Differences in microbial metabolites in urine headspace of subjects with Immune Thrombocytopenia (ITP) detected by volatile organic compound (VOC) analysis and metabolomics

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Abstract

ITP is an organ specific autoimmune disorder characterised by a low platelet count whose cause is uncertain. A possible factor is food intolerance, although much of the information linking this with ITP is anecdotal. The role of food intolerance in ITP was studied by replacing a normal diet with an elemental diet (E028) but this did not increase platelet counts. Clear differences, however, were apparent between the volatile organic compounds (VOCs) in the urine headspace of patients with ITP and those present in healthy volunteers, which leads to speculation that abnormal metabolic activity of the intestinal microbiome may be a factor causing ITP. However further work is needed to confirm this. There were also differences between the VOCs of patients on a normal diet and those on the elemental diet, and in this case, the VOCs involved are very likely to be of bacterial origin, as their production is affected by dietary manipulation. Many of these VOCs are known to be toxic.

Keywords

Immune Thrombocytopenia, ITP, Metabolomics, Volatile Organic Compounds, VOCs, Selective Ion Flow Tube Mass Spectrometry (SIFT-MS)

1. Introduction

1.1 Immune Thrombocytopenia (ITP)

ITP is an organ specific autoimmune disorder (1) characterised by a low platelet count. In diagnostic terms, ITP is defined as a platelet count of 'less than 100 x 10^9/L (100,000/µL)' (2). Its prevalence has been examined in a number of countries and ranges from 2.64 per 100,000 patients/year in Denmark to 9.5 per 100,000 in the USA. A predominant number of cases in childhood are in males with this changing to middle-aged women in adulthood. The overall ratio male to female ratio is thought to be 1:1.9 (3i). Most cases of ITP are classed as being idiopathic but some cases are secondary to coexisting conditions as mentioned in section 1.2 (4).

1.2 Causes of ITP

Numerous theories have been put forward as to the cause of ITP but a definitive answer remains elusive. Definitive diagnosis of the condition is still extremely difficult and is often related to numerous other factors, for example SLE, HIV infection, hepatitis C, drug induced or Helicobacter pylori infection. It also means that diagnosis is therefore usually made by exclusion of other disorders (3).

One of the major hypotheses is that ITP is characterised by increased platelet destruction either by antibody mediated platelet destruction or platelet lysis due to cytotoxic T-lymphocytes (1,4,5). Another suggestion is 'molecular mimicry' which is defined as 'similar structures shared by molecules produced by dissimilar genes' (1). Here, by a complementary mechanism, a pathogen (such as Helicobacter pylori or HIV) can induce cellular injury and release self-antigens. This generates an immune response that cross-reacts with additional self-antigens which are genetically distinct. The

theory is that a pathogen could induce antibody production in response to antigens that cross-react against various platelet glycoprotein antigens. Therefore, specific antibodies might be capable of causing thrombocytopenia. This mimicry of host proteins enables the pathogens to escape host surveillance (1,6,7). The final major theory is that there is decreased platelet production. In healthy people, when platelet production equals platelet destruction and consumption, a stable platelet count will occur (8). In patients with ITP, shorter platelet lifespan is consistently seen. One explanation for this is that some surface antigens that are co-expressed on platelets, megakaryocyte precursors, and megakaryocytes are recognised by autoantibodies. Reduced platelet production is then assumed to be due to the direct effect of antibodies on the maturation of megakaryocytes or platelet release (6,9).

Certainly ITP occurs by a number of complex processes involving multiple components of the immune system. Apart from decreased platelet production, a continual 'self-antigen-stimulated' response ultimately leads to a deficiency in the central and/or peripheral tolerance (failure to mount an immune response to an antigen) which triggers an autoreactive lymphocyte response. Evidence suggests that both environmental and genetic factors are the key to this issue (1).

1.3 Linking ITP to food intolerance?

Food intolerance has been suggested as a cause of ITP, however much of the information linking the two is anecdotal. The link has been recognised and documented in a few cases as early as 1936 (10), but little has been documented specifically related to ITP and food intolerance between then and now, except in individual case reports (11,12,13).

Most recently, in 2012, a case study was published in the Lancet by Achterbergh, et al. which detailed the case of a 70 year old man who was admitted with vomiting, nausea and fever and found to have a platelet count of 32,000 per μL. After investigation and a second admission, the causative factor was determined to be the walnut, and to test this, a subsequent walnut challenge was carried out. His initial platelet count was 233,000 per μL, and 4 hours after being given 100g of walnuts, he developed fever, nausea and vomiting, bled from a small wound and developed large haematomas around venepuncture sites. Approximately 15 hours after ingestion his platelet count had dropped to 4,000 per μL. Following exclusion of nuts from his diet he had no further episodes and maintained a normal platelet count. Although walnuts were clearly related to the sudden drop in platelet count, this remains a rare case, and is likely the result of an acute ITP episode.

Bacteria produce numerous metabolites, some of which are volatile, others which are semi volatile or not volatile at all. These may all vary depending on the food sources available in the gut. In addition, as the human body produces a steady stream of metabolic waste (ammonia, lactic acid, urea, bilirubin etc.) and exotoxins (produced by the microbiota in the gut) the body carries out biotransformation in the liver to remove toxins by converting them to less toxic substances which may then be excreted in urine or the bile (14).

It is to be expected, therefore, that these volatile organic compounds (VOCs) may be detected in urine headspace. Approximately 279 VOCs have been identified in urine in apparently healthy individuals (14i). Urinary VOCs for example, have been shown to discriminate tuberculosis patients from healthy subjects (14ii) and some have shown differences in the female reproductive cycle during ovulation (14iii). The headspace of urine of a group of ITP patients and healthy control subjects were therefore studied using selected ion flow tube mass spectrometry (SIFT-MS) to look at initial overall differences between urine headspace from the ITP and healthy control groups; this was then extended to seeing how the urinary VOC profile was affected by diet.

The data were compared using univariate and multivariate statistical analysis. The aim of this project was to discover if ITP subjects and healthy controls could be easily differentiated by looking at urinary profiles using SIFT-MS and to determine whether food intolerance is involved in ITP.

2. Methods

2.1 Initial Phase: Patient recruitment

The initial volunteer population group for this part of the study was recruited via the ITP Support Association.

Ethical permission for the study was obtained from the Cambridge Local Research Ethics committee (LREC) reference 11/EE/0084. Patients of either sex aged 18-65 years suffering from chronic primary immune thrombocytopenia were recruited.

The diagnosis of ITP was based on the criteria set out by Rodeghiero *et al* (2009) with a platelet count of less than 100,000/µL, and a history of this condition for at least 12 months. Patients who had undergone splenectomy were included, provided the operation was not recent and that they were taking no other antibiotics than penicillin to reduce the risk of infection. Patients on other drugs were included if their platelet count remained low and provided that the drug was started before the longest time to peak response quoted by Rodeghiero *et al.*, 2009 and that they were willing to continue that treatment until their participation in the trial was concluded.

On recruitment, a urine sample was taken and was tested with a multistix® urianalysis (Siemens UK) test before being frozen and sent for SIFT-MS headspace analysis.

A healthy control group of 50 people aged 18-65 years was also selected; these had the same initial tests performed.

Subjects were excluded from the study according to the following criteria:

a) Subjects outside 18-65 yrs

b) Pregnancy and lactation

c) Acute thrombocytopenia

d) Thrombocytopenia related to underlying disease or drugs

e) Patients who have received broad spectrum antibiotics in the previous 6 weeks

f) Patients who had started treatment with Rituximab, Danazol, Eltrombopag or Azathioprine in the previous 6 months

g) Patients who had started treatment with Corticosteroids, Vincristine or AMG531 (Romiplostim) in the previous 8 weeks

h) Patients who have undergone splenectomy within previous 8 weeks

i) Patients known to have Inflammatory Bowel Disease (IBD) or Coeliac disease

2.2 Sampling

Participants were recruited from around the UK and travelled from their homes to attended Addenbrooke's Hospital. Here they provided written informed consent. They were asked to complete a symptom questionnaire and provided a sample of urine and blood. The urine samples were collected

in 20ml Fisherbrand™ scintillation vials (Fisher Scientific, Loughborough) while the subjects were present at the hospital and were immediately frozen at -80°C. All samples were anonymised and labelled with a specific number; the code was only known by the principal investigator (PI). Urine samples were frozen for a maximum of 3 months before being thawed and immediately tested. Blood was taken into Thromboexact® (Sarstedt AG & Co, Germany) containers to ensure accuracy of platelet counts and was immediately transferred to the laboratory for testing.

2.2.1 Demographics

All initial samples were collected over a 12 month period. The mean age in years (± s.d) of the healthy controls was 48.2 ± 10.9 with a percentage of female sex being 54.3%. The mean age in years (± s.d) of the ITP volunteers was 47.6 ± 12.9 with the percentage female sex being 66.7%.

From the initial ITP results, a group of participants with the lowest platelet counts throughout the whole ITP group were selected to undertake dietary modification using the elemental diet E028 extra (Nutricia, Trowbridge, UK). A group of healthy controls also volunteered to limit their nutrition to elemental E028 extra for 7 days.

2.3 Dietary modification - Elemental Diet

Elemental 028 Extra diet is a nutritionally complete liquid formula containing a mix of essential and non-essential amino acids, a single fatty oil, minerals, maltodextrins, vitamins and trace elements. It was administered in 250ml ready-made cartons in a choice of flavours. E028 Extra has been shown to be of therapeutic value in Crohn's disease and refractory coeliac disease (15,16).

Each participant gave a blood and urine sample taken prior to beginning the diet and had a consultation with a registered dietician to give advice on what the diet entailed, and what to expect. They were given written details of the diet and asked to complete a symptom diary. E028 extra was introduced gradually in half quantities over the first few days and then fully for the rest of the 4-week period. A blood sample for platelet count was taken every week either by the patient's General Practitioner (GP), or at Addenbrooke's Hospital. Urine samples were taken before the diet was started and after the diet was completed and were immediately frozen at -80°C before being sent for analysis by SIFT-MS.

2.4 Control Group – Elemental Diet

The healthy volunteers were people who were known to be healthy with no blood or gastrointestinal disorders or food intolerances. Due to time restrictions with the project and the difficulties encountered when being on the E028 Extra, only 3 members of the research team took part. As these subjects had to follow their normal lives, it was not appropriate for them to continue E028 Extra for the full four weeks, and they remained on it for 5-8 days.

A blood sample was taken prior to starting the diet and on the last day of the diet. A urine sample was also taken prior to starting the diet and on the last day. Following the collection of urine samples, they were frozen at -80°C before being processed using SIFT-MS.

2.5 Urine Sample Preparation

Urine samples were removed from the freezer and placed in a room temperature environment to fully defrost. To 5ml of each sample, which was pipetted from the scintillation vial, 125µl of 13M hydrochloric acid was added to aid in the generation of VOCs (20). Exactly 3g of sodium chloride was added to the empty Nalophan sample bags (Foodpak®) that had been made up to 40cm long and sealed at one end with a cable tie. The acidified urine was then added to each bag. The Nalophan

bag was then sealed around silicone tubing with a Swagelok® fitting, and the urine mixed by gently shaking to ensure even distribution of the acid and the salt. Each sample bag was then filled with hydrocarbon-free air and sealed with a Swagelok® nut.

The samples were placed into an incubator at 40°C. The door was sealed and then the samples were left for one hour to generate headspace prior to headspace analysis by selected ion flow tube mass spectrometry (SIFT-MS).

2.6 SIFT-MS Analysis

In this case a SIFT-MS MkII from PDZ Europa was used, with a flow rate through the heated capillary giving a pressure of 0.008 Torr in the flow tube. Samples were analysed for T5 x 6 at unit resolution. Full details of how SIFT-MS may be used to analyse trace gases and volatile organic compounds may be found elsewhere (18), however, a brief explanation is warranted here. In SIFT-MS, precursor ions (H_3O^+, NO^+ and O_2^+) are generated in a microwave discharge and may then be selected by a quadrupole mass filter. The selected ion is then injected into a fast flowing helium carrier gas, and down a flow tube. A sample is then introduced into the flow tube at a rate of 15ml/min, and the precursor ion reacts with the trace gases and volatile organic compounds in the sample. Each sample is analysed for 5 seconds, on 6 separate scans, giving a total of 30 seconds analysis on each scan. The precursor and product ions in the carrier gas are separated in a second quadrupole mass spectrometer and subsequently counted in a detector. Data may be obtained through scanning a spectrum at a user-defined range of mass-to-charge ratio (m/z) values and quantification is carried out using a kinetics database stored in the instrument. In this case the user defined m/z range was 10-140 m/z.

2.7 Statistical Analysis

Data were analysed using univariate and multivariate statistical techniques. The univariate technique involved the non-parametric Mann-Whitney U test analysis because the data were not normally distributed. Multivariate data analysis was performed by custom-built scripts written in MATLAB R2011a (MathWorks Inc., Nattick, USA) using functions from the PLS Toolbox (version 3.5, EigenVector Research Inc., USA). A variety of multivariate statistics were tested to determine the most appropriate for these data sets. Prior to multivariate data analysis, data generated by the H_3O^+, NO^+ and O_2^+ precursor ions were optionally combined into one large dataset. The intensity values at the range of m/z values within the data were normalised against the intensity values of the H_3O^+ precursor ions (m/z value of 19). The m/z values pertaining to the known adducts (isotopologues) of the H_3O^+ precursor ion were removed; these had the following m/z values: 19, 21, 32, 37, 39, 55, 57, 73, 75, and 91. If combined, then m/z values of 30, 34, 48 and 66 were also removed. Finally, m/z values pertaining to data columns consisting only of zeros were also removed.

Exploratory data analysis using principal components analysis (19) revealed no outlying samples. Multivariate classification via partial least squares discriminant analysis (PLS-DA) was performed. This is a supervised pattern recognition technique in which the computer is trained to recognise patterns in the data that will help distinguish between low and high risk patients. To ensure a robust and confident result was attained, a two-step bootstrapping process was employed in an average performance of all models created. To further improve the classification accuracy attained, feature selection via either the parametric Student t test (STT) or the non-parametric Wilcoxon T test (WTT), was performed prior to the classification. The number of features in each sample following feature selection via the student t test was 9. The statistical significance of the classification accuracy was determined by performing permutation testing which involved randomising the class assignation 300

times, and for each random assignation, the two-step bootstrapping process was performed. The statistical z-test was employed to determine the significance ($p < 0.05$) (20).

3. Results

3.1 Initial Platelet count

Results of the platelet counts showed a highly significant difference between the ITP and the control groups. Although the subject groups were relatively large, they were still assessed with non-parametric testing as there were fewer than 100 samples in each group and were not normally distributed.

The data were analysed using Mann Whitney U testing. With this analysis the two sets of data were found to be significantly different. The z-score was found to be -6.98 with a p-value of <0.00001. All the control samples had normal platelet counts with a mean platelet count of 236,200 per µL and a range of 147,000-341,000 per µL. The ITP volunteer group had a mean platelet count of 58,000 per µL with a range of 5,000- 281,000 per µL.

There were some outliers in the ITP group which were closer to normal platelet levels. These were 117,000, 144,000 and 281,000 per µl and although these data are included in these initial results, all were subsequently excluded from the study.

3.2 Platelet Counts before and after E028 Diet

In total 10 volunteers with ITP and 3 healthy control volunteers went onto the E028 diet. Platelet counts were assessed prior to and after dietary modification with the E028 diet.

When ITP platelet counts were assessed, no significant increase in platelet count occurred while on or initially after taking the E028 diet (Figure 1).

3.4 Analysis of VOCs in Urine Headspace using SIFT-MS

3.4.1 Univariate Analysis of urine headspace of whole cohort of ITP and control participants

In total 35 controls and 42 ITP subjects were included in this study to compare the groups of ITP participants and healthy control participants. Univariate analysis of urine headspace of the H_3O^+ data using the Mann-Whitney U test, found two m/z values to be significantly different between ITP sufferers and healthy controls. These m/z values were m/z 18 and m/z 43. Using box and whisker plots (Figure 2) these differences are shown. These ions are likely to represent ammonia and 1 or 2 propanol respectively.

3.4.2 Multivariate Analysis of urine headspace of whole cohort of ITP and control participants

When analysing the H_3O^+ data alone, it was found that PLS-DA in conjunction with feature selection via the Student t test (STT) offered the best result when comparing the headspace of urine from ITP suffers and healthy controls. Overall the percentage of correctly classified (%CC) samples was 70% with a specificity of 53% and a sensitivity of 84%. The sensitivity suggests that SIFT-MS is able to show there are clear differences in the volatile metabolome of subjects with ITP. However, the relatively low specificity suggests that data pertaining to healthy control subjects is more complex; this could be attributed to some underlying features or a wide variation in the urinary volatile metabolome of healthy volunteers. The area under the ROC (AUROC) curve was calculated as 0.80.

In order to determine whether the overall classification value was statistically significant, permutation testing was carried out (Figure 3) which involves randomising the class assignations 300 times. Though

overlap between the two distributions exists, the z-test calculation, which compares the means of the distributions, rejected the null hypothesis thus suggesting that the %CC attained at 70% is statistically significant at the 95% confidence ($p<0.05$). Furthermore, the number of variables (m/z values) which were found to be significant following the Student t-test was 8 with the corresponding m/z values of 34, 43, 53, 72, 74, 76, 89, and 112. The influential variables further indicated by the PLS-DA loadings (not shown) appear to be, m/z 43, m/z 53 and m/z 76.

Of interest is that the PLS-DA loadings also showed that m/z 43 was in the opposite direction to the other m/z values. This can be interpreted that ITP sufferers had strong correlations with higher counts of m/z 43 using H_3O^+ and lower counts of m/z, 34, 53, 72, 74, 76, 89 and 112 than the control subjects. It also correlates with the earlier univariate analysis to show that m/z 43 is significant.

Combination of the abundances from all three pre-cursor ions in conjunction with the normal approach of removing the precursor ions and their adducts had resulted in good performances by the PLS-DA models in conjunction with feature selection by both Wilcoxon T test and Student t-test (88% and 87% respectively).

The classification accuracy of 88% pertaining to WTT (Control versus ITP) produced a specificity of 87% and a sensitivity of 89%, which is considerably better than just using H_3O^+ alone.

The permutation testing demonstrated that the result was statistically significant and not due to chance. Furthermore, the PLS-DA loadings (not shown) suggests that the following m/z values could be influential to the distinction between ITP patients and healthy controls:

- H_3O^+: 43, 46, 53, 70, 76, 89
- NO^+: 54, 56, 59, 76, 78, 92, 111
- O_2^+: 31, 41, 54, 86, 105, 106

3.5 Identifying the compounds related to m/z values

A number of m/z values have been identified above. The potential identifications indicated in table 1 shows potential compounds these m/z values may relate to but this would need to be verified using GC/MS.

The m/z values for products using the O_2^+ precursor ion are much more difficult to identify, and therefore would need another technique such as GC-MS for identification.

These results demonstrate that there is a clear difference between urine headspace from ITP patients and healthy volunteers.

3.6 Dietary Modification – E028 Diet

3.6.1 Univariate Data H_3O^+

Although sample groups were small, some analysis was possible when looking at the differences that the E028 diet made on the headspace analysis of both the ITP sufferers and the healthy controls. On comparing the SIFT-MS data, two compounds were found to be statistically significant in the ITP group of pre and post diet. These were methanol and propanol. Figure 4 and 5 illustrate the ITP group results for the VOC methanol along with the corresponding control group results. Although the control group was too small to be statistically compared it is illustrated that the controls showed a similar pattern of reduction of methanol following E028 diet as in the ITP group.

Individual ion data were also analysed and two m/z values were found to be statistically significant in the ITP group. These were m/z 51 and 69. These m/z values most likely represent methanol, with water hydrates ($CH_4OH.H^+.H_2O$ and$CH_4OH.H^+.2H_2O$). Table 2 illustrates these data.

When propanol data were analysed it can be observed that in the ITP group, the concentration in parts-per-billion (ppb) in urine headspace decreases significantly after E028 diet. Conversely in the control group, although not statistically proven, indicates an opposite pattern where the propanol levels increase after E028 diet (Figure 5).

3.6.2 Multivariate statistical analysis

Due to too few control subjects, the E028 diet data were only analysed for the ITP volunteers. Initial multivariate analysis used data from the H_3O^+ precursor ions. The method showing the best resulting classification was PLS-DA with WTT. This had an overall correct classification of 80% with a specificity of 70% and a sensitivity of 90%. The AUROC was calculated to be 0.9360. Combining the precursor ions as previously described and subjecting to PLS-DA with WTT produced an overall classification (%CC) of 95% (95% specificity and 95% sensitivity). The calculated AUROC was 0.9554.

This suggests that the effects of the E028 Extra via SIFT-MS and pattern recognition are very clearly visible, thus demonstrating the effect on urine headspace VOCs following elemental diet.

4. Discussion

This study investigated ITP and urine headspace changes in relation to dietary changes and food intolerance. There were no increases in platelet counts when ITP subjects maintained an E028 Extra diet, implying that it was unlikely that ITP is caused by food intolerance. This is in contrast to Crohn's disease, where the E028 extra brings about long term remission of the condition and therefore long term dietary therapy is practicable (21).

Although much research has been carried out looking at the effects certain drugs have in causing ITP, it is still unknown if these mechanisms could also explain why some foods may cause similar reactions. However, it has been noted that drug induced reactions can take up to 7 days to appear (22), which would imply a different reaction than foods as these have generally been reported, to occur very rapidly (23).

Research looking at actual platelet counts in relation to food intolerance is limited and mostly relate to complementary therapies, supplements and drinks, rather than a full scale look at specific foods (24). It is difficult to know if there have been any other experiences of these type of results when looking at chronic ITP and not just isolated or acute episodes as is often reported as by Royer *et al.,* in 2010. It is also unknown if diet or dietary modification has ever been assessed in relation to actual platelet count data.

What could be hypothesised is that there are certain groups of people, or certain medical conditions that lead to a person having a predisposition for the way in which they process their food. This is then ultimately influenced by bacteria in the gut and may be related to factors including the type of bacteria in the population, the number of those bacteria and their responses to the food presented to them.

4.1 Explaining significant m/z values

Analysis of the headspace using SIFT-MS showed differences in propanol in ITP compared with healthy subjects, but cannot distinguish between 1-propanol, or 2-propanol. Propanol is a fermentation product in the gut, for example and Hosseini *et al* in 2011 found that *Clostridium neopropionicum* X4

was able to ferment (1-^{13}C)-ethanol and CO_2 to (2-^{13}C)-Propanol, suggesting that this compound is potentially produced via fermentation by bacteria.

Ions of m/z 18 and 36 most likely correspond to the compound ammonia. Ammonia is ubiquitous in urine due to the fact it is a breakdown product of urea, and as a simple nitrogenous product it is of interest biologically as it can be used by living organisms for protein synthesis (25,26). Bacteria can also utilise it for their nitrogen requirements and bacteria can also be major producers of ammonia too (27). Ammonia can be found in many tissues in the body including muscle and kidneys but it also enters the circulation from the gastrointestinal tract. During deamination, significant ammonia is released and endogenously it is also released by the kidney. The third source is formed in the gastrointestinal tract by the action of amino-acid oxidase and urease derived from bacteria (28). The micro-organisms responsible for urease activity include *enterobacteria*, *bacterioides*, *clostridia* and *Klebsiellaaerobacter* (29).

Overall it is extremely difficult to pinpoint exactly why the ammonia levels were significantly different between ITP and healthy controls but again, one of the more likely causes is gut fermentation - considerable amounts of ammonia have been shown to be produced by bacteria from non-urea sources. Ammonia has been shown to be a potentially toxic product of protein breakdown in the large intestine and it seems to be a compound that is repeatedly indicated in a number of diseased states (30). Richardson suggests that a functional group on a 'deaminative' bacteria exists, which is similar to the 'hyper-ammonia producing' bacteria in the rumen of cattle and sheep, and that if these exist in the human colon they may have a similar significance with fermentation, and may also be manipulated via dietary modification (30). This repetitive indication of the compound suggests it is not unique to any one disease and therefore would not alone, be useful as an indicator of a specific disease.

Add bit here on breakdown of platelets?

SIFT-MS analysis following dietary changes proved that the size of the sample groups were too small to offer convincing evidence of potential compounds that may be useful. However, it showed how the counts per second of different m/z values changed before and after the diet.

4.2 Linking to metabolism and the gut microbiome

What multivariate analysis indicated is a metabolic or metabolomic difference in the profiles of ITP sufferers and healthy controls. Multivariate data analysis showed that with a specificity of 87% and a sensitivity of 88%, samples from ITP sufferers can be distinguished from healthy controls with a percentage correctly classified with an accuracy of 88%. This means that the test could correctly differentiate 88% of the samples into either the ITP group or the control group. This is potentially useful due to the fact ITP is a multifactorial disease and so difficult to diagnose. If a method was available to help identify a certain metabolomic pattern in a person to suggest ITP – along with their presenting symptoms – it could help faster and less invasive diagnosis. Aside from the diagnostic possibilities, the differences in the headspace of urine in ITP patients and controls demonstrates a clear metabolic difference in these subjects and this could then lead to a greater understanding of the origin of this debilitating condition.

One possibility is that these differences result from changes in gut flora in ITP patients. The 'microbiomial relationship' can become imbalanced via various internal and external factors. Evidence suggests that much of this impact is mediated through diet and also suggests that the gut microbes can influence what the human host is able to extract from its diet, including energetically (31). The intestinal microbiome has a high level of metabolic activity and diet, genetic factors, bodily changes (e.g. pregnancy), ageing, environment, chemical therapies and pharmacology can all affect it (32). In

turn, the microbiome can have an effect not only on the gut itself but also on the immune response and immune system.

These bacteria coexist in a complex mutually beneficial relationship which is physiologically important to host-microbe interactions in the gut in both directions (33). If the bacterial profile changes, it can lead to added issues for the host including possible changes in fermentation in the gut. If the microbial community is maladapted to its environment, it will actually impair all these processes and result in disease states including allergy, obesity, diabetes, rheumatoid arthritis (RA), cancer and inflammatory bowel disease (34).

Cordain *et al*. in 2000 review how dietary lectins interact with enterocytes and lymphocytes to facilitate the translocation of dietary and gut-derived pathogen antigens into the peripheral tissues. This in turn causes persistent peripheral antigenic stimulation. In genetically susceptible individuals, this antigenic stimulation could result in expression of overt RA via molecular mimicry, a factor which is also thought to play a role in ITP.

It is therefore possible that such factors can also be implicated in conditions such as ITP. One case of ITP highlighted was show by Borody *et al* in 2011. Here a patient had presented with chronic relapsing ulcerative colitis with concomitant ITP. This patient was offered faecal microbiota transplant (FMT) and accepted the offer. The mean platelet count prior to FMT was below 100,000 per µl, following recurrent FMT the platelet count increased to 168,000 per µl then onto a steady progression to a normal range (mean 195,000 per µl, range 153,000-261,000 per µl). They concluded from this 'unexpected' side effect of FMT that some cases of ITP should be reassessed as arising from an 'infection' in the gut microbiota (35).

The findings of this study suggest further research is required into the gut microbiome of ITP patients. With work having been carried out by van der Waaji *et al*. in 2004 which showed that patients with IBD had an increased percentage of immunoglobulin-coated faecal anaerobic bacteria in both active disease, and just after remission, this area would be extremely pertinent to follow up with in ITP.

It is also pertinent to mention the fact the ITP group was compared to a healthy volunteer group. The intent of this was to examine differences and similarities between groups not suggest it is suitable at this point as a diagnostic test. A further study to compare the ITP group to a group with co-related diseases would be beneficial to also show how these metabolomic changes occur in diseased groups and if they follow a similar pattern to the ITP group.

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6. References

1. Zhou B, Zhao H, Yang RC, Han ZC. Multi-dysfunctional pathophysiology of ITP. Critical Reviews in Oncology and Haematology. 2005; 54: p. 107-116.
2. Stasi R, Amadori S, Osbourn J, Newland AC, Provan D. Long-term outcome of otherwise healthy individuals with incidentally discovered borderline thrombocytopenia. PLOS Medicine. 2006; 3(3): p. 388-394.
3. George JN. Definition, diagnosis and treatment of immune thrombocytopenic purpura. Haematalogica. 2009; 94(6): p. 759-762.
    a. Segal, Powel

4. Cines DB, Bussel JB, Liebman HA, Prak ETL. The ITP syndrome: pathogenic and clinical diversity. Blood. 2009; 113(26): p. 6511-6521.
5. Wang L, Yuan L, Hou M. Idiopathic Thrombocytopenic Purpura and dysmegakaryocytopoiesis. Critical Reviews in Oncology/Haematology. 2007; 64: p. 83-89.
6. Catani L. Recent advances in the pathology of Idiopathic Thrombocytopenic Purpura. Haematology Meeting Reports. 2009; 3(3): p. 55-63.
7. Stasi R, Provan D. Helicobacter pylori and Chronic ITP. Haematology. 2008; 2008(1): p. 206-211.
8. Aster RH. Molecular mimicry and Immune Thrombocytopenia. Blood. 2009; 113(17): p. 3887-3888.
9. Gernsheimer T. Chronic Idiopathic Thrombocytopenic Purpura: Mechanisms of Pathogenesis. The Oncologist. 2009; 80(Supplement 69): p. 12-21.
10. Squier TL, Madison FW. Thrombocytopenic Purpura due to Food Allergy. The Journal of Allergy. 1936; 8(2): p. 143-154.
11. Lavy R. Thrombocytopenic purpura due to lupinus termis bean. Journal of Allergy. 1964; 35: p. 386-389.
12. Caffrey EA, Sladen GE, Isaacs PET, Clark KGA. Thrombocytopenia caused by cow's milk. The Lancet. 1981; 318(8241): p. 316.
13. Davies JK, Ahktar N, Ranasinge E. A Juicy Problem. The Lancet. 2001; 358: p. 2126.
14. Dancygier H. Hepatic Biotransformation. In Clinical Hepatology: Principles and Practice of Hepatobiliary Diseases: Volume 1. Berlin: Springer; 2010. p. 127-130.
    a. De Lacey Costello, Ratcliffe
    b. Banday
    c. Diskin
15. Olaussen RW, Løvik A, Tollefson S, Andresen PA, Vatn MH, De Lange T, et al. Effect of elemental diet on mucosal immunopathology and clinical symptoms in type 1 refractory celiac disease. Clinical Gastroenterology and Hepatology. 2005; 3(9): p. 875-885.
16. Johnson T, MacDonald S, Hill SM, Thomas A, Murphy MS. Treatment of active Crohn's disease in children using partial enteral nutrition with liquid formula: a randomised controlled trial. Gut. 2006; 55: p. 356–361.
17. Smith S, Burden H, Persad R, Whittington K, deLacy Costello B, Ratcliffe NM, et al. A comparative study of the analysis of human urine headspace using gas chromatography–mass spectrometry. Journal of Breath Research. 2008; 2: p. 1-10.
18. Smith D, Španěl P. Seleted ion flow tube mass spectrometry (SIFT-MS) for online trace gas analysis. Mass Spectrometry Reviews. 2005; 24: p. 661-700.
19. Wold S, Esbensen K, Geladi P. Principle Component Analysis. Chemometric and Intelligent Laboratory Systems. 1987; 2: p. 37-52.
20. Brereton RG, Lloyd GR. Partial least squares discriminant analysis: taking the magic away. Journal of Chemometrics. 2014; 28(4): p. 213-225.
21. King TS, Woolner JT, Hunter JO. Review Article: The Dietary Management of Crohn's Disease. Alimentary Pharmacology and Therapeutics. 1997; 11: p. 17-31.
22. Aster RH, Curtis BR, McFarland JG, Bougie DW. Drug-Indiced Immune Thrombocytopenia: Pathogenesis, Diagnosis, and Management. Journal of Thrombosis and Haemostasis. 2009; 7(6): p. 911-918.
23. Achterbergh R, Vermeer HJ, Curtis BR, Porcelijn L, Aster RH, Deenik W, et al. Thrombocytopenia: In a Nutshell. The Lancet. 2012; 379: p. 766.

24. Royer DJ, George JN, Terrell DR. Thrombocytopenia as an adverse effect of complementary and alternative medicines, herbal remedies, nutritional supplements, foods, and beverages. European Journal of Haematology. 2010; 84: p. 421-429.
25. Richards P, Metcalfe-Gibson A, Ward EE, Wrong O, Houghton BJ. Utilisation of ammonia nitrogen for protein synthesisin man, and the effect of protein restriction and uraemia. The Lancet. 1967; 290(7521): p. 845-849.
26. Pisulewski PM, Okorie AU, Buttery PJ, Haresign W, Lewis D. Ammonia concentration and protein synthesis in the rumen. Journal of the Science of Food and Agriculture. 1981; 32(8): p. 759-766.
27. Wrong OM, Vince A. Urea and ammonia metabolism in the human large intestine. Proceedings of the Nutritional Society. 1984; 43: p. 77-86.
28. McDermott WV, Adams RD, Riddell AG. Ammonia Metabolism in Man. Annals of Surgery. 1954; 140(4): p. 539-554.
29. Summerskill WHJ, Wolpert E. Ammonia metabolism in the gut. The American Journal of Clinical Nutrition. 1970; 23(5): p. 633-639.
30. Richardson AJ, McKain N, Wallace RJ. Ammonia production by human faecal bacteria, and the enumeration, isolation and characterization of bacteria capable of growth on peptides and amino acids. BMC Microbiology. 2013; 13(6): p. 1-8.
31. Pray L, Pillsburt L, Tomayko E. The Human Microbiome, Diet, and Health Washington D.C.: The National Academies Press; 2013.
32. Thomas LV, Ockhuizen T. New insights into the impact of the intestinal microbiota on health and disease: A symposium report. British Journal of Nutrition. 2012; 29(6): p. s1-s13.
33. Purchiaroni F, Tortora A, Gabrielli M, Bertucci F, Gigante G, Ianiro G, et al. The role of intestinal microbiota and the immune system. European Review for Medical and Pharmacological Sciences. 2013; 17: p. 323-333.
34. Holmes E, Li JV, Athanasiou T, Ashrafian H, Nicholson JK. Understanding the role of the gut microbiome - host metabolic signal disruption in health and disease. Trends in Microbiology. 2011; 19(7): p. 349-359.
35. Borody T, Campbell J, Torres M, Nowak A, Leis S. Reversal of Idiopathic Thrombocytopenic Purpura (ITP) with Fecal Microbiota Transplant (FMT). The American Journal of Gastroenterology. 2011; 106(Supplement 2): p. S352.
36. Rodeghiero F, Stasi R, Gernsheimer T, Michel M, Provan D, Arnold DM, et al. Standardisation of terminology, definitions and outcome criteria in Immune Thrombocytopenic Purpura of adults and children: report from an international working group. Blood. 2009; 113(11): p. 2386-2393.
37. Hosseini E, Grootaert C, Verstraete W, Van de Wiele T. Propionate as a health-promoting microbial metabolite in the human gut. Nutrition Reviews. 2011; 69(5): p. 245-258.
38. Cordain L, Toohey L, Smith MJ, Hickey MS. Modulation of immune function by dietary lectins in rheumatoid arthritis. British Journal of Nutrition. 2000; 83: p. 207-217.
39. van der Waaij LA, Kroese FG, Visser A44, Nelis GF, Westerveld BD, Jansen PL, et al. Immunoglobulin coating of faecal bacteria in inflammatory bowel disease. European Journal of Gastroenterology & Hepatology. 2004; 16(7): p. 669-674.