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GENETIC AND SEROLOGICAL CONCORDANCE IN PATIENTS WITH SUSPECTED COELIAC DISEASE

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2020-2021

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This is the final submission of this dissertation, August 2022, to the School of Pathology, University of Witwatersrand, Johannesburg, as per the requirements for the degree of Master of Science in Medicine (Immunology)

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Candidate's Declaration

I, Anastasia Gandini (1348734), declare that this Dissertation is my own unaided work. It is being submitted for the degree of Master of Science in Medicine (Immunology) at the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



Anastasia Gandini 29th day of August 2022 in NHLS Braamfontein

Dedication

I would like to dedicate this project to my parents for giving me everything I have and my sister for always keeping me grounded.

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Conference Proceedings/Publications

Conference	Location	Date	Title of Abstract
South African Society of Immunology	Online	August 2021	GENETIC CONCORDANCE IN PATIENTS WITH SUSPECTED COELIAC DISEASE

Publications
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Anastasia Gandini, Georginah Chiloane, Ying X. Liao, Nakampe Mampeule, Sarika Jugwanth, Nontobeko Zwane, Maemu P. Gededzha, June Fabian, Irma Mare, Jean Botha and Elizabeth Mayne. Human Leukocyte Antigen Typing with Sequence Specific Oligonucleotides for Renal Transplantation in South Africa. Clin Lab November 2021; 67: 2560-2567 DOI: 10.7754/Clin.Lab.2021.210336
Anastasia Gandini, Maemu P. Gededzha, Tim De Maayer, Peter Barrow, Elizabeth Mayne. Diagnosing coeliac disease: A literature review. Human Immunology (2021) https://doi.org/10.1016/j.humimm.2021.07.015. (In press)
Gandini A, Van Wyk N, De Maayer T, Gededzha MP, Mayne E. Coeliac disease high-risk HLA alleles in a South African type one diabetic population. Submitted to Human Immunology 07.03.2021
Gandini A, De Maayer T, Munien C, Bertrand K, Cairns R, Landau G, Mayne A, Gededzha MP, Mayne E. I-FABP and CXC3L1 evaluation as biomarkers for South African patients at high-risk for coeliac disease. Accepted for publication in Cytokine 13/06/2022.

Abstract

Introduction: Coeliac disease (CD) is an autoimmune gastroenteropathy strongly associated with Human Leukocyte Antigen (HLA) DQ2 and DQ8 genes. The DQ2/8 alleles are also associated with other autoimmune diseases including type 1 diabetes (T1D), which puts these patients at a higher risk of developing CD. CD serology is used to screen these patients but has limitations. Intestinal Fatty Acid Binding Protein (I-FABP) and CX3CL1 (Fractalkine) are two promising biomarkers for CD but have yet to be examined in patients at a high-risk (HR). This study, therefore, aimed to examine genetic and serological concordance in South African patients with T1D and high-risk CD alleles.

Methods: A total of 38 paediatric and adult participants were recruited from an endocrinology clinic and gastroenterologists in the Johannesburg region. Clinical details were collected, and all patients were HLA typed at the DQ loci. I-FABP and CX3CL1 serum levels were then determined using ELISA and Luminex® technology respectively.

Results: Of our patients with T1D, 63.6% had at least 1 HLA allele associated with CD. I-FABP serum levels were significantly higher in CD-positive patients compared to CD-negative individuals ($p=0.03$). No significant differences in the CX3CL1 serum levels were detected although this may reflect the impact of the comorbid autoimmune diseases on the CX3CL1 levels.

Conclusions: This study provides preliminary insight into the HLA-DQ2/8 type frequency in a South African population with T1D and is the first to assess the levels of I-FABP and CX3CL1 in a multi-ethnic population with comorbid autoimmune diseases. It determined I-FABP to be the more promising biomarker in such clinical contexts compared to CX3CL1.

List of Abbreviations and Acronyms

- 4-PL: Four parameter logistic curve fit
- APCs: Professional antigen-presenting cells
- CD: Coeliac disease
- DGP: Deaminated gliadin peptide
- ELISA: Enzyme-linked Immunosorbent assay
- GCD: Gluten-containing diet
- GIT: Gastrointestinal tract
- GFD: Gluten-free diet
- HR: High-risk
- HLA: Human Leukocyte Antigen
- IEL: Intraepithelial lymphocytes
- I-FABP: Intestinal Fatty Acid Binding Protein
- IFU: Instruction For Use
- Ig: Immunoglobulin
- IL: Interleukin
- RMMCH: Rahima Moosa Mother and Child Hospital
- RCD: Refractory coeliac disease
- SPE: Solid-phase extraction
- SSO: Sequence Specific Oligonucleotide
- SD: Standard Deviation
- T1D: Type 1 Diabetes mellitus
- tTG: Tissue transglutaminase
- TH: T-helper cells

Chapter 1: Introduction

1.1 Coeliac disease background

Coeliac disease (CD) is an inflammatory autoimmune disorder, primarily directed by CD4+ T-cells with T-cell receptors specific for tissue transglutaminase (tTG) bound to gliadin (1-12). Gliadin is a non-digestible component of gluten that ultimately causes inflammation and villous atrophy in CD patients through presentation on professional antigen presenting cells (APCs) (8, 9, 13). Although the main trigger for CD is gluten, other factors, including the type of gluten products consumed, gut microbiome, gastrointestinal tract (GIT) infections, and infant diet, have been hypothesised to have a key role in gluten tolerance (8, 14, 15).

Statistics on the CD prevalence in South Africa are currently unavailable because no wide-scale epidemiological studies have been conducted, although outside Africa it is considered a common pathology with an approximate 0.5-1% prevalence in the world (5, 8, 16). CD affects largely Caucasian populations but the type 1 diabetes mellitus (T1D) incidence in other ethnicities has risen, some of which have developed CD (4, 8, 17). The popular maize-based diets have been hypothesised as protective against CD in South Africa but the recent shift to a more wheat-based diet is increasing the quantities of gluten in diets of different ethnic groups and subsequently increasing their risk of developing CD (2, 4-8, 14, 15).

1.2 Clinical presentations and complications

The clinical presentation of CD varies significantly (18). The symptoms can be categorised as either classical, non-classical, subclinical, seronegative, or refractory coeliac disease (RCD) (5, 8, 19-24). The clinical variability has created a diagnostic dilemma as the required biopsies are invasive and costly, highlighting the need for accurate diagnostic and prognostic markers for

CD and RCD especially since late diagnosis increases the risk of complications including malignant transformation (18, 25-27).

1.3 Diagnosing CD

There is no consensus diagnostic algorithm for CD, but a number of guidelines are available from Europe, North America, New Zealand, and Australia which are largely similar (5, 8, 23, 28-34). Diagnosis rests on tTG and deaminated gliadin peptide (DGP) serology followed by a confirmatory duodenal biopsy. When serology and biopsy results are ambiguous, Human Leukocyte Antigen (HLA) typing is used as an exclusionary test and has recently been more widely used to screen high-risk patients for CD (5, 8, 29, 35-42).

1.3.1 HLA genotyping

The HLA-DQA1*05:01-DQB1*02:01 alleles (DQ2 molecule) are found in nearly 90% of CD patients (8, 43). The HLA-DQA1*03:01-DQB1*03:02 alleles (DQ8 molecule) are expressed in about 5% of CD patients (8, 44-46). The remainder of CD patients have at least one of the DQ2/DQ8 alleles (47). Previous work by our laboratory and others confirmed that CD high-risk alleles are present in a substantial number of black South Africans (48, 49).

In addition to CD, HLA alleles predispose the development of other autoimmune conditions like T1D, autoimmune thyroid disease and Addison's disease (6, 50-52). For this reason, CD is seen more commonly in these disorders with 8% of patients with T1D developing CD and a reported hazard ratio of 2.4 (CI = 95) (4, 17, 53, 54). The possession of these alleles is required but not enough to cause CD which makes HLA genotyping a useful screening test, particularly for T1D and other high-risk patients who receive annual CD serology to monitor for arising comorbidities (8). T1D patients who lack the HLA DQ2/8 alleles would not require regular serological monitoring for CD which could make HLA-DQ genotyping an attractive first-line

screening test. The frequency of HLA DQ2/8 alleles in South African T1D patients is, however, unknown.

1.3.2 Novel biomarkers

There are a limited number of gastroenteropathies with accurate, minimally invasive serological testing, including CD (8, 55). CD autoantibody levels decline rapidly after initiation of a gluten free diet (GFD) although the correspondence with mucosal inflammation and healing is not always commensurate (8, 56-59). This has prompted research into other biomarkers including microRNA and phospholipid profiling (8, 60-64).

Intestinal Fatty Acid Binding Protein (I-FABP) and CX3CL1 (Fractalkine) are two promising biomarkers in diagnosing CD and other autoimmune diseases, such as ulcerative colitis, rheumatoid arthritis, and Crohn's disease (60-62, 65-68). I-FABP is intracellularly expressed by enterocytes and binds to long fatty acid chains to promote absorption and digestion (65, 69, 70). 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 (61, 65). Research into I-FABP diagnostic testing has only compared healthy individuals with CD patients although the prognostic value of I-FABP levels has not been investigated (61, 63, 71, 72). CX3CL1, a chemokine that binds to CX3CR1, is primarily expressed on damaged or activated endothelial cells where it promotes tight adhesion (73-75). Metalloproteinases can cleave the transmembrane form of CX3CL1 to create the soluble chemoattractant for the recruitment of immune cells and inflammation in CD (74, 76). Increased serum levels of CX3CL1 are associated with chronic inflammatory processes including CD, rheumatoid arthritis, cardiovascular diseases, and T1D (68, 77-79). The widely accessible and semi-automated techniques used to detect these biomarkers makes them an attractive option for ancillary testing and are more efficient compared to other biomarker testing techniques. I-FABP and CX3CL1, however, have yet to be studied in populations at high-risk for CD or in a South African population (4, 80).

Aims and objectives

This study aimed to investigate different strategies for diagnosing coeliac disease in an at-risk cohort of patients and to investigate novel tests in this population.

This study's aim was supported by the following objectives:

1. To perform an extensive review of the literature in CD diagnosis specifically focusing on current diagnostic algorithms and novel biomarkers (publication 1).
2. To assess CD-associated HLA expression in a cohort of children with T1D to assess the potential risk of development of CD (publication 2).
3. To assess the expression of selected serological biomarkers of CD in high-risk and CD-diagnosed patients (publication 3)

Chapter 2: Synopsis of Publications

2.1 Publication 1: Diagnosing coeliac disease: A literature review

As a component of the project planning, a decision was taken to perform an extensive literature review on CD diagnostics. During this review, the different algorithms were compared, and the utility of serology and genetic typing was assessed (8). The importance of identifying CD associated high-risk HLA alleles in populations was highlighted. Two promising biomarkers were identified which showed promise in larger studies for further evaluation in a cohort of children at-risk in Johannesburg

Candidate's contribution: First author, reviewing of literature, manuscript writing and editing

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Review

Diagnosing coeliac disease: A literature review

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ABSTRACT

Coeliac disease (CD) is an autoimmune gastroenteropathy triggered by gliadin and gliadin-tissue transglutaminase (tTG) complexes. CD is one of the few autoimmune diseases with an accurate, non-invasive serological test. Anti-endomysial, anti-tTG and anti-deaminated gliadin peptides (DGP) antibodies are currently used for serological tests with tTG ELISAs being the superior test. Duodenal biopsy, although invasive, is the gold standard for CD diagnosis. HLA genotyping and flow cytometry can also be used as supplementary tests.

The incidence of CD is rising globally although the reasons for this remain unclear. In addition, the true incidence of coeliac disease in African populations remains unknown although recent work suggests that South African populations express the alleles associated with this disease.

This review examines the pathogenesis and diagnosis of coeliac disease and considers novel and innovative biomarkers in its diagnosis specifically in an African population.

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1. Introduction

Coeliac disease (CD) is an autoimmune enteropathy with an estimated prevalence of 0.5–1% worldwide and a rising incidence [1,2]. It is one of the commonest autoimmune diseases affecting

Abbreviations: T1DM, Type 1 diabetes mellitus; DGP, Deaminated gliadin peptide; tTG, Tissue transglutaminase; EMA, Anti-endomysial antibodies; RCD, Refractory Coeliac Disease; GFD, Gluten-free diet.

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predominantly Caucasian populations. Recently there has been a rise in the incidence of type 1 diabetes mellitus (T1DM) in other population groups, including black and mixed-race patients, some of which have developed CD [3,4]. It has been hypothesized that maize-based diets, popular in South Africa, maybe protective against CD but that recently, with a shift to a more wheat-based diet, increased gluten ingestion may increase the risk of developing CD [5,6]. In addition to gluten ingestion, CD has been associated with several HLA alleles found to be well represented in our South African (SA) population [7]. Despite the potential risk, studies of CD in African populations are small and the prevalence of CD in South Africa is unknown [4].

1.1. Immunopathogenesis of coeliac disease

CD is a CD4 + T-cell mediated inflammatory disorder [2,8]. The CD4 + T cells are specific to a gliadin fragment complexed to tissue transglutaminase (tTG) [1,9–11]. Gliadin (a non-digestible, immunogenic component of gluten) binds with CXCR3 receptors on enterocytes (Fig. 1) [9,12]. The binding transiently increases gastrointestinal tract (GIT) permeability and allows gliadin fragments to move into the lamina propria, triggering an inflammatory response with subsequent intestinal epithelial apoptosis and the release of cellular contents, including the enzyme, tTG [8,13,14]. tTG deaminates positively charged glutamines into negatively charged glutamic acid residues [8,9,15]. The deaminated gliadin and the tTG-gliadin complexes are endocytosed by antigen-presenting cells (APCs), such as macrophages and dendritic cells which present processed forms of these molecules on HLA-DQ2 and -DQ8 to gliadin-specific helper T-cells (TH) causing activation [8,16]. TH2 cells activate B-cells, which produce anti-tTG and anti-gliadin antibodies (Table 1). TH1 cells produce IFN-γ and TNF-α which further increases gut permeability, inflammation and causes an enteropathy which ultimately results in villous atrophy [1]. TH17 cells and the accompanying cytokines have also been associated with an inflammatory role CD [17,18]. CD8 cytotoxic lymphocytes (CTLs) are important contributors to CD immunopathogenesis, particularly in patients with CD autoantibodies without intestinal damage [19]. The potential antigen specificity of these CD8 CTLs in CD intestinal epithelium was recently identified as A-gliadin 123–132 and further studies indicate that CD8 CTL and T-cell activation are similarly triggered by dietary gluten [20,21]. The CD8 + T-cells have αβ or γδ T-cell receptor and form a component of the intestinal intraepithelial lymphocytes (IELs) in addition to CD4 + T-cells (especially regulatory CD4 + T-cells and Th17 CD4 + T-cells) and B-cells. [21] Th17 cells and regulatory T-cells are usually present in the inverse proportions with a high preponderance of regulatory CD4 + T-cells. Inflammation in CD is associated with CD4 + T-cell polarization to a Th1 or Th17 phenotype resulting in tissue damage and destruction either directly or through the activation of autoreactive CD8 + T-cells [17]. Other IELs which may potentiate inflammatory changes in coeliac disease include mucosa-associated invariant T-cell, regulatory T-cells and NK T-cells which respond to non-peptide auto-antigens [22,23].

1.2. Clinical presentation and aetiology

Understanding the CD immunopathogenesis is key to its diagnosis and the improvement of CD testing. CD has a variable clinical presentation and can be categorized as classic (intestinal), non-classic (extraintestinal), subclinical, refractory (RCD), and seronegative CD [1,24,41,42]. This can result in delayed diagnosis [43]. Patients with classic CD often present with intestinal symptoms, such as diarrhoea, decreased appetite, malabsorption, abdominal pain and distention, but may also have extraintestinal symptoms, such as iron-deficiency anaemia, arthritis, and dermatitis herpetiformis [41,44]. Subclinical CD is often only detected during the screening of susceptible individuals for CD because the symptoms

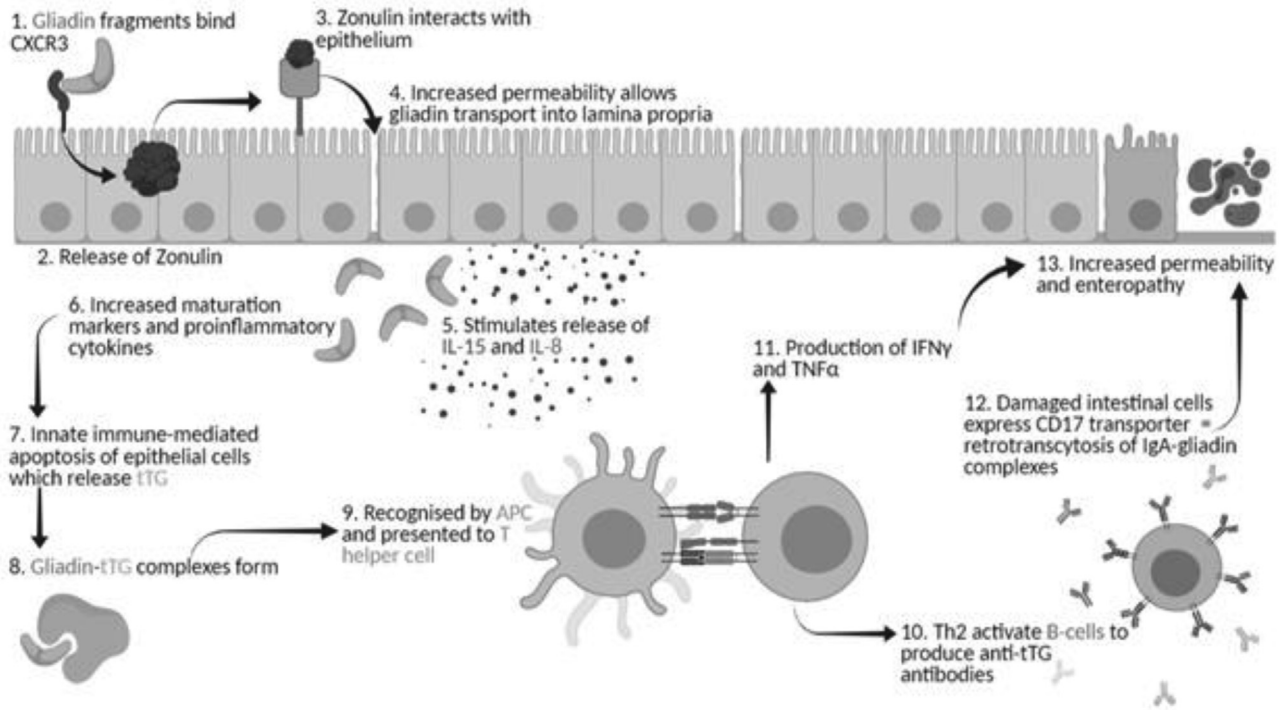

Fig. 1. The immunopathogenesis of CD is complex and multifactorial (Created with BioRender.com). CD is initiated by the binding of gliadin to CXCR3 (1) and the release of zonulin (2–3). This increases GIT permeability (4) with production of pro-inflammatory cytokines (5) and the presentation of gliadin to CD4 + T-cells by APCs (8–9) to initiate an adaptive immune response (10–12) further increases permeability and enteropathy (13).

Table 1
Commonly measured antibodies in CD[1].

ANTIBODY	*CURRENT USE*	*ACCURACY*
Anti-gliadin antibodies	Historical test now superseded [27–30]	
Anti-reticulin antibodies		
Anti-endomysial antibodies (EMA)	Routine	Low specificity High sensitivity [30,31]
Anti-tTG antibodies	tTG is the autoantigen of CD and a valuable diagnostic marker.[15,32–34] tTG-based serology tests have a Several comparisons with other antigens suggest that tTG is the superior diagnosis marker for CD.[35–37]	High sensitivity, accuracy, and efficiency.[1,34,35,38,39]
Anti-DGP antibodies	CD patients have T-cells specific for DGP. Antibodies against DGP are now a diagnostic marker.	High specificity High sensitivity [9,10,40]

[1] Selective IgA deficiency occurs in approximately 2–3% of CD patients and can render the IgA-based tests inaccurate. [24,25] It is recommended that total IgA is tested with every CD antibody test performed. In the presence of IgA deficiency, IgG antibodies against tTG and DGP are typically used.[26]

are not clinically significant [5]. There have been clinical cases of seronegative CD where serological markers are absent but there is severe malabsorption and villous atrophy [24,45]. Patients with RCD experience symptoms and villous atrophy after maintaining a gluten-free diet for a year or more [5]. The 2 subtypes of RCD are distinguished histologically by differences in IEL populations and have different prognoses [46].

Although gluten is the principal trigger for CD, several other environmental and genetic factors influence CD development [1,4,8,47,48]. These aspects of CD are not well understood but factors hypothesized to have a key role in gluten intolerance include intestinal infections, quantity and quality of gluten consumed, gut microbiota and infant diet [49,50].

1.3. Treatment and management

The only treatment for CD currently is a lifelong gluten-free diet (GFD) [1,5,8]. Although this has shown to relieve symptoms, decrease autoantibodies, and promote villous regrowth, a GFD can also cause poorer quality of life, mineral and vitamin deficiency, and increase cardiovascular risk [51,52]. Potential future therapeutics include dietary supplements to assist in digesting gluten, targeted therapies to induce tolerance, and inhibiting deamination of gluten by tTG [2]. Endopeptidases and zonulin inhibitors have been suggested as potential supplements to assist in gluten digestions and epithelial barrier restoration [2,8].

Misdiagnosis or late diagnosis of CD can increase morbidity and mortality because of complications. Common complications include iron deficiency and lowered bone mineral bone density leading to osteoporosis while lesson common complications include hyposplenism, refractory CD, intestinal lymphoma, small bowel adenocarcinoma and ulcerative jejunoileitis [53,54]. This provides additional motivation to establish biomarkers for CD as, currently, these complications require biopsies for diagnostic and prognostic information. This review addresses the current diagnostic algorithm for CD and examines potential future directions, specifically in the South African population.

2. Discussion

2.1. Serological testing

Serological testing for CD is accurate with sensitivities, specificities, and diagnostic accuracies greater than 90%, although testing is more accurate when individuals are tested whilst on a gluten-containing diet [55,56]. Autoantibody titers rapidly decline after GFD initiation in CD patients typically within a year. This is useful in monitoring dietary adherence and can be suggestive of GIT recovery and is associated with improved clinical symptoms [57]. Serological tests may have decreased accuracy for mucosal inflammation and recovery with RCD [58–60]. These challenges have resulted in the development of ancillary diagnostics like microbead-based technology, chemiluminescence, autoantibody profiling and regression tree analysis which aim to improve sensitivity and predict the requirements for biopsy [61–64].

2.2. Duodenal biopsies

Intestinal biopsy and histological examination confirms the diagnosis of CD in the context of positive serology [1,65]. Two to three biopsy samples are typically collected from the duodenal bulb and 4–6 biopsies from the distal duodenum using endoscopy [56,66]. The biopsies are graded histologically according to the degree of villous disruption [67].

There are different histological classification systems but the Marsh-Oberhüber histological classification method is the most widely used [65,68]. This system grades GIT disruption as Grade 1 (infiltration of IELs), Grade 2 (crypt hyperplasia), Grade 3 (partial (A), subtotal (B) or complete (C) villous atrophy) or Grade 4 (hypoplasia of the small bowel architecture) [1,65]. Duodenal biopsies are invasive but necessary confirmatory assays to establish the degree of disease and mucosal recovery following GFD. [18,69,70]

2.3. Supplemental testing

CD has a strong genetic component with a 10–15% familial recurrence and 75–80% concordance amongst monozygotic twins [8,71]. The HLA-DQA1*05:01-DQB1*02:01 haplotype, encoding the DQ2 molecule, is present in 90–95% of CD patients [72,73]. In the remaining approximately 5% of CD patients, the HLA-DQA1*03:01-DQB1*03:02 haplotype is expressed, encoding the DQ8 molecule. The remaining 5% of patients express at least one of the DQ2/DQ8 alleles [74]. This association provides a useful screening tool for CD through genotyping. The HLA DQ2/DQ8 genes, however, are required but not sufficient to cause CD limiting its utility as a standalone diagnostic test [8,75].

In the context of CD diagnosis, flow cytometry is mainly used to detect and count IELs to diagnose RCD when serology is unhelpful [76–78]. RCDII IEL characterization is typically indicated by the presence of clonal T-cells on flow cytometry [5,79]. These clonal T-cells typically express no surface CD3, CD4 or CD8 but have both cytoplasmic CD3 and CD103 but the lack of CD3 and CD8 with preservation of cytoplasmic CD3 and monoclonal rearrangement of T-cell receptor chain are the primary factors considered when diagnosing [5,77,80]. Although identifying IELs is primarily used to diagnose RCD, IELs can also be useful in diagnosing difficult cases of CD with inconclusive serology and histopathology [81,82].

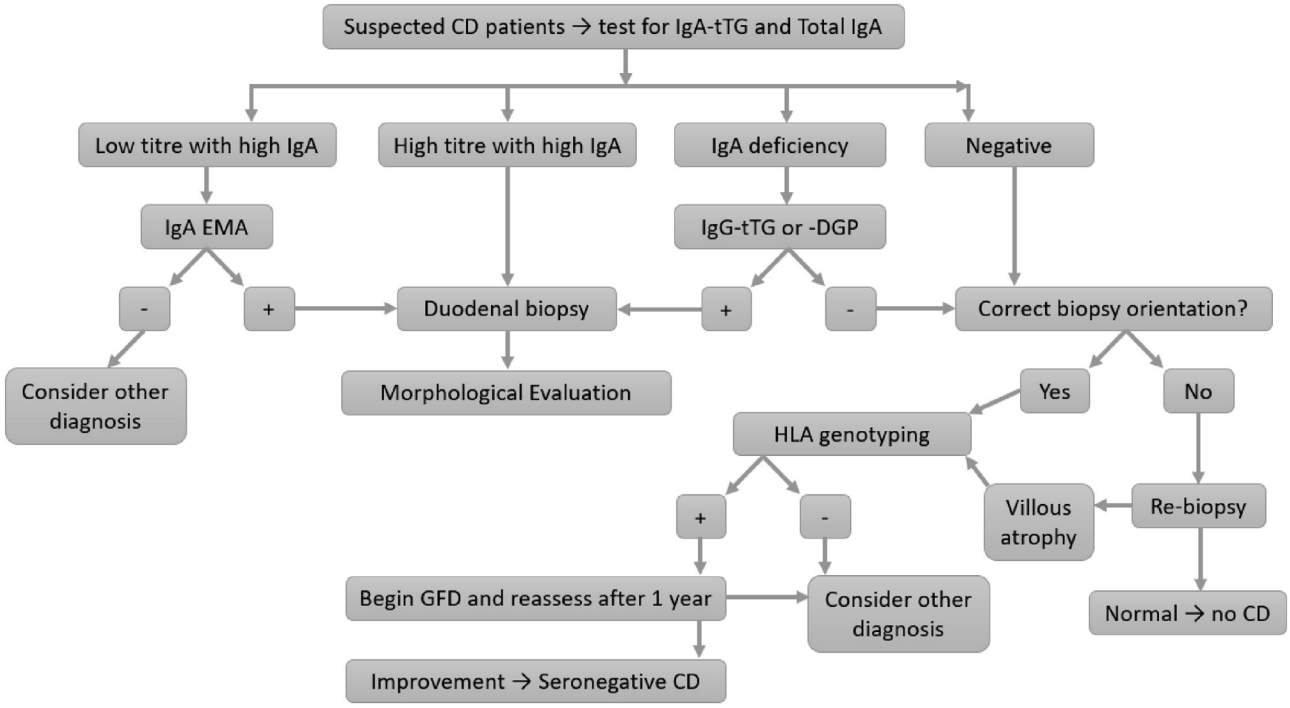

Fig. 2. The golden standard for the diagnosis of CD is serology with confirmatory histological features on duodenal biopsy. Currently the recommend serological test is IgA-tTG although this may be unreliable in patients with selective IgA deficiency. HLA typing may be used as a non-invasive screening test for CD.

Table 2
Potential diagnostic biomarkers identified in the last 10 years for CD.

Biomarker	Alterations seen in coeliac disease	Size	Population
Inflammatory Cytokines			
Serum levels IFN-γ, IL-6 and IL2	Increased	110	Iran [88]
		50	Australia [89]
Serum CX3CL1 levels	Increased	100	Spain [90]
Micro-RNA (mi-RNA)			
miRNA-146a and miRNA-155	Increased with high sensitivity and specificity	30	Chile [91]
miRNA-21 and −31	Increased	70	Egypt [92]
Faecal Components			
Volatile Organic Compounds (VOCs)	Different profiles	30	Netherlands [46]
Faecal Calprotectin	Increased	29	Germany [93]
Lipidomics			
Lipid and phospholipid profiles	Certain triacylglycerols are increased	233	Finland [94]
		256	Italy [95]
T-cells			
CD38 expression on gliadin-specific T-cells	Increased with gluten exposure	13	Norway [96]
Meta-analysis of TCRγδ + counting with flow cytometry[97]	High diagnostic accuracy	519	–
Oxidative Stress			
Levels of ROS and other biomarkers of oxidative stress	Correlates with the degree of villous atrophy	54	Italy [98]
Gene Expression			
Gene expression data from RT-PCR assays	Defined discriminant equations which could objectively and accurately classify duodenal biopsies into Marsh score categories	36	Australia [99]
Intestinal-derived Serum Proteins			
I-FABP[33]	Increased in untreated CD	20	Netherlands [100]
		141	Netherlands [101]
		108	Netherlands [102]
UBE2L3	Increased expression of (a ubiquitin ligase) predicts CD	9451	Spain [103]
Neo-epitopes of the tTG-DGP complex	Identify CD and mucosal healing	90	USA [104]
REG I α	Increased levels in CD and decreased with GFD	113	Spain [105]
Transcriptomics			
Transcriptomic signatures	Unique signature linked to increased cell proliferation, nuclear division, and cell cycle activity	121	Israel [106]

2.4. Diagnostic algorithms for CD

Global standards on CD diagnosis have been influenced mainly by European, British and North American guidelines which are widely published and have been summarized in Fig. 2 [1,5,56,83,84]. This diagnostic algorithm uses five antibodies for serology, IgA-tTG, total IgA, IgA-endomysial antibodies (EMA), IgA-DGP, and IgG-tTG or IgG-DGP (Fig. 2). Serology is followed up with a duodenal biopsy as final confirmation and HLA genotyping as an exclusion test when serology and biopsies are ambiguous. Canada, New Zealand and Australia follow similar guidelines [85–87]. The new European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) guidelines recommend no biopsy if tTG levels are 10 times greater than the normal limit and a second sample is EMA positive [83]. There is, however, little to no guidelines published in developing countries. The South African algorithm consists mainly of IgA-tTG and IgA-DGP serology with a follow-up biopsy. Diagnostic challenges in a resource-limited setting, such as South Africa, include loss of patient follow-ups and decreased availability of diagnostic testing.

2.5. Novel directions

Several new biomarkers are under development and could potentially improve the diagnostic, prognostic, and monitoring capabilities of CD testing (Table 2). The majority of these studies, however, were conducted outside of Africa. Key biomarkers include markers of inflammation (cytokines, reactive oxygen species (ROS) and genetic signatures of lymphocyte activation), biochemical markers of intestinal destruction (such as IFABP), and characterization of IEL populations. Recently, there has also been increased interest in the GIT microbiome because of its potential pathogenic role in CD.

Monitoring and prognostication are key in RCD because of the risk of malignant transformation [5,107]. Promising avenues of investigation include phospholipids profiling and mi-RNA detection [91,95]. Auricchio *et. al.* (2019) recently identified a serum phospholipid profile that distinguishes individuals who will develop CD before they present with symptoms or antibodies [95]. mRNA-146a, miRNA155, miRNA-21 and miRNA-125b have high specificity and sensitivity for active and inactive CD irrespective of treatment [91]. Investigations into these biomarkers in South African populations are needed as the novel research into CD diagnostics has occurred mainly in Europe and USA.

3. Conclusions

In conclusion, the expanding knowledge on CD pathology has enabled the construction of a complex diagnostic algorithm capable of accurately identifying individuals with CD, whereas the same cannot be said for many other autoimmune diseases. Regardless, several limitations need to be addressed through further investigations into the numerous avenues of research with the potential to improve the diagnosis, prognosis, and monitoring of patients with CD. In South Africa, the current diagnostic algorithm requires modification to better use resources and identify high-risk individuals.

Declaration of Competing Interest

The authors have no competing interests to declare.

None of the authors has any potential financial conflict of interest related to this manuscript.

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2.2 Publication 2: Coeliac disease high-risk HLA alleles in a small type one diabetic population, Johannesburg, South Africa

The extensive literature review, conducted as a part of this project, highlighted the potential of HLA DQ genotyping as an initial screening tool for CD high-risk individuals, for example, T1D patients (81-84). There is, however, very little data on HLA types in South Africa and CD-associated HLA alleles have only been investigated in healthy individuals (49). The HLA DQ2/8 frequency was, therefore, examined in T1D patients from a high-risk endocrinology clinic in Johannesburg, South Africa. Briefly, 22 paediatric participants were recruited and HLA-DQ genotyped with informed consent and assent. This confirmed that approximately 37% of the cohort lacked CD-associated HLA alleles. This subset of the South African T1D population without the DQ2/8 alleles is currently receiving CD serological monitoring unnecessarily which suggests a re-examination of the diagnostic algorithm is required. The substantial portion of our cohort that did have the DQ2/8 alleles agrees with the published literature and motivates the examination of potential CD biomarkers in high-risk populations.

Candidate's contribution: First author, sample collection, laboratory investigations, data analysis, manuscript writing and editing.

Coeliac disease high-risk HLA alleles in a small type one diabetic population, Johannesburg, South Africa

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Keywords: Coeliac disease, HLA, South Africa

Abbreviations: Coeliac disease (CD); Human Leukocyte Antigen (HLA); type 1 diabetes (T1D); tissue transglutaminase (tTG)

Abstract

Coeliac disease (CD) is an autoimmune disorder strongly associated with Human Leukocyte Antigen (HLA) genes DQ2 and DQ8. These alleles are also associated with other autoimmune diseases including type 1 diabetes (T1D) and these patients are consequently at a higher risk of developing CD. We examined the frequency of high-risk HLA alleles in a cohort of South African T1D paediatric patients. A total of 22 paediatric participants were recruited at a tertiary endocrinology clinic in public sector hospital in Johannesburg, South Africa (ethics number M200337). Clinical details were collected, and all patients were HLA typed at the DQ loci. In our patients with T1D, 63,6% had at least 1 high-risk HLA type alleles associated with CD. Of the patients with high-risk alleles, DQ2 was found in the majority which is consistent with CD literature. In conclusion, this study provides preliminary insight into the frequency of the HLA-DQ types associated with CD in a South African population. The high prevalence of high-risk alleles in our T1D population motivates for continual monitoring and further investigations into CD in South Africa.

1. Introduction

Coeliac disease (CD) is an autoimmune disorder triggered by gluten ingestion. The key effector cells are CD4+ T-cells specific for tissue transglutaminase (tTG) bound to gliadin, an immunogenic component of gluten (1-5). The global prevalence of CD is 0,5-1% but the prevalence in South Africa is unclear. CD can be diagnosed with autoantibody detection and duodenal biopsies. It can also be excluded from differential diagnoses with HLA genotyping (6-8).

The HLA-DQA1*05:01-DQB1*02:01 haplotype, encoding the DQ2 molecule, is present in 90-95% of CD patients. In about 5% of CD patients, the HLA-DQA1*03:01-DQB1*03:02

haplotype is expressed, encoding the DQ8 molecule (9, 10). The remaining 5% of CD patients express at least one of the DQ2/DQ8 alleles (11). The presence of these alleles is necessary but not sufficient to cause CD which makes HLA genotyping a useful screening application (12). The prognostic value is less clear although individuals homozygous for HLA-DQ2 (DQA1*05:01-DQB1*02:01) are at a higher risk of early-onset CD during infancy (11, 12).

In addition to the association with CD, these alleles are associated with the development of other autoimmune conditions like type 1 diabetes mellitus (T1D), autoimmune thyroid disease and Addison's disease (13-15). For this reason, CD is seen more commonly in these disorders with 8% of patients with T1D developing CD and a reported hazard ratio of 2.4 (CI = 95) (16-19). A recent study reported a 10,2% serology-positive CD in South African T1D individuals but only a 1,9% prevalence of biopsy-confirmed CD (20). The true prevalence of T1D from the International Diabetes Foundation is 4,5 in 1000 patients but is considered an underestimation (21). Epidemiological data on other autoimmune diseases in CD is also unclear although preliminary studies suggest a 3,5% prevalence of Hashimoto's thyroiditis and a 0,031% prevalence of Addison's disease in Africa (22, 23).

Previously, our laboratory retrospectively described HLA types and haplotypes in a South African organ donor population (24). The DQ2 haplotype was found in 10% of the population and 14% expressed the DQ8 haplotype but the participant's ethnicities were unknown. The high-risk alleles were observed in the population, namely HLA-DQA1*03 (18%), HLA-DQA1*05 (25%), HLA-DQB1*02 (36%), suggesting that subsections of the South African population are at a higher risk of developing CD. A recent study by Mrubata et al. (2021) confirmed that the CD high-risk alleles are present in a substantial number of black South African organ donors (25). This study aimed to examine the frequency of high-risk CD HLA alleles in South African T1D patients.

2. Methods and Materials

2.1 Ethical Statement

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

2.2 Study population

Whole peripheral blood samples were collected from T1D patients at Rahima Moosa Mother and Child Hospital (RMMCH) during their routine clinical management. The blood was collected by the nurse/physician drawing blood at the time of routine testing. All patients and volunteers provided informed consent. The demographic information of each participant was recorded from patient files, including age, gender, diet, self-reported ethnicity, comorbid autoimmune diseases, current BMI, FBC (to detect iron deficiency anaemia) and HbA1c to gauge long-term blood glucose control in T1D patients. HbA1c results, expressed in %, were collected with permission from the NHLS laboratory information system and the result at the time of patient recruitment was utilised. The Bio-Rad VARIANT™ II TURBO HbA1c kit was used by the NHLS laboratory to determine HbA1c levels (%).

2.3 HLA genotyping

DNA was extracted using the High Pure PCR Template Extraction kit (Roche, Geneva) in accordance with the manufacturer's instructions. The eluted DNA was quantified using a Nanodrop Spectrometer (Thermo Fisher Scientific, UK) to confirm the concentration and purity of the samples. The concentration and purity acceptability criteria followed Immucor^{TM} (Immucor, USA) recommendations: a DNA concentration of between 10 and 200 ng/µl and a A260nm/280nm ratio of between 1,6 and 2 to be acceptable. Sequence Specific Oligonucleotide (SSO) PCR was used to determine the HLA genotypes as a 2- to 4-digit resolution. The SSO typing kits were purchased from Immucor^{TM} (Immucor, USA) and contain the reagents needed for PCR. The Immucor^{TM} IFU (Immucor, USA) recommends a DNA concentration of 15 ng/µl for optimal PCR reactions. This was adjusted to 20ng/µL during the validation study in 2019.

3. Results and Discussion

A total of 22 patient samples were collected in a one-year period between November 2020 and November 2021. Demographic information is recorded in Table 1. The sample population had a median age of 10 years and had a median HbA1c level of 11,5%, suggesting poor glucose control.

CD has one of the highest HLA associations with all patients expressing at least one of the high-risk alleles (6). These alleles show association with other autoimmune diseases like T1D (13-15). The disease has been reported more commonly in Caucasian populations and the risk in SA is unclear even in high-risk populations. Fourteen (63,6%) of our sample population had at least one of the alleles associated with CD. In this sample population, The CD-associated alleles most commonly found were DQA1*05:01, DQB1*02:01 and DQB1*03:02 which were present in 36,4% of the population (Table 2). Other alleles not associated with CD were also identified in patients with T1D, most commonly DQA1*03:02 (45,5%) (Table 2). There was 27,3% heterozygosity for HLA DQ2 in this population and 18,2% for HLA DQ8 but none were homozygous for either DQ2/8. The presence of one high-risk allele is necessary but not sufficient to cause CD and different haplotypes do not substantially increase this risk, although HLA DQ2 homozygosity has been associated with an increased risk of early-onset CD in infants with a first-degree family member with CD, including a parent, full sibling, or child (11, 12). Other common alleles observed in the population include, DQA1*03:02 (45,5%), DQA1*01:02 (31,8%), and DQB1*02:02 (22,7%).

Most of the participants in this study were non-Caucasian with over 50% self-reporting as black. Of note, two of the black patients were of Somalian descent which may skew the data. Here we build on the previous literature confirming the finding that CD high-risk alleles are present in black T1D South Africans. It has been hypothesised that a shift towards a more wheat-based

diet in South Africa could be triggering CD in populations where the high-risk alleles are present (26).

In South Africa, patients with T1D receive regular serology testing to ensure that CD is detected early, particularly because T1D patients are often asymptomatic (27, 28). The determination of HLA-DQ genotypes in T1D is important for better identification of individuals with high-risk alleles and who, therefore, required continual serological testing. This study suggests that a large part of the South African T1D population (36,4%) are not at risk for CD and, therefore, do not require the regular serological testing they receive. This has implications for the CD diagnostic and screening guidelines for South African T1D patients as a first-line HLA DQ screening test may improve cost efficiency freeing resources to investigate at-risk patients more comprehensively. The early identification of at-risk T1D patients is key to effective early management of CD which can improve outcomes for this disease and other autoimmune pathologies (29).

This study was a small pilot project and samples sizes were small. In addition, use was made of self-reported ethnicity which may lack accuracy. The study does, however, suggest that more active screening is warranted in this subgroup of patients and suggests that HLA DQ typing may be a cost-effective screen to ensure early diagnosis and management of CD.

4. Concluding remarks

In summary, this study provides preliminary insight into the frequency of the HLA-DQ types associated with CD in a South African T1D population. The high prevalence of high-risk alleles in our South African T1D population suggest that screening by HLA DQ typing may have utility to stratify the T1D population into low-risk individuals who should not receive serological testing and high-risk patients who must be continuously monitoring for CD autoantibodies and receive duodenal biopsies where appropriate.

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Table 1. A summary of the demographic information of the participants.

Characteristic	
Median age in years (±SD)	10 (±3,07)
Gender	
Female – n (%)	13 (59,1)
Male – n (%)	9 (40,9)
Self-reported ethnicity	
Black – n (%)	13 (59,1)
White – n (%)	1 (4,5)
Coloured – n (%)	6 (27,3)
Indian – n (%)	2 (9,1)
Median HbA1c level % (±SD) Normal HbA1c <7.5%	11,5 (±3,48)
Total	22

Table 2. HLA genotype summary. The most common HLA DQA1 and DQB1 types found in this population were DQA1*03:02, DQA1*05:01, DQB1*02:01 and DQB1*03:02, three of which are considered high-risk alleles for CD.

HLA genotype	n (%)
High-risk CD two-alleles haplotypes (heterozygous)	
DQA1*05:01- DQB1*02:01 (DQ2)	6 (27,3)
DQA1*03:01- DQB1*03:02 (DQ8)	4 (18,2)
High-risk CD one-alleles haplotypes	
DQA1*03:01	4 (19,1)
DQA1*05:01	8 (36,4)
DQB1*02:01	8 (36,4)
DQB1*03:02	8 (36,4)
Other DQ alleles present	
DQA1*01:01	3 (13,6)
DQA1*01:02	7 (31,8)
DQA1*01:03	1 (4,6)
DQA1*02:01	4 (18,2)
DQA1*03:02	10 (45,5)
DQA1*03:03	1 (4,6)
DQA1*05:05	2 (9,1)
DQA1*05:08	2 (9,1)
DQB1*02:02	5 (22,7)
DQB1*03:01	1 (4,6)
DQB1*03:03	3 (13,6)
DQB1*04:01	1 (4,6)
DQB1*05:01	3 (13,6)
DQB1*05:05	1 (4,6)
DQB1*06:02	2 (9,1)
DQB1*06:03	1 (4,6)
DQB1*06:09	1 (4,6)

2.3 Publication 3: Intestinal fatty acid binding protein (I-FABP) and CXC3L1 evaluation as biomarkers for patients at high-risk for coeliac disease in Johannesburg, South Africa

Biomarkers represent an attractive, non-invasive option to monitor CD patients. Many of the biomarkers identified require further investigations in CD patients but none of them have been examined in the context of populations at high-risk for CD nor in South African populations. I-FABP and CX3CL1 are two promising biomarkers for CD and can be easily detected. For these reasons, these biomarkers were chosen for investigation in high-risk T1D patients and CD-diagnosed patients for this project. A total of 38 participants were recruited from Rahima Moosa Mother and Child Hospital and Wits Donal Gordon Medical Centre and grouped according to diagnosis and HLA type. Participants with the DQ2/8 alleles and CD-confirmed diagnosis were grouped as CD-positive. Those with the DQ2/8 alleles and T1D were grouped as high-risk and any individual without the DQ2/8 alleles was categorised as CD-negative. I-FABP levels were significantly higher in the CD-positive compared to the CD-negative cohort ($p=0.03$). There was no statistically significant difference noted in the CX3CL1 levels which may be caused by the comorbid autoimmune and inflammatory conditions present across the groups. This study is the first to examine the levels of these potential CD biomarkers in a multi-ethnic population with comorbid autoimmune diseases.

Candidate's contribution: First author, sample collection, laboratory investigations, data analysis, manuscript writing and editing.

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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

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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.

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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

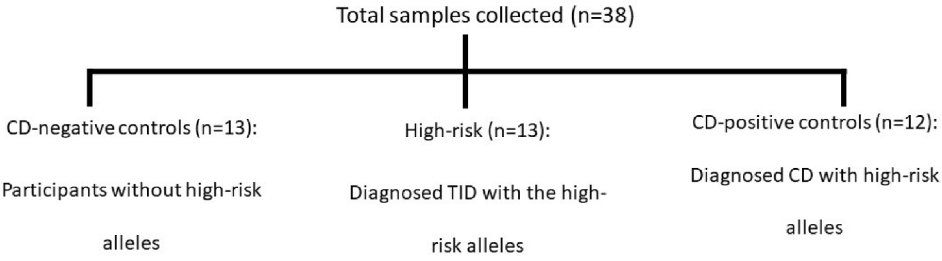

Fig. 1. Cohort selection. A total of 38 samples were collected from November 2020 until November 2021 and were stratified into CD-positive, high-risk, and CD-negative groups. The groups were stratified according to the presence or absence of high-risk HLA DQ2/8 and diagnoses.

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).

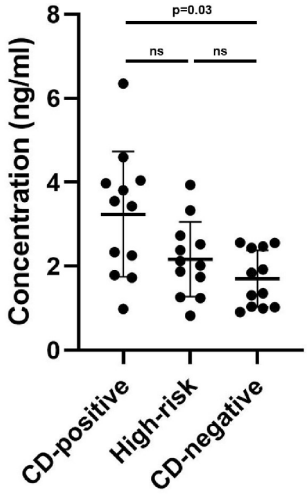

Fig. 2. I-FABP levels were significantly higher in patients diagnosed with CD and with TID at a high-risk for CD compared to CD-negative patients.

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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Chapter 3: Conclusions and Future Directions

Current algorithmic approaches for CD diagnoses in South Africa are poorly defined and limited. High-risk populations, such as T1D patients, are only monitored for CD antibodies sporadically with many patients being lost to follow-up. Furthermore, CD has not been sufficiently researched in South African populations, particularly in high-risk T1D patients, and improved biomarkers have yet to be fully investigated and introduced into any CD diagnostic algorithm. This study, therefore, aimed to determine genetic and serological concordance in patients with suspected CD in a South African population after a comprehensive literature review.

Our data provided preliminary insight into CD-associated HLA-DQ type frequencies in a South African T1D population. The high prevalence of high-risk alleles detected in this cohort suggests that HLA-DQ screening in high-risk populations may have value in order to stratify these populations into low-risk individuals who should not receive serological testing and high-risk patients who must be continuously monitored for CD autoantibodies and receive duodenal biopsies when indicated. This has implications for the South African CD screening and diagnostics as a first-line HLA-DQ screening test will improve cost efficiency and the comprehensive investigation of at-risk patients. HLA-DQ screening may also assist in mortality prediction in at-risk and asymptomatic patients because of the association with lymphoproliferative disorders (46, 85). Data regarding the clinical course in CD patients in a South African population should, however, be collected.

CD is one of the few gastroenteropathies which has accurate serological diagnostic tests, but certain limitations remain which have prompted investigation of additional diagnostic biomarkers. As part of this study, two different serological biomarkers were measured – I-FABP and CX3CL1 in the high-risk cohort and compared to individuals both with and without CD. Levels of I-FABP, a gastrointestinal tract epithelial marker, were only significantly

different between CD-positive patients and CD-negative patients. 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 (62, 71). The reason in this cohort is unclear but further correlations between I-FABP levels and gastrointestinal recovery may be of benefit.

CX3CL1, a chemokine ligand associated with autoimmune pathology, did not accurately distinguish the groups although average levels were highest in the CD-positive cohort and the lowest in the CD-negative cohort. This may reflect the high number of patients in this study with comorbid autoimmune pathology and the impact of African polymorphisms could not be excluded (68, 76-79, 86). The inclusion of CX3CL1 in a multianalyte profile in CD may show utility. Interestingly, although only a few patients with confirmed CD included gluten in their diet, the CX3CL1 levels were higher than those on a GFD. This may suggest utility in monitoring dietary compliance.

This study was the first to assess levels of these biomarkers in a multi-ethnic population with comorbid autoimmune diseases as well as contribute to the knowledge on HLA frequencies in South African populations. Certain limitations are acknowledged including a small sample size. In addition, most CD-positive patients were on GFD which may have impacted the biomarkers. The study was cross-sectional, and the impact of longitudinal intestinal damage could not be assessed. This was, however, an important pilot study and future directions including a diverse biomarker panel, longitudinal follow-up of high-risk patients and a larger sample size are recommended. These data will inform a meta-analysis, currently in process, looking at algorithms internationally and the use of HLA DQ screening in T1D patients. An amended algorithm is likely to minimise the impact of CD on the health care system through early diagnosis (42, 87).

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Appendix

1. Ethics Approval Certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Ms A Gandini
School of Pathology
Department of Immunology
Medical School
University

E-mail: anastasiagandini@gmail.com

CC: Supervisor: Prof E Mayne; Drs M Gededzha and T De Maayer
<Elizabeth.Mayne@nhls.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 2020/08/04

REF: *R14/49*

PROTOCOL NO: **M200337** *(This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)*

PROJECT TITLE: *Genetic and serological concordance in patients with suspected coeliac disease*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2000/Iain0007/Clearscan.wps

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Ms A Gandini

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M200337

NAME: (Principal Investigator)	Ms A Gandini
DEPARTMENT:	School of Pathology Department of Immunology Medical School University
PROJECT TITLE:	Genetic and serological concordance in patients with suspected coeliac disease
DATE CONSIDERED:	2020/03/27
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof E Mayne; Drs M Gededzha and T De Maayer
APPROVED BY:	CB Penny Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2020/08/04

This clearance certificate is valid for 5 years from the date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the 3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **March** and will therefore reports and re-certification will be due early in the month of **March** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

05/08/20
Date

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

2021/09/06

Ms A Gandini
School of Pathology
Department of Immunology
Medical School
University

Sent by e-mail to: anastasiagandini@gmail.com

Dear Ms Gandini

Re: **Protocol Ref No:** M200337
Protocol Title: *Genetic and serological concordance in patients with suspected coeliac disease*
Principal Investigator: Ms A Gandini

Thank you for your letter of 2021/08/26

I confirm that we have noted and approve of your proposed study amendments, as follows:

1. Dr G Landau will become an additional investigator on the study
2. a project-specific blood draw will be introduced for Dr Landau's patients, who will receive compensation for the travel costs involved
3. a Facebook post will be used to draw the study to the attention of potential additional participants

Thank you for keeping us informed.

Yours Sincerely

G. Burns
.....
Mr I Burns
For the Human Research Ethics Committee (Medical)

CB Penny
.....
Dr CB Penny, Chairperson, Human Research Ethics Committee (Medical)

2. Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, Anastasia Gandini, student number [1348734], declare that this Dissertation is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Dissertation.

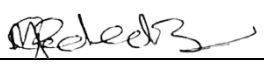
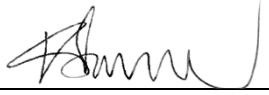
Signature of Student …… **Date**……24/03/2022……..

Name of Primary Supervisor……Elizabeth Mayne……..

Signature of Primary Supervisor ……**Date**……24/03/2022……..

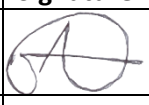
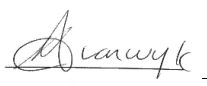
Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of her Dissertation. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for his/her degree purposes.

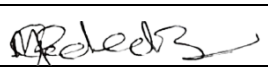
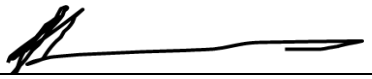
1. **Article 1: Title:** Diagnosing coeliac disease: A literature review
   Journal name, year, volume and page numbers: Human Immunology. 2021;82(12):930-6

Authors	Name	Signature	Date
1st author	Anastasia Gandini		01/03/2022
2nd author	Maemu P. Gededzha		01/03/2022
3rd author	Tim De Maayer		01/03/2022
4th author	Peter Barrow		01/03/2022
5th author	Elizabeth Mayne		01/03/2022

Comments by primary supervisor: N/A

2. **Article 2: Title:** Coeliac disease high-risk HLA alleles in a South African type one diabetic population
   Journal name, year, volume and page numbers: Submitted to Clinical Laboratory 16.08.2022

Authors	Name	Signature	Date
1st author	Anastasia Gandini		24/03/2022
2nd author	Nicole Van Wyk		24/03/2022
3rd author	Tim De Maayer		24/03/2022

4th author	Maemu P. Gededzha		24/03/2022
5th author	Elizabeth Mayne		24/03/2022

Comments by primary supervisor: N/A

3. **Article 3: Title**: Intestinal fatty acid binding protein (I-FABP) and CXC3L1 evaluation as biomarkers for patients at high-risk for coeliac disease in Johannesburg, South Africa
   Journal name, year, volume and page numbers: Cytokine. 2022;157 (in press).

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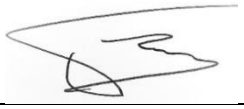
2nd author	Tim De Maayer		08/03/2021
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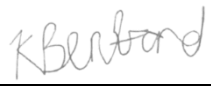
4th author	Katherine Bertrand		08/03/2021
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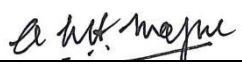
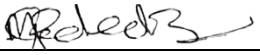
6th author	Anthony Mayne		08/03/2021
7th author	Maemu P. Gededzha		08/03/2021
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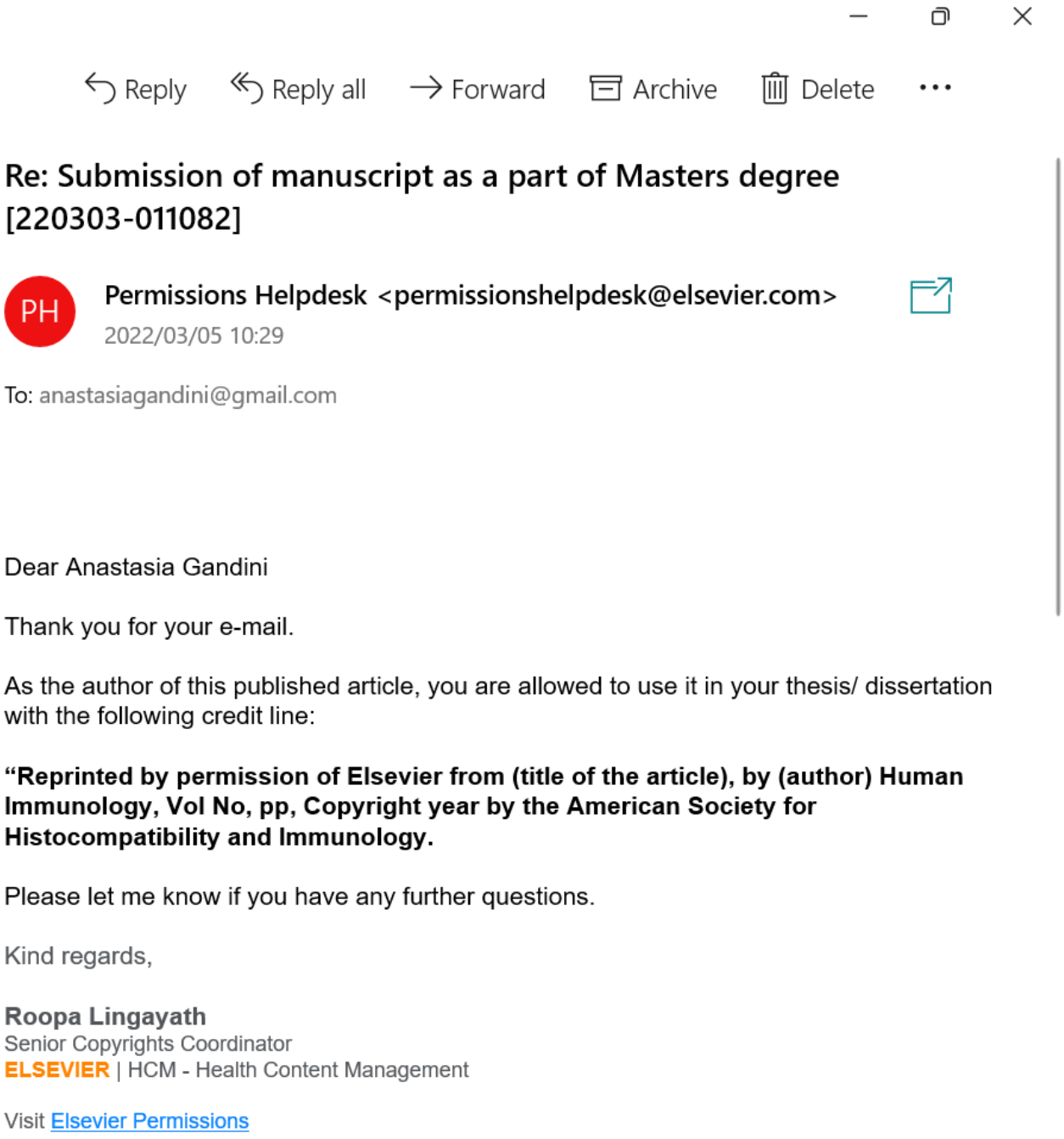

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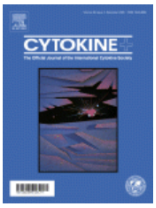

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