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

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8

9 **Abstract**

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

20 **Keywords**

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

23 **1. Introduction**

24 *1.1 Immune Thrombocytopenia (ITP)*

25 ITP is an organ specific autoimmune disorder (1) characterised by a low platelet count. In diagnostic
26 terms, ITP is defined as a platelet count of 'less than 100×10^9/L (100,000/µL)' (2). Its prevalence has
27 been examined in a number of countries and ranges from 2.64 per 100,000 patients/year in Denmark
28 to 9.5 per 100,000 in the USA. A predominant number of cases in childhood are in males with this
29 changing to middle-aged women in adulthood. The overall ratio male to female ratio is thought to be
30 1:1.9 (3i). Most cases of ITP are classed as being idiopathic but some cases are secondary to coexisting
31 conditions as mentioned in section 1.2 (4).

32 *1.2 Causes of ITP*

33 Numerous theories have been put forward as to the cause of ITP but a definitive answer remains
34 elusive. Definitive diagnosis of the condition is still extremely difficult and is often related to
35 numerous other factors, for example SLE, HIV infection, hepatitis C, drug induced or Helicobacter
36 pylori infection. It also means that diagnosis is therefore usually made by exclusion of other disorders
37 (3).

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

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

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

58 *1.3 Linking ITP to food intolerance?*

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

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

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

79 It is to be expected, therefore, that these volatile organic compounds (VOCs) may be detected in urine
80 headspace. Approximately 279 VOCs have been identified in urine in apparently healthy individuals
81 (14i). Urinary VOCs for example, have been shown to discriminate tuberculosis patients from healthy
82 subjects (14ii) and some have shown differences in the female reproductive cycle during ovulation
83 (14iii). The headspace of urine of a group of ITP patients and healthy control subjects were therefore
84 studied using selected ion flow tube mass spectrometry (SIFT-MS) to look at initial overall differences
85 between urine headspace from the ITP and healthy control groups; this was then extended to seeing
86 how the urinary VOC profile was affected by diet.

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

90 **2. Methods**

91 *2.1 Initial Phase: Patient recruitment*

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

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

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

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

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

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

109   a) Subjects outside 18-65 yrs

110   b) Pregnancy and lactation

111   c) Acute thrombocytopenia

112   d) Thrombocytopenia related to underlying disease or drugs

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

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

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

118   h) Patients who have undergone splenectomy within previous 8 weeks

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

120 *2.2 Sampling*

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

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

2.2.1 Demographics

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

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

2.3 Dietary modification - Elemental Diet

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

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

2.4 Control Group – Elemental Diet

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

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

2.5 Urine Sample Preparation

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

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

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

172 *2.6 SIFT-MS Analysis*

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

186 *2.7 Statistical Analysis*

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

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

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

3. Results

3.1 Initial Platelet count

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

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

222 There were some outliers in the ITP group which were closer to normal platelet levels. These were
223 117,000, 144,000 and 281,000 per μl and although these data are included in these initial results, all
224 were subsequently excluded from the study.

3.2 Platelet Counts before and after E028 Diet

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

228 When ITP platelet counts were assessed, no significant increase in platelet count occurred while on or
229 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

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

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

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

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

249 overlap between the two distributions exists, the z-test calculation, which compares the means of the
250 distributions, rejected the null hypothesis thus suggesting that the %CC attained at 70% is statistically
251 significant at the 95% confidence (p<0.05). Furthermore, the number of variables (m/z values) which
252 were found to be significant following the Student t-test was 8 with the corresponding m/z values of
253 34, 43, 53, 72, 74, 76, 89, and 112. The influential variables further indicated by the PLS-DA loadings
254 (not shown) appear to be, m/z 43, m/z 53 and m/z 76.

255 Of interest is that the PLS-DA loadings also showed that m/z 43 was in the opposite direction to the
256 other m/z values. This can be interpreted that ITP sufferers had strong correlations with higher counts
257 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.
258 It also correlates with the earlier univariate analysis to show that m/z 43 is significant.

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

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

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

268 • H_3O^+: 43, 46, 53, 70, 76, 89
269 • NO^+: 54, 56, 59, 76, 78, 92, 111
270 • O_2^+: 31, 41, 54, 86, 105, 106
271

3.5 Identifying the compounds related to m/z values

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

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

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

3.6 Dietary Modification – E028 Diet

3.6.1 Univariate Data H_3O^+

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

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

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

296 *3.6.2 Multivariate statistical analysis*

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

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

305 **4. Discussion**

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

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

316 Research looking at actual platelet counts in relation to food intolerance is limited and mostly relate
317 to complementary therapies, supplements and drinks, rather than a full scale look at specific foods
318 (24). It is difficult to know if there have been any other experiences of these type of results when
319 looking at chronic ITP and not just isolated or acute episodes as is often reported as by Royer *et al.,* in
320 2010. It is also unknown if diet or dietary modification has ever been assessed in relation to actual
321 platelet count data.

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

326 *4.1 Explaining significant m/z values*

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

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

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

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

352 Add bit here on breakdown of platelets?

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

356 *4.2 Linking to metabolism and the gut microbiome*

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

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

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

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

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

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

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

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

5. Acknowledgements

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