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Intestinal fatty acid binding protein (I-FABP) and CXC3L1 evaluation as biomarkers for patients at high-risk for coeliac disease in Johannesburg, South Africa

Anastasia Gandini [a,*], Tim De Maayer [b], Cameron Munien [e], Katherine Bertrand [e], Ross Cairns [e], Anthony Mayne [c], Maemu P. Gededzha [a], Elizabeth S. Mayne [d]

[a] Department of Immunology, University of Witwatersrand and National Health Laboratory Service, Johannesburg, South Africa
[b] Department of Paediatrics, Rahima Moosa Mother and Child Hospital and University of Witwatersrand, Johannesburg, South Africa
[c] Department of Molecular Medicine and Haematology, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa
[d] Division of Immunology, Department of Pathology, Faculty of Health Sciences, University of Cape Town and National Health Laboratory Service, South Africa
[e] School of Clinical Medicine, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

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ABSTRACT

Coeliac disease (CD) is an autoimmune disorder and one of the few gastroenteropathies with accurate serological testing. CD serology has decreased accuracy for patients on a gluten-free diet and for monitoring mucosal healing. New ancillary tests would, therefore, be useful. Intestinal Fatty Acid Binding Protein (I-FABP) and CX3CL1 (Fractalkine) are two promising biomarkers for CD but haven't been examined in patients who are at a high-risk for CD such as patients with type one diabetes (TID). This study, therefore, aimed to investigate serum levels of I-FABP and CX3CL1 in a cohort of South African patients with TID at a high-risk of developing CD. The serum I-FABP levels were significantly higher in CD-positive patients compared to CD-negative individuals ($p = 0.03$). No significant differences in the serum CX3CL1 levels were detected although this may reflect the impact of the comorbid autoimmune diseases had on the serum CX3CL1 levels. In conclusion, this study is the first to assess the levels of these biomarkers in a multiethnic population with comorbid autoimmune disease and determined I-FABP to be the more promising biomarker in such clinical contexts. Future research should focus on a diverse biomarker panel and longitudinal follow-up of patients at a high-risk for CD.

1. Introduction

Coeliac disease (CD) is an autoimmune disorder, presenting with gastrointestinal symptoms and associated autoimmune manifestations [1]. A CD4 + T cell population in CD patients is specific to immunogenic gliadin fragments complexed to tissue transglutaminase (tTG)[2]. Gluten is digested to form gliadin which binds with CXCR3 receptors on the gastrointestinal tract (GIT) epithelial lining and perpetuates gut permeability [1]. Gliadin fragments then move into the lamina propria, triggering an inflammatory response followed by enterocyte apoptosis and an immunological response to the endothelial cellular contents, specifically tTG [3,4]. This autoimmune response, comprising both B-cell and T-cell responses, perpetuates gut permeability, inflammation and apoptosis causing villous atrophy [5].

There is an estimated 0.5–1% global prevalence of CD making it one of the commonest autoimmune diseases [6,7]. CD can be defined symptomatically as classic (intestinal), non-classic (extraintestinal), subclinical, refractory (RCD), or seronegative CD [8,9]. There are several laboratory tests in the CD diagnostic algorithm, including duodenal biopsy, serology and Human Leukocyte Antigen (HLA) typing. The HLA DQ2 (HLA-DQA1*05:01-DQB1*02:01) and DQ8 (HLA-DQA1*03:01-DQB1*03:02) are associated with a higher risk for CD,

Abbreviations: CD, Coeliac disease; HLA, Human Leukocyte Antigen; TID, type 1 diabetes; tTG, tissue transglutaminase; RCD, refractory coeliac disease; GFD, gluten-free diet; I-FABP, intestinal fatty acid binding protein; GIT, gastrointestinal tract; GCD, gluten-containing diet.

* Corresponding author at: Department of Immunology, Faculty of Health Sciences, University of Witwatersrand, De Korte and Hospital Street Braamfontein 2001 Johannesburg, South Africa.

E-mail addresses: anastasiagandini@gmail.com (A. Gandini), tim.DeMaayer@wits.ac.za (T. De Maayer), cameron.munien@gmail.com (C. Munien), 1354473@students.wits.ac.za (K. Bertrand), rosshcairns@gmail.com (R. Cairns), amayne@absamail.co.za (A. Mayne), maemu.gededzha@nhls.ac.za (M.P. Gededzha), Elizabeth.mayne@uct.ac.za (E.S. Mayne).

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with all CD patients expressing at least one allele [10-12]. The presence of these alleles is necessary but not sufficient to cause CD which makes HLA genotyping a useful screening application to identify individuals at risk but who have not yet developed clinical manifestations [1].

The mainstay of CD diagnosis remains tTG autoantibody detection although this has limitations including reduced accuracy in patients on a gluten-free diet (GFD) [13-15]. CD serology is also used in screening patients with other autoimmune diseases who are considered at a high-risk for developing CD. Duodenal biopsies and histology are used for monitoring villous healing and screening for potential malignant transformation but are invasive and expensive [6,16]. For these reasons, the development of ancillary testing would be useful. A number of biomarkers for CD are under investigation, including microRNA and phospholipid profiling [1,17-21]. Intestinal Fatty Acid Binding Protein (I-FABP) and CX3CL1 (Fractalkine) have been highlighted as promising biomarkers in diagnosing CD and other autoimmune disorders, such as Crohn's disease, rheumatoid arthritis, and ulcerative colitis [17-19,22-25].

I-FABP is an intracellular protein, expressed by enterocytes, which binds to long fatty acid chains to promote absorption and digestion. It is generally detectable at low levels in the serum of healthy individuals but is released with enterocyte damage [26,27]. In CD, proinflammatory gluten-tTG complexes trigger anti-enterocyte immune responses which compromise the integrity of the enterocyte cellular membrane allowing the release of cytoplasmic contents including I-FABP [18,22]. Serum levels of I-FABP predict 68% of CD diagnoses and correlate with more severe intestinal damage (intestinal villous atrophy of grade 2 or more by Marsh classification) [18,28]. Levels of I-FABP appear to reduce with compliance to a GFD suggesting that it may have utility as a marker of villous healing [18,20]. The prognostic capabilities of I-FABP have yet to be examined.

CX3CL1, a chemokine that binds to CX3CR1, is primarily expressed on damaged or activated endothelial cells where it promotes tight adhesion [29-31]. This transmembrane form of CX3CL1 can be cleaved by metalloproteinases to produce the soluble chemoattractant for the recruitment of immune cells and inflammation in CD [30,32]. The binding of CX3CL1 to CX3CR1 activates the receptor associated-G protein, causing the α subunit to dissociate from the βγ complex. The mechanism activates the PI3K and MAPK kinase pathways to trigger leukocytes migration [32]. Increased levels of CX3CL1 are associated with chronic inflammatory processes including CD, rheumatoid arthritis, cardiovascular diseases, and type one diabetes (TID) [24,33-35]. Techniques for measuring CX3CL1 levels are widely available making this an accessible potential diagnostic biomarker [36,37].

Three proinflammatory cytokines are primarily associated with TID - interferon γ, tumour necrosis factor α, and interleukin-1 β [36,37]. I-FABP and CX3CL1 are raised in type 2 diabetes mellitus but have not been widely studied in patients with TID [38,39]. This study, therefore, aimed to investigate levels of I-FABP and CX3CL1 in a cohort of South African patients with TID at a high-risk of developing CD.

2. Materials and methods

2.1. Ethics statement

The study was approved by the University of Witwatersrand Human Research Ethics Committee for Medical Research in Johannesburg, South Africa (M200337).

2.2. Cohort selection and sample collection

Whole peripheral blood samples and fresh clotted blood samples were collected from patients with TID at Rahima Moosa Mother and Child Hospital (RMMCH) and from adult CD-positive patients at Wits Donald Gordon Medical Centre (WDGMC) recruited during their routine clinical management. The blood was collected by the recruiting nurse/physician drawing blood at the time of routine testing with informed consent/assent. Patients were required to have a diagnosis of TID or CD to be included in this study. Participants were excluded if the blood samples were over a week old or if no whole peripheral blood samples were taken at the time of recruitment as this was required for HLA typing.

The residual blood samples (1 EDTA tube and 1 clotted tube) were centrifuged at 3,500 rpm for 15 min and the serum was aliquoted and stored at −80 °C. Serum samples were thawed and centrifuged at 15,000 rpm for 3 min immediately before testing. Patients were typed at the HLA DQB1 and DQA1 loci and based on the results were classified as CD-negative patients or patients at a high-risk of CD. Biopsy confirmed patients with CD were utilised as a CD-positive controls (Fig. 1).

2.3. Serum I-FABP biomarker

The serum I-FABP levels were tested with the Quantikine® Human FABP2/I-FABP Immunoassay kit (R&D Systems, USA) which is a solid-phase sandwich Enzyme-linked immunosorbent assay. Patient serum was diluted 5-fold dilution with Calibrator Diluent and then add to the 96-well plate. Human I-FABP Conjugate, Substrate solution and Stop solution were added. The optical density of each well was determined using a EL_X800 universal microplate reader (Bio-Tek Instruments Inc., USA) at 450 nm and 540 nm, and the results were calculated from a standard curve, created by generating a four-parameter logistic (4-PL) curve-fit on Microsoft Excel® (Microsoft, USA).

2.4. Serum CX3CL1 biomarker

The Luminex® Human Premixed Multi-Analyte kit (R&D Systems, USA) was used to detect serum CX3CL1 levels. Analyte-specific antibodies are pre-coated onto magnetic beads containing fluorophores at unique ratios. Samples were prepared with a 2-fold dilution with patient serum and Calibrator Diluent and then mixed thoroughly. Following incubation and addition of Biotin-Antibody cocktail and Streptavidin-PE, samples were analysed on a Luminex xMAP 200 instrument (Bio-Rad, USA). Results were calculated by averaging the duplicate readings for each standard and sample, and then subtracting the average blank mean fluorescent intensity. A standard curve was created by Luminex xPONENT® Software by generating a 4-PL curve-fit.

2.5. Statistical analysis

All demographic data were recorded in Microsoft Excel® 2016 and descriptive statistics were computed for medians and distributions. Duplicate values for serum I-FABP and CX3CL1 levels were recorded for patients in three groups, (1) CD-negative controls (negative), (2) patients with TID and high-risk alleles (high-risk), and (3) CD-positive patients (positive). A Wilcoxon (Mann-Whitney) rank-sum test for equal medians was applied.

For the three groups in both I-FABP and CX3CL1, a one-way ANOVA was performed. A Bonferroni correction for multiple groups was applied and a Bartlett's test for equal variances was computed. A p-value for the ANOVA was computed and a p-value ≤ 0.05 was considered significant. Formal statistical analysis was performed with STATA® 15.1 StataCorp, College Station, Texas software. The impact of diet on the biomarker levels in CD-positive patients was determined in a similar method.

3. Results

3.1. Population demographics

Blood samples (n = 38) were collected from November 2020 until November 2021. Of the samples collected from patients with TID (n = 19), 13 were identified as high-risk for developing CD by the presence of HLA DQ2/8. The remainder of patients with TID lacked the HLA DQ2/8

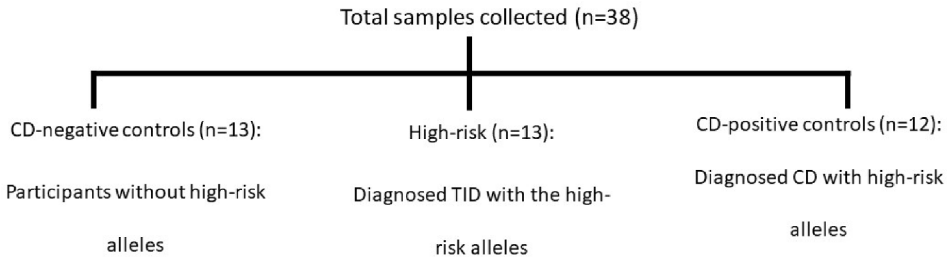

Fig. 1. Cohort selection. A total of 38 samples were collected from November 2020 until November 2021 and were stratified into CD-positive, high-risk, and CD-negative groups. The groups were stratified according to the presence or absence of high-risk HLA DQ2/8 and diagnoses.

alleles and were classified as CD-negative. The majority of the sample population were female (66%) and all patients at a high-risk had gluten in their diet (Table 1). Several comorbid autoimmune diseases were reported across the cohorts including TID, Rheumatoid arthritis, Hashimoto's thyroiditis, and Vitiligo.

3.2. I-FABP and CX3CL1 biomarkers

3.2.1. I-FABP can distinguish CD-positive patients from CD-negative individuals

Although I-FABP is a promising biomarker for CD, I-FABP serum concentrations in patients at a high-risk have not been investigated. The average serum levels of I-FABP found in this study were 3.24 ng/mL vs 2.36 ng/mL and 2.18 ng/mL in the CD-positive, high-risk and CD-negative groups respectively. The one-way ANOVA for the three groups showed no statistical significance ($p = 0.055$) and the one-way ANOVA for the two groups showed statistical significance between only the positive and negative groups ($p = 0.03$) with a p-values of 0.12 and 0.42 being reported for the positive and high-risk groups, and the negative and high-risk groups analyses respectively (Fig. 2). The median I-FABP levels in patients on GFD and gluten-containing diet (GCD) were not statistically significant (2.47 and 2.07 ng/mL respectively) and did not differ significantly between ethnic groups ($p > 0.05$).

Table 1
Demographic data. Our data was grouped according to their diagnosis and HLA DQ type. Of the patients with TID, 13 were determined as high-risk and 13 as CD-negative. 12 participants were established as CD-positive. The demographic information, including age, gender, diet, self-reported ethnicity and autoimmune comorbidities were recorded and statistics calculated at a 95% CI.

Group	CD-negative	High-risk	CD-positive
Age years – median; IQR	14; 12	9.5; 4	44; 25
	n (%)	**n (%)**	**n (%)**
Gender			
Female	8 (61.5)	7 (53.8)	10 (83.3)
Male	5 (38.5)	6 (46.2)	1 (8.3)
Not disclosed	0	0	1 (8.3)
Diet			
Gluten-containing	12 (92.3)	13 (100)	3 (25)
Gluten-free	1 (7.7)	0 (0)	8 (66.7)
Not disclosed	0	0	1 (8.3)
Self-reported ethnicity			
Black	4 (30.8)	7 (53.8)	2 (16.7)
White	4 (30.8)	0 (0)	9 (75)
Coloured	4 (30.8)	4 (30.8)	0 (0)
Indian	1 (7.7)	2 (15.4)	0 (0)
Not disclosed	0	0	1(8.3)
Comorbidities			
TID	6 (46.2)	13 (100)	0 (0)
Rheumatoid arthritis	1 (7.7)	0 (0)	2 (16.7)
Hashimoto's thyroiditis	1 (7.7)	1 (7.7)*	1 (8.3)*
Vitiligo	0 (0)	0 (0)	1 (8.3)
None	5 (38.5)	0 (0)	8 (66.7)

* Two patients had multiple comorbidities (TID and Hashimoto's thyroiditis, and Vitiligo and Hashimoto's thyroiditis).

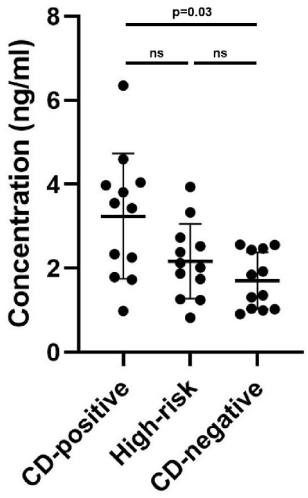

Fig. 2. I-FABP levels were significantly higher in patients diagnosed with CD and with TID at a high-risk for CD compared to CD-negative patients.

3.2.2. CX3CL1 is a general inflammatory marker and is not specific to CD

The average concentration from the CD-positive group was 1,461.41 ng/mL. 1,164.85 ng/mL and 842.45 ng/mL were the serum levels detected in the high-risk and CD-negative groups respectively. The one-way ANOVA for the three groups showed no statistical significance ($p = 0.15$) and the one-way ANOVA for the two groups showed no statistical significance between the positive/negative groups ($p = 0.37$), positive/high-risk groups ($p = 0,18$) and the negative/high-risk ($p = 0.16$) analyses. Median concentration of 1,432.55 ng/mL on a GCD compared to 877.33 ng/mL on a GFD ($p = 0.17$, CI = 95%) nor were the CX3CL1 levels significantly different between ethnicities ($p > 0.05$).

4. Discussion

Potential CD biomarkers have not been extensively investigated in populations at a high-risk. In this study, we report findings on two promising biomarkers in a multi-ethnic cohort. CD is generally reported in Caucasian patients but previous work by us and others suggests that patients in South Africa possess HLA-DQ alleles which predisposes them to the condition particularly with the shift to a wheat-based diet [40-42]. Gluten is the primary trigger for CD but other factors, such as intestinal infections, contribute to the inflammation and villous atrophy [1]. Factors, such as BMI and age, have little research examining their impact on CD and the biomarkers examined in this study.

I-FABP, an intracellular protein for transporting long-chain fatty acids through cell membranes and chaperone intracellular transport, is expressed by gastrointestinal epithelial cells mainly in the duodenum and jejunum [43]. It is released with enterocyte damage and its utility as an ancillary test in GIT autoimmune pathology is recognised [18-20,22,23,28,44]. Serum I-FABP levels were significantly different

between the CD-positive and CD-negative groups. The median I-FABP levels in patients on GFD was higher compared to those on a GCD although this was not statistically significant. This contrasts with other published literature which reports a rapid decline in I-FABP levels after successful GFD initiation [19,28]. It would, therefore, be useful to examine I-FABP levels more closely in this population particularly in relation to duodenal biopsy confirmation of villous healing and GFD adherence.

CX3CL1 is a chemokine that is expressed by inflamed intestinal epithelial cells and has potential as a biomarker for intestinal pathologies, such as CD and Crohn's disease [17,45]. Levels are increased in patients with widescale epithelial disruption, such as in CD [17]. In our study, the average CX3CL1 serum levels were highest in the CD-positive cohort and the lowest in the CD-negative cohort, but this was not statistically significant. This may reflect high numbers of patients in this study with comorbid autoimmune pathologies [24,33-35,46]. Additionally, polymorphisms in the CX3CL1 gene have shown to produce functionally different variants that could impact pathologies, although this has yet to be investigation in CD-positive patients and in non-Caucasian individuals [32]. The inclusion of CX3CL1 in a multianalyte profile of CD may show utility. Interestingly, although numbers of CD-positive patients on a GCD diet in the study were small, the levels of CX3CL1 were higher. This may suggest utility in monitoring dietary compliance.

This study had certain limitations including a small sample size, incomplete hospital records, and absence of biopsy results as a standard variable. In addition, most CD-positive patients were on GFD which may have impacted the biomarkers and the impact of age could not be assessed as the majority of the population was paediatric. The study was cross-sectional, and the impact of longitudinal intestinal damage could not be assessed. This is, however, the first study to assess the levels of these markers in a multi-ethnic population with comorbid autoimmune diseases. Future research should focus on a diverse biomarker panel, longitudinal follow-up of patients at a high-risk and larger sample size.

5. Conclusions

In conclusion, this study has shown that I-FABP is more promising as a CD biomarker than CX3CL1 in clinical contexts where patients have comorbidities with other autoimmune diseases or inflammatory conditions. Further research and larger samples are required to assess the prognosis of patients with comorbidities and other autoimmune diseases or inflammatory conditions. Both biomarkers show potential as ancillary tests in the context of CD.

CRediT authorship contribution statement

Anastasia Gandini: Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Writing – original draft, Writing review & editing. Tim De Maayer: Supervision, Writing review & editing. Cameron Munien: Project administration. Katherine Bertrand: Project administration. Ross Cairns: Project administration. Anthony Mayne: Data curation, Formal analysis, Writing review & editing. Maemu P. Gededzha: Conceptualization, Supervision, Funding acquisition, Methodology, Writing review & editing. Elizabeth S. Mayne: Conceptualization, Supervision, Funding acquisition, Methodology, Writing review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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