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The diagnosis of coeliac disease
by flow cytometry of intraepithelial lymphocytes
– a new ‘gold’ standard?

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conceived the project, carried out the gastroscopies and duodenal biopsies and contributed to the writing.

1 **Abstract**

2 **Objective**

3 The analysis of intraepithelial lymphocytes (IEL) by flow cytometry of duodenal biopsies – the
4 ‘IEL’ lymphogram - has been proposed as a diagnostic test for coeliac disease. However, its
5 clinical applicability has been limited due to variability in methods and definitions. This study
6 set out to define useful parameters for the application of the IEL lymphogram to the diagnosis
7 of coeliac disease.

8 **Design**

9 Flow cytometry was performed on 117 sets of duodenal biopsies in 107 adult patients with
10 active coeliac disease, long-term coeliac disease on a gluten free diet and a control group. The
11 initial 95 samples were used for hypothesis generation for the subsequent samples comprising
12 12 patients with coeliac disease and 10 controls.

13 **Results**

14 Rather than using single linear cut-offs for CD3 and TCR + IELs, a discriminant function was
15 identified as $\%CD3+ve\ IELs + 2x(\%TCR + IELs) \geq 100$. This differentiated coeliac disease from
16 control biopsies in the hypothesis generating group. These results were replicated in the
17 validation group and found to be independent of histology in patients on long term gluten free
18 diet up to 12 years (combined sensitivity, 98.5%; specificity 97.7%).

19 **Conclusions**

20 Flow cytometric analysis of intraepithelial lymphocytes is a highly sensitive and specific
21 adjunct to serology and histological examination for the diagnosis of coeliac disease, even in
22 individuals with coeliac disease following a gluten free diet who exhibit normal duodenal
23 histology.

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- **What is already known about this subject?**

Duodenal intra epithelial lymphocyte (IEL) populations are altered in coeliac disease compared to normal. This is the basis of the 'IEL' lymphogram using flow cytometry of fresh intestinal biopsies. It has been applied to the diagnosis of coeliac disease using specified cut offs for CD3-ve and gamma delta T cell receptor ($\gamma\delta$ TCR) +ve cells.

- **What are the new findings?**

This study demonstrates that CD3+ve and $\gamma\delta$ TCR+ve IELs are dependently variable such that a simple linear function combining both can discriminate coeliac from non-coeliac individuals with ~98% sensitivity and specificity. This is independent of gluten ingestion or histological appearances.

- **How might it impact on clinical practice in the foreseeable future?**

The use of flow cytometry can strengthen the diagnosis of coeliac disease where it is not clear cut. Flow cytometry could be used on a follow up biopsy on diet to both assess response and confirm the diagnosis on a single endoscopic procedure where the diagnosis has been made by serology alone, as occurred during the COVID-19 pandemic.

31 Introduction

32 The diagnosis of coeliac disease requires the identification of serum immunoglobulin-A (IgA)
33 antibodies targeting tissue-transglutaminase 2 (TTG) or deamidated gliadin peptides (DGP),
34 confirmed by the finding of characteristic changes on histological examination of biopsies
35 taken from the duodenum. Both tools require ongoing gluten ingestion¹⁻³.

36 However, neither provides a 'gold standard' for the diagnosis of coeliac disease. Antibody
37 levels may be sufficient to make the diagnosis when present in high titre in both children and
38 adults^{4,5}, but low levels may be associated with marginal or absent histological changes in
39 duodenal biopsies⁶. Coeliac disease-associated antibodies may be absent altogether from the
40 serum but detectable within the lamina propria complexed with TTG⁷.

41 Similarly, the interpretation of biopsies may be hampered by sampling error, cross-cutting of
42 sections and minimal changes, and there is wide inter-observer variability between reporting
43 pathologists. The characteristic histological features associated with coeliac disease are also
44 found in other conditions^{8,9}. The presence or absence of symptoms is an unreliable indicator
45 of coeliac disease¹⁰, and assessment of human leucocyte antigen haplotype (HLA) is only helpful
46 to rule out the condition when non-compatible¹¹.

47 The increase in numbers of intestinal intraepithelial lymphocytes (IELs) is well reported in the
48 active coeliac lesion and often persists long-term on a gluten free diet⁸. Recently, it has
49 become clear that in addition, the phenotypic composition of the IELs remains permanently
50 altered^{12,13}. Studies of duodenal IELs reveal reduced CD3^{-ve} cells and an increase in $\gamma\delta$ T cells
51 and it has been suggested that this could be used as a tool for the diagnosis of coeliac disease¹⁴.
52 ¹⁶ A recent study using the proportion of CD3^{+ve} cells expressing the $\gamma\delta$ T cell receptor to
53 differentiate coeliac individuals from normal controls resulted in a 66.3% sensitivity with a
54 96.6% specificity at a cut off of 14%¹⁷.

55 In this study we set out to determine whether this 'IEL lymphogram' could be further refined
56 for diagnostic application in coeliac disease.

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58 Patients and Methods

59 Patients considered likely to require mucosal biopsy were recruited into the study and gave
60 their consent for additional biopsies to be taken from the second part of the duodenum to be

61 used for flow cytometric analysis. Patients were categorized into three groups: control (CON)
62 - subjects with normal duodenal histological appearances referred for gastroscopy for diverse
63 reasons; Active coeliac disease (ACD) - subjects with positive serum anti-tissue
64 transglutaminase antibodies and characteristic duodenal biopsy features of coeliac disease at
65 time of diagnosis or ongoing villous atrophy with or without elevated anti-TTG antibody
66 titres; Long-term coeliac disease (LTCD) - subjects diagnosed previously with coeliac disease
67 on long term gluten free diet and with normal mucosal appearances on duodenal histological
68 examination.

69 At the time of upper gastrointestinal endoscopic examination biopsies (n=5) were taken into
70 formalin for histological examination and additional biopsies (n=10) into normal saline for
71 flow cytometry. IELs were isolated using an adaption of a standard technique^{18,19} as follows:
72 the epithelium was separated and disaggregated by vigorous mechanical disaggregation using
73 a vortex mixer (VWR) in the presence of dithiothreitol (1 mM) and ethylene diamine tetra-
74 acetic acid, EDTA (1 mM). The cellular extract was centrifuged and washed in phosphate
75 buffered saline supplemented with 0.45% human albumin. Washed cells were incubated at
76 room temperature in the dark with fluorochrome-conjugated antibodies to the cell surface
77 antigens. Following a further 2 wash cycles, cell permeabilisation was then performed
78 according to manufacturers' instructions (Fix and Perm kit Nordic-MUbio) before incubation
79 with the intracellular CD3 conjugated antibody. The antibody panel was established for
80 diagnostic purposes in refractory coeliac disease and included detection of intracellular
81 (cytoplasmic) CD3 expression separately from cell surface CD3 expression. The full panel
82 comprised the following antibody markers: CD2, CD3, CD4, CD5, CD7, CD8, CD30, CD38,
83 CD45, CD56, CD103, CD335, T-cell receptor $\alpha\beta$ and T cell receptor $\gamma\delta$. Data was acquired using
84 a BD FACSCantoII three laser configuration flow cytometer, and analysed using BD FACS diva
85 software (v 6.1.3). The lymphocytes were gated by CD45 and low side scatter characteristics.
86 Cytoplasmic and surface staining of CD3 was included in the common backbone across all
87 panels and used for selectively identify the different IEL populations - surface/cytoplasmic
88 CD3+, surface CD3-/cytoplasmic CD3+ and CD3-.

89 Results from the first 95 samples were analysed to generate a hypothesis for appropriate cut
90 off values for subsequent lymphogram categorization. The subsequent 22 samples were used
91 as a 'validation' group to assess the validity of the discriminant parameters for the test.

92 A student t-test was used at significance of $p < 0.01$ between datasets. Ethical permission for
93 this study was granted by the research ethics service (14/WA/1270, January 2015) and the local
94 research and development department.

95

96 **Results**

97 Patient details are tabulated in table 1. The commonest indications for gastroscopy in the
98 control group (CON) were iron deficiency anaemia and functional bowel symptoms (39% in
99 each). Other indications (one or two patients each) included unexplained weight loss,
100 unexplained diarrhoea, abnormal cross-sectional radiology, and two patients had undergone
101 small intestinal transplantation with biopsies taken of the proximal graft.

102 The active group (ACD) of 44 subjects included eight patients with seropositive type 1
103 refractory coeliac disease at diagnosis, yet despite becoming seronegative over a period of
104 years; on follow up still had ongoing villous atrophy. These eight patients underwent routine
105 follow up with flow cytometric analysis on an annual basis with the results remaining
106 consistent on separate occasions demonstrating intra-individual reproducibility.

107 Of those in the LTCD group, an IgA Anti-TTG antibody titre was available for only 12
108 individuals at initial diagnosis, although a further nine were reported from elsewhere as
109 'positive' and two were not done. Six patients underwent initial diagnostic biopsy elsewhere
110 and were reported as showing confirmatory changes, 14 were carried out at diagnosis in
111 Cambridge and all showed villous atrophy of which 10 were sub-total. IgA anti-TTG antibody
112 titre at follow up biopsy was available for 13 patients as it is not standard practice in
113 Cambridge to use this assay during follow up. The median duration of adherence to a gluten
114 free diet was 5.5 years, with a range of 1 – 50 years. Two patients with the longest duration of
115 gluten free diet maintained since diagnosis had been diagnosed at a time when confirmatory
116 duodenal biopsies and antibody tests were not available (44 and 50 years respectively). Of the
117 remainder in this group, nine had followed a strict gluten free diet for three years or more, and
118 three had done so for 10 years or more (up to 12 years).

119 The proportions of cells by flow cytometry expressing surface CD3 and T-cell receptor $\gamma\delta$ (TCR
120 $\gamma\delta$) are shown in figure 1 (a,b) by category. The proportions of CD3+ cells are the proportion of
121 all gated lymphocytes – whereas the proportion of TCR $\gamma\delta$ + cells is the proportion of CD3+ cells
122 expressing the TCR $\gamma\delta$ receptor.

123 There is significant overlap between the CON, ACD and LTCD groups with regard to both
124 CD3 and TCR $\gamma\delta$ proportions. The sensitivity of using a diagnostic cut off for TCR $\gamma\delta$ of 14% in
125 the ACD group was 64% and in the LTCD group, 57%. The specificity based on the one
126 patient with a high TCR $\gamma\delta$ proportion in the CON group is 97%.

127 However, when surface and cytoplasmic CD3 positive cell proportions are charted against TCR
128 $\gamma\delta$ positive T cell proportions, there is a clear separation of ACD and CON groups as shown in
129 figure 2 (a,b). The discriminant function between the non-coeliac and the coeliac groups is a
130 simple linear equation which corresponds to $[\%CD3 + 2x(\%TCR \gamma\delta) \geq 100]$ (fig 1c).

131 The validation cohort comprised 12 samples from patients with active coeliac disease and 10
132 without. None of these samples were from the individuals in the ACD cohort that had
133 previously undergone flow cytometric analysis. Of those with coeliac disease in the validation
134 group, 58% were male with a median age of 56 years (compared with 56% and 60 years in
135 the hypothesis generating group). Of those without coeliac disease, 40% were male with
136 median age 34 years (compared with 40% and 59.5 years in the hypothesis generating group).
137 The average discriminant function was respectively 131 (range 104-151) for the active coeliac
138 patients and 65.5 (range 54-96) for non-coeliac patients, thereby correctly identifying all
139 subsequent patients in each group. The validation and hypothesis-generating groups have
140 been combined in subsequent data analysis.

141 It can be noted that (due to the long-term persistence of altered IEL phenotypes in coeliac
142 disease) the IEL lymphogram is indistinguishable between the LTCD (fig 2c) and the ACD (fig
143 2a) groups, of which 63/67 (94%) show a discriminant function of ≥ 100 . However, on closer
144 inspection of the four outlying cases, one was borderline (99.2) and two were those that had
145 been diagnosed before any diagnostic tests were available, 44 and 50 years previously. Both of
146 these were challenged by prolonged (>three months) gluten ingestion and re-biopsied. Both
147 remained symptom free, seronegative and with normal repeat duodenal biopsies and chose to
148 eat gluten thereafter. One further patient had been diagnosed 10 years previously in a
149 children's hospital on the basis of anti-gliadin antibody positivity alone, but did not undergo
150 histological confirmation and was negative for both anti-TTG and endomysial antibodies.
151 This patient chose to continue a gluten free diet. Therefore, on the assumption that two of
152 these three patients did not have coeliac disease, and the evidence for the third patient having
153 the condition was extremely weak, the sensitivity of flow cytometry in the remaining 64 cases
154 increases to 98.44% (95% confidence intervals 91.60% - 99.96%).

155 In terms of specificity, 1/40 non-coeliac/control patients had a discriminant function >100.
 156 This patient had no reported symptoms or family history and was seronegative for anti-TTG
 157 antibodies. The HLA DQ status was not known. This gives a specificity of 97% for flow
 158 cytometry in this setting (97.67% including the three deemed unlikely to have coeliac disease
 159 as above, with 95% confidence intervals of 87.7% - 99.94%).

160 Discussion

161 The utility of the IEL lymphogram in the diagnosis of coeliac disease has been described in a
 162 recent meta-analysis²⁰. Of the six studies included, only five reported an 'IEL lymphogram'
 163 based on proportions of CD3-ve and TCR $\gamma\delta$ +ve IELs^{12,14-16,21}. Two of these studies were
 164 specifically in children^{12,16} and the other three were mixed paediatric and adult populations.
 165 Methods varied between studies including gating strategies: three additionally gated cell
 166 populations for CD103 positivity, and one for CD7 positive cells. There is great diversity of IEL
 167 phenotype, especially within the CD3-ve population which also comprises a subset expressing
 168 cytoplasmic CD3 but lacking surface CD3 and this may add to the variability between studies
 169 relying on CD3-ve populations.

170 In addition, of those five studies using measurement of CD3-ve IELs by flow cytometry (by
 171 various definitions), only one provided the relevant cut-offs applied for their 'lymphogram'¹⁴.
 172 In this case a lymphogram comprising $\geq 8.5\%$ TCR $\gamma\delta$ and $\leq 10\%$ CD3-ve IELs gave a sensitivity
 173 and specificity of 85% and 100% respectively. This demonstrates a better sensitivity than the
 174 use of TCR $\gamma\delta$ proportions alone²⁰. Applying these 'IEL lymphogram' criteria to our data would
 175 provide a sensitivity of 64% and a specificity of 92.5%. Despite lacking information on the cut-
 176 offs applied, a further study reported the sensitivity and specificity of the IEL lymphogram in
 177 adults as 89% and 96% respectively¹⁵.

178 In our study, in order to simplify the IEL lymphogram and to remove possible confounding
 179 variables, we selected lymphocytes by their CD45 high/low side scatter properties, measuring
 180 proportions of CD45+CD3+ and CD45+CD3+TCR $\gamma\delta$ IELs. The plot of %CD3+ve IELs against
 181 %TCR $\gamma\delta$ +ve IELs was able to differentiate the samples from controls and those with active
 182 coeliac disease very effectively according to whether they lay above or below a line
 183 corresponding to the discriminant function: $\%CD3+ve + 2x(\%TCR\gamma\delta) \geq 100$. This gave a high
 184 sensitivity of 100% and a specificity of 97%. The discriminant function in this instance was
 185 derived through charting and identification of separate populations. With larger datasets it
 186 may be possible to define regions of interest mathematically with greater accuracy.

187 Application of our discriminant function to the LTCD group with normal histology on gluten
188 free diet showed that four patients would have an IEL lymphogram considered incompatible
189 with coeliac disease. However, one of these was borderline, and on examination of diagnostic
190 records and subsequent gluten challenge and biopsy, the other three were highly unlikely to
191 have the condition. The overall specificity and sensitivity of this test after combining the ACD
192 and LTCD groups were 98.3% and 97.5% respectively. The results from the LTCD group
193 would suggest that this is an effective way of making – or refuting – the diagnosis of coeliac
194 disease in individuals following a gluten free diet over many years without any changes on
195 microscopic examination of duodenal biopsies, and without the need for undergoing a gluten
196 challenge.

197 The use of a separate validation cohort following the generation of a hypothesis ensured that
198 the discriminant function used for diagnosis was reproducible within the single centre.
199 However, the main weakness of this study is that it is from only one centre and laboratory and
200 the findings will require corroboration. Of note, transferability of results from studies of the
201 IEL lymphogram between sites has not been possible to date given the different methods and
202 definitions of IEL lymphogram applied. It is hoped that this simplified test will provide the
203 basis for comparison with results from other centres. However, it is notable that using the
204 same cut off just for TCR $\gamma\delta$ + cells in this study as those from another recent study¹⁷ gave
205 equivalent values of sensitivity and specificity suggesting a degree of transferability of results
206 between sites.

207 Intra-individual reproducibility was also demonstrated in this study by the eight patients who
208 underwent repeated flow cytometry analyses - the discriminant function differed by less than
209 10% between tests (data not shown) and did not result in a change of diagnosis in any case.

210 In this study we have demonstrated that the greatest utility of the IEL lymphogram is when a
211 discriminant function is used that provides for adjustable, mutually dependent cut offs to be
212 applied rather than simple independent linear cut-off levels for each variable as used in other
213 IEL lymphograms. It is unclear why many patients with coeliac disease do not exhibit an
214 increase in TCR $\gamma\delta$ +ve IELs and why this should be compensated by TCR $\alpha\beta$ cells, such that the
215 combination of proportions of CD3+ve and TCR $\gamma\delta$ +ve cells becomes diagnostic rather than
216 either alone. In our data we were unable to find any difference in IEL subsets (using a variety
217 of different cell surface markers) between those patients with coeliac disease in whom
218 TCR $\gamma\delta$ + proportions were low and those in which they were high. It has previously been
219 postulated that the age of the patient may dictate the TCR $\gamma\delta$ response, however we were unable

220 to demonstrate any such association, in either the ACD or LTCD groups. There were notable
221 differences in gender distribution between the study groups. It is unlikely that this skewed
222 the data in this study as IEL subtypes are not thought to differ between sexes.

223 Our method involved taking 10 additional biopsies. This resulted in a prolongation of the
224 procedure by under three minutes as the biopsies were taken as ‘double bites’ – there being no
225 requirement for architectural interpretation. This is a much larger amount of tissue than is
226 strictly necessary as we applied our standard immunostaining protocols used for analysis of
227 biopsies for refractory coeliac disease and to look for additional potential biomarkers. However, in
228 the longer term, the number of additional biopsies could be reduced to just one or two for
229 flow cytometry if limited to analysis of surface CD3 and TCR $\gamma\delta$ markers for diagnostic
230 purposes, as used in other centres (15).

231 The potential clinical utility of the IEL lymphogram has been demonstrated in this study but
232 in view of the relatively small sample sizes will require larger scale studies to validate. Many
233 cases of coeliac disease are ‘challenging’ to diagnose on the basis of weak seropositivity and
234 low-grade changes in the biopsies. The addition of flow cytometry as an additional tool can
235 strengthen the diagnosis. It is notoriously difficult for patients to undergo gluten challenge
236 for re-biopsy and results may not be definitive due to poor compliance with the challenge
237 protocol. The use of flow cytometry also obviates this requirement. This may be of particular
238 relevance in the COVID-19 period when gluten free diets were started on the basis of
239 seropositivity alone. Indeed, for those centres where routine practice includes a confirmatory
240 diagnostic biopsy and a subsequent follow up biopsy for assessment of response, the use of
241 flow cytometry could abolish the requirement for a diagnostic biopsy and be carried out on
242 the follow up biopsy alone. The laboratory cost of flow cytometry in our institution is
243 equivalent to that of a gastroscopy and therefore this would be a cost-effective pathway to
244 both confirm the diagnosis and to assess the response to diet in one procedure.

245

Category	Number (% male)	Median age, yrs (IQR)	IgA anti-TTG titre, mean iu (range)	HLA DQ 2/8?	Duodenal Histology
CON	n=40 (40%)	53 (40-68)	0.62 (0.1-1.3)	Yes = 8 No = 2 (n=10)	39/40 normal (1/40 showed 'possible mild patchy increase in IELs')
ACD	n=44 (57%) (n=54 biopsies)	60 (50-68.5)	42 (0.4 ->128)	Yes = 14 (n=14)	Sub-total villous atrophy =29; Partial villous atrophy =15.
LTCD	n=23 (17%)	56 (42.5-62.5)	75.5 (9.6->128) at time of diagnosis; 2.2 (0.6-6.5) at time of follow up biopsy	DQ2 = 7	Normal = 19/23; Patchy increase in IELs = 4/23

Table 1 Patient characteristics

Figure Legends

246

247 **Figure 1:** Box and whisker plots of values of %CD3+ IELs as a proportion of all CD45+
248 lymphocytes (a), % TCR $\gamma\delta$ + IELs as a proportion of all CD3+ IELs (b) and 'discriminant
249 function' values (%CD3+ IELs + 2x(%TCR $\gamma\delta$ + IELs)) (c) for control (CON), active coeliac
250 disease (ACD), long-term coeliac disease (LTCD) and LTCD and ACD groups combined.

251 **Figure 2:** Scatter plots of %CD3+ IELs as a proportion of all CD45+ lymphocytes (X-axis)
252 charted against % TCR $\gamma\delta$ + IELs as a proportion of CD3+ IELs (Y-axis), showing the
253 discriminant function (%CD3+ IELs + 2x(%TCR $\gamma\delta$ + IELs)) as a line. a) Active coeliac disease
254 (ACD), b) control (CON), c) Long-term coeliac disease on diet (LTCD).

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