnostic delay seen in PIBD.

Tables and Figures

*Figure 1*- Percentage of patients presenting with abnormal blood tests for all IBD, Crohn’s disease and ulcerative colitis

*Figure 2*- Heatmap showing normalised data for all 256 patients presenting with IBD. Data were normalised by mean value and standard deviation. Red indicates a higher value, blue indicates a lower value and white indicates a value of 0 (mean value). Black represents missing data. For example a high CRP has a brighter red colour, whereas a lower albumin has a brighter blue colour.

Data were grouped by hierarchical clustering (average linkage, Euclidian distance). The diagnosis of the patient is annotated on the Y axis. The dendrogram represents the similarity of the patients, with a shorter distance indicating a more similar blood result profile at diagnosis. Outlying groups at the top (highlighted in yellow, characterised by high CRP and low albumin) and bottom (highlighted in green characterised by normal albumin and low haemoglobin) of the heatmap are enriched for Crohn’s disease and ulcerative colitis respectively. IBDU occurs throughout the heatmap and does not cluster together. Nine patients highlighted in pink cluster due to an isolated increase in white cell count and represent a mix of subsequent diagnoses.

*Figure 3*- Box and whisker plot displaying normalised data for all 256 patients presenting with IBD. Data were normalised by mean value and standard deviation. Significant differences between Crohn’s disease and ulcerative colitis are indicated on the graph.

*Table 1*- Percentage of patients presenting with abnormal blood tests for all IBD, Crohn’s disease and ulcerative colitis. Sensitivity of each blood test for diagnosis of IBD in this cohort.

*Table 2-* Median results for each blood test for all IBD, Crohn’s disease and ulcerative colitis.

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