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CHARACTERISATION OF GUT MUCOSA IN PATIENTS WITH POTENTIAL COELIAC DISEASE

A thesis submitted for the degree of Doctor of Philosophy

by

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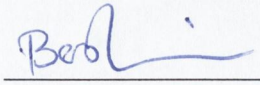
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Bashir Mustafa Y. Mohamed

DEDICATION

THIS THESIS IS DEDICATED TO LAILA, ABRAR, MOHAMED, AND MY PARENTS

TABLE OF CONTENTS

LIST OF FIGURES i

LIST OF TABLES v

LIST OF ABBREVIATIONS vi

PRESENTATIONS viii

PUBLICATIONS x

ACKNOWLEDGEMENTS xi

SUMMARY xii

CHAPTER 1.0 GENERAL INTRODUCTION

1.1 THE GUT-ASSOCIATED LYMPHOID TISSUE 1

1.2 COELIAC DISEASE 3

1.2.1 History of coeliac disease 3

1.2.2 Epidemiology 4

1.2.3 Clinical presentation of coeliac disease 5

1.2.4 Diagnosis of coeliac disease 9

1.3 PATHOGENESIS OF COELIAC DISEASE 12

1.3.1 Genetics of coeliac disease 13

1.3.2 Gliadin 15

1.3.3 Tissue transglutaminase 20

1.4 IMMUNOLOGICAL CHANGES IN COELIAC LESION 22

1.4.1 Intestinal histological changes 22

1.4.2 Enterocytes 24

1.4.3 Intraepithelial lymphocytes 26

1.4.4 Lamina propria lymphocytes 28

1.5 CYTOSKELETAL PROTEINS 29

1.5.1 Cytoskeletal proteins in coeliac disease 30

1.6 EXTRACELLULAR MATRIX 30

1.6.1 Matrix metalloproteinases and their inhibitor 30

1.6.2 Matrix metalloproteinases and their inhibitor in Inflammatory Conditions 32

1.6.3 Matrix metalloproteinases and their inhibitor in coeliac disease 33

1.7 COMPLICATIONS 33
1.7.1 Refractory coeliac disease 33
1.7.2 Reduced bone minerals 34
1.7.3 Infertility 35
1.7.4 Malignancy and mortality 36
1.8 MANAGEMENT 36
1.9 PURPOSE AND RATIONALE OF THESIS 37

CHAPTER 2.0 IMMUNOHISTOCHEMICAL CHARACTERISATION OF IELs AND CRYPT CELL PROLIFERATION IN PATIENTS WITH POSITIVE EMA TEST AND NORMAL DUODENAL MUCOSA

2.1 INTRODUCTION 40
2.2 MATERIALS AND METHODS 41
2.2.1 Patients 41
2.2.2 Small intestine biopsy specimens 43
2.2.3 Routine histology 45
2.2.4 Immunohistochemistry 45
2.2.5 Quantification of T cell markers on IELs and Ki-67 marker in the crypt cells 45
2.2.6 Statistical analysis 46
2.3 RESULTS 47
2.3.1 Detailed analysis of the original duodenal biopsies 47
2.3.2 Enterocyte proliferation 49
2.3.3 Follow-up data on 14 patients attended for reassessment 50
2.3.4 Immunohistochemistry findings on follow-up duodenal biopsies 53
2.4 DISCUSSION 61

CHAPTER 3.0 QUANTIFICATION OF ENTEROCYTE STRUCTURES AND TUBULIN FIBRE PROFILES IN PATIENTS WITH POSITIVE EMA TEST AND NORMAL DUODENAL MUCOSA AND IN PATIENTS WITH GLUTEN-SENSITIVE ENTEROPATHY

3.1 INTRODUCTION 66
3.2 MATERIALS AND METHODS 69
3.2.1 Patients 69
3.2.2 Immunofluorescent technique 71
3.2.3 Slide holder modification 71
3.2.4 Quantification and image acquisition 73
3.2.5 Optimisation of the image acquisition 73
3.2.6 Interpretation of the results 75
3.2.7 Statistical analysis 79
3.3 RESULTS 80
3.3.1 Enterocyte length 80
3.3.2 Enterocyte width 80
3.3.3 Nuclear shape 80
3.3.4 Nuclear length and width ratio 81
3.3.5 Enterocyte tubulin fibre angles 81
3.3.6 Enterocyte tubulin fibre staining intensity 82
3.3.7 Enterocyte tubulin fibre density 82
3.4 DISCUSSION 90

CHAPTER 4.0 IMMUNOHISTOCHEMICAL CHARACTERISATION OF MATRIX METALLOPROTEINASES -1, -3, -9 AND TIMP-1 IN PATIENTS WITH POSITIVE EMA TEST AND NORMAL DUODENAL MUCOSA AND IN PATIENTS WITH GLUTEN-SENSITIVE ENTEROPATHY

4.1 INTRODUCTION 96
4.2 MATERIALS AND METHODS 98
4.2.1 Patients 98
4.2.2 Control tissue 99
4.2.3 Standardisation of techniques 99
4.2.4 Immunohistochemistry 99
4.2.5 Quantification of MMPs and TIMP-1 expression 100
4.2.6 Statistical analysis 100
4.3 RESULTS 101

4.3.1 Details of MMPs –1, -3, -9 and TIMP-1 expression in the duodenal mucosa of gluten-sensitive enteropathy patients.... 101

4.3.2 Details of MMPs –1, -3, -9 and TIMP-1 expression in the duodenal mucosa of the study group subjects..... 102

4.4 DISCUSSION..... 114

4.4.1 MMPs –1, -3, -9 and TIMP-1 expression and the gluten sensitive enteropathy patients..... 114

4.4.2 MMPs –1, -3, -9 and TIMP-1 expression and the study group subjects..... 117

CHAPTER 5.0 GENERAL DISCUSSION..... 121

5.1 Evidence of increased intraepithelial lymphocytes in the study group..... 122

5.2 Evidence of increased crypt cell proliferation in the study group..... 123

5.3 Enterocyte morphological changes detected by cellomics (HCA)..... 124

5.4 The intestinal protein expression of matrix metalloproteinases and their inhibitor in gluten-sensitive enteropathy and study group..... 126

5.5 Conclusions..... 128

5.6 Key findings of the thesis..... 129

5.7 Future work..... 130

REFERENCES..... 131

APPENDICES..... 160

APPENDIX I- REAGENTS..... 161

i-IMMUNOPEROXIDASE REAGENTS 161

ii-IMMUNOFLUORESCENT REAGENTS 162

APPENDIX II..... 163

MONOCLONAL POLYCLONAL ANTIBODIES..... 163

STANDARDISATION..... 165

APPENDIX III..... 166

i- Cellomics® Kineticscan (high content analysis HCA)..... 166

ii- Scanned duodenal section using Cellomics®Kineticscan... 166

iii- Identified duodenal section using Virtual scan 167

iv- Individualised enterocytes using the Bioapplication tools. 167

v- Data was generated using Virtual scan..... 168

vi- Data exported to Microsoft Excel sheet..... 168

LIST OF FIGURES

Figure 1.1	The fate of ingested antigen.	2
Figure 1.2	The iceberg model of coeliac disease.	5
Figure 1.3	The structure of HLA-DQ2 molecule.	14
Figure 1.4	Diagrammatic representation of the interaction between the DQ2 molecule and an epitope from γ-gliadin.	18
Figure 1.5	**a.** Gluten digestion, deamidation and recognition.	19
Figure 1.5	**b.** Immunofluorescence staining of tTG, HLA-DQ and T cells.	19
Figure 1.6	Identified gluten epitopes in key studies.	20
Figure 1.7	The Marsh classification of intestinal damage in coeliac disease.	23
Figure 2.1	Patient flow diagram.	44
Figure 2.2	Percentage of IELs expressing the CD2 molecule in the study group subjects, treated coeliac disease, untreated coeliac disease patients, and normal controls.	54
Figure 2.3	Intraepithelial lymphocytes expressing CD3 in the duodenal mucosa of the study group subjects, normal control group, untreated and treated coeliac disease patients.	55
Figure 2.4	Percentage of IELs expressing the CD3 molecule in the study group subjects, treated coeliac disease, untreated coeliac disease patients, and normal controls.	56
Figure 2.5	Percentage of IELs expressing the CD7 molecule in the study group subjects, treated coeliac disease, untreated coeliac disease patients, and normal controls.	56

Figure 2.6 Intraepithelial lymphocytes expressing CD8 in the duodenal mucosa of the study group subjects, normal control group, untreated and treated coeliac disease patients. 57

Figure 2.7 Percentage of IELs expressing the CD8 molecule in the study group subjects, treated coeliac disease, untreated coeliac disease patients, and normal controls. 58

Figure 2.8 Percentage of IELs expressing the CD69 molecule in the study group subjects, treated coeliac disease, untreated coeliac disease patients, and normal controls. 58

Figure 2.9 Crypt cells expressing Ki67 in duodenal biopsies of the study group subjects, normal control group, untreated and treated coeliac disease patients. 59

Figure 2.10 Percentage of crypt cells expressing Ki67 marker in the study group subjects, treated coeliac disease, untreated coeliac disease patients, and normal controls. 60

Figure 2.11 Percentages of CD7 in the original and current duodenal biopsies of the study group subjects. 60

Figure 3.1 Patient flow diagram. 70

Figure 3.2 The modification of a 96 well plate to be used as a slide holder for HCA. 72

Figure 3.3 Segmented enterocytes. 74

Figure 3.4 Field map. 74

Figure 3.5 Length and width of enterocytes. 75

Figure 3.6 Nuclear shape (Roundness and LWR). 76

Figure 3.7 **a.** The higher the SD of the fibre angles the less fibres are aligned to each other. 77

Figure 3.7 **b.** The smaller the SD of fibre angle, the more aligned the fibre. 77

Figure 3.8 The mean number of pixels of a labelled fibre intensity distribution. 78

Figure 3.9 Tubulin fibre density. 79

Figure 3.10 Enterocyte length (red) and width (yellow) in small intestinal mucosa of normal controls, untreated coeliac disease, and study group subjects. 84

Figure 3.11 The mean of the enterocyte length in the initial and current duodenal section of the study group subjects (SGS), treated (TCD) and untreated (UTCD) coeliac patients and the normal control group. 85

Figure 3.12 The mean of the enterocyte width in the initial and current duodenal section of the SGS, UTCD, TCD patients and the normal control group. 85

Figure 3.13 The examined enterocytes' nuclei (red) in intestinal mucosa of normal control, untreated coeliac disease and study group. 86

Figure 3.14 The mean of the enterocyte nuclear roundness in the initial and current duodenal section of the SGS, UTCD, TCD patients and the normal control group. 87

Figure 3.15 The mean of the enterocyte nuclear length width ratio in the initial and current duodenal section of the SGS, UTCD, TCD patients and the normal control group. 87

Figure 3.16 The SD of the enterocyte tubulin fibre angles in the initial and current duodenal section of the SGS, UTCD, TCD patients and the normal control group. 88

Figure 3.17 The mean of the enterocyte tubulin fibre intensity in the initial and current duodenal section of the SGS, UTCD, TCD patients and the normal control group. 88

Figure 3.18 The mean of the enterocyte fibre density in the initial and current duodenal section of the SGS, UTCD, TCD

patients and the normal control group. 89

Figure 4.1 Lamina propria cells expressing MMP-1 in the normal control, study group and gluten sensitive-enteropathy patients with grade I-III lesions. 106

Figure 4.2 Percentages of lamina propria cells expressing MMP-1 in the normal controls, study group subjects and gluten sensitive enteropathy patients (grade I-III). 107

Figure 4.3 Lamina propria cells expressing MMP-3 in the normal control, study group and gluten sensitive-enteropathy patients with grade I-III lesions. 108

Figure 4.4 Percentages of lamina propria cells expressing MMP-3 in the normal controls, study group subjects and gluten sensitive enteropathy patients (grade I-III). 109

Figure 4.5 Lamina propria cells expressing MMP-9 in the normal control, study group and gluten sensitive-enteropathy patients with grade I-III lesions. 110

Figure 4.6 Percentages of lamina propria cells expressing MMP-9 in the normal controls, study group subjects and gluten - sensitive enteropathy patients (grade I-III). 111

Figure 4.7 Lamina propria cells expressing TIMP-1 in the normal control, study group and gluten sensitive-enteropathy patients with grade I-III lesions. 112

Figure 4.8 Percentages of lamina propria cells expressing TIMP-1 in the normal controls, study group subjects and gluten-sensitive enteropathy patients (grade I-III). 113

LIST OF TABLES

Table 2.1 Age, sex, clinical symptoms and laboratory results in the study group subjects. 42

Table 2.2 T cell markers and Ki67 expression in the duodenal biopsies of the study group subjects, untreated coeliac disease patients and treated coeliac disease patients. 50

Table 2.3 Age, sex, clinical and laboratory data of the 14 patients who attended for reassessment. 52

Table 2.4 T cell markers and Ki67 expression in the original and the current biopsies of the study group subjects who developed small intestinal enteropathy. 53

Table 3.1 Characteristics of the enterocyte morphology and tubulin profiles. 83

Table 4.1 Characteristics of MMP-1, -3, -9 and TIMP-1 expression in the study group subjects and patients with gluten sensitive enteropathy. 105

LIST OF ABBREVIATIONS

Abbreviation	Meaning
AAA	Anti-actin antibody
AGA	Anti-gliadin antibody
ARA	Anti-reticulin antibody
APCs	Antigen presenting cells
BSA	Bovine serum albumin
CD	Coeliac disease
CI	Confidence intervals
D2	Duodenal biopsy
DAB	Diaminobenzidine tetrahydrochloride dihydrate
DC	Dendritic cells
DH	Dermatitis herpetiformis
EATL	Enteropathy associated T cell lymphoma
ECM	Extracellular matrix
EDTA	Ethylene diaminetetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
EMA	Endomysial antibody
ESPGAN	European society of pediatric gastroenterology and nutrition
GALT	Gut-associated lymphoid tissue
GFD	Gluten free diet
GSE	Gluten sensitive enteropathy
HCS	High Content Screening
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
ICAM-1	Intracellular adhesion molecule-1
IELs	Intraepithelial lymphocytes
Ig	Immunoglobulin
IL	Interleukin
INF	Interferon
LFA	Leukocyte function associated antigen

LP	Lamina propria
LPLs	Lamina propria lymphocytes
LWR	Length and width ratio
MALT	Mucosal associated lymphoid tissue
min	Minute
ml	Millilitre
MMPs	Matrix metalloproteinases
MT-MMPs	Membrane type matrix metalloproteinases
P^2A	Perimeter square area
PBMC	Peripheral Blood Mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PVA	Partial villous atrophy
RA	Rheumatoid Arthritis
RCD	Refractory coeliac disease
RT	Room temperature
SD	Standard deviation
SGS	Study group subjects
TBS	Tris buffered saline
TCD	Treated coeliac disease
TCR	T cell receptor
TGF	Transforming growth factor
TIA	T-cell intracellular antigen
TIMPs	Tissue inhibitor of matrix metalloproteinases
TNF	Tumour necrosis factor
tTG	Tissue transglutaminase
TVA	Total villous atrophy
UC	Ulcerative colitis
UTCD	Untreated coeliac disease

PRESENTATIONS

Oral presentations

- **Bashir Mohamed,** Con Feighery, Jacinta Kelly, Christian Coates, Una O'Shea, Mohamed Abuzakouk. The assessment of matrix metalloproteinases (MMPs) -1, -3 and -9 in patients with gluten sensitive enteropathy. Irish Society for Gastroenterology (Dublin, Ireland, Winter 2003).

- **Bashir Mohamed**, Feighery C, O'Shea U, Kelly J. Delaney D, Briain DS. Cooney R. Abuzakouk M. Investigation of individuals with positive endomysial antibodies and normal duodenal biopsy for evidence of coeliac disease. The eleventh international symposium on coeliac disease (Belfast, northern Ireland, April 2004).

- **Bashir Mohamed**, Con Feighery, Jacinta Kelly, Christian Coates, Una O'Shea, Mohamed Abuzakouk. The assessment of matrix metalloproteinases (MMPs) -1, -3, -9 and TIMP-1 in patients with gluten sensitive enteropathy. The eleventh international symposium on coeliac disease (Belfast, northern Ireland, April 2004).

- **Bashir Mohamed**, Feighery C, O'Shea U, Kelly J. Delaney D, O'Briain DS. Cooney R. Abuzakouk M. Investigation of individuals with positive endomysial antibodies and normal duodenal biopsy for evidence of coeliac disease. Irish Society for Gastroenterology (Kilkenny, Ireland, summer 2004)

Poster presentation

- **Bashir Mohamed**, Con Feighery, Jacinta Kelly, Christian Coates, Una O'Shea, Mohamed Abuzakouk. The assessment of matrix

metalloproteinases (MMPs) -1, -3, -9 and TIMP-1 in patients with gluten sensitive enteropathy. The eleventh international symposium on coeliac disease (Belfast, northern Ireland, April 2004).

- **Bashir Mohamed**, Feighery C, O'Shea U, Kelly J. Delaney D, O'Briain DS. Cooney R. Abuzakouk M. Investigation of individuals with positive endomysial antibodies and normal duodenal biopsy for evidence of coeliac disease. The eleventh international symposium on coeliac disease (Belfast, northern Ireland, April 2004).

- **Bashir M. Mohamed,** Conleth Feighery, Dermot Kelleher, Yuri Volkov, Tony Davies, Yvonne Williams, Jacinta Kelly and Mohamed Abuzakouk**.** Quantitative analysis of enterocyte structure of patients with positive coeliac disease serology and normal duodenal mucosa. Cellomics 2006 European User Group Meeting (Dublin, Ireland, 8-9 June 2006).

PUBLICATIONS

- **Mohamed BM**, Feighery C, Kelly J, Coates C, O'Shea U, Barnes L and Abuzakouk M. Increased Protein Expression of Matrix Metalloproteinases -1, -3, and -9 and TIMP-1 in Patients with Gluten-Sensitive Enteropathy. Dig Dis Sci 2006. Oct, 51 (10): 1862-8.

- **Mohamed BM**, Conleth Feighery, Christian Coates, Una O'Shea, David Delaney, Seán O'Briain, Jacinta Kelly and Mohamed Abuzakouk. The absence of a mucosal lesion on standard histological examination does not exclude a diagnosis of coeliac disease (Accepted Dig Dis Sci 2007).

- **Mohamed BM,** Conleth Feighery, Yvonne Williams, Tony Davies, Dermot Kelleher, Yuri Volkov, Jacinta Kelly and Mohamed Abuzakouk. The use of Cellomics to study enterocyte cytoskeletal proteins in coeliac disease patients (submitted).

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SUMMARY

Coeliac disease is a gluten dependent enteropathy with a high incidence and increased risk of mortality. Diagnosis of coeliac disease is based on a biopsy of small intestine. However, it is now believed that many individuals have gluten-sensitive inflammation without an overt small intestinal lesion. Endomysial antibodies (EMA) are widely used to screen for coeliac disease with 100% specificity and 89-100% sensitivity. These antibodies do not only support the diagnosis, but also predict the severity of mucosal damage in patients with gluten-sensitive enteropathy (GSE).

The aim of this thesis was to examine a group of patients (25 patients) with a positive EMA test in the absence of supportive histological evidence for coeliac disease: these patients are known throughout the manuscript as the study group subjects (SGS). Immunohistochemical techniques were used to characterise intestinal T cell subpopulations in these patients and the results were compared with patients with established coeliac disease and with normal control population. The study group subjects showed significantly increased numbers of intraepithelial lymphocytes (IELs) expressing CD2, CD3, CD7, CD8, and CD69 compared with the normal control group. In addition, the number of proliferating crypt cells in the study group subjects detected by the Ki67 antibody was evaluated. It was shown that crypt cell proliferation in the study group subjects was increased compared with the normal control group, suggesting active cell proliferation.

Follow-up clinical evaluation and histological investigation was performed on 14 of the 25 patients. Of the 14 patients, nine underwent upper gastrointestinal endoscopy and duodenal biopsies. Six of these nine patients had developed the classical small intestinal lesion characteristic of coeliac disease. These six patients had the highest counts of IELs expressing CD2, CD3, CD7, CD8 and CD69 and enterocytes expressing the Ki67 measured in their original biopsies. The second set of duodenal biopsies showed similar increase in these examined markers to those found in the original biopsies. It was concluded, based on the results of this study, that immunohistochemical characterisation of IELs and

crypt cell populations in the small intestinal mucosa may be used to identify patients with early gluten-sensitive enteropathy, particularly those with positive coeliac serology and no overt histological evidence of the disease.

The second set of investigations was performed by employing the high content analysis (HCA) approach. This involved a detailed examination of tubulin profile and enterocyte structure in the original and the current duodenal biopsies of the study group subjects who had developed a coeliac lesion (n=6). This study is the first to use this system to examine small intestinal biopsies. It was found that enterocyte structure could be assessed in greater detail using this approach. It was demonstrated that the enterocyte shape, nuclei and the tubulin fibre structures were significantly changed, when compared to the initial and current duodenal tissue sections of the study group subjects and coeliac patients with the normal control group. It was demonstrated, that the HCA could be used to analyse the enterocyte's morphological properties, intracellular structures, and the tubulin fibre texture. It was found that enterocytes of the initial duodenal sections from the study group subjects had cellular morphological changes similar to enterocyte changes detected in the untreated coeliac patients. It was concluded, based on these findings, that the quantification of the enterocyte shape, nuclei, and tubulin fibre in duodenal tissue sections could be used for identification of patients with an early gluten sensitive enteropathy.

Subsequently, the protein expression of matrix metalloproteinases (MMP-1, -3, -9) and their tissue inhibitor (TIMP-1) was investigated in the GSE patients and in the study group subjects. It was demonstrated that the expression of MMPs-1, -3 and -9 and TIMP-1 was significantly increased in all subjects examined compared with normal controls. This correlated with the grade of mucosal damage. Based on our findings of increased MMPs -1, -3, -9 and TIMP-1 expression in the gut mucosa of patients with GSE, it was proposed that these enzymes have an important role in the process of tissue remodelling and/or destruction in GSE.

The presence of serum IgA-EMA is the most sensitive and specific marker for detecting individuals who have early immune responses to dietary gluten. The

findings of this thesis demonstrated that individuals with positive EMA and normal histology have subtle tissue immunological changes. This should help clinicians and histopathologists to closely monitor such patients and make an appropriate early decision with regard to diagnosis.

CHAPTER 1.0

GENERAL INTRODUCTION

1.1 THE GUT-ASSOCIATED LYMPHOID TISSUE

The mucosal associated lymphoid tissue (MALT) is a peripheral lymphoid organ found in the mucosal tissue of respiratory, urogenital or gastrointestinal tracts. In the gut, the immune defence system is known as the gut-associated lymphoid tissue (GALT), and it is estimated that GALT constitutes approximately 40% of the body's immune effector cells (O'Dorisio *et al.*1986) (Figure 1.1). The GALT which functions to protect against luminal antigens, includes the Peyer's patches, mesenteric lymph nodes and the lymphocytes of the lamina propria and epithelium, which are dispersed throughout the intestinal tract (Köhne *et al.* 1996).

Antigens can access the GALT via two routes; through M cells, microfolded epithelial cells overlying the Peyer's patches and through villous enterocytes (Köhne *et al.* 1996). Peyer's patches are structurally similar to other secondary lymph nodes i.e. they consist of a varying number of follicles with a central dome of B cells surrounded by T cells and macrophages (Janeway and Travers 1997). Naive T and B cells encounter antigen in the Peyer's patch and become activated. They then leave via mesenteric lymph nodes, the lymphatic system and the thoracic duct and re-circulate back to mucosal surfaces where they act as effector cells of the intestine mucosa (Köhne *et al.* 1996; Mowat and Viney 1997; Mowat *et al.* 2003).

Lymphocyte homing to the mucosa is achieved by the upregulation of adhesion molecules belonging to the integrin family, namely $\alpha4\beta7$ and $\alpha E\beta7$ (Shaw and Brenner 1995). $\alpha4\beta7$ is expressed on most lamina propria lymphocytes (LPLs). This integrin binds to its endothelial ligand, mucosal addressing cell adhesion molecule 1 (MAdCAM-1), which is selectively expressed by venules in mucosal tissues (Shaw and Brenner 1995; Butcher *et al.* 1999). $\alpha4\beta7$ is important for the movement of blasts from the Peyer's patches into the intestinal mucosa (Hamann *et al.* 1994). $\alpha E\beta7$ is expressed on 40% of LPLs and nearly all intraepithelial cells (IELs). This integrin ligates to E cadherin, which is expressed on enterocytes (Kilshaw 1999), and is thought to be involved in maintaining IELs within the epithelial layer (Cepek *et al.* 1994).

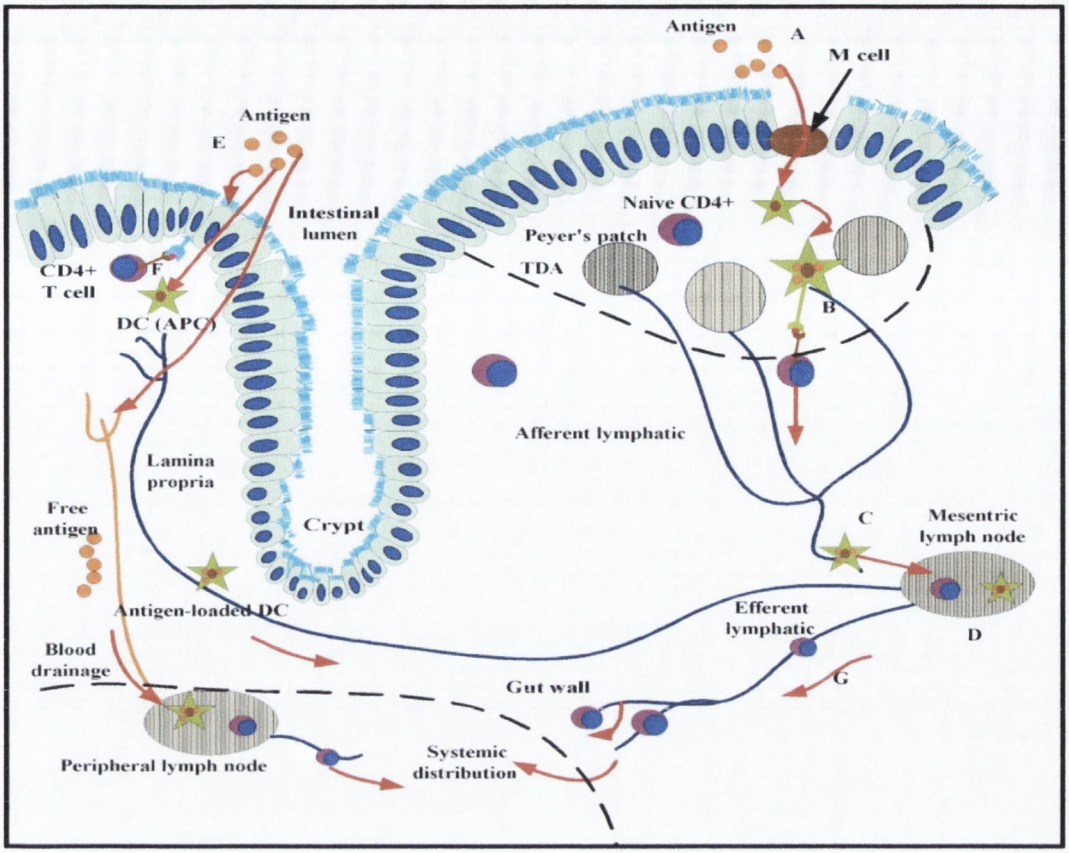


Figure 1.1 The fate of ingested antigen. Antigen may enter the GALT via the M cell route (**A**) and be processed by local antigen presenting cells such as dendritic cells (**B**). Antigen loaded dendritic cells leave the Peyer's patch via the draining lymph (**C**) and migrate to the mesenteric lymph node (**D**). Antigen may also enter through the intestinal epithelium (**E**) with the enterocyte acting as an antigen presenting cell (**F**). In either case, T cells leave the mesenteric lymph nodes via the efferent lymph (**G**). Antigen may gain direct access to the bloodstream (**H**) and interact with T cells in the peripheral lymphoid tissues (Image adapted from Mowat *et al.* 2003).

Chemokines also regulate the directional trafficking of lymphocytes and endothelial cell activation essential to lymphocyte migration (Dwinell *et al.* 2003). After the adhesion molecules have tightly bound the lymphocytes, chemoattractants direct the migration of the lymphocytes which crawl along the endothelium where chemokines are deposited in a solid phase, and move into the tissue along an immobilised gradient of chemotactic signals (Brandtzaeg *et al.* 1999). CCR9 is expressed by almost all small intestinal lymphocytes in humans. CCR9 allows T cells to respond to the chemokine CCL25, which is expressed by the enterocytes (Campbell and Butcher 2002; Kunkel *et al.* 2000). Dendritic cell (DC) migration is thought to be largely under the control of chemokines and chemokine receptors (Bilsborough and Viney 2004).

1.2 COELIAC DISEASE

1.2.1 History of coeliac disease

Coeliac disease is a chronic inflammatory disease of the intestine that is triggered in genetically susceptible individuals by ingestion of gliadin. Galen wrote the first description of childhood and adult coeliac disease around the 2nd century A.D. Much later in 1888, Samuel Gee described the symptoms of coeliac disease in Britain and hinted that manipulations of diet could have an effect on the disease (Gee S, 1888). Dicke and colleagues reported that certain dietary cereal grains were harmful to children with coeliac disease and they reported that coeliac patients improved during the 2nd world war as a result of a shortage of grain products (Dicke, *et al.* 1950). Dicke also demonstrated that wheat flour and not starch was responsible for the increased faecal fat seen in coeliac disease patients (Dicke *et al.* 1953). While other starch-containing foods were shown to be harmless, Dicke showed that rye, flour and oats had unfavourable effects on patients (Dicke *et al.* 1953).

In 1952 Anderson *et al.* demonstrated that the re-introduction of wheat into the diet of a coeliac disease patient resulted in loss of appetite, diarrhoea, loss of weight and change in temperament, thus inventing the "gluten challenge" that became a tool for coeliac disease diagnosis (Anderson *et al.* 1952). In 1954, Paulley first described the intestinal lesion, with its characteristic villous atrophy and inflammation (Paulley 1954). The existence of this inflammatory change was

confirmed in several patients. In the 1980s, Marsh and co-workers emphasized the role of the immune system in causing the intestinal injury in coeliac disease (Marsh 1980).

1.2.2 Epidemiology

Coeliac disease is a disorder that occurs primarily in Caucasians, particularly from countries of Western Europe and those countries to which Europeans have emigrated, specifically North America and Australia. Coeliac disease patients have been reported in the Middle East (Rostami *et al.* 2004), South America (Trevisiol *et al.* 2004), North Africa (Bouguerra *et al.* 2005) and Asia (Malekzadeh *et al.* 2005). The highest recorded prevalence ranges from 1:100 to 1: 200 in the European population (Grodzinsky *et al.* 1996; Catassi *et al.* 1996; Kolho *et al.* 1998; Csizmadia *et al.* 1999; Hill *et al.* 2000; Ciclitira *et al.* 2005). Coeliac disease is sometimes compared to an iceberg model (Figure 1.2). The tip of this iceberg represents clinical cases but the submerged portion represents silent and subclinical cases (Collin *et al.* 1997). The size of the submerged iceberg appears to vary from country to country and may reflect differences in access to health care or in recognition of symptoms. The majority of people with coeliac disease are asymptomatic or have mild symptoms and do not approach health care professionals for a diagnosis. Individuals with positive coeliac disease serology and flat biopsy but no symptoms, are described as having "silent" coeliac disease.

It is clear from epidemiological studies that there are a substantial number of undiagnosed cases in the general population; possibly 10 times as many as actually have been diagnosed (Catassi *et al.* 1994; Green and Jabri 2003). Individuals with abnormal serological results but no mucosal lesion have been described as having latent or potential coeliac disease (Wong *et al.* 2003). In adults the peak incidence of coeliac disease diagnosis is in the fifth decade (Feighery 1999). This might be due to the delayed introduction of gluten into the diet in childhood and increased diagnosis of older children and adults with atypical symptoms (Murray *et al.* 1999; Kennedy and Feighery 2000). Females are more commonly affected than males with a female to male ration of 3:1 having been reported (Feighery 1999).

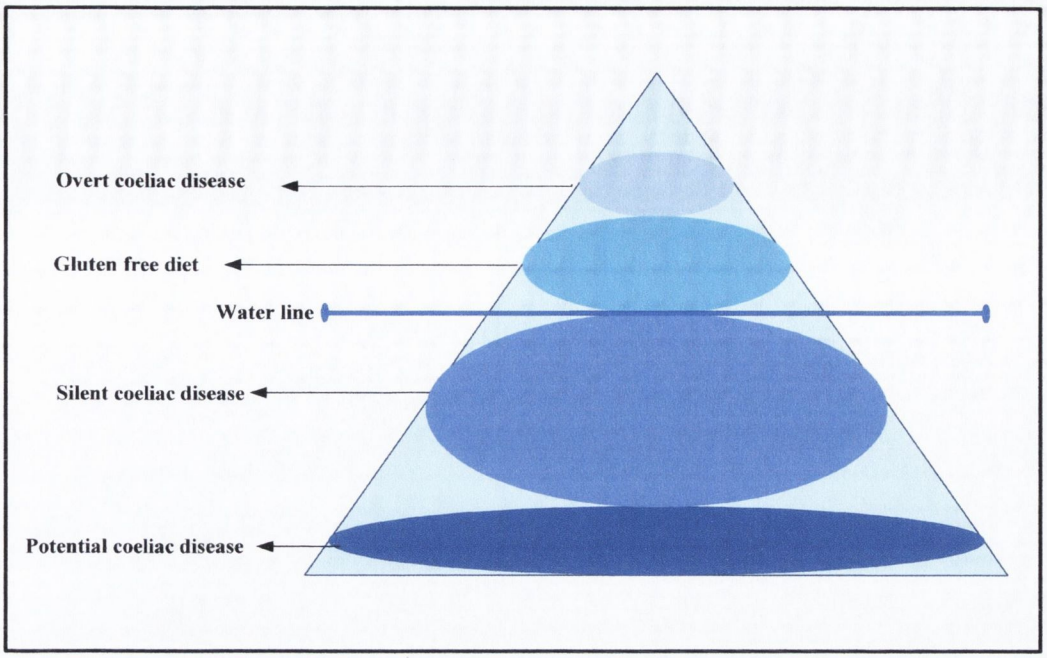


Figure 1.2 The iceberg model of coeliac disease.

1.2.3 Clinical Presentation of Coeliac Disease

The clinical presentation of coeliac disease is highly variable and can include diarrhoea, steatorrhoea, abdominal bloating, cramps, flatulence, weight loss, weakness and fatigue. Coeliac disease can also present with anaemia, reduced bone mineral density, infertility, mouth ulcers, angular stomatitis, failure to thrive, dermatological and neurological disorders, as an incidental finding during an unrelated endoscopic investigation and increasingly by screening of asymptomatic individuals on the basis of family history (Green and Jabri 2003). Those affected suffer intestinal mucosal damage to the villi and crypt region of the intestinal mucosa when they eat specific food-products containing wheat, rye and barley (Dessi *et al.*1993).

1.2.3.1 Potential coeliac disease

Individuals who have typical coeliac disease serology but with normal or minimal small intestinal mucosal changes have been described as having latent or potential coeliac disease (Wong *et al.* 2003). The natural history of this presentation is not understood but anecdotally some individuals have been reported to progress to villous atrophy and clinical manifestations compatible with coeliac disease. Traditionally, a diagnosis of coeliac disease was made on finding mucosal

abnormalities equivalent to a Marsh III lesion. It is now clear that many individuals have gluten-sensitive inflammation without villous atrophy (Kaukinen *et al.* 2001; Mino *et al.* 2003; Settakorn *et al.* 2004). Patients have borderline histological abnormalities that improve on a gluten-free diet (Tursi *et al.* 2003). Although some of these patients developed the typical coeliac lesion, it is not yet known whether these individuals have the same adverse health risks as the traditional coeliac patient with villous atrophy (Collin *et al.* 1993; Paparo *et al.* 2005). Clearly, if an individual has symptoms or clinical manifestations attributable to coeliac disease, a gluten-free diet should be advised (Kaukinen *et al.* 1998).

The decision is more difficult in the case of an apparently healthy person with positive coeliac serology and macroscopically normal intestinal mucosa. It may be difficult to convince such a person to follow a gluten free diet, although some asymptomatic individuals report unexpectedly feeling better on gluten free diet (Tursi *et al.* 2003). Further work is required to characterise the natural history and relative health risks for borderline lesions. However, it may be prudent at least to follow-up these patients in clinical practice to look for the development of potential complications such as anaemia and osteoporosis. An increase of IEL number is a non-specific finding for patients with coeliac disease, as increased IEL count is found in other conditions such as inflammatory bowel disease.

In one study, approximately 10% of individuals with an unexplained increased IEL count went on to be diagnosed with coeliac disease, although suspected coeliac patients already had been excluded (Kakar *et al.* 2003). An increased IEL count in itself is insufficient to diagnose coeliac disease and requires correlation with clinical and serologic parameters. However, not all individuals with these minor abnormalities will be identified using coeliac antibody testing. Many early studies reporting on the sensitivity of coeliac antibody tests focused on Marsh III lesions and did not include many cases with minor gut changes. The literature suggests that the sensitivity of antigliadin, anti-tissue transglutaminase (anti-tTG), and anti-endomysial antibodies (anti-EMA) may be much lower in Marsh I and II lesions (Rostami *et al.* 1999), which further adds to the diagnostic dilemma.

1.2.3.2 Coeliac disease in children

Classic coeliac disease typically presents between six and 24 months of age with impaired growth, abnormal abdominal distension, muscle wasting, hypotonia, poor appetite, and irritability. Vomiting commonly occurs in very young infants (Catassi *et al.*1997). Numerous other symptoms may result in the patient being brought for advice. Untreated coeliac children typically pass soft, bulky clay-coloured stools, but watery diarrhoea or constipation have occasionally been reported. In addition, recurrent abdominal pain, hypertransaminasemia, recurrent aphthous stomatitis, or dental enamel defects have also been reported (Aine *et al.* 2000). Older children tend to have more varied symptoms and present with symptoms of anaemia, rickets or failure to thrive, resulting in their falling below the third percentile for weight. Once a gluten-free diet is commenced, it has been well documented that the patient's growth rate returns to the normal. Previously, coeliac disease was thought to be a disease primarily of infancy; however, with the widespread delay in introduction of wheat gluten into the infant diet, the clinical manifestations have become more subtle, and diagnosis is now typically made in older children and adults (Maki *et al.* 1988; Murray *et al.* 2003; Fasano and Catassi 2001)

The prevalence of childhood coeliac disease has been reported to be between 0.35% and 1.16% in Sweden (Cavell *et al.* 1992; Carlsson *et al.* 2001), between 1% and 1.5% (HLA, anti-EMA, anti-tissue transglutamenaise; tTG) in Finland (Maki *et al.* 2003), between 0.44% (biopsy proven) and 0.94% in Italy (Catassi *et al.* 1995; Tommasini *et al.* 2004), and between 1% and 4% (IgA anti-tTG) in the US (Hoffenberg *et al.* 2003). Other non-European Caucasian populations such as Israel, Argentina, New Zealand and Australia have reported similar rates (Shamir *et al.* 2002; Gomez *et al.* 2001; Cook *et al.* 2000; Hovell *et al.* 2001).

1.2.3.3 Coeliac disease in adults.

Many adult patients have no previous symptoms of disease, suggesting that coeliac disease may develop for the first time in adult life (Corazza *et al.* 1995). Proportions of these adult patients have short stature or give a history consistent with undiagnosed childhood coeliac disease. The finding of the typical lesion in asymptomatic relatives of coeliac disease patients suggests that adults may have

clinically unapparent coeliac disease for some time (MacDonald *et al.* 1965). Coeliac disease is being increasingly diagnosed in the elderly; currently about 20% of cases are diagnosed after the age of 60 years (Mulder and Cellier 2005). However, in adults the classic signs of steatorrhoea, weight loss and fatigue are now rarely encountered.

The small intestinal mucosal lesion in the coeliac small intestine results in the malabsorption of nutrients (Jewell *et al.* 2000). Coeliac disease patients are at a high risk of developing osteopenia or osteoporosis as a result of impaired calcium and vitamin D uptake (Mora *et al.* 1999; Ciclitira *et al.* 2001). Iron deficiency anaemia is common among coeliac disease patients that may result in fatigue (Corroccio *et al.* 1998; Hin *et al.* 1999). Enteropathy associated T cell lymphoma (EATL) is reported in coeliac disease patients with poor compliance with gluten free diet (GFD) (O`Mahony *et al.* 1996).

1.2.3.4 Dermatitis herpetiformis

Dermatitis herpetiformis (DH) is an extremely itchy blistering skin rash associated with gluten sensitive enteropathy (Murray *et al.* 1999). The incidence of dermatitis herpetiformis was described as 11 per 100,000 population in a North American study but reports of incidence vary (Zone 2005). DH affects men slightly more frequently than women (Nicolas *et al.* 2003). The genetic similarity to coeliac disease, response of the skin rash to a gluten free diet and recently the reproduction of DH in a HLA transgenic animal model of intestinal gluten sensitivity has confirmed that DH is part of the spectrum of gluten sensitivity (Marietta *et al.* 2004).

The gluten sensitive enteropathy associated with DH is similar to but usually less severe than that found in coeliac disease patients (Egan *et al.* 1997; Gawkrodger *et al.* 1991). The enteropathy in DH is patchy in distribution and the histological lesion is often less marked with raised IEL count the only abnormality in one-third of patients (Fry *et al.*1995). Evidence shows that most patients with DH without small intestinal villous atrophy have mild mucosal lesions consistent with Marsh I–II (Reunala *et al.* 1984). The association of subtle changes (Marsh I–II) in some patients with DH supports the hypothesis that these patients represent a subtle

manifestation of gluten-sensitive enteropathy. Immunohistochemical staining of IELs also shows a pattern similar to that observed in coeliac disease (Reunala *et al.* 1984). Ferguson and co-workers showed that the number of IELs increased by additional gluten challenge in patients with DH and normal small intestinal architecture (Ferguson *et al.* 1987).

It has been demonstrated that the predominant autoantigen of dermatitis herpetiformis is epidermal transglutaminase (Sardy *et al.* 2002). The targeting of epidermal transglutaminase by DH IgA helps explain the skin manifestations seen in this subclass of gluten-sensitive disease. DH patients also produce autoantibodies directed against tTG and EMA (Chorzelski *et al.* 1983; Dieterich *et al.* 1999; Kumar *et al.* 2001; Karpati 2004). Epidermal transglutaminase and tTG share an overall homology of 38% at the amino acid level, but with up to 64% homology in certain regions (Kim *et al.* 1993).

1.2.5 Diagnosis of Coeliac Disease

The diagnosis of coeliac disease is based on clinical symptoms (discussed earlier), serological markers and intestinal biopsies.

1.2.5.1 Serological markers

Anti-reticulin antibodies

Coeliac patients were found to have serum antibodies that bound to antigens in rat kidney, liver and stomach, which are known as anti-reticulin antibodies (ARA) (Seah *et al.* 1971). Immunofluorescence staining produced a distinctive staining pattern (Hallstrom *et al.* 1989). A study by Collin and colleagues reported that IgA ARA antibody titre might identify coeliac disease in asymptomatic patients (Collin *at al.*1990). IgA ARA gave a sensitivity of 97% and a specificity of 98% (Maki *et al.*1995). However, this antibody has been superseded by anti-EMA and tTG antibodies tests.

Anti-gliadin antibodies

Coeliac patients produce antibodies that are directed towards gliadin (Maki *et al.*1995). However, the anti-gliadin antibody (AGA) test was one of the first tests

developed for the serological diagnosis of coeliac disease and was introduced in the late 1970s (Haeney and Asquith 1978). Enzyme linked immunosorbent assays (ELISA) detecting serum IgA and IgG antibodies were first used in the early 1980s to diagnose coeliac disease. AGA had only a moderate sensitivity (IgA; 75-80%, IgG; 69-80%) and specificity (IgA; 80-90%, IgG; 80-90%) (Farrell and Kelly 2001; Rostom A *et al.* 2005). Positivity for gliadin antibodies seems to increase with age in the normal population (Uibo *et al.* 1993). AGA can also be detected in other gastrointestinal conditions such as inflammatory bowel disease or milk protein intolerance as well as occurring in normal individuals (Uibo *et al.* 1993). AGA tests only retain their diagnostic usefulness in cases of IgA deficiency (IgG-AGA only) or patients younger than 2 years old. There is a better correlation between gliadin antibodies and villous atrophy in children than in adults (Maki *et al.* 1995).

Anti-endomysial antibodies

The development of serologic testing to evaluate people with suggestive symptoms of gluten sensitive enteropathy (GSE) and high-risk populations greatly facilitated the study of coeliac disease. Anti-endomysial antibodies (EMA) were first tested for in 1983 (Chorzelski *et al.* 1983). The presence of IgA EMA is very sensitive and specific predictor of coeliac disease (Chorzelski *et al.* 1983; Dieterich *et al.* 1998; Feighery *et al.*1998; Burgin-Wolff *et al.*1997; Burgin-Wolff *et al.* 2002). By using monkey oesophagus as substrate, EMA can be visualised in an indirect immunofluorescence assay and produce a brilliant green fishnet pattern. The test has high sensitivity and specificity (90% -100% and 100% respectively) (Pearce *et al.* 2002; Collin *et al.* 2005; Rostom *et al.* 2005).

These antibodies do not only support the diagnosis, but also predict the severity of mucosal damage in patients with GSE (Szaflarska-Szcepanik *et al.* 2001). It is of particular use in diagnosing latent coeliacs since EMA positive individuals with normal mucosa often later develop a flat mucosa (Collin P *et al.*1993; James and Scott 2000). Like IgA AGA, IgA EMA will be absent in individuals with coeliac disease associated with IgA deficiency. Selective IgA deficiency affects 2-5% of patients diagnosed with coeliac disease (Cataldo *et al.* 1997). Most of the serological tests measure antibodies of the IgA immunoglobulin class therefore, a negative result will occur in patients with coeliac disease who are IgA deficient.

IgG tests for gliadin and endomysial antibodies will usually be positive in IgA deficient patients with coeliac disease (Cataldo *et al.* 2000).

Anti-tissue transglutaminase antibodies

The identification of the tissue transglutaminase (tTG) as an auto-antigen of EMA has made possible the development of quantitative, reproducible immunoassays for anti-tTG-antibody providing a reliable alternative to immunofluorescence for EMA (Dieterich *et al.*1997). Subsequently, ELISA, for diagnosing coeliac disease was established using guinea- pig tTG as an antigen (Sulkanen *et al.* 1998). However, it has been reported that the sensitivity of guinea pig tTG assay in screening for coeliac disease ranges from 84% to 98% and the specificity was 98%. The specificity and sensitivity for human-tTG was 99% and 96% respectively (Hansson *et al.*2000; Rostom *et al.* 2005), correlating more closely with the EMA test.

These serologic tests (EMA and tTG antibodies) are very sensitive and specific and may detect affected individuals before intestinal injury characteristic of coeliac disease occurs. While the IgA anti-tTG is a good predictor of coeliac disease, the IgG anti-tTG test has been reported to have lower sensitivity and specificity (Dieterich *et al.* 1998; Sulkanen *et al.* 1998). In one study, the IgG anti-tTG sensitivity was only 13% (Feighery *et al.* 2003). Because the IgG classes of the EMA and tTG antibodies tests are not well validated, patients with clinical evidence suggestion of coeliac disease and who are IgA deficient should undergo small intestinal biopsy to confirm the diagnosis.

Anti-actin antibodies

Recently, it has been suggested that the presence of anti-actin antibodies (AAAs) correlates with severe mucosal damage in coeliac disease (Clemente *et al.* 2000; Clemente *et al.* 2004). AAAs have been detected using both immunofluorescence (Clemente *et al.* 2003) and ELISA techniques (Granito *et al.* 2004). Actin is one of the major components of cytoskeletal microfilaments and is regarded as the target of smooth muscle antibodies detected by indirect immunofluorescence on kidney sections and is closely associated with type I autoimmune hepatitis (Bottazzo *et al.* 1976). A multi-centre study showed that the AAA ELISA test might represent a serological marker that correlates well with mucosal damage in

coeliac disease patients (Clemente *et al.* 2004). AAAs is a reliable marker of severe intestinal mucosa damage in coeliac disease patients, and a simple ELISA technique offers an accurate method for their determination (Carroccio *et al.* 2005).

1.2.5.2 Small intestinal biopsy

Initially the diagnosis of coeliac disease was based on the identification of the classical symptoms such as steatorhea, weight loss, and malnutrition. Later, after the development of the duodenal biopsy technique, the requirement for histological confirmation was added. The requirement for three biopsies to diagnose coeliac disease was proposed by European Society for Pediatric Gastroenterolgy and Nutrition (ESPGN) in 1970 (Meeuwisse *et al.*1970). In 1990, the revised ESPGN criteria required a single biopsy with characteristic coeliac disease lesions and unequivocal clinical response to gliadin (ESPGN 1990). The diagnosis of coeliac disease currently rests on the histological demonstration of the characteristic lesion in the small intestine and the subsequent clinical response after the introduction of gluten free diet. There may be only minimal changes on biopsy even when significant symptoms are present. The initial event observed is an increase in IEL count, followed by infiltration of the lamina propria with lymphocytes. Crypt hyperplasia precedes villous atrophy and is only observed in the presence of lamina propria lymphocytosis, suggesting that intraepithelial lymphocytosis is not sufficient for intestinal transformation in coeliac disease (Marsh 1989). Biopsies should always be performed while ingesting a liberal unrestricted diet or after gluten challenge to ensure expression of histological abnormality. Patients may come for biopsy as a result of clinical suspicion, positive serological tests for unrelated reasons (Green and Jabri *et al.* 2003).

1.3 PATHOGENESIS OF COELIAC DISEASE

The mechanisms responsible for tissue damage in coeliac disease are only partially understood. It is not clear how gluten interacts with the intestinal mucosa and induces injury, what genetic defects or abnormal gene predispose to the disorder, or what other exogenous antigens induce this condition. However,

the following three factors may influence the development of coeliac disease, namely genetic factors including gliadin and tissue transglutaminase.

1.3.1 Genetics of coeliac disease

A high prevalence rate (10%) among first-degree relatives of coeliac disease patients indicates a strong genetic influence on predisposition to coeliac disease (Myllotte *et al.* 1974). The strong genetic influence in coeliac disease is further supported by a high concordance rate of 75% in monozygotic twins (Walker-Smith *et al.*1973). The sibship aggregation attributable to HLA is estimated to be 2.3-5.5 (Petronzelli *et al.* 1997). Looking at these estimates and assuming a multiplicative model of disease predisposing genes, the overall importance of non- HLA genes has been calculated to be greater than that of HLA genes.

The HLA and non-HLA genes shape the immune response to gluten so that immunopathology is produced in the small intestine. In the 1970s, it was reported that coeliac disease was associated with the HLA class I molecule B8 (Stokes *et al.* 1972). Later strong associations were found to the HLA class II molecules DR3 and DQ2 (Tosi *et al.* 1983). The genes for DR3 and DQ2 are both contained within the B8-DR3-DQ2 or the D18 –DR3-DQ2, extended haplotypes. The B8-DR3-DQ2 and B18-DR3- DQ2 haplotypes are both associated with coeliac disease (Congia *et al.* 1992). This is significant, as these two haplotypes are conspicuously dissimilar in the region outside the DR-DQ region.

The function of the DQ2 molecules is to present exogenous peptide antigens (gliadin peptides in the case of coeliac disease) to helper T cells (Eisenlohr and Rothstein 2005) and expressed as antigen presenting cells (APCs) such as monocytes, macrophages and dendritic cells. The evidence for the involvement of DQ2 provided by initial genetic association studies was strengthened by subsequent functional studies. An immuno-dominant gliadin peptide was shown to be recognised by DQ2-restricted T cells in the intestine (Arentz-Hansen *et al.* 2000) and in peripheral blood after antigen challenge (Anderson *et al.* 2000). The crystal structure of dominant epitope-DQ2 binding was also resolved (Kim *et al.* 2004).

The HLA-DQ heterodimer can be encoded in *cis* (on the same haplotype) or more rarely in *trans* where the monomer subunits are encoded on separate haplotypes (van Heel *et al.* 2005). Although the *cis* and *trans* variants are known to vary by one amino acid in each subunit, the ability of the variants to present antigen is thought to be identical (Figure 1.3).

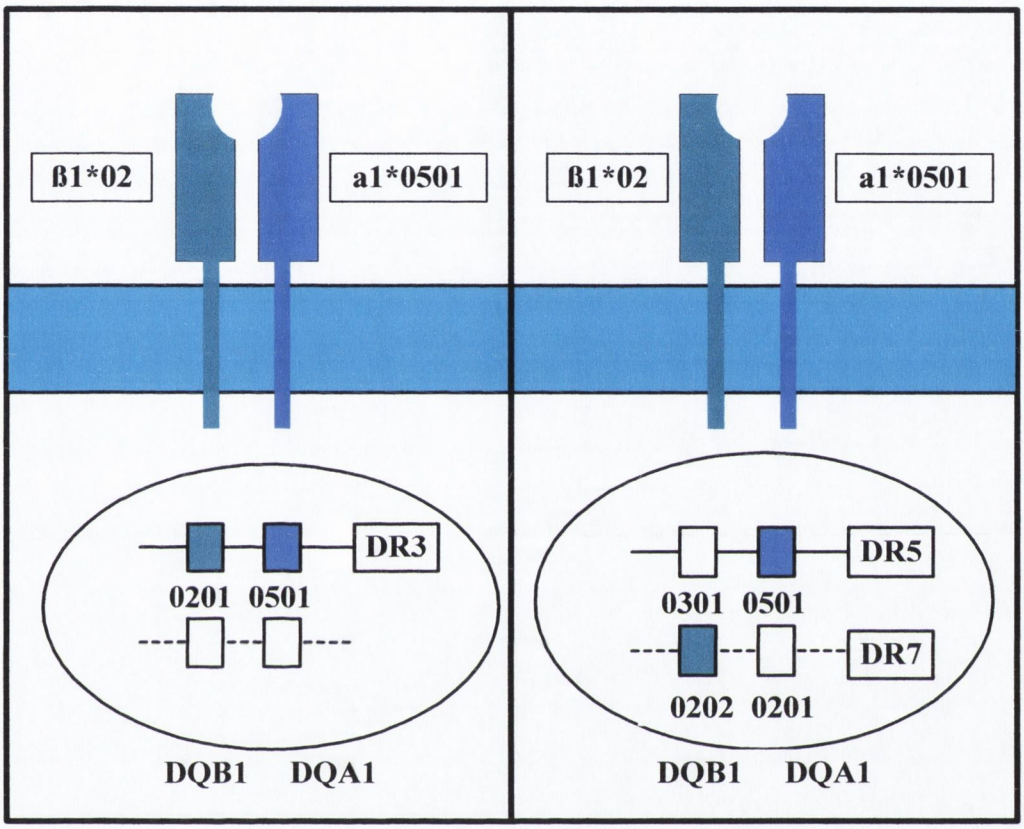


Figure 1.3 The structure of HLA-DQ2 molecule. Individuals who are DR3 or DR5/DR7 heterozygous express the same HLA-DQ2 molecule, HLA-DQ (α1*0501, β1*02). The DQA1*0501 and DQB1*02 genes are located in *cis* (on the same chromosome) in DR3 individuals, whereas they are located in *trans* (on opposite chromosomes) in DR5/DR7 heterozygous individuals. (Image adapted from Sollid *et al.* 2000).

Coeliac disease is also associated with DR7 (Betuel *et al.* 1980). But this association is seen almost only when DR7 occurs together with DR3 or DR5 (Trabqce *et al.* 1984; Tighe *et al.*1993). However, most coeliac patients carry the DR3-DQ2 haplotype or alternatively are heterozygous DR5-DQ7 / DR7-DQ2 (Sollid and Thorsby 1993). Coeliac disease patients with these DR-DQ

combinations share the genetic information conferring coeliac susceptibility. The DQ α1*0501 and DQ β1*0201 alleles of the DR3-DQ2 haplotype are also found when the DR5-DQ7 and DR7- DQ2 haplotypes are combined (Shenning *et al.* 1984). Further more, the DQ α1 and DQβ1 are good candidates because their products interact by forming class II heterodimer, suggesting that most coeliac disease patients share a particular pair of DQα1 and DQβ1 genes, so DQα1*0501 and DQβ1*0201 alleles jointly confer susceptibility to coeliac disease by coding for the DQ (α1*0501,β1*0202) heterodimer. However, more than 90% of coeliac patients carry the DQ (α1*0501,β1*02) heterodimer, compared to 20%-30% in healthy controls (Sollid *et al.* 1989; Peña *et al.* 1998). Depending on the populations studied, about 2%-10% of coeliac disease patients do not carry the DQ (α1 *0501 and β1 *02) heterodimer. The great majority of these patients carry different subtypes of DR4 (Sollid *et al.* 1993).

1.3.2 Gliadin

The exact structure of the part of gluten that causes the damage in coeliac disease remains unclear. Wheat grains have three major components that are separated by milling: the outer husk or bran, the germ and the endosperm. Three types of cereal protein are known: structural, metabolic and storage proteins. Storage proteins make up 70-80% of total grain protein and occur exclusively in starchy endosperm. There are two main groups of wheat endosperm proteins; the prolamins (ethanol-soluble fraction) and the glutenins. Wheat proteins are divided into classes according to their solubility characteristics: gliadins are soluble in 40–90% ethanol, whilst glutenins are insoluble in neutral aqueous solutions, saline and ethanol. The gliadins may be further subdivided into α, β, γ and Ω subfractions, either according to their relative electrophoretic mobility, or according to their N-terminal amino acid sequences. Molecular masses from gliadins range from of 32–58 kDa.

Dicke discovered that the toxic fraction in wheat resided in the flour fraction of wheat (Dicke *et al.* 1953). Frazer and colleagues demonstrated that a soluble extract resulting from a peptic-tryptic digest of gluten- denoted as Frazer's Fraction III- remained toxic to coeliac patients (Frazer *et al.* 1959). He showed that the majority of these peptides were toxic to coeliac patients in remission. It

has been reported that α-gliadin shares an eight amino acid sequence homology, in a span of 12 amino acids, with an identical pentapeptide with the 54 kDa E1b protein of the human adenovirus 12, an adenovirus commonly isolated from the gastrointestinal tract (Kagnoff *et al.* 1984; Kagnoff *et al.* 1987).

Glutamine comprises 35% of the amino acids in gliadin and may play a central role in its toxicity. α-gliadin sequences were published by Kasarda *et al.* (1984). Following this, the toxicity of various synthetic peptide sequences from α-gliadin has been assessed using coeliac small bowel biopsy organ culture techniques (De Ritis *et al.* 1988; Shidrawi *et al.* 1995). Toxicity was shown using peptide sequences corresponding to residues 3–24, 25–55, 31–55, and 1–30 of α-gliadin. Gjertsen and colleagues reported that a peptide corresponding to amino acid residue 31–49 was recognised by $CD4^+$ T-cells obtained from the peripheral blood of a patient with coeliac disease when presented by HLA-DQ2 heterodimer (Gjertsen *et al.* 1994).

Coeliac disease patients on a gluten-containing diet have increased levels of serum antibodies specific for various antigens, including gliadin and the autoantigen tissue transglutaminase (tTG) (Maki *et al.* 1995, Dieterich *et al.* 1997; Molberg *et al.* 1998). Recently it has been shown that tTG causes selective deamidation of gluten proteins, which increases their stimulating effect on gluten-sensitive T-cells obtained from the small intestine of patients with coeliac disease. Immunohistochemical staining has shown that in the active coeliac lesion, tTG is expressed at the epithelial brush border, as well as being expressed extracellularly in the subepithelial region (Molberg *et al.* 1998). Interestingly, active tTG enzyme is expressed at the surface of macrophages and monocytes after activation (Akimov *et al.* 2001) and its expression is induced in dendritic cells during their maturation (Le Naour *et al.* 2001). It has been postulated that this results in neoepitopes in wheat proteins, which are then involved in the disease pathogenesis (Molberg *et al.* 2003). The cereals rye and barley belong to the same tribe as wheat and are known to be toxic in coeliac disease. Oats, taxonomically derived from a different group within the cereal family, appear to be safe with the result of a recent study concluding that patients with coeliac disease remained well whilst ingesting oats daily over a 5-year period (Janatuinen *et al.* 1995; Kilmartin *et al.* 2003; Lundin *et al.* 2003; Storsrud *et al.* 2003).

Since then, a vast number of epitopes within α- and γ-gliadin have been discovered which in most cases require prior deamidation, although the focus has been placed on epitopes within the 51-89 fraction of α-gliadin (Arentz-Hansen *et al.* 2000; Anderson *et al.* 2000; Arentz-Hansen *et al.* 2002; Shan *et al.* 2002; Martucci *et al.* 2003). An algorithm was designed to predict epitopes within gluten, which described the specificity of deamidation based on preferential spacing between glutamine and proline (Vader *et al.* 2002a). This algorithm confirmed the previously described stimulatory α-gliadin peptide 57-68 as well as finding many more stimulatory peptides.

The antigenicity of these epitopes has been confirmed and examined further by another group. A series of synthetic analogues of immunodominant epitopes was produced, by substituting alanine for each amino acid in turn, and then these were tested using coeliac-derived T-cell clones. These single alterations abolished or dramatically attenuated T-cell stimulation indicating the high specificity of MHC binding affinity (Ellis *et al.*, 2003). Key anchor residues are required at positions 1, 4, 6, 7 and 9 of the HLA-binding groove but it was noted that any alteration within the groove led to reduced reactivity, suggesting that T cell receptor interaction is critically sensitive to amino acid structure (Figures 1.4, 1.5 and 1.6).

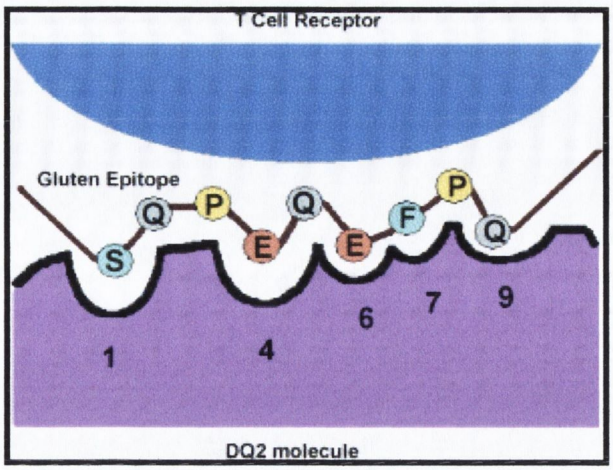


Figure 1.4 Diagrammatic representation of the interaction between the DQ2 molecule peptide-binding groove and an epitope from γ-gliadin. Key anchor points are at positions 1, 4, 6, 7 and 9. Negative charge is preferred at positions 4, 6 and 7. **E** represents deamidated residues with production of glutamic acid. **Q** and **P** denote glutamine and proline, respectively (Adapted from Dewar *et al.* 2004).

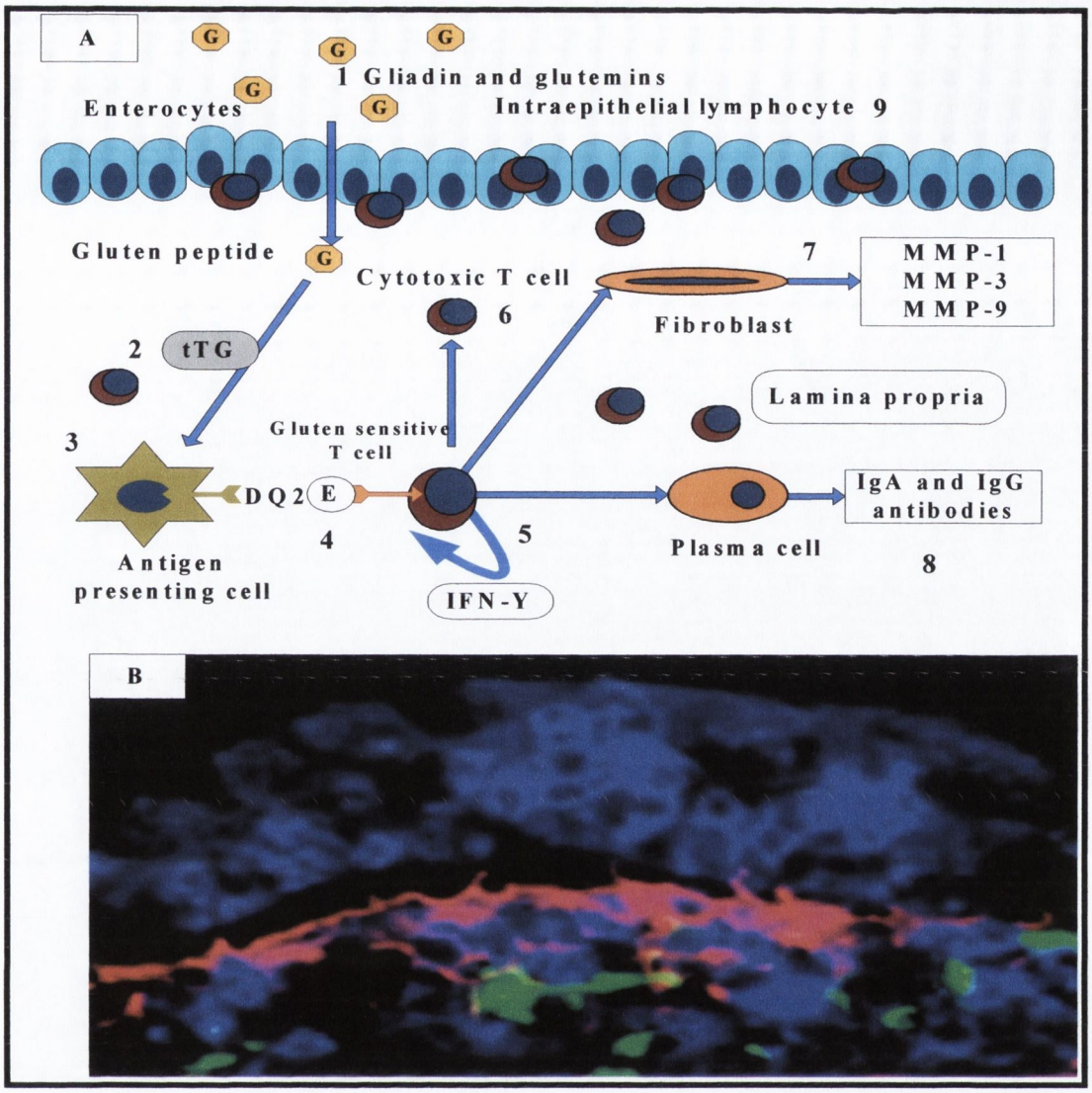


Figure 1.5 A. (1) Gluten is digested to yield peptides, which are transported into mucosa. (2) Glutamine residues are deamidated by tissue transglutaminase. (3) Epitope processing and presentation with DQ2 by antigen presenting cells. (4) Gluten-sensitive T cells recognize epitope and are stimulated. (5) Lamina propria lymphocytes proliferate and recruit cellular infiltrate. (6) CD8 T cells with cytotoxic markers increase in mucosa. (7) Fibroblasts are activated and produce metalloproteinases to degrade matrix. (8) Plasma cells produce disease-specific coeliac antibodies. (9) The role of primitive intra-epithelial lymphocytes remains unclear. B-Immunofluorescence staining of tTG (pink), HLA-DQ (green) and T cells (CD3; purple) in the small-intestine mucosa of an untreated coeliac-disease patient. Note that there is a close spatial relationship between tTG, APCs that express HLA-DQ and T cells just beneath the epithelium. (Immunofluorescent image adapted from Sollid *et al.* 2002).

Peptide sequence	Origin	Notes
LQLQPFPQPQLPY	α-gliadin	original overlapping immunodominant epitopes*
PQPQLPYPQPQLPY	α-gliadin	
QPQQSFPQQQ	γ-gliadin	minor epitope
VQGQGHQPPQQPAQL	γ-gliadin	deamidation not required[§]
QQPFQQQQQPLPQ	glutenin	no response in adults[†]
QQQQPPFSQQQQSPFSQQQQ	glutenin	no response in adults[†]
QPQPFPQQSEQSQQPFQPQPF	unknown	‡
QQXSQPQXPQQQQXPQQPQQF	unknown	deamidation not required[§]
PFRPQQPYPQPQPQ	α-gliadin	epitope unknown; deamidation not essential[†]
PYPQP QLP Y	α-gliadin	illustrates optimal tTG substrate[‖]
FPQQPQQPYPQQP	γ-gliadin	‡
FSQPQQQFPQPQ	γ-gliadin	optimal tTG substrate[‖]

Figure 1.6 Identified gluten epitopes in key studies. **Q** designates glutamine residues deamidated by tTG. The epitopes are clustered in proline and glutamine rich regions of the gluten genome and sequence homology can be seen between epitopes. Many Q × P sequences can be seen which act as optimal substrates for tTG. The minimum requirement for T cell reactivity is nine residues although optimal peptide size is 10–15 residues (Adapted from Dewar *et al.* 2004).

1.3.3 Tissue transglutaminase

Tissue transglutaminase (tTG) is one of six enzymes, called the transglutaminase family, which catalyse the post-translational modification of proteins in a calcium-dependent manner. tTG is found in many different tissue types and can be expressed both intracellularly and extracellularly (Piacentini and Colizzi 1999; Reif and Lerner 2004). Intracellularly, tTG was found to be involved in regulation

of several biological processes including cellular proliferation, differentiation and apoptosis (Griffin *et al.* 2002; Piacentini *et al.* 2002). tTG can be released extracellularly, particularly in response to tissue wounding and stress (Aeschilmann *et al.* 1994; Patel *et al.* 1985).

tTG catalyses the cross-linking of proteins by catalysing the formation of ε-(γ-glutamyl)-lysine or ε-(γ-glutamyl) polyamine bonds. This is known as transamidation (Freitag *et al.* 2004). In the absence of an appropriate glutamine acceptor, tTG can deamidate certain glutamine residues of donor proteins (Freitag *et al.* 2004). The ratio of deamidation to transamidation in the presence of primary amines is markedly increased when pH is less than 7.3, which is the case in the small intestine (pH 6.6) (Fleckenstein *et al.* 2002). Gliadin has been shown to act as a donor substrate for tTG, with glutamine residues being deamidated to glutamic acid, creating a negative charge on the protein (Caputo *et al.* 2004).

Transglutaminases are involved in the pathogenesis of many diseases through their cross-linking function such as neurodegenerative disease, coeliac disease and dermatitis herpetiformis (Karpuj and Steinman 2004; Kim *et al.* 2002; Dieterich *et al.* 1997; Ardy *et al.* 2002). tTG is the predominant autoantigen in the gluten-sensitive enteropathy, coeliac disease (Dieterich *et al.* 1997). Antibodies directed against tTG provide a very sensitive and specific test for coeliac disease (Reif and Lerner 2004). These antibodies are thought to occur when tTG cross-links to gliadin, acting as a hapten to generate autoantibodies (Caputo *et al.* 2004).

In recent years a role has been assigned to tTG in the molecular pathogenesis of coeliac disease (van de Wal *et al.* 1998). Anti-tTG antibodies from coeliac patients were found to inhibit fibroblast-induced differentiation of epithelial cells *in vitro*, suggesting a role for tTG in the mucosal remodelling seen in coeliac disease (Haltunnen *et al.* 1999). tTG has been shown to be necessary for activation of TGF-β which is also involved in the differentiation of intestinal epithelium (Kurokawa *et al.* 1987). TGF-β suppresses T-cell activity; hence, decreased TGF-β could lead to increased activation of T_H1 cells producing inflammatory cytokines (Letterio *et al.* 1998; Hansson *et al.* 2002).

1.4 IMMUNOLOGICAL CHANGES IN THE COELIAC LESION

1.4.1 Intestinal histological changes

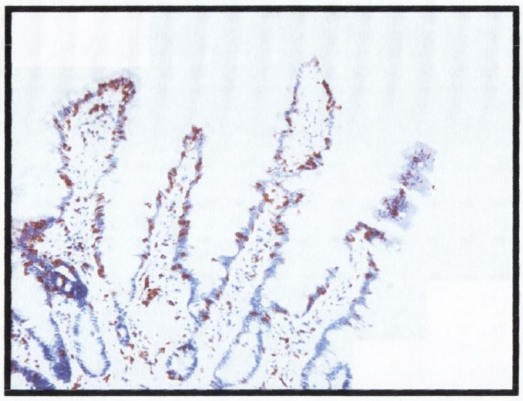
The inflammatory lesion in patients with coeliac disease is restricted to the small intestinal mucosa, because this is the site ordinarily in contact with gliadin. The earliest histological changes take place in the lamina propria beneath the epithelial cell layer (Shiner *et al.* 1973). There is evidence of increased vascular permeability, including swelling of capillary endothelia, tissue oedema and extravascular fibrinogen deposition, which is followed by an influx of leukocytes that cause a second round of vascular permeability changes (Halstensen *et al.* 1992).

In 1954, Paulley described the histological abnormalities in the intestinal lesion of the coeliac disease patients (Paulley 1954). In 1992, Marsh identified several histopathologic forms of the coeliac lesion that define the development of disease and its severity. Furthermore, he has classified the mucosal lesion into three stages; the infiltrative lesion (Marsh I) which is characterised by infiltration of IELs in the villous epithelium with normal mucosal architecture (Figure 1.8 A and B). A hyperplastic lesion (Marsh II) is characterised by enlarged crypts in which immature epithelial cells are being generated at an increased rate, accompanied by inflammatory cells, but the villi remain normal in length (Figure 1.8 C). The destructive lesion (Marsh III), in this stage the typical villous atrophy is the principal morphological feature (Marsh 1992; Marsh 1992 Oxford).

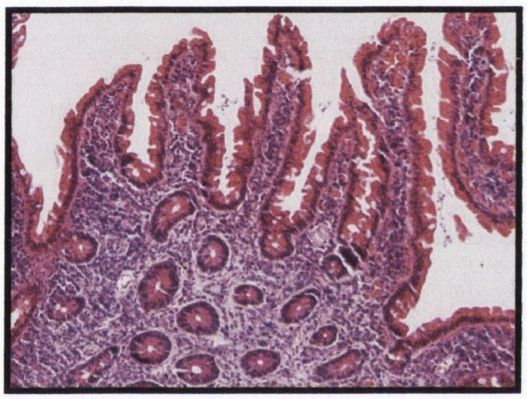
A more recent study by Rostami and colleagues, which further classified Marsh III stage into three subgroups including Marsh IIIa partial villous atrophy, this is characterised by shortened blunt villi and mild infiltration of lymphocytes in epithelial layer. Marsh IIIb subtotal villous atrophy, which is characterised by atrophic villi and Marsh IIIc describes a total absence of villi with, severe atrophic, hyperplastic, and infiltrative lesions (Figure 1.8 D, E and F) (Rostami *et al.* 1999; Amsterdam 2001). Thus, biopsy of the proximal part of the small intestine remains the gold standard for coeliac disease diagnosis (Green *et al.* 2005).



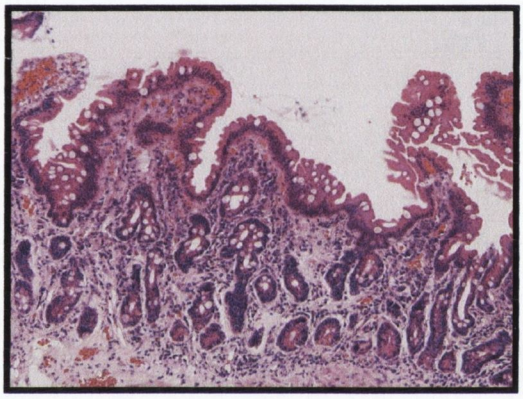
A.Marsh I: Infiltrative lesion (increased IELs).



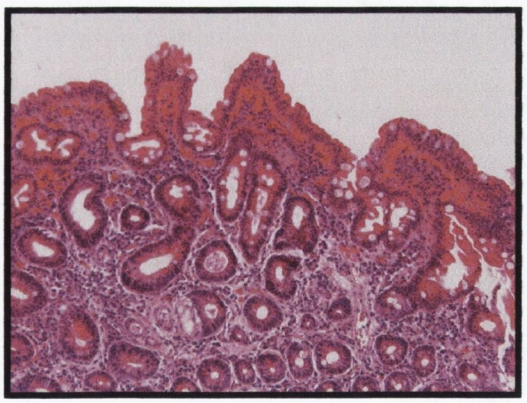
B. Intraepithelial lymphocytes stained with CD3



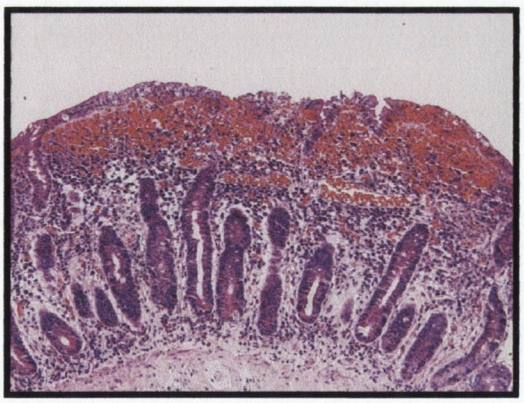
C. Marsh II: Hyperplastic lesion (villous blunting and increased IELs).



D. Marsh IIIa: Partial villous atrophy.



E. Marsh IIIb: Subtotal villous atrophy.



F. Marsh IIIc: Total villous atrophy

Figure 1.7 The Marsh classification of intestinal damage in coeliac disease.

1.4.2 Enterocytes

Increased loss and proliferation of epithelial cells are used to help try to explain the pathology involved in the villous atrophy in coeliac disease patients (Walker-Smith *et al.* 1989). It was suggested that the increased epithelial cell loss reflects increased apoptosis of enterocytes, whereas the increased enterocyte proliferation appears to be due to an increased production of keratinocyte growth factor by stromal cells (Bajaj-Elliott *et al.* 1998). Two opposing kinetic views have been proposed in an attempt to explain the changed morphology that is characteristic of gluten sensitive enteropathy. Firstly, the considerable crypt hyperplasia has been described as an increased proliferation response needed to accumulate a greatly increased mass of proliferating and maturing cells (Trier *et al.* 1970). Secondly, the mucosal derangement has been attributed to an impaired proliferative response resulting in a slower output of cells from the crypts (Creamer *et al.* 1962). This second view has now been largely discredited, as both indirect and direct evidence exists for enhanced epithelial proliferative states in coeliac disease. It was shown that the proliferating crypt cell number is increased in coeliac disease patients using Ki67 antibody (Savidge *et al.* 1995; Moss *et al.* 1996; Przemioslo *et al.* 1995).

Apoptosis, programmed cell death or 'cell suicide' is, in distinction to passive cell death, an active physiological process. In tissues that undergo continual cellular turnover, such as the gut, homeostasis is dependent upon the balance between cell proliferation and apoptosis (Thompson *et al.* 1995). In the normal gut mucosa, apoptotic cells are seen rarely at the villous tip (Hall *et al.* 1994; Moss *et al.* 1996). Moss and colleagues reported that the number of apoptotic enterocytes is increased and mainly located at the surface epithelium and in the crypts in coeliac disease patients. They also reported that the apoptotic cells were present in the adjacent lamina propria (Moss *et al.* 1996).

1.4.2.1 Enterocytes as antigen presenting cells

The epithelium of the intestine is a single cell layer that separates the highest concentration of foreign antigen from the largest population of lymphocytes in the body. Numerous reports have described the expression of a low level of HLA class II antigens on the surface of normal intestinal epithelial cells (Mayer *et al.*

1991; Madrigal-Estebas *et al.* 1993; Byrne *et al.* 2002) and have demonstrated that increased expression of these molecules is associated with a diverse group of pathological conditions including inflammatory bowel disease (IBD) (Mayer *et al.* 1991), graft versus host disease (Bland *et al.* 1992) and coeliac disease (Ciclitira *et al.* 1986). Since the expression of HLA class II molecules is a prerequisite for cells that function as antigen presenting cells (APCs) to $CD4^+$T lymphocytes (Germain *et al.* 1994), these observations suggested that the intestinal epithelium might function in the initiation and/or regulation of $CD4^+$T cell responses in the mucosa of the intestinal tract.

The early reports describing antigen presentation by enterocytes in human (Mayer *et al.* 1987) and rat (Bland *et al.* 1988) models reported the proliferation of $CD8^+$ T lymphocytes with suppressor activity after stimulation of primed T cells with antigen pulsed, class II expressing enterocytes. However, HLA class II molecules are unlikely to stimulate $CD8^+$ T cell responses. Indeed, it has been reported that in humans the HLA class I like CD1d molecule, which is expressed on enterocytes (Blumberg *et al.* 1991), is involved in the stimulation of $CD8^+$ T cells in the mucosa (Panja *et al.* 1993). Enterocytes are thought to play an important role in coeliac disease in transporting gliadin to the underlying lamina propria. There are two possible mechanisms by which gliadin reaches lamina propria T cells; between the epithelium (paracellularly) or through the epithelium (transcellularly).

Paracellular transport

It is well established that there is increased gut permeability in the coeliac disease (Fasano *et al.* 2000). Recently, it was discovered that gliadin could increase permeability in the intestine by controlling the protein zonulin, which is a regulator of tight junctions in the small intestine (Fasano *et al.* 2000). It was shown that gliadin induces zonulin dependent actin polymerisation, which results in tight junction disassembly in coeliac and control intestinal tissues (Fasano *et al.* 2000). In rat intestine, gliadin administration results in increase permeability of tight junctions, mediated by zonulin (Clemente *et al.* 2003). Immunofluorescence staining and immunoblotting showed enhanced zonulin expression in the coeliac submucosa compared to controls. It is possible that this increase may cause intestinal permeability and the potential for increased movement of gliadin into the submucosa (Fasano *et al.* 2000).

Transcellular transport

Gliadin uptake by enterocytes has been demonstrated, although the mechanism by which this occurs is not yet known. Enterocytes express HLA class II molecules, with HLA-DR expression being greater than DP and DP greater than DQ (Marley *et al.* 1987; Madrigal-Estebas *et al.* 1993; Byrne *et al.* 2002). Expression is greatest in villous enterocytes and declines towards the crypt cells. In active coeliacs HLA class II expression is increased, with both surface and crypt enterocytes staining positive for all class II molecules (Marley *et al.* 1987). Gliadin molecules can upregulate HLA-DR expression on enterocytes *in vitro* within 2 hours (Maiuri *et al.* 1996). Theories that enterocytes might present gliadin via MHC class II molecules were expanded when gliadin was found co-localised with HLA-DR in endocytic vesicles in coeliac patients (Zimmer *et al.* 1995; Zimmer *et al.* 1998).

1.4.3 Intraepithelial lymphocytes (IELs)

The intestinal epithelial layer normally contains a large population of T lymphocytes known as IELs composed mainly of $CD8^+$ expressing αβT-cell receptor (TCR) (Cerf-Bensussan *et al.* 1993; Arato *et al.* 1998). Only about 10% of human intestinal intraepithelial cells express the γδ TCR and do not recognize MHC associated peptide antigens (Brandtzaeg *et al.* 1989; Abbas *et al.* 2000).

A working hypothesis for the specificity of γδ T cells is that they may recognize antigens that are frequently encountered at epithelial boundaries between the host and the external environment (Abbas *et al.* 2000). It has been suggested that these cells function in immune surveillance and in repair of damaged epithelial tissues (Boismenu *et al.*1994). IELs have an activated phenotype expressing HLA-DR (Abuzakouk *et al.* 1996) and the memory T cell marker, CD45RO (Cerf-Bensussan *et al.* 1993).

IELs are increased in the mucosa of untreated coeliac disease patients (Ferguson A, *et al.* 1971; Kelly *et al.*1987; Catassi *et al.* 1993; Jarvinen *et al.* 2003). In coeliac disease, IELs were markedly increased in the small intestinal epithelium (Catassi *et al.* 1993; Arato *et al.* 1998). In general, these IELs are $CD3^+$/CD8 αβ TCR (Catassi *et al.* 1993). Recently, it has been shown that IEL subsets from

coeliac disease mucosa and controls produce Type I cytokines and that TCRαβ are the main contributors of INF-γ and TNF-α cytokines (Leon *et al.* 2005).

Careful quantitation of IELs suggested that a considerable proportion of $CD3^+$ IELs had neither CD4 nor CD8 antigens on their surfaces (Verkasalo *et al.* 1990). In coeliac disease patients, the mean proportion of this $CD3^+$ $CD8^-CD4^-$ T cell population was 28% (Spencer *et al.* 1989). It had been suggested that this T cell population represents the $CD3^+$ $CD4^-$ $CD8^-\gamma\delta$ T cell receptor bearing cells. In other studies, emphasis was given to raised numbers of intraepithelial T cells which expressed the gamma delta T cell receptor, and this was considered a specific finding in coeliac disease (Spencer *et al.* 1999; Maki *et al.* 1990; Arranz *et al.* 1994; Savilahti *et al.* 1990; Savilahti *et al.* 97). The number of $\gamma\delta^+$ T cells has been reported to be increased in coeliacs regardless of dietary treatment and intestinal morphology (Savilahti *et al.* 1990; Kutlu *et al.*1993). Furthermore, an increase of $\gamma\delta^+$ T cells number in a patient with normal mucosal morphology was observed to precede the development of small bowel villous atrophy and crypt hyperplasia (Maki *et al.* 1990). Sturgess and co-workers have shown that there is a significant difference between $\gamma\delta^+$ T cell counts in treated and untreated coeliacs (Sturgess *et al.* 1993).

IELs are characterised by discrete homing, activation and phenotypic characteristics (Trejdosiewicz *et al.* 1992; Schieferdecker *et al.* 1992). In coeliac patients stimulation of peripheral blood T cells with alpha-gliadin showed increased percentage of CD69 positive cells. This increase was found only in untreated coeliac disease patients. A positive response to the gliadin was found also in coeliac patients with IgA deficiency and anti-endomysial antibody negative (Perticarari *et al.* 2002). CD7 is another marker expressed on T cells subsets such as CD3, CD4, or CD8 (Haynes *et al.* 1990). CD2 receptor is a T cell activation marker and adhesion molecule that binds to its ligand, LFA-3, which is expressed on a variety of leucocytes (Meuer *et al.* 1984). Overall, information about activation markers such as CD2, CD69 and CD7 in coeliac disease is limited in the literature. However, another T-cell activation marker HLA-DR was found to be enhanced in coeliac disease patients (Marsh 1992). Furthermore, the intensity of expression of HLA-DR in the small intestinal mucosa of patients with

coeliac disease has been shown to be gluten dependent (Scott *et al.* 1981; Marley *et al.* 1987).

1.4.4 Lamina propria lymphocytes

The lamina propria is composed of a variety of cells. These cells include T and B-lymphocytes, NK cells, macrophages, dendritic cells, neutrophils, and mast cells. The majority of lamina propria T-cells are $CD4^+$ being TCR $\alpha\beta^+$ (Brandtzaeg *et al.* 1998). However, they differ from peripheral blood by being in a more activated state and express CD25 and HLA-DR antigen. These cells have a mature or memory phenotype as indicated by their surface markers $CD44^{high}$, $CD62^{low}$, $CD45RO^+$ and high levels of the integrin $\alpha_4\beta_7$ (James *et al.* 1986; Schieferdecker *et al.* 1992). The $CD4^+$ subset is predominantly Th2 but Th1 cells also exist (Simecka *et al.*1998). The high levels of Th2 cells help the B-cell production of IgA. Lamina propria T-cells have been demonstrated to produce IL-2, IL-4, IL-5, IL-10 and IFN-γ. T cell lines derived from this population show varying types and amounts of cytokines produced (Braunstein *et al.* 1997; Harriman *et al.* 1992). Although a significant number of IFN-γ-producing CD4+ T cells are present in the lamina propria, IL-5-producing T cells have been shown to be more frequent (Taguchi *et al.* 1990).

Lamina propria B cells synthesise IgA, IgM and IgG, with IgM and IgG plasma cells increased relative to IgA plasma cells. 5-10% of the B cells produce antigliadin antibodies, especially of the IgG isotype (Brandtzaeg *et al.* 1989). The second cellular component in the coeliac disease lesion is a T cell infiltrate that is evident both in the lamina propria and in the epithelial layer.

Quantification of mRNA levels by reverse transcription polymerase chain reaction (RT.PCR) showed that the expression of IFN-γ was remarkably increased in the duodenal mucosa of coeliac disease patients after gluten exposure (Kilmartin *et al.* 2003). In peripheral blood mononuclear cells (PBMC), gliadin stimulated the secretion of IL-6 and IL-10 in coeliac disease and normal samples, while only coeliac disease PBMC secreted IFN-γ (O'Keefe *et al.* 1999). Increased production of several cytokines, in particular IFN-γ, has been described in the

coeliac mucosa but it is not clear how this leads to the coeliac lesion (Forsberg *et al.* 2002).

In addition, IFN-γ causes activation of macrophages that produce cytokines such IL-10, IL-12, and TNF-α as well as metalloproteinases that can degrade the mucosal matrix which may contribute to the gut damage in coeliac disease (Pender *et al.* 1996; Pender *et al.*1998). It is known that IFN-γ can direct T lymphocytes towards a Th1 rather than a Th2 cytokine response. IFN-γ can also activate macrophages, thus leading to the secretion of IL-6 and TNF-α. IFN-α may be responsible for the development of a Th1 cytokine profile in coeliac disease (Monteleone *et al.* 2001). It was reported that TNF-α was directly toxic to intestinal epithelial tissue and induced increased expression of MHC class I and class II (Pober *et al.* 1988; Kvale *et al.* 1988).

1.5 CYTOSKELETAL PROTEINS

The cytoskeletal system consists of several filamentous networks that extend from the plasma membrane to the nuclear envelope and even the interior of the nucleus. The cytoskeleton also plays a role in attaching the cell to its neighbours and to the extracellular matrix via specialised cell junctions that span the plasma membrane. A major fraction of total cellular protein, over 80% in some cells, is of cytoskeletal origin. The cytoskeleton is now known to consist of numerous of different proteins collaborating in the organisation of the complex machinery that is involved in essentially all structural and dynamic aspects of living cells, including maintenance of cell shape, cell movement, cell replication, apoptosis, cell differentiation, and cell signalling (Fuchs *et al.* 1998).

The cytoskeleton of eukaryotic cells is composed of three major protein families that form filamentous structures running throughout the cell, i.e. microfilaments consisting of different actin isoforms, microtubules made of α- and β-tubulin, and the intermediate filaments, together with their associated molecular motors and regulatory protein complexes. The micro- filaments and microtubules are assembled from highly conserved globular proteins, while the intermediate filaments are built from extended proteins with a central α-helical domain.

Although these different proteins have a conserved substructure, they are characterised by considerable divergence with respect to their amino acid sequences in the non-α-helical head and tail domains.

Cytoskeletal protein abnormalities are thought to be the underlying reason for many pathological changes. It is no surprise that modifications in such a crucial cellular structure lead to pathological conditions. In fact, many diseases have now been associated with abnormalities in cytoskeletal proteins, including several cardiovascular disease syndromes, neurodegeneration, cancer, liver cirrhosis, pulmonary fibrosis, and skin diseases (Lane *et al.* 2004; Zatloukal *et al.* 2004; Chaponnier *et al.* 2004; Rottner *et al.* 2004; Cairns *et al.* 2004).

1.5.1 Cytoskeletal proteins in coeliac disease

Few studies have examined the cytoskeletal proteins in coeliac disease patients. Actin network specifically has been shown to play an important role in the gliadin-induced effects on intestinal epithelial cells, demonstrated by both *in vivo* and *in vitro* study (Oberhuber *et al.*1999; Oxentenko *et al.* 2002). Gliadin exposure in coeliac disease patients on a gluten free diet has been shown to cause rapid disarrangement of intestinal mucosa actin filaments *in vivo* (Holmgren-Peterson *et al.* 1995; Bailey *et al.*1989). Recently Clemente and colleagues reported that gliadin has an instant effect on the enterocyte intracellular cytoskeleton. They found that gliadin induced an immune independent increase in actin polymerisation and a redistribution of actin filaments mainly in the intracellular subcortical compartment (Clemente *et al.* 2003).

1.6 EXTRACELLULAR MATRIX

1.6.1 Matrix metalloproteinases and their inhibitors

In the normal small intestine, extracellular matrix (ECM) formation by stromal cells balances ECM degradation mediated by matrix metalloproteinases (MMPs) (Birkedal-Hansen *et al.* 1995). These MMPs are a group of zinc dependent neutral endopeptidases collectively capable of degrading all components of the ECM (Nagese *et al.* 1999). The human matrix metalloproteinases gene family contains more than twenty types of structurally related members (de Coingnae *et*

al. 2000). MMPs can cleave all components of the ECM and in health act in harmony as part of normal tissue turnover, so it is suggested that they play an important role in the normal physiology of angiogenesis and tissue repair.

MMPs are secreted as inactive proenzymes and require proteolytic processing of their amino terminal domain to become active. All MMPs contain several various domains, each of which is responsible for a certain function: the maintenance of the latent form of the enzyme, secretion, substrate recognition, and catalysis (Khasigov *et al.* 2001). The propeptide is thought to keep the enzyme in latent form by the interaction of a cysteine residue with the zinc moity in the enzyme active site. Disruption of this interaction triggers the cysteine switch mechanism and results in activation of the enzyme (Vu TH and Werb 2000).

The catalytic domain includes a motif with three conserved histidine residues that complex with zinc. The C-terminal region of the MMP molecule contains a hemopexin-like domain that seems to determine substrate specificity or the interaction with the cell surface receptor. Gelatinase molecules contain an additional fibronectin-like domain that helps during their binding to their substrate, gelatin. Furthermore, molecules of gelatinase B (MMP-9) contain a domain with a sequence similar to α-2-chain of collagen V. Membrane-type matrix metalloproteinases (MT-MMPs) are characterised by a specific transmembrane domain in the C-terminal region, and also by a sequence recognisable by a convertase furin that has also been found in molecules of stromolysin-3 (Uasigov *et al.* 2001).

As MMPs can degrade ECM components, their function was supposed to be remodelling of the ECM. They are thought to play important roles during embryonic development, as ECM remodelling is a critical component of tissue growth and morphogenesis (Vu and Werb 2000). MMPs are classified according to their substrate specificity into four groups: collagenases (MMP-1, -8 and -13), gelatinases or type IV collagenases (MMP-2 and-9), stromelysins (MMP-3,-10,and-12), and membrane-type MMP (MT-MMPs) (de Coignac *et al.* 2000; Grant *et al.* 1999; Welgus *et al.* 1990). The catalytic activity of MMPs can be differentially regulated during transcription and secretion, as well as specifically activated and inhibited in the extracellular space (Massova *et al.* 1998).

MMP activity is controlled by their natural inhibitor; tissue inhibitors of metalloproteinases (TIMPs), or by synthetic MMP inhibitors (de Coignac *et al.* 2000). TIMPs can bind to the catalytic domain of MMPs in a 1:1 stochiometry to form complexes, thus inhibiting the enzymatic activity of the MMPs (Brew *et al.* 2000). Currently, four different TIMPs have been identified (Goetzl *et al.* 1996), revealing different tissue and cell type specific expression and regulation patterns (von Lampe *et al.* 2000; Gomez *et al.* 1997). MMPs and TIMPs are produced by various cell populations, including myofibroblasts, fibroblasts, and inflammatory cells such as macrophages and lymphocytes (Daum *et al.* 1999; Salmela *et al.* 2002).

1.6.2 MMPs and TIMPs in Inflammatory conditions

MMPs have recently been shown to be important in tissue injury in the gut. A number of recent studies suggest that MMPs are the most important group of proteolytic enzymes responsible for the collapse of ECM in inflammatory bowel disease (Vaalamo *et al.* 1998; Heuschkel *et al.* 2002; von Lampe *et al.* 2000) and rheumatoid arthritis (Klimiuk *et al.* 2002) and may also play a role in malignancy (Zhang *et al.* 2003). In many of these studies, the findings were based on estimation of mRNA transcripts, using techniques such as *in-situ* hybridisation or PCR (Heuschkel *et al.* 2002; von Lampe *et al.* 2000). Elevated mRNA levels for the three metalloproteinases investigated in this study were reported in inflammatory bowel disease (Heuschkel *et al.* 2002) and transcript levels correlated positively with the degree of inflammation in some studies (Stallmach *et al.* 2000). Furthermore, MMP protein expression examined by immunohistochemistry was shown to be present extracellularly in areas of mucosal damage in both Crohn's disease and ulcerative colitis (Salmela *et al.* 2004; von Lampe *et al.* 2000). In rheumatoid arthritis, raised levels of metalloproteinase protein were described in both serum and synovial fluid and found to correlate with clinical indicators of disease activity (Yoshihara *et al.* 2000; Klimiuk *et al.* 2002). It was observed that mRNA expression of MMP-1, and MMP-3 was significantly increased in inflamed compared with non-inflamed colonic mucosa of patients with IBD (Salmela *et al.* 2002). Using immunofluorescence, MMP-3 was shown to be present extracellularly in regions

of mucosal damage in ulcerative colitis (UC) and Crohn's disease (Stallmach *et al.* 2000).

1.6.3 MMPs and TIMPs and coeliac disease

Only three studies have investigated mRNA expression of MMPs and TIMPs in the intestinal mucosa of patients with coeliac disease and dermatitis herpetiformis (Daum *et al.* 1999; Salmela *et al.* 2001; Ciccocioppo *et al.* 2005). Employing *in situ* hybridisation, Daum *et al.* reported increased expression of MMP-1, MMP-3 and the inhibitor TIMP-1 in untreated coeliac disease (Daum *et al.* 1999). Using a similar technique, Salmela *et al.* described elevated expression of MMP-12 in the mucosa of patients with dermatitis herpetiformis, another gluten sensitive disorder (Salmela *et al.* 2001). In a more recent study, using real-time RT-PCR, increased MMP-1, MMP-12 and TIMP-1 mRNA levels were described in patients with untreated coeliac disease (Ciccocioppo *et al.* 2005).

1.7 COMPLICATIONS

1.7.1 Refractory coeliac disease

Refractory coeliac disease (RCD) is considered to be primary when there is no response to a GFD following diagnosis and secondary when there is an initial response to a GFD, but recurrence of symptoms and villous atrophy or overt intestinal lymphoma despite adherence to the diet (Trier *et al.* 1978; Trier *et al.* 1998). RCD shares genetic, biological, and histological features with coeliac disease (Cellier *et al.* 1998). Recent evidence suggests that RCD is a low-grade intraepithelial lymphoma, characterised by massive expansion of IELs with normal cytology but with clonal TCRγ rearrangements and an aberrant phenotype containing intracellular CD3ε (iCD3ε$^+$) but lacking surface expression of CD3$^-$ TCR complexes (sCD3ε, CD8, TCR) (Cellier *et al.* 1998; Cellier *et al.* 2000; Verkarre *et al.* 2003).

This clonal population is easily detected by analysing DNA extracted from intestinal biopsy specimens, and the aberrant phenotype can be detected with a simple immunohistochemistry method on formalin fixed paraffin embedded

biopsy samples (Patey-Mariaud *et al.* 2000). Detection of such an aberrant clonal IEL population in patients with manifestations of RCD is associated with poor outcome and a higher risk of overt T cell lymphoma than in coeliac disease (Cellier *et al.* 2000). This has led to RCD being considered a cryptic form of enteropathy type intestinal T cell lymphoma (Carbonnel *et al.* 1998; Daum *et al.* 2001).

Enteropathy-associated T-cell Lymphoma (EATL) is rare but it is the most serious complication of coeliac disease. It has a peak incidence in the sixth decade of life, indicating that many years of latency need to occur between the onset of coeliac disease and the development of EATL (Catassi *et al.* 2005). The neoplastic cell of EATL is most commonly $CD3^+$, $CD4^-$, $CD8^-$, and $CD103^+$ and contains cytotoxic granules recognized by the TIA-1 (T-cell intracellular antigen) antibody (Bagdi *et al.* 1999). EATL commonly develops in the jejunum but may also be found in the ileum and lymph nodes and less frequently in the stomach and the colon. It is often multifocal with ulcerative lesions because EATL is often disseminated at diagnosis; extraintestinal presentations are not uncommon in the liver, spleen, thyroid, skin, nasal sinus, and brain (Catassi *et al.* 2005). The prognosis of the disease is poor, although it may improve with chemotherapy and surgery. In a recent study of EATL patients, survival at 30 months was only 13% (Howdle *et al.* 2003).

1.7.2 Reduced bone mineral density

Patients with coeliac disease are at high risk of developing low bone mineral density and bone turnover impairment. The reduction in bone density is more severe in symptomatic coeliac disease than in the silent form (Corazza *et al.* 1996) In osteoporosis, the risk of fractures has been shown to be increased two-to threefold for every z-score decrease in bone mineral density (Fickling *et al.* 2001). The most common sites of osteoporotic fractures are at the distal forearm, spine, hip, ankle, humerus, and pelvis (Lips *et al.* 1997).

In adult patients responsive to GFD, the bone density seems comparable to that of healthy individuals. Patients who followed a GFD for at least 5 years had normal bone mineralization and bone turnover (Valdimarsson *et al.* 1996, Meyer *et al.* 2001). All metabolic studies suggest that osteopenia in coeliac disease is a

consequence of secondary hyperparathyroidism (Mautalen *et al.* 1997). Recently, proinflammatory and anti-inflammatory cytokines (IL-1-β, IL-6 and IL-1 receptor antagonist) were suggested as playing an active role in coeliac disease associated ostepenia (Fornari *et al.* 1998).

1.7.3 Infertility

The association between coeliac disease and infertility is a controversial area. It has previously been suggested that coeliac disease, along with other chronic inflammatory diseases, may be associated with reduced fertility and increased risk of adverse pregnancy related events (Wood *et al.* 2003; Alstead *et al.* 2003). Some have accepted that infertility is indeed a complication of coeliac disease and if true this is of importance to both women with coeliac disease and to those who manage their care (Green *et al.* 2003). Reported associations between coeliac disease and a higher incidence of termination, miscarriage, and having babies with low birth weight or intrauterine growth retardation, have also raised concern (Catassi *et al.* 1994; Rostami *et al.* 2001; Smecuol *et al.* 1996; Martinelli *et al.* 2000).

Recent work compared the fertility experience of UK women with coeliac disease to women in the general population using the General Practice Research Database (Tata *et al.* 2005). Women with coeliac disease had similar fertility rates to the general female population but tended to have babies at a later age. As the patterns of later fertility for the untreated group versus the treated group were similar, when restricted to incident patients, it seems unlikely that the cause of this later fertility is due to the degree of disease activity. These results indicate that the risks of adverse pregnancy related outcomes for women with coeliac disease are not markedly raised.

These findings are in contrast with those recently reported by Ludvigsson and co-workers who examined adverse foetal outcome in a large population based cohort study (Ludvigsson *et al.* 2005). They found that women who had undiagnosed coeliac disease at the time of delivery were more likely to have a pre term birth, caesarean section, or have an offspring with intrauterine growth retardation, low birth weight, or very low birth weight. In contrast, maternal coeliac disease diagnosed before birth was not associated with adverse foetal outcomes. Infertility

among men is also common (Collin *et al.* 1996; Sher *et al.* 1994). Furthermore, men with the disease also tend to have children with a shorter gestation and lower birth weight than those without the disease (ludvigsson *et al.* 2001).

1.7.4 Malignancy and mortality

Early studies of the risk of malignancy and mortality in patients with coeliac disease suggested a twofold increase in mortality rate, and increased risks of lymphoproliferative malignancies. Most studies have been small or not population based, and their findings probably do not reflect the risks in contemporary coeliac disease (Corrao *et al.* 2001; Cottone *et al.* 1999). More recent data have suggested more modest increases in the risks but still found that people with coeliac disease were at excess risk of certain malignancies and death (Peters *et al.* 2003; Askling *et al.* 2002; Mearin *et al.* 2006).

1.8 MANAGEMENT

As soon as the disease is diagnosed, the implications of the condition and the important of gluten free diet have to be carefully explained to the patients. Patients often react with grief and many have a hard time accepting that something so fundamental to their diet could be injuring them. Mostly younger patients have difficulty in accepting the dietary restrictions and may have partial suppression of symptoms even when they consume gluten in their diets, increasing the chances that they will be noncompliant (Holmes *et al.* 1989). However, patients can be motivated by the expectation of dramatic improvements in their general gastrointestinal symptoms. On the other hand, patients need to know that they cannot depend on their reactions to questionable food as a measure of safety. An active interest on the part of the clinician can improve compliance as can patient knowledge that follow up blood test can detect gross gluten ingestion.

Recovery is more rapid and complete in children than in adults (Kumar *et al.* 1988). The most common reason for delayed recovery of the small intestinal mucosa in the treatment of coeliac disease patients is the poor compliance with a GFD (O`Mahony *et al.* 1996). However, when GFD is definite and symptoms and villous atrophy have persisted after ruling out other reasons for malabsorption and villous atrophy, the term refractory coeliac disease may be considered.

1.9 PURPOSE AND RATIONALE OF THESIS

There is no doubt that immunological mechanisms play an important role in the development of the characteristic lesion in coeliac disease. Since endomysial antibody (EMA) has high sensitivity (89-100%) and specificity (100%) for coeliac disease, a positive test should identify patients with this condition. Traditionally, a diagnosis of coeliac disease was made on finding mucosal abnormalities equivalent to a Marsh III lesion. It is now clear that many individuals have gluten-sensitive inflammation without villous atrophy. We identified a group of individuals with positive EMA test and normal duodenal mucosa by routine histology. The main aim of this thesis was to examine these patients in detail for evidence of coeliac disease.

The scientific studies carried out are presented in chapter 2-4. Initially 25 patients with positive EMA test and without villous atrophy were identified. (These patients were known throughout the thesis as the study group subjects). Duodenal biopsies from 20 of these 25 patients were available for further histological evaluation. All 20 biopsies were reviewed by routine histology and subjected to immunohistochemical evaluation. At the time of this study, all patients were invited to attend for review, and 14 returned. A further clinical and laboratory assessment was performed and this included small intestinal biopsy in nine patients. An advanced high content screening technology (Cellomics) was used to examine enterocyte morphology in the duodenal mucosa of these patients.

To date, up to 24 different MMPs have been identified. These are subdivided into four families based on their structure and substrate specificity, including collagenases and interstitial collagenases (MMP-1, MMP-8 and -13), stromelysins (MMP-3, -7, -10, -11, -12, and –26), gelatinises (MMP-2 and -9), and membrane-type MMPs. In this thesis, the MMP-1, -3, and –9 molecules were chosen to be examined because; each of which represents a different family, their protein expression had not been examined in the small intestinal mucosa in patients with coeliac disease before and because of their possible physiopathological role in tissue remodelling and destruction. Details of these are presented in this thesis in the following chapters.

Chapter 2.0: Immunohistochemical characterisation of intraepithelial lymphocytes and crypt cells in the study group subjects

The duodenal mucosa of the study group subjects was examined using detailed immunoperoxidase technique. The first aim of the study was to investigate the expression of intraepithelial lymphocyte markers such as CD2, CD3, CD7, CD8 and CD69 and crypt cell proliferation marker (Ki67) in the study group subjects. The second aim was to perform a follow-up study with further clinical and laboratory assessment.

Chapter 3.0: Quantification of enterocyte structures and tubulin fibre arrangement in the study group subjects and coeliac disease patients

A Cellomics® Kineticscan reader was used to examined, acquire and process the images and to quantify the specific tubulin fibre changes of the enterocytes based on an optimised set of output features. The aim of this study was to perform detailed examination of enterocyte morphology, nuclear and cytoskeletal structure in the study group subjects utilising Kineticscan reader.

Chapter 4.0: Immunohistochemical characterisation of matrix metalloproteinases -1, -3, -9 and their tissue inhibitor in the study group subjects and patients with gluten-sensitive enteropathy.

An optimised immunoperoxidase technique was used to examine the protein level of the MMP-1, -3, -9 and TIMP-1. The aim of this study was to examine the expression of the MMP-1, -3, -9 and TIMP-1 in the duodenal mucosa from the study group subjects and gluten-sensitive enteropathy patients.

Chapter 5.0: General discussion

In chapter five, the data presented in this thesis in chapters 2-4 are summarised and discussed in the light of previous work. Suggestions of future work are presented.

CHAPTER 2.0

IMMUNOHISTOCHEMICAL CHARACTERISATION OF INTRAEPITHELIAL LYMPHOCYTES AND CRYPT CELLS IN THE STUDY GROUP SUBJECTS

2.1 INTRODUCTION

Coeliac disease is an inflammatory disorder of the small intestine, caused by the ingestion of gluten in genetically susceptible individuals (Cooke and Holmes 1984; Marsh 1992). Although a common clinical disorder, perhaps affecting 0.5 to 1% of the population, many patients remain undiagnosed because they have few symptoms suggestive of coeliac disease (Logan *et al.* 1992; Feighery *et al.* 1998; Sanders *et al.* 2002; Rampertab *et al.* 2006). Evidence of the presence of coeliac disease is often suggested by the finding of antibodies to tissue transglutaminase (Dieterich *et al.* 1997) and to endomysium (Chorzelski *et al.* 1983). Multiple publications confirm the high specificity and sensitivity of these serological assays (Chorzelski *et al.* 1983; Dieterich *et al.* 1998; Sulkanen *et al.* 1998; Ferreira *et al.* 1992; Feighery *et al.* 1998; Burgin-Wolff *et al.* 1997). However, confirmation of the presence of enteropathy, revealed by histological examination of small intestinal biopsies, remains the traditional method of making the diagnosis (Marsh 1992; Walker-Smith *et al.* 1990; Oberhuber *et al.* 1999).

With the increased use of serological tests for coeliac disease, it became evident that some patients with positive coeliac autoantibodies had apparently normal small intestinal histology (Holmes *et al.* 2001; Corazza *et al.* 1996; Troncone *et al.* 1996; Weinstein *et al.* 1974; Maki *et al.* 1990; Maki; *et al.* 1991). In many such patients, the diagnosis of coeliac disease was rejected, because of the absence of a histological lesion. However, in a proportion of these patients, more detailed histological analysis may reveal subtle histological abnormalities such as an increased number of IELs (Kaukinen *et al.* 2001; Mino *et al.* 2003; Settakorn *et al.* 2004) with this increase sometimes being confined to the villous tip (Biagi *et al.* 2004; Jarvinen *et al.* 2004).

In our institution, over a ten-year period (1991-2001), twenty-five patients were identified with a positive endomysial antibody test in whom routine small intestinal histology showed no evidence of enteropathy. These patients had been discharged from hospital to their primary care physicians. The purpose of the study was to re-evaluate the clinical status of these patients by detailed immunohistological examination of their original biopsies, by repeat serological investigation and by inviting patients to return for complete clinical review.

2.2 MATERIALS AND METHODS

2.2.1 Patients

Between 1991 and 2001, a total of 25 patients with a positive endomysial antibody test but reported normal duodenal mucosal histology were identified in our institution. These patients will be referred to throughout the manuscript as the study group subjects. These patients include 11 males (age range 22-75 years) and 14 females (age range 20-86 years). The majority of these patients presented with symptoms consistent with malabsorption, including iron deficiency anaemia, chronic diarrhoea, weight loss, dyspepsia, abdominal pain, fatigue and bloating. Demographic features of all 25 patients are presented in Table 2.1. Based on the normal histological findings, none of these patients was considered to have coeliac disease and all were discharged to their primary care physicians. At the time of this study, all patients were invited to attend for review, and 14 returned. A further clinical and laboratory assessment was performed and this included small intestinal biopsy in nine patients (Figure 2.1).

Pt.	*Age (years)*	*Sex*	*Symptoms*	*EMA*	*TTG*	*Hb*	*Ferritin*
1	20	F	Anaemia	+	+	4.9	
2	49	F	Bloating, dyspepsia, weight loss	+		13	3.9
3	67	F	Bloating, diarrhoea, dyspepsia	+	+		8.7
4	54	M	Fatigue	+	+		
5	34	F	Anaemia, diarrhoea, fatigue	+	+	11	3.9
6	67	F	Weight loss	+	+	12	
7	31	M	Abdominal pain, weight loss	+			
8	62	M	Anaemia, dyspepsia	+		10	89
9	22	M	Abdominal pain, weight loss	+		14	40
10	75	M	Loose bowel motion	+		14	
11	73	M	Abdominal pain, fatigue, weight loss	+			
12	41	M	Anaemia, diarrhoea, mouth ulcer	+		14	
13	55	F	Abdominal pain	+			71
14	59	F	Abdominal pain, fatigue, weight loss	+			
15	22	F	Abdominal pain, weight loss	+		12	
16	27	F	Abdominal pain, fatigue	+	+		3.9
17	81	F	Diarrhoea, fatigue, weight loss	+	+	11	5.7
18	22	F	Abdominal pain	+	+		
19	31	F	Abdominal pain	+			
20	35	F	Anaemia, fatigue, weight loss	+			7.5
21	29	M	Diarrhoea, fatigue	+		13	
22	54	M	Abdominal pain, dyspepsia	+			280
23	86	F	Anaemia, vomiting	+		12	
24	47	M	Diarrhoea, weight loss	+	+		72
25	26	M	Dyspepsia	+	+		60

Table 2.1 Age, sex, clinical symptoms and laboratory results in the study group subjects.

Note. Normal range: Ferritin = 20-300 μg/l

Hb; Female = 11-16 g/dl & Male = 13-18 g/dl

EMA, anti-endomysial antibody; tTG, anti-tissue transglutaminase antibody

2.2.2 Small intestine biopsy specimens

2.2.2.1 Study group subjects

Duodenal biopsies from 20 of these 25 study group subjects were available for further histological evaluation. All 20 biopsies were reviewed by routine histology and subjected to immunohistochemical evaluation (Figure 2.1). Of the 14 patients who returned for review, duodenal biopsies were obtained in nine and these biopsies were similarly investigated.

2.2.2.2 Untreated coeliac disease patients

Sixteen untreated coeliac disease patients were investigated. Four males with a mean age of 56 years (range, 50-66 years) and 12 females were studied with a mean age of 45 years (range, 17-69 years). These were divided into two groups depending on their mucosal abnormality reports. Ten patients had severe intestinal damage. The remaining six had subtotal villous atrophy and increased IELs.

2.2.2.3 Treated coeliac disease patients

Thirteen treated coeliac disease patients were investigated. This group consisted of four males with a mean age of 43 years (range, 27-52 years) and nine females, with a mean age of 40 years (range, 21-65years).

2.2.2.4 Normal control group

Twenty individuals were screened for evidence of coeliac disease and found to be negative for endomysial and tissue transglutaminase antibodies were used as normal controls. These consisted of five males with a mean age of 53 years (range, 27-83 years) and fifteen females with a mean age of 55 years (range, 23-83 years) were studied. These underwent upper gastrointestinal endoscopy and duodenal examination showed normal duodenal mucosa.

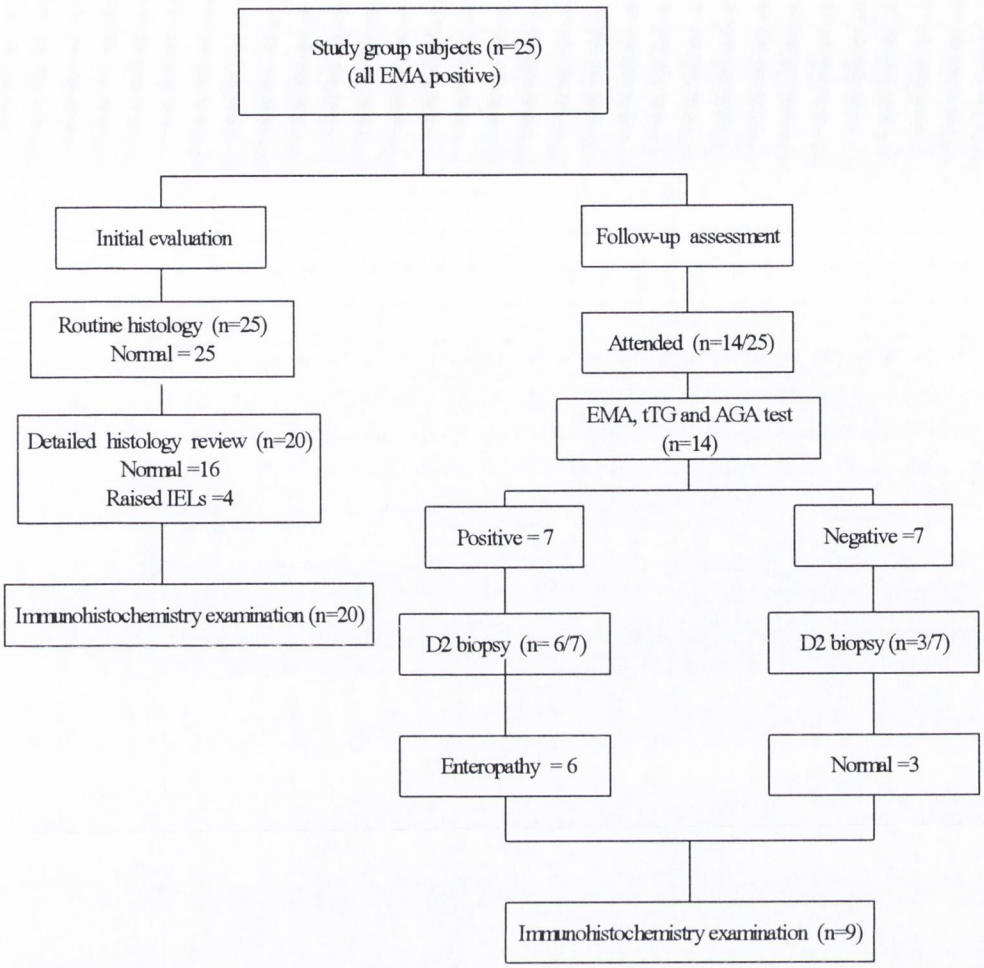


Figure 2.1 Patient flow diagram

AGA: anti-gliadin antibody

D2: duodenal biopsy

IELs: intraepithelial lymphocytes

2.2.3 Routine histology

Haematoxylin and eosin stained duodenal sections from all patients were examined in detail by a trained pathologist for features suggestive of coeliac disease including villous atrophy, increased IELs, enterocyte nuclear disarray, crypt hyperplasia and increased lamina propria cellular infiltrate.

2.2.4 Immunohistochemistry

Immunohistochemical investigation was carried out on 5μm thick, formalin fixed, paraffin-embedded duodenal sections using the avidin-biotin-peroxidase complex detection procedure (Vector Labs ABC technique, USA). Deparaffinized and rehydrated sections were heated in a domestic microwave oven for at least 20 minutes (min) in either 0.1 M citrate buffer, (pH 6.0) or EDTA (pH 8.0) to unmask the antigens. To block the endogenous peroxidase activity, sections were then immersed in 0.3% hydrogen peroxide in 100% methanol for 10 min. Following incubation in normal horse serum for 20 min, sections were incubated with anti-CD2, CD3, CD7, CD8, CD69 (mouse monoclonal antibodies; Novocastra laboratories Ltd UK) and Ki67 antibodies (rabbit polyclonal antibody) at the optimised dilution in Tris buffered saline (TBS pH 7.6). Afterwards sections were incubated with biotinylated rabbit anti-mouse IgG (secondary antibody) for 30 min, followed by peroxidase-conjugated streptavidin (vector Labs ABC technique, USA) for 30 min. at room temperature. After each antibody application, sections were washed in Tris buffered saline containing 0.05% Tween (TBS pH 7.6). Diaminobenzidine tetrahydrochlorid dehydrate (DAB; Sigma) was used for specific colour development and slides were counterstained with haematoxylin. In our immunohistochemical studies we used both TBS and an irrelevant, isotype control.

2.2.5 Quantification of T cell markers on intraepithelial lymphocytes and Ki67 marker in the crypt cells

Intraepithelial lymphocytes (IELs) expressing specific surface markers were determined by counting the number of positive cells per 500 enterocytes at high power magnification (x40) using light microscope (Olympus Bx41). Results were expressed as the percentage of positive IELs within 500 enterocytes.

Stained crypt cells for Ki67 were counted per five fields at high power magnification (x40) using an eyepiece graticule. The number of positive crypt cells was expressed as the percentage of stained cells of all crypt cells. All sections were coded and the parameters were assessed without prior knowledge of the clinical and serological information.

2.2.6 Statistical analysis

Mann-Whitney U test was used to assess the statistical significance of differences in mean number of IELs, LPLs and Ki67 expression and 95% confidence intervals (CI) among the study subjects group and the three control groups. A p-value of <0.05 was considered to be statistically significant.

2.3 RESULTS

2.3.1 Detailed analysis of the original duodenal biopsies

2.3.1.1Clinical review.

The medical charts of 25 patients were examined. In two patients, prior evidence of gluten sensitive disease was noted: patient number 7 had a history of childhood coeliac disease; and patient number 4 had a diagnosis of dermatitis herpetiformis made ten years earlier. In 12 patients, stored serum was available for repeat serological testing and raised anti-tTG antibodies were detected in 7 patients (Table 1). In three additional patients (number 18, 24 and 25) positive anti-tTG results had previously been noted. Six of 12 patients in whom serum ferritin results were available had a reduced level.

2.3.1.2 Light microscopy re-evaluation of the original duodenal biopsies

Haematoxylin and eosin stained slides for 20 of the 25 individuals were available for review by light microscopy. The number of IELs per 100 enterocytes was counted and a value in excess of 25%, regarded as elevated by others (Mahadeva *et al.* 2002; Hayat *et al.* 2002), were found for four patients- 37%, 32%, 34%, and 43% respectively for patients 3, 15,16 and 17 in Table 2.1. All these patients were regarded as having normal villous architecture. No abnormality was observed in the remaining 16 biopsies.

Immunohistochemical studies were used to examine these 20 duodenal biopsies for subtle small intestinal abnormalities. The mean percentage and 95% CI of the markers assessed in the study group subjects, untreated coeliac disease patients, treated coeliac disease patients and normal control group are seen in Table 2.2.

2.3.1.3 Immunophenotyping of CD2 IEL

The number of stained IELs expressing CD2 molecule was found to be increased in the study group subjects in comparison with the normal control group. The mean percentage of this marker in the study group subjects was 22% (range: 15-29%) in comparison with the normal control group (12%, range: 7-16%; $p<0.02$). Although the percentages of IELs expressing CD2 in treated coeliac disease patients were greater (mean 28%, range: 20-37%) than in the study group subjects, this difference was not statistically significant. In the untreated coeliac

disease group, the expression of $CD2^+$ IEL was significantly higher (38%, range: 31-45%) compared with the normal control group ($p<0.0001$) and the study group subjects ($p<0.002$) (Figure 2.2).

2.3.1.4 Immunophenotyping of CD3 IELs

The number of T cells, which stained positive for CD3, was found to be significantly higher in the study group subjects compared with normal controls (Figure 2.3). The mean percentage of $CD3^+$ IELs in the study group subjects was 43% (range: 35-51%) compared with the normal control group, with a mean percentage of 14% (range: 11-18%; $p<0.0001$). In contrast, the study group subjects had lower $CD3^+$IEL numbers compared with untreated coeliac disease patients, in whom the mean percentage was 60% (range 50-70%; $p<0.01$) (Figure 2.4). Although treated coeliac disease patients had higher number of IELs expressing the CD3 marker (mean 48%, range: 40-56%) compared with the study group subjects, this difference was not statistically significant (Table 2.2).

2.3.1.5 Immunophenotyping of CD7 IELs

Further phenotyping of IELs was carried out using anti-CD7 to detect activated cells. The mean percentage of $CD7^+$ IELs in the study group subjects was 36% (range: 30-42%) in comparison with the normal control group, with a mean percentage of 13% (range: 9-16%; $p<0.0001$). The treated coeliac disease patients had a similar percentage of IELs expressing CD7 to the study group subjects (mean 40%, range: 28-52%). However, in the untreated coeliac disease patients the mean percentage of $CD7^+$ IELs was significantly higher (mean 53%, range: 42-63%) compared with the study group subjects ($p<0.008$) and the normal controls ($p<0.0001$) (Figure 2.5).

Interestingly, the numbers of $CD7^+$ IELs were similar to the CD3 and CD8 expressing IELs, suggesting that all $CD3^+$ and $CD8^+$ cells might be activated.

2.3.1.6 Immunophenotyping of CD8 IELs

An increase of IELs expressing CD8 marker was observed in the duodenal sections of the study group subjects (Figure 2.6). The mean percentage of $CD8^+$

IEL counts in the study group subjects was 43% (range: 35-52%) compared with the normal control group, with a mean percentage of 14% (range: 11-19%; $p<0.0001$). The study group had a lower percentage of $CD8^+$ IELs compared with the untreated coeliac disease patients (mean 61%, range: 53-69%; $p<0.003$). Treated coeliac disease patients had a similar percentage of IELs expressing $CD8^+$ (mean 42%, range: 32-53%) compared with the study group subjects (Figure 2.7)

The results were similar to the $CD3^+$ IEL counts, suggesting that most of the $CD3^+$ IELs are also $CD8^+$ IELs.

2.3.1.7 Immunophenotyping of CD69 IEL

The number of IELs expressing the CD69 marker was examined and found to be significantly higher in study group subjects (mean 38%, range: 29-48%) in comparison with the normal control group (mean 13%, range: 9-15; $p<0.0001$). In addition, significant percentages of IELs expressing CD69 were detected in the untreated coeliac disease patients (mean 29%, range 24-35%), and treated coeliac disease patients (mean 39%, range: 30-48%) compared with the normal control group (Figure2.8). However, there was no statistically significant difference found between the study group subjects and either the untreated coeliac disease or treated coeliac disease patients (Table 2.2).

2.3.2 Enterocyte proliferation

Stained crypt cells were counted per five fields at high power magnification, and the number of positive crypt cells was expressed as the percentage of stained cells of all crypt cells.

2.3.2.1 Ki67 expression

Using the proliferation marker Ki67, the majority of the study group subjects showed a marked increase of positive crypt cells (Figure 2.9). The mean percentage of the crypt cells expressing Ki67 in the study group subjects was 50% (range: 41-59%) in comparison with the normal control group, with a mean percentage of 15% (range: 13-18; $p<0.0001$). The untreated coeliac disease group demonstrated a significant increase of Ki67 positive crypt cells (mean 66%, range: 62-70; $p<0.0001$) compared with the normal control group. Furthermore, raised

Ki67 positive crypt cell numbers in the treated coeliac disease group were observed (mean 55%, range: 48-61) compared with the normal controls ($p<0.0001$). There was no statistically significant difference in Ki67 expression between the treated coeliac disease group and the study group subjects (Figure 2.10).

Markers	*Study group subjects (n=20)*	*Untreated CD patients (n=16)*	*Treated CD Patients (n=13)*	*Normal controls (n=20)*
CD2	22% (15-29%)	38% (31-45%)	28% (20-37%)	12% (7-16%)
CD3	43% (35-51%)	60% (50-70%)	48% (40-56%)	14%(11-18%)
CD7	36% (30-42%)	53% (42-63%)	40% (28-52%)	13% (9-16%)
CD8	43% (35-52%)	61% (53-69%)	42% (32-53%)	14%(11-19%)
CD69	38% (29-48%)	29% (24-35%)	39% (30-48%)	13% (9-15%)
Ki67	50% (41-59%)	66% (62-70%)	55% (48-61%)	15%(13-18%)

Table 2.2 T cell markers and Ki67 expression in the duodenal biopsies of the study group subjects, untreated coeliac disease (CD) patients and treated coeliac disease patients. Results are expressed as mean and 95% CI of CD2, CD3, CD7, CD8, CD69 and Ki67 expression in the study group subjects, untreated coeliac disease patients, treated coeliac disease patients and normal control group.

2.3.3 Follow-up data on 14 patients who attended for reassessment

All study group subjects were invited to attend for reassessment. Of the 25 patients, fourteen responded and agreed to further evaluation including clinical review and laboratory tests. Details of their current symptoms, haematology, coeliac serology and biopsy findings are summarised in Table 2.3. All patients had continuing symptoms consistent with a diagnosis of coeliac disease. Interestingly, of the 14 patients, only seven continued to have positive endomysial, tissue transglutaminase and antigliadin antibody tests. The remaining seven patients had negative antibody tests.

Nine of the 14 patients agreed to undergo upper gastrointestinal endoscopy and duodenal biopsy. Six of the nine patients (numbers 1-6) had persistently positive coeliac serology tests and all had histological changes consistent with coeliac

disease: total villous atrophy in two and partial villous atrophy in four patients. These patients had the highest counts of IELs expressing the lymphocyte markers measured (Figures 2.2, 2.4, 2.5, 2.7, 2.8, 2.10). In one of these patients (number 3) a raised IEL count was noted at review of the original histological sample. The duodenal biopsies in the remaining three patients (numbers 8,9,10) who had negative tTG, EMA and AGA antibodies on re-evaluation, all demonstrated normal intestinal mucosa. Interestingly patient number 10 was discovered to have ulcerative colitis.

Pt.	*Age (years)*	*Sex*	*Symptoms*	*EMA*	*tTG*	*AGA*	*Hb*	*F*	*H*
1	27	F	Anaemia, bloating, fatigue	+	+	+	14	19	TVA
2	53	F	Abdominal pain, dyspepsia, weight loss	+	+	+	13		TVA
3	70	F	Anaemia, depression, skin rash	+	+	+	11	3.4	PVA
4	59	M	Fatigue	+	+	+	15	9	TVA
5	40	F	Anaemia, fatigue	+	+	+	9	3.9	TVA
6	77	F	Bloating, dyspepsia	+	+	+			PVA
7	34	M	Bloating	+	+	+	15	160	
8	63	M	Diarrhoea, dyspepsia	-	-	-	10	32	Nor
9	25	M	Abdominal pain, vomiting, weight loss	-	-	-	15	40	Nor
10	78	M	Diarrhoea, weight loss	-	-	-	14	77	Nor*
11	75	M	Diarrhoea	-	-	-	14	377	
12	45	M	Mouth ulcer	-	-	-	12		
13	57	F	Abdominal pain	-	-	-	14	71	
14	64	F	Diarrhoea	-	-	-	13	293	

Table 2.3 Age, sex, clinical and laboratory data of the 14 patients who attended for reassessment.

Note. TVA, Total villous atrophy PVA, Partial villous atrophy

* Found to have ulcerative colitis

Normal range: Ferritin= 20-300 μg/l Hb; Female =11-16 g/dl & Male =13-18g/dl

EMA, anti-endomysial antibody tTG, anti-tissue transglutaminase antibody

AGA, anti-gliadin antibody

2.3.4 Immunohistochemistry findings on follow-up duodenal biopsies

Immunohistochemical studies were performed on biopsies obtained from all nine patients who attended for follow-up biopsies. Results were compared with immunohistochemical data obtained from the original biopsy of the same patients. Data from both original and current biopsies from these six patients were compared with data from 20 normal controls. The mean percentage and 95% CI of different markers assessed in the normal control group and both the current and the original biopsies from the six patients who developed villous atrophy are presented in Table 2.4. A significant increase in the number of IELs expressing CD2, CD3, CD7, CD8, CD69 molecules and the crypt cells expressing Ki67 proliferation marker was noted in the original and the current biopsies of the six patients in comparison with the normal control group ($p<0.0001$). On the other hand, in these six patients CD7 expression on IELs was the only molecule that was markedly increased ($p<0.01$) in the current biopsies compared with its expression in the original biopsies (Figure 2.11). There was no significant difference in IEL expression of CD2, CD3, CD8, CD69, and the crypt cell expression of Ki67 between the initial and current biopsies.

The expression of all markers was similar in the current biopsies and the original biopsies in the three patients who tested negative for EMA and tTG antibodies on reassessment (data not shown).

Molecules	*Normal controls*	*Original biopsies (n=6)*	*Current biopsies (n=6)*
CD2	12% (7-16%)	37% (28-45%)	39% (30-48%)
CD3	14% (11-18%)	62% (53-71%)	72% (65-79%)
CD7	13% (9-16%)	49% (42-56%)	59% (53-65%)
CD8	14% (11-19%)	53% (47-59%)	61% (49-72%)
CD69	13% (9-15%)	20% (11-29%)	27% (15-39%)
Ki67	15% (13-15%)	57% (43-71%)	62% (54-71%)

Table 2.4 T cell markers and Ki67 expression in the original and the current biopsies of the study group subjects who developed small intestinal enteropathy. Results are expressed as mean and 95% CI of CD2, CD3, CD7, CD8, CD69 and Ki67 molecules.

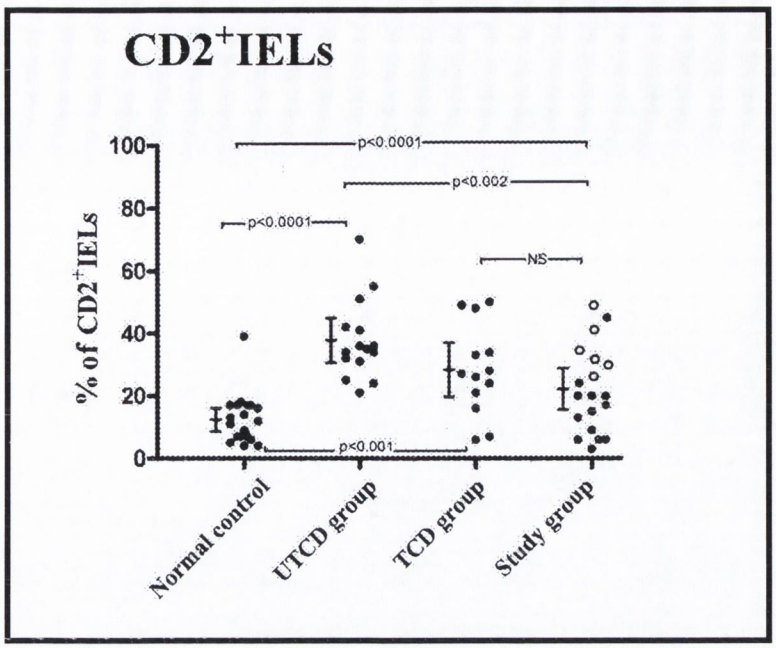


Figure 2.2 Percentage of IELs expressing the CD2 molecule in the study group subjects, treated coeliac disease (TCD), untreated coeliac disease (UTCD) group, and normal control group. Open circles denote patients who later developed a classical coeliac lesion. Bars represent the mean and 95% CI.

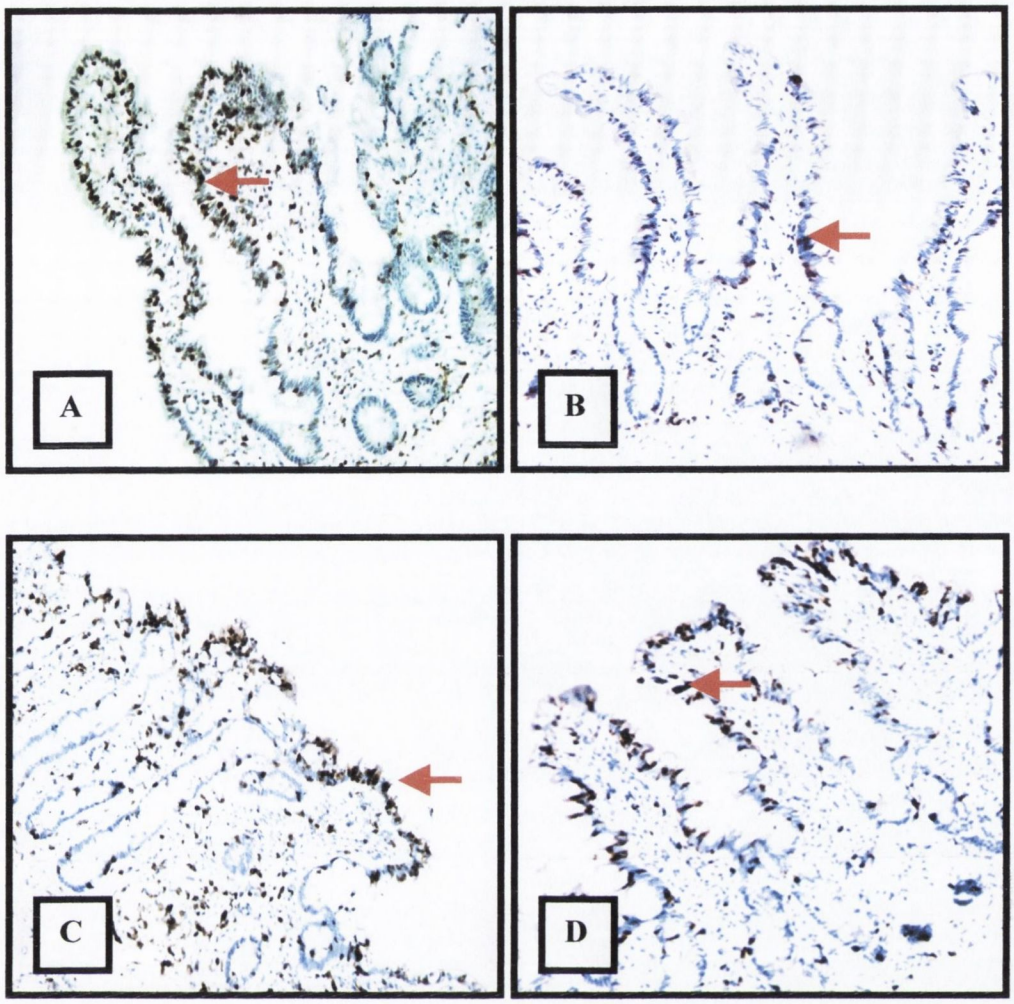


Figure 2.3 Intraepithelial lymphocytes expressing CD3 in the duodenal mucosa of the study group subjects (A), normal control group (B), untreated coeliac disease patients (C) and treated coeliac disease patients (D). Arrows represent the stained IELs.

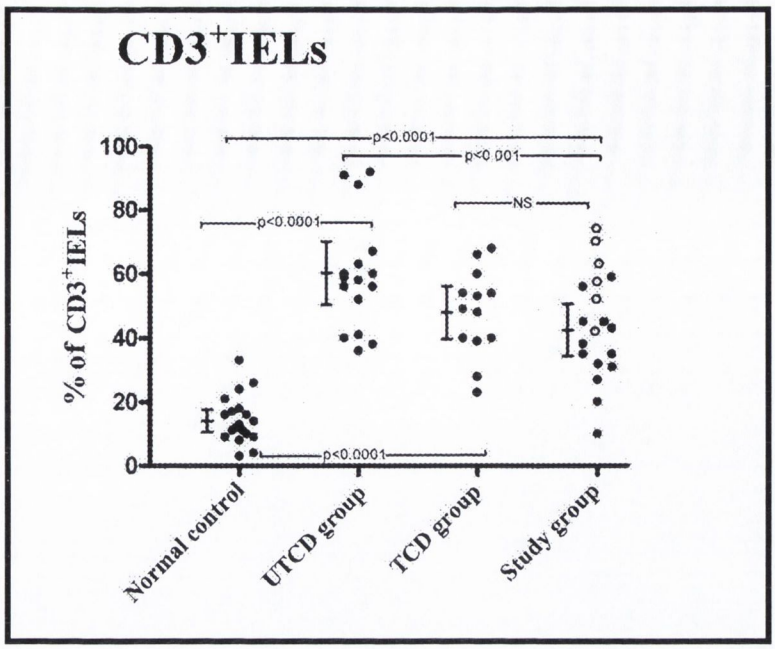


Figure 2.4 Percentage of IELs expressing the CD3 molecule in the study group subjects, treated coeliac disease group (TCD), untreated coeliac disease (UTCD) group, and normal control group. Open circles denote patients who later developed a classical coeliac lesion. Bars represent the mean and 95% CI.

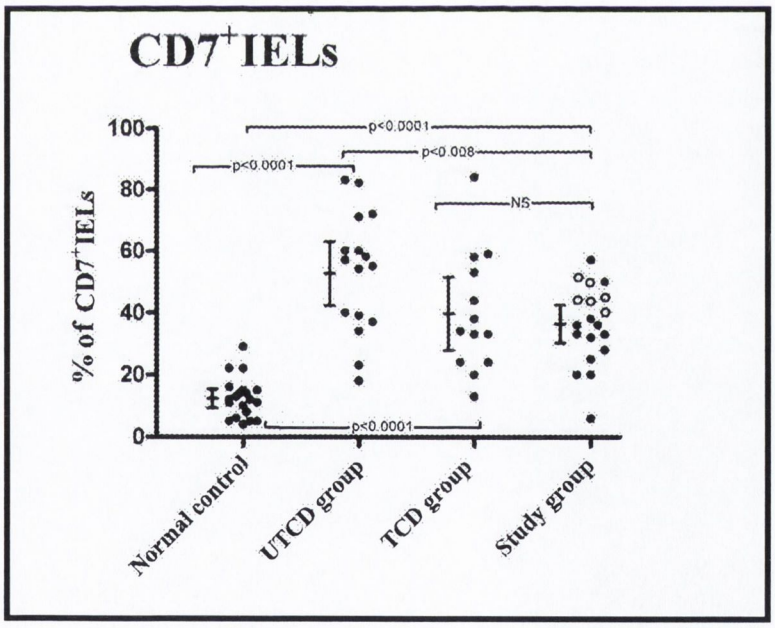


Figure 2.5 Percentage of IELs expressing the CD7 molecule in the study group subjects, treated coeliac disease group (TCD), untreated coeliac disease (UTCD) group, and normal control group. Open circles denote patients who later developed a classical coeliac lesion. Bars represent the mean and 95% CI.

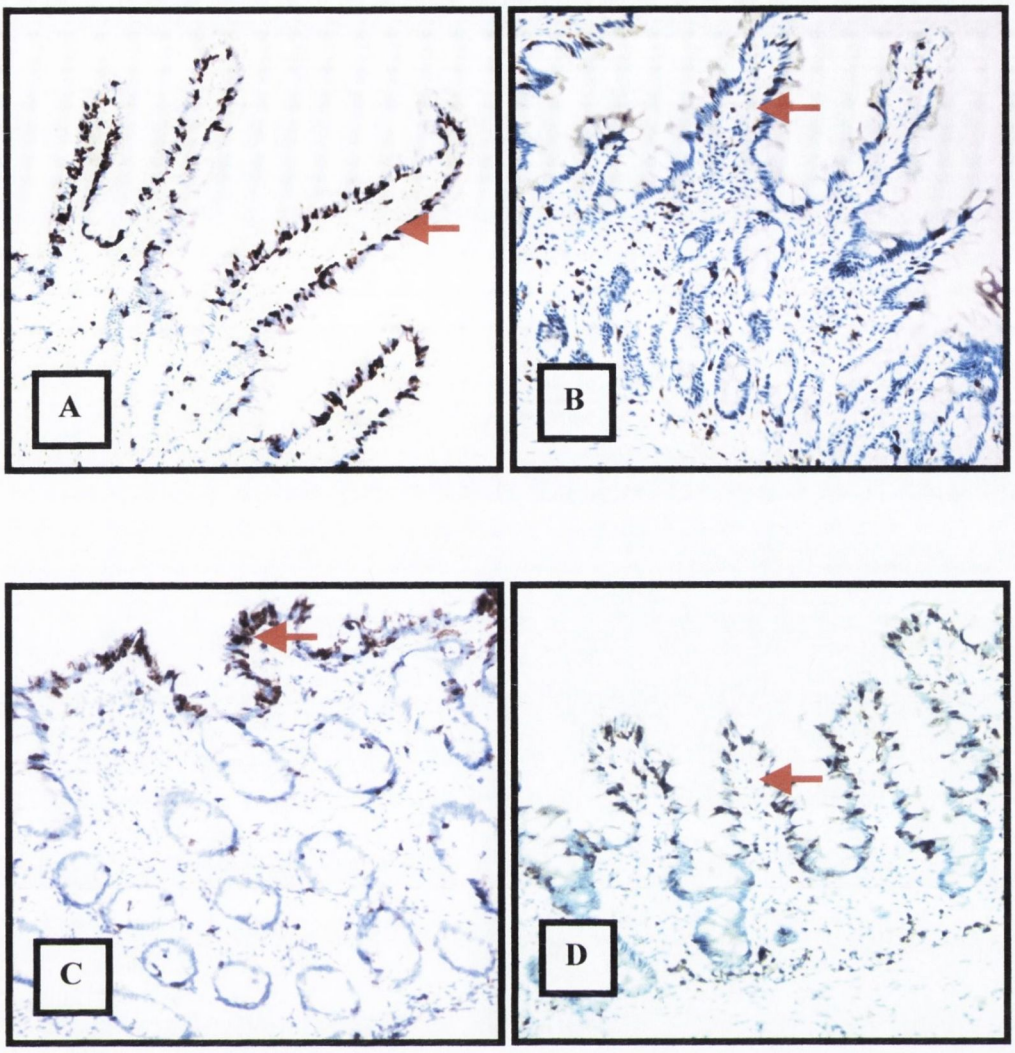


Figure 2.6 Intraepithelial lymphocytes expressing CD8 in the duodenal mucosa of the study group subjects (A), normal control group (B), untreated coeliac disease patients (C) and treated coeliac disease patients (D). Arrows represent the stained IELs.

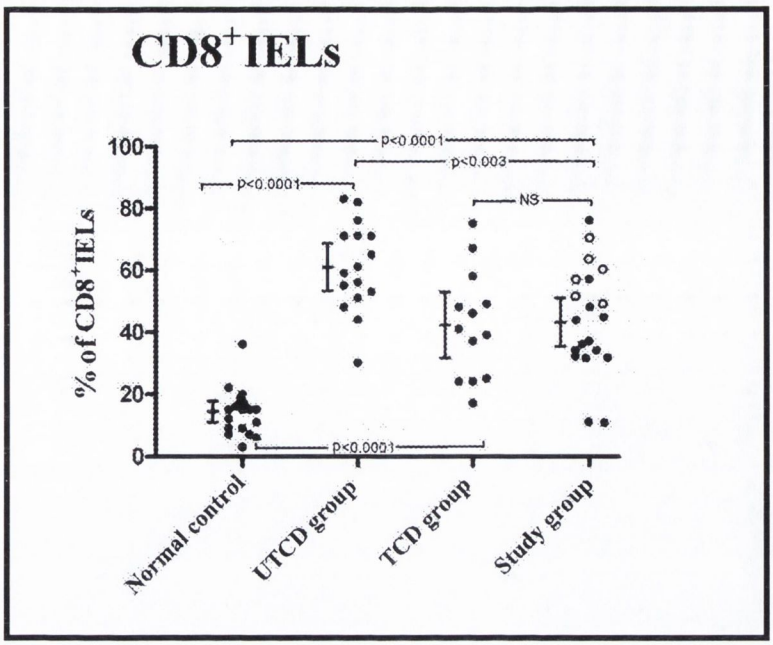


Figure 2.7 Percentage of IELs expressing the CD8 molecule in the study group subjects, treated coeliac disease group (TCD), untreated coeliac disease (UTCD) group, and normal control group. Open circles denote patients who later developed a classical coeliac lesion. Bars represent the mean and 95% CI.

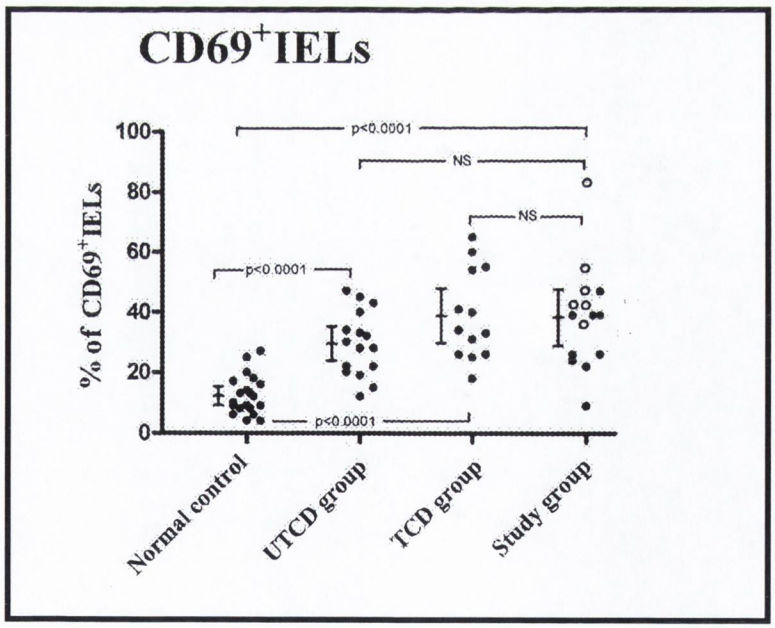


Figure 2.8 Percentage of IELs expressing the CD69 molecule in the study group subjects treated coeliac disease group (TCD), untreated coeliac disease (UTCD) group, and normal control group. Open circles denote patients who later developed a classical coeliac lesion. Bars represent the mean and 95% CI.

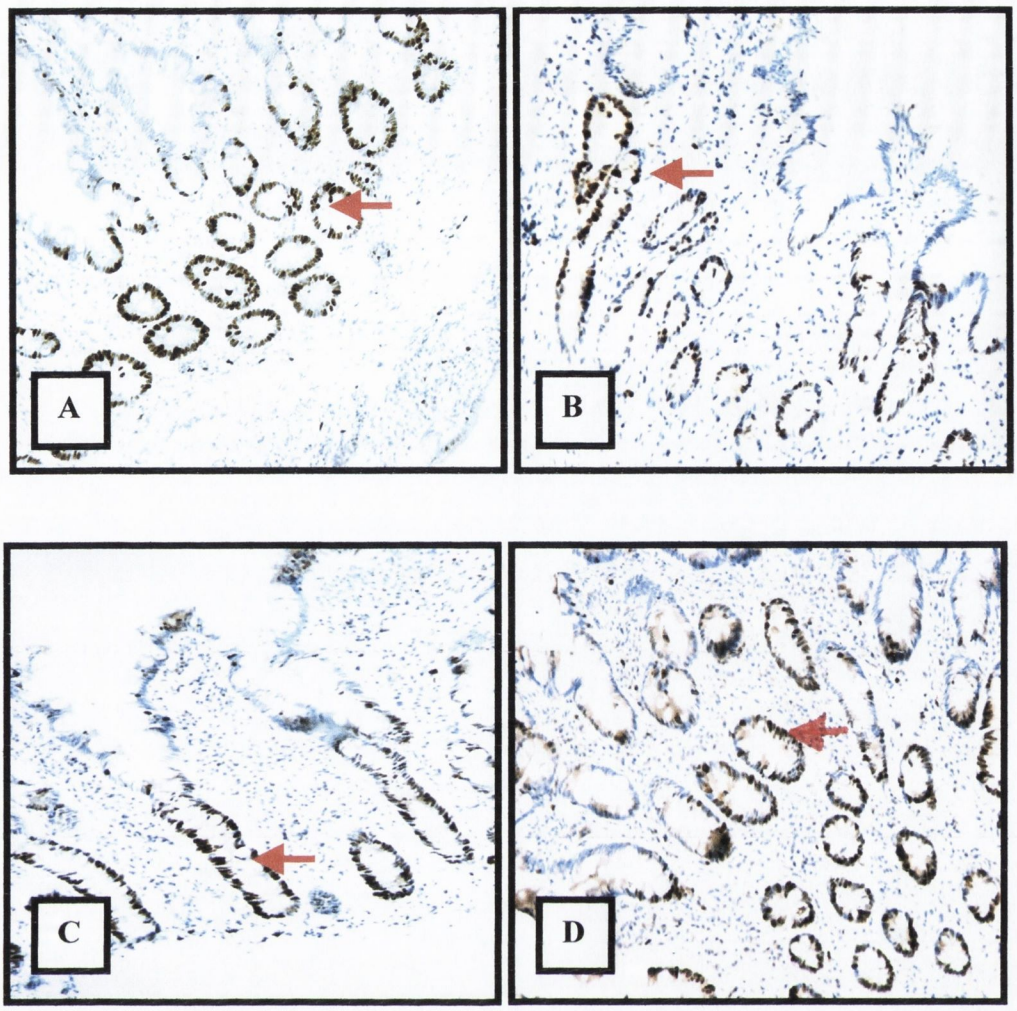


Figure 2.9 Crypt cells expressing Ki67 in duodenal biopsies of the study group subjects (A), normal control group (B), untreated coeliac disease patients (C) and (D) treated coeliac disease patients. Arrows represent the stained crypt cells.

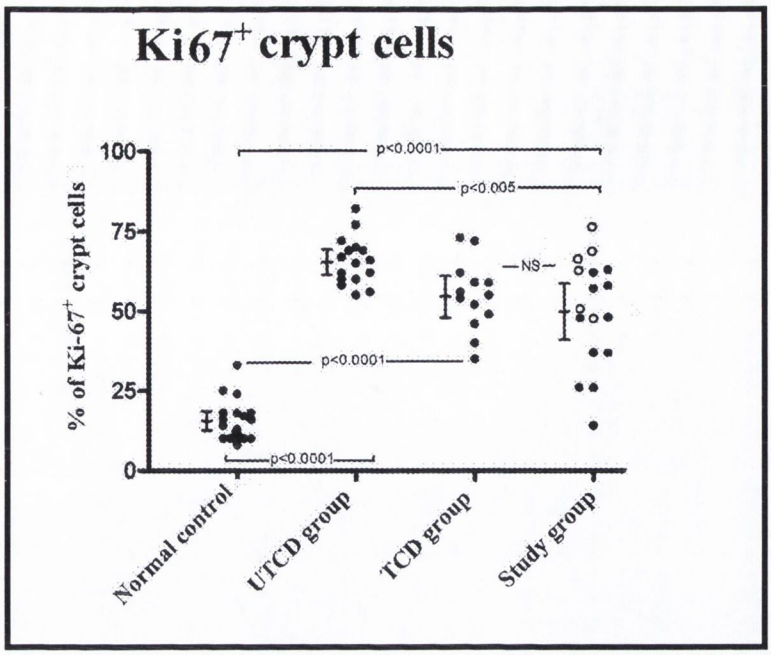


Figure 2.10 Percentage of crypt cells expressing Ki67 marker in the study group subjects, treated coeliac disease group (TCD), untreated coeliac disease (UTCD) group, and normal control group. Open circles denote patients who later developed a classical coeliac lesion. Bars represent the mean and 95% CI.

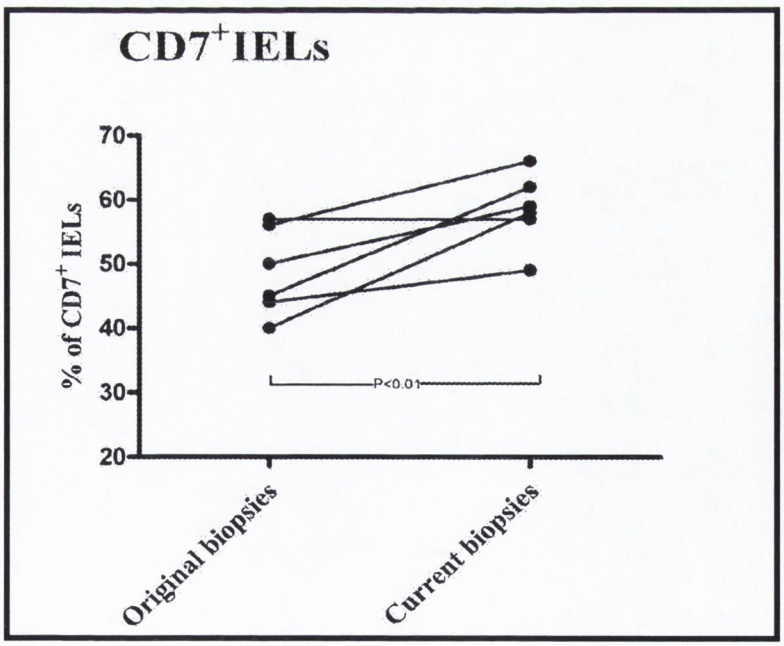


Figure 2.11 Percentages of CD7 in the original and current duodenal biopsies in six of nine of the study group subjects who attended for follow-up examination.

2.4 DISCUSSION

The diagnosis of coeliac disease is currently based on the finding of an inflammatory histological lesion in the small intestinal mucosa (Cooke and Holmes 1984; Marsh 1992; Walker-Smith *et al.* 1990; Oberhuber G *et al.* 1999). However, some coeliac patients have only a minimal mucosal lesion and this may not be observed during routine histological analysis (Kaukinen *et al.* 2001; Mino *et al.* 2003; Settakorn *et al.* 2004). The presence of on-going gluten sensitive enteropathy in these patients may be indicated by the finding of coeliac related auto-antibodies (Kaukinen *et al.* 2001; Dickey *et al.* 2005; Collin *et al.* 1993). This clinical dilemma was addressed in this study. A group of 25 patients with a positive endomysial antibody test, but who were considered not to have coeliac disease based on normal duodenal histological findings, were further evaluated. Detailed analysis of the original biopsies, repeat serology and further clinical evaluation revealed that six of these patients had developed clear-cut evidence of coeliac disease, and this diagnosis was also very likely in a least three further patients.

The original duodenal biopsy samples were available from 20 individuals. Further studies were performed on these biopsies to see if there was evidence of gluten sensitive pathology. Repeat examination of haematoxylin and eosin stained tissue sections revealed that the IEL count was elevated in four subjects, but no other morphological abnormalities were noted. In contrast, when immunohistochemical staining of the sections was performed, this revealed a significant increase in intraepithelial T cells expressing CD2, CD3, CD7, CD8, and CD69 antigens (Figures 2.2 –2.8). In addition, based on Ki67 staining, a significant increase in enterocyte proliferation was found in these patients. Thus, despite the initial finding that no histological lesion was evident in these patients, more detailed investigation did show abnormalities to be present.

Follow-up clinical evaluation and investigation was performed on 14 of the 25 patients, 5 to 10 years after their initial presentation. Duodenal biopsies showed that six of these patients had developed the classical small intestinal enteropathy characteristic of coeliac disease. In these patients, the finding of positive coeliac

serological tests (anti-endomysial, anti-tissue transglutaminase and anti-gliadin antibodies) with low serum ferritin levels was entirely consistent with this diagnosis (Feighery *et al.* 1998; Chorzelski *et al.* 1983; Dieterich *et al.* 1998; Sulkanen *et al.* 1998). In addition, in a seventh patient who failed to have a repeat biopsy, a history of childhood coeliac disease was revealed. This patient continued to have positive coeliac serology. Thus, of the 14 patients available for further clinical review, seven had coeliac disease. In contrast, it is unlikely that any of the remaining seven reviewed patients had coeliac disease: in all of them, coeliac serology was now negative and in the three patients who were biopsied, the duodenal histology was normal. The possible explanation of these findings is that the initial results were false positive even though the given rate of false positivity of the anti-endomysial antibodies in our lab over the past decade was low. It is also possible that these patients reduced their dietary gluten intake since their initial evaluation and that resulted in negative coeliac disease serology when they subsequently attended for reassessment.

The current gold standard investigation for gluten sensitive enteropathy is small intestinal biopsy (Marsh 1992; Walker-Smith *et al.* 1990; Oberhuber *et al.* 1999). However, the sensitivity of this investigation has rarely, if ever, been formally investigated (Feighery *et al.* 1998). Moreover, when minor, subtle or patchy histological lesions are present, these may not be noted by the histopathologist (Kaukinen *et al.* 2001; Mino *et al.* 2003; Settakorn *et al.* 2004; Biagi *et al.* 2004; Jarvinen *et al.* 2004). Nonetheless, some such patients are symptomatic and have gluten sensitive malabsorption. Indeed, several of the patients in this study were likely to have established coeliac disease when they initially presented: these patients had typical symptoms, positive coeliac auto-antibodies and reduced serum ferritin levels. However, because no histological lesion was identified, they were considered not to have coeliac disease and discharged from hospital.

An increase in IELs, called a Marsh grade 1 lesion, is the most common mild histological lesion found in coeliac disease (Marsh 1992). Moreover, in recent publications it was reported that the raised IEL numbers may only be evident at the villous tip (Biagi *et al.* 2004; Jarvinen *et al.* 2004). This abnormality may be missed on routine histological examination of biopsies unless a formal count of these cells is performed. In this study, to enhance the recognition of IELs, tissue

sections were stained with a range of monoclonal antibodies, including antibodies to CD2, CD3, CD7, CD8, and CD69. A significant increase in IELs was noted in the study population with each of these antibodies. Those patients (n=6) who developed definite evidence of coeliac disease had the highest counts of IELs expressing the lymphocyte markers measured (Figures 2.2, 2.4, 2.5, 2.7,2.8 and 2.10). In earlier publications, emphasis was given to raised numbers of intraepithelial T cells which expressed the gamma delta T cell receptor, and this was considered a specific finding in coeliac disease (Spencer *et al.* 1999; Maki *et al.* 1991; Arranz *et al.* 1994). However, currently available monoclonal antibodies to the gamma delta T cell receptor do not react satisfactorily on formalin fixed tissue and frozen tissue is not routinely available for examination (Maki *et al.* 1991).

The findings reported in this study are in keeping with earlier reports of raised IEL counts in coeliac mucosa, when biopsies were stained with anti-CD3 or anti-CD8 monoclonal antibodies (Kelly *et al.* 1987; Catassi *et al.* 1993; Jarvinen *et al.* 2003). There are few previous immunohistochemical studies examining IEL staining with antibodies to CD2, CD7 and CD69. In one study, CD3 negative/CD7 positive cells were found to be either unchanged or reduced in coeliac disease (Spencer *et al.* 1989) and a marked reduction in this subset of cells was also reported in a study employing flow cytometery (Eiras *et al.* 1998). The results of this current study suggest that investigation of small intestinal tissue with any of these monoclonal antibody reagents may be of value in identifying an increase in the IEL population in gluten sensitive mucosa.

Enterocyte proliferation is a reported finding in coeliac disease (Savidge *et al.* 1995; Przemioslo *et al.* 1995; Moss *et al.* 1996) and assessment of this parameter might be useful in detecting evidence of a minimal inflammatory lesion in this disorder. In this study the Ki67 antibody, which detects a nuclear antigen associated with cell proliferation, was employed for this purpose (McCormick *et al.* 1993). In the study group, expression of Ki67 was increased, and this was also found in patients with untreated and treated coeliac disease. These findings concur with a recent study, which reported a raised Ki67 index in individuals with positive coeliac auto-antibody serology and normal small intestinal mucosa (Settakorn *et al.* 2004).

In the early years of the study (1991 to 1997), the anti-tissue transglutaminase test had yet to be developed. Patients who presented in those years had anti-endomysial antibody investigations alone performed. However, stored samples were available on 12 patients, enabling repeat serology to be performed, including anti-tissue transglutaminase antibodies. In seven of these patients, repeat serology testing confirmed the presence of anti-endomysial antibodies and in addition raised anti-tissue transglutaminase antibodies: this group included five patients in whom later biopsies showed the presence of enteropathy. The two additional sero-positive patients (Table 1, nos.16, 17) had low serum ferritin levels and symptoms compatible with coeliac disease. In contrast, the stored serum samples in the remaining five patients were negative for both anti-tissue transglutaminase and endomysial antibodies and remained negative when these patients returned for review in the follow-up study. This would suggest that the original serological findings in these latter patients were false positive results: such a finding is perhaps not surprising when it is considered that in excess of 50,000 samples were tested over this decade, giving an approximate false positive rate of 0.014%. It is probable that this false positive rate is even lower now, as positive samples are first detected employing the anti-tissue transglutaminase assay and then re-examined with the anti-endomysial antibody test.

In conclusion, the findings of this study emphasise the value of auto-antibody serological tests in the diagnosis of coeliac disease. Absolute reliance on the finding of a mucosal lesion, when standard histological examination of biopsy tissue is performed, is likely to under-diagnose gluten sensitive enteropathy. If more subtle abnormalities are present, including increased numbers of IELs and increased enterocyte proliferation, these can be detected by immunohistological staining of tissue sections. When a diagnosis of coeliac disease is being considered in a patient with positive coeliac auto-antibodies but apparently normal small intestinal mucosa, it is important that continued clinical follow-up be performed.

CHAPTER 3.0

QUANTIFICATION OF ENTEROCYTE STRUCTURES AND TUBULIN FIBRE ARRANGEMENT IN THE STUDY GROUP SUBJECTS AND COELIAC DISEASE PATIENTS

3.1 INTRODUCTION

Coeliac disease is a gluten sensitive enteropathy characterised by inflammation of the small intestinal mucosa, with a range of abnormal villous architecture and crypt hyperplasia (Trier *et al.* 1998; Cooke and Holmes 1984; Marsh 1992). However, several studies have reported only minimal intestinal changes in some patients with positive coeliac disease serology (Kaukinen *et al.* 2002; Mino *et al.* 2003; Settakorn *et al.* 2004). In a proportion of these patients, detailed histological analysis reveals subtle histological abnormalities such as nuclear disarray, irregular cell shape (Magliocca *et al.*1992), and increased number of intraepithelial lymphocytes (IELs) (Kaukinen *et al.* 2002; Mino *et al.* 2003; Settakorn *et al.* 2004) with this increase sometimes being confined to the villous tip (Jarvinen *et al.* 2004; Biagi *et al.* 2004). In this clinical setting, the diagnosis of coeliac disease may be missed, unless the patient is regularly monitored for more definitive evidence of gluten sensitive enteropathy.

Cytoskeleton proteins are involved in essentially all structural and dynamic aspects of the living cell, including maintenance of cell shape, movement, replication, apoptosis, differentiation and intracellular signalling (Fuchs *et al.* 1998; Banan *et al.* 1999; Fenteany *et al.* 2004). The cytoskeleton of eukaryotic cells is composed of three major protein families that form filamentous structures running throughout the cell, i.e. microfilaments consisting of different actin isoforms, microtubules made of α- and β-tubulin, and the intermediate filaments, together with their associated molecular motors and regulatory protein complexes (Fuchs *et al.* 1998; Banan A *et al.* 1998; Banan A *et al.* 1999).

The requirement for microtubules to regulate cell morphology, cell polarity and maintain the plane of cell division has been demonstrated (MacRae *et al.* 1992; Parczyk *et al.* 1989). Disruption of the cytoskeleton can severely limit cell function, and if not reversed can adversely affect its integrity and viability (Banan *et al.* 1998; Banan *et al.* 1999; Banan *et al.* 2000). It has been demonstrated *in vitro* that disrupting microtubule function either by chemical interference or by genetic deficiencies affects the intracellular trafficking of antigens peptide loading onto MHC class I molecules, and MHC-class II endosomal sorting (Faigle *et al.* 1998). Cytoskeletal protein defects may be the underlying cause of many

pathological phenotypes. Indeed, many pathological conditions have now been shown to be associated with abnormalities in cytoskeletal proteins, including several cardiovascular disease syndromes, neurodegeneration, cancer, liver cirrhosis, pulmonary fibrosis, and skin diseases (Lane *et al.* 2004; Zatloukal *et al.* 2004; Chaponnier *et al.* 2004; Rottner *et al.* 2004; Cairns *et al.* 2004).

It has been shown that gluten changes the organisation of the cytoskeleton in cell line (Intestine 407 cells) (Sjolander *et al.*1988). The actin network has been shown to play an important role in the gliadin-induced effects on intestinal epithelial cells, demonstrated by both *in vivo* and *in vitro* studies (Oberhuber *et al.*1999; Oxentenko *et al.* 2002). Exposure of coeliac disease patients on a gluten-free diet to gliadin has been shown to cause rapid disarrangement of intestinal mucosal actin filaments *in vivo* (Holmgren-Peterson *et al.* 1995; Bailey *et al.* 1989). More recently, Clemente and colleagues reported that gliadin has a direct effect on the enterocyte cytoskelton *in vitro*. They found that gliadin induced an immune independent increase in actin polymerisation and caused redistribution of actin filaments in the intracellular compartments (Clemente *et al.* 2003).

Nuclear disarray and irregular enterocyte shape are common features of the coeliac lesion. These changes are thought to be caused by rearrangement of the cytoskeletal proteins. The high content analysis (HCA) approach, based on automated extraction of data from cellular images is proving to be an invaluable tool for the investigation of cytoskeletal networks, allowing for the study of their complex dynamic changes at a qualitatively new level of detail. Using the morphology explorer bioapplication tool (Cellomics®), HCA technology was deployed to investigate and quantify fluorescent images of the cytoskeleton in the NIH 3T3 cell line (Grove, Poster presentation, Cellomics meeting, Pittsburgh 2004). One of the key components of an HCA assay is an automated image analysis algorithm, which is used to extract biologically meaningful multiplexed measurements from a large number of fluorescent microscopic images.

In this study, we used this new technology (Cellomics ®) to perform detailed examination of tubulin profiles and enterocyte structure in a group of patients with positive coeliac disease serology and normal duodenal mucosa (Patients are referred to throughout the manuscript as the study group subjects). Our study is

the first to provide detailed quantitative characterisation of the enterocyte morphology and cytoskeletal structures utilizing the power of HCA technology.

3.2 MATERIALS AND METHODS

3.2.1 Patients

3.2.1.1 Study group subjects

Over the past ten years (1991-2001), we have identified 25 patients with positive endomysial antibodies, but apparently without the duodenal morphological changes suggestive of gluten sensitivity. These included 11 males with mean age of 46 years (range 22-75 years) and 14 females with mean age of 47 years (range 20-86 years). These patients presented with abdominal pain, diarrhoea, weight loss, anaemia, fatigue, bloating and dyspepsia. Based on the routine histology findings, none of these patients was considered to have a gluten sensitive enteropathy. In 2002, all patients were invited to attend for review. Fourteen patients returned and further clinical and laboratory assessment were performed on these 14 patients. This included small intestinal biopsy in nine patients (Figure 3.1). Six of these nine patients had developed villous atrophy compatible with untreated coeliac disease patients (refer chapter 2 for more details). In this chapter we examined the earlier (original) and current duodenal biopsies obtained from these six patients (Figure 3.1).

3.2.1.2 Untreated coeliac disease patients

Fourteen untreated coeliac disease patients were investigated. Four males with a mean age of 59 years (range, 49-69 years) and 10 females were studied with a mean age of 52 years (range, 35-74 years). All the 14 showed positive endomysial and tissue transglutaminase antibodies and had duodenal partial villous atrophy.

3.2.1.3 Treated coeliac disease patients

This group of patients were on a gluten free diet. All showed negative coeliac disease serology and had normal duodenal mucosal based on routine histology. This group consisted of four males with a mean age of 54 years (range, 31-57 years) and six females, with a mean age of 41 years (range, 29-47years).

3.2.1.4 Normal control group

Twenty individuals screened for evidence of coeliac disease and found to be negative for endomysial and tissue transglutaminase antibodies were used as

normal controls. These consisted of nine males with a mean age of 56 years (range, 35-79 years) and 11 females with a mean age of 55 years (range, 33-82 years). These underwent upper gastrointestinal endoscopy and duodenal biopsy examination, which showed normal duodenal mucosa.

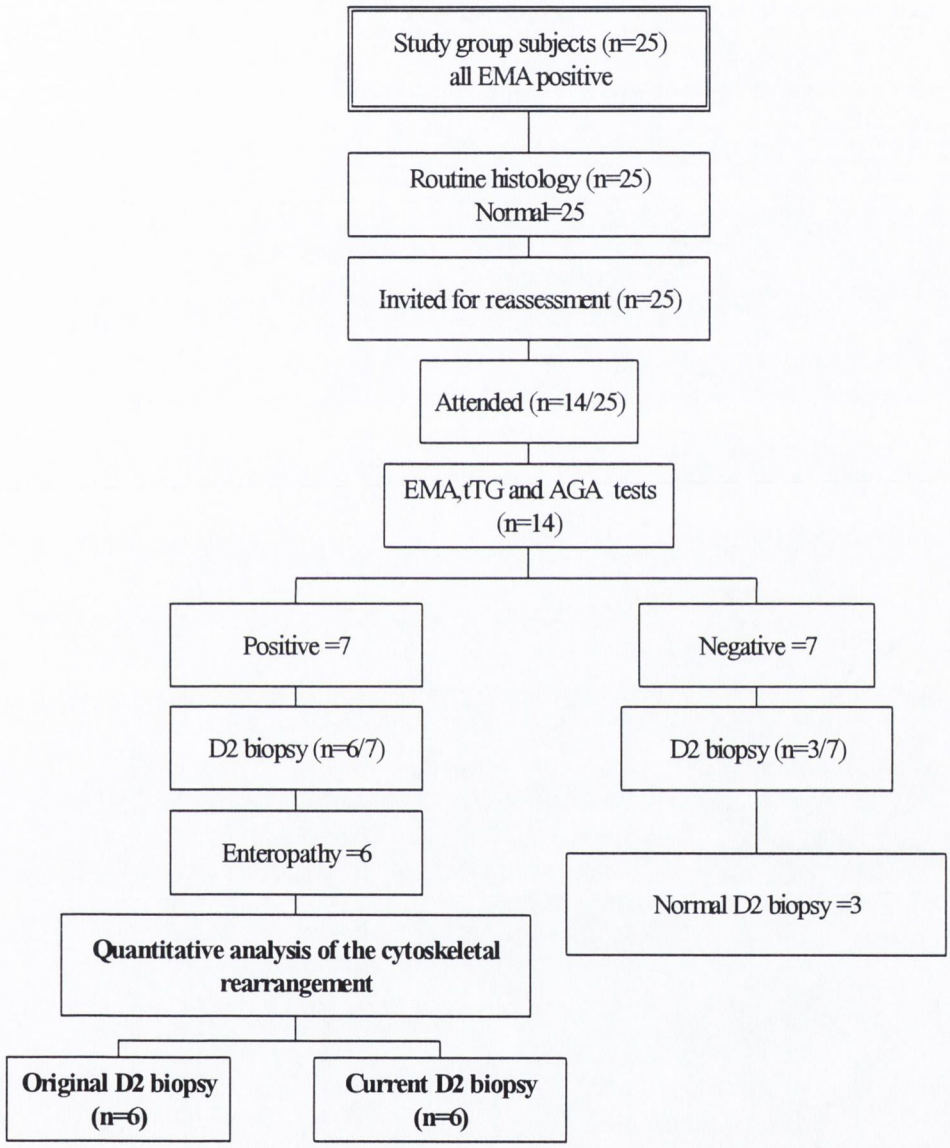


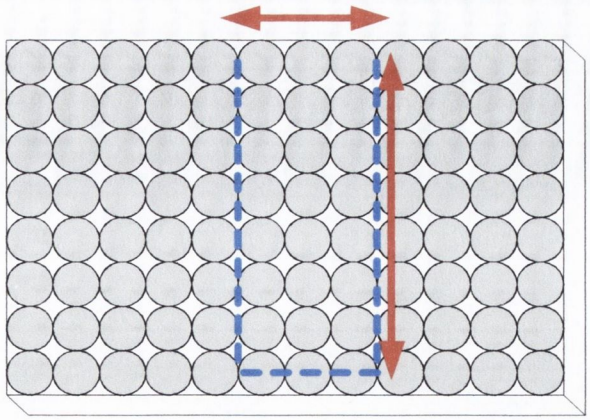
Figure 3.1 Patient flow diagram

3.2.2 Immunofluorescent technique

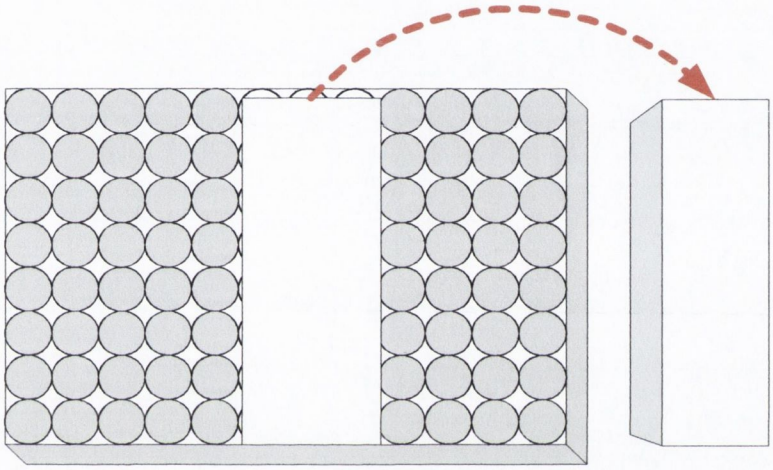
2μm-thick duodenal sections from paraffin-embedded blocks were placed on the uncoated glass slides and dried overnight at 52°C. Sections were dewaxed and rehydrated in a series of graded xylene and alcohol solutions and subsequently immersed in phosphate-buffered saline solution (PBS) at pH=7.4. Sections were then preheated for antigen retrieval in a microwave oven in 0.1 M citrate buffer pH 6.0 for 20 minutes on high power and cooled for 20 minutes at room temperature. After immersing the prepared samples in PBS, the sections were next incubated with alpha tubulin antibody (clone B-5-1-2, Sigma, St Louis, MO) at 1:100 dilution in antibody buffer (0.05% bovine serum albumin and 0.1 sodium azide in PBS) for 60 minutes at 4°C. Sections were then incubated with Alexa® (Molecular Probes, Eugene, OR) fluorescent conjugated secondary antibody at 1:500 in dilution buffer for 30 minutes. Cell nuclei were counterstained with 1μM Hoeschst 33258 (Sigma, St Louis, MO). After each antibody application, sections were washed twice in PBS. Sections were then mounted with DAKO fluorescent mounting media and kept in the dark at 4°C until used in analysis. Negative control samples were incubated with isotype-matched control immunoglobulins instead of monoclonal antibodies.

3.2.3 Slide holder modification

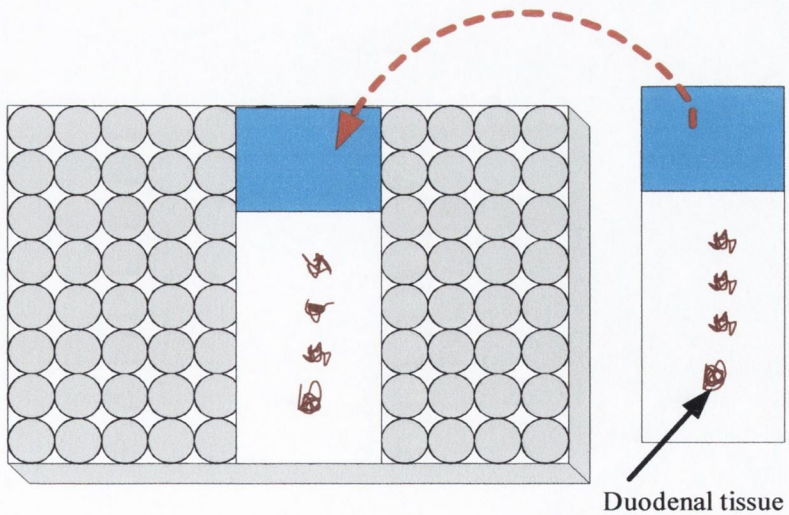
As there was no slide holder commercially available at the time of this study, we had to modify a 96 well plate for use as a slide holder. We measured the slide length and width and then we cut the base of the plate based on these measurements. We removed the base of the plate corresponding to the slide size and then replaced with the slide that had stained duodenal tissue fixed on it (Figure 3.2 A-C).



A. Dimensions of a glass slide (length and width) were measured and applied to the base of a 96 well plate.



B. The measured section was cut and removed



C. A slide with stained sections was fixed into the frame made

Figure 3.2 The modification of a 96 well plate to be used as a slide holder for HCA.

3.2.4 Quantification and image acquisition

Once sections were stained and dried, slides were attached onto the custom-modified slide holder developed for imaging on the Cellomics® Kineticscan reader (Figure 3.2 C). Three-colour image sets were simultaneously acquired at 40x dry lens magnification. Quantitative data from the stored digital images of the enterocytes were processed using the morphology explorer bioapplication tool (Cellomics®). This bioapplication has been optimised for cell morphology analysis, including cytoskeleton rearrangements and Hoechst-stained nuclei. "Virtual" scanning was carried out using specific assay parameters of fluorescent images of the enterocytes in each histological section. These were acquired and analysed by proprietary image processing algorithms.

3.2.5 Optimisation of image acquisition

The morphology explorer bioapplication has been originally designed to be a general tool that can perform a wide range of measurements related to cell morphology. The features and capabilities provide the researchers with the flexibility to design their own assays. However, this bioapplication has previously been used to only analyse cell lines. We have optimised it for use with paraffin embedded duodenal sections, so that individual cells within selected tissue areas could be analysed *in situ*. In order to examine duodenal sections on the HCA reader, we modified a 96 well plate to employ as a slide holder (Figure 3.2) and developed a system for identification of the region of interest (middle third of the villi) of the duodenal tissue using a field map.

To examine enterocytes individually (Figure 3.3), we used three different fluorescent channels, each determining a specific morphological feature of the enterocytes. Furthermore, we set up the parameters to examine the whole cells and cytoskeletal fibre arrangement. These parameters (Background correction, smooth factor in channel 1 and 2, object segmentation in channel 1 and 2, fibre detection and fibre alignment in channel 3) were activated simultaneously. Thus, we were able to measure the cell length, width, nuclear shape and the tubulin fibre profiles in each individual enterocyte from the duodenal sections of the normal control and the study groups.

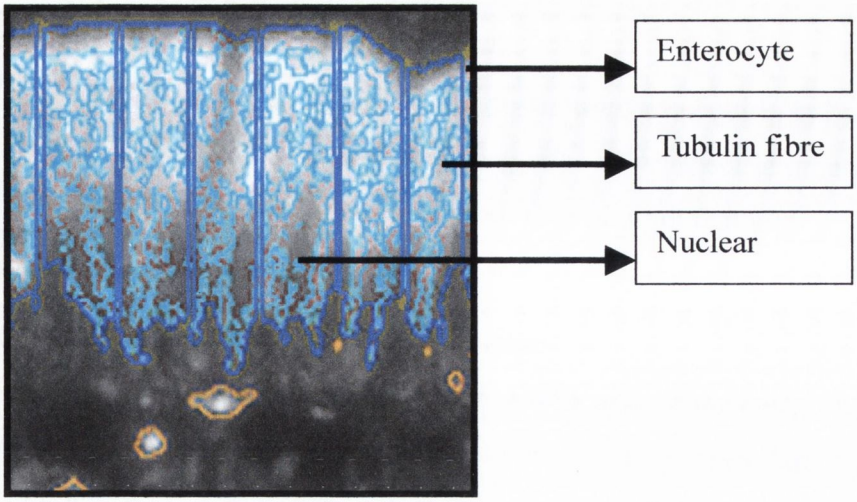


Figure 3.3 Segmented enterocytes

3.2.5.1 Field map

Based on the object magnification, there are defined grid references. As the object power is increased, these grids are subdivided (Figure 3.4). Following initial location of tissue using our adapted system, we further defined structure location using this field map tool.

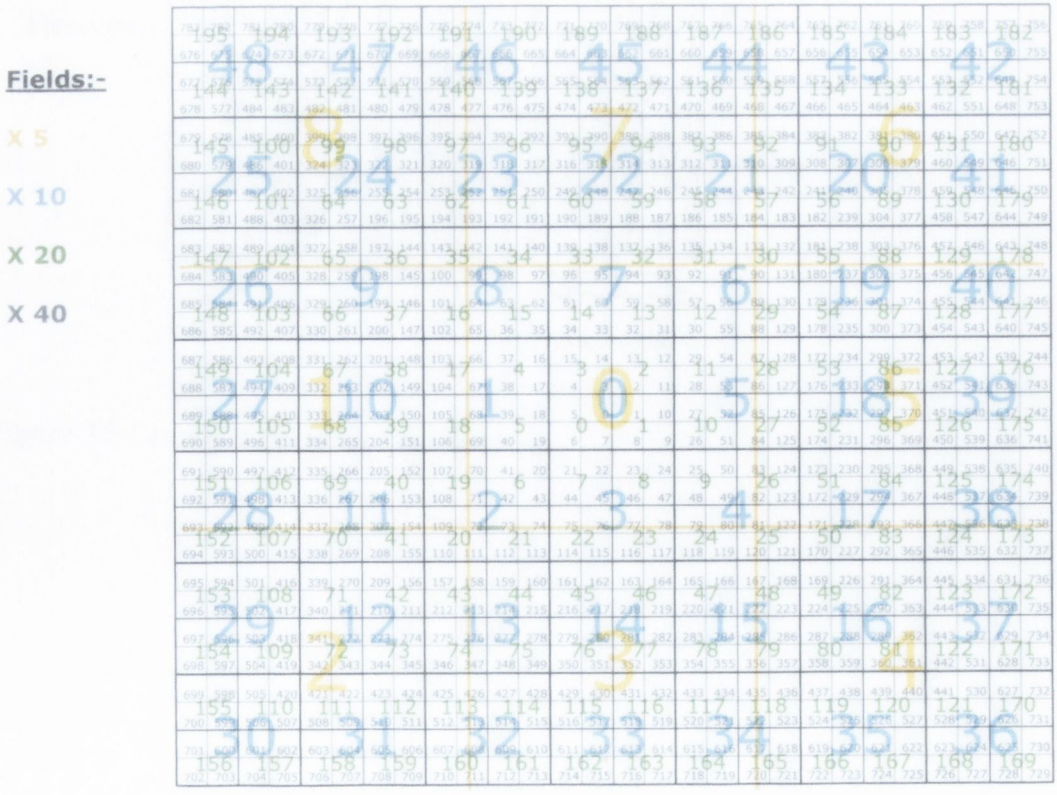


Figure 3.4 Field map

3.2.6 Interpretation of the results

The high content analysis approach was used to acquire and process the images and to quantify the overall morphology as well as specific tubulin fibre changes of the enterocytes based on an optimised set of output features.

3.2.7.1 Length and width of enterocytes

The length was measured along the middle axis of the cell. These measurements are approximations of the elongated cell, so that it resembles a fibre as shown in Figure 3.5. The width was measured perpendicular to the middle axis of the enterocytes. These measurements were based on a single enterocyte boundary (Figure 3.5).

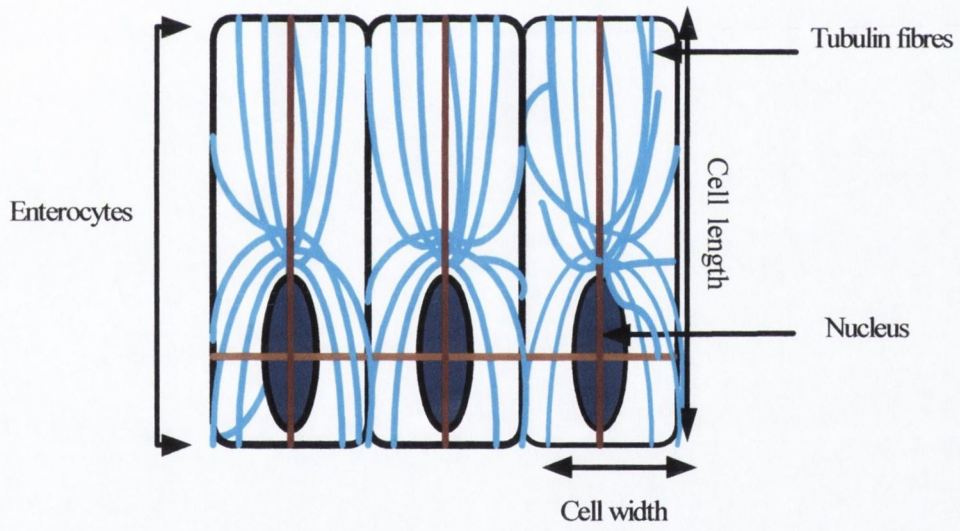


Figure 3.5 Length and width of enterocytes

3.2.7.2 Nuclear shape

The area and perimeter of each nucleus was investigated. The area is proportional to the total number of pixels covered by the nucleus. Thus, we used pixels to measure the nuclear shape parameters such as the nuclear roundness (perimeter 2 area; P^2A) and nuclear length and width ratio (LWR). The more rounded the nucleus, the closer the (P^2A) values are to one. A round nuclear length and width are the same, and thus its LWR is equal to one (i.e Figure 3.6).

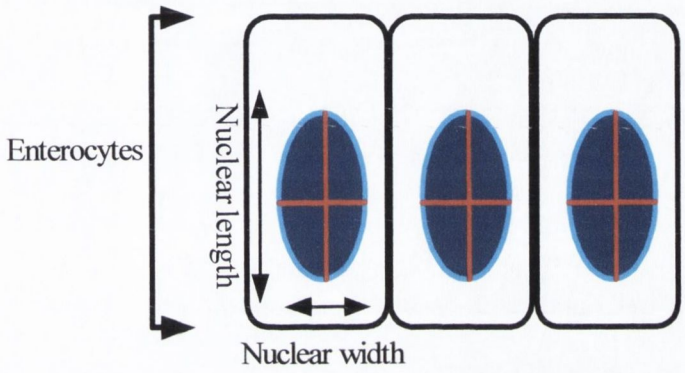


Figure 3.6 Nuclear shape (Roundness and LWR)

3.2.7.3 Fibre angles of tubulin

This is the standard deviation (SD) of the angles of the algorithmic image of the fibres within the enterocytes. These measurements were done based on the algorithmic referenced angles. The higher the SD numbers the greater the degree of radial distribution of the fibres (i.e. Figure 3.7 a-b).

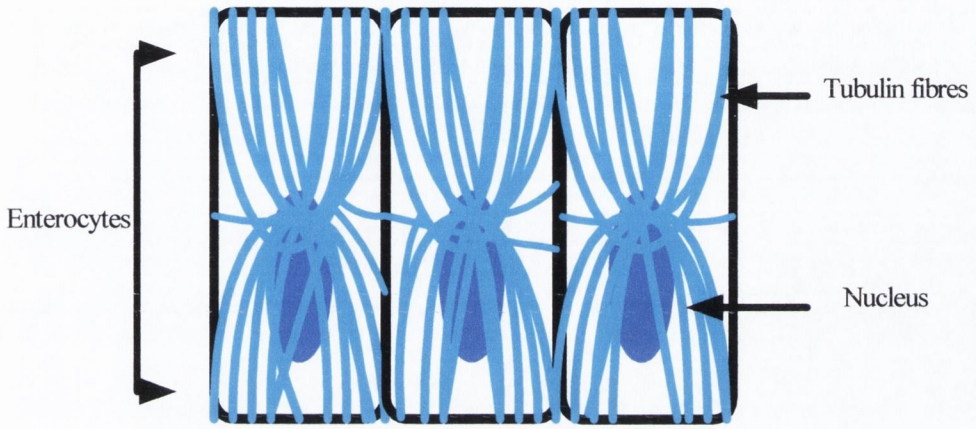


Figure 3.7 (a) The higher the SD of the fibre angles the less fibres are aligned to each other.

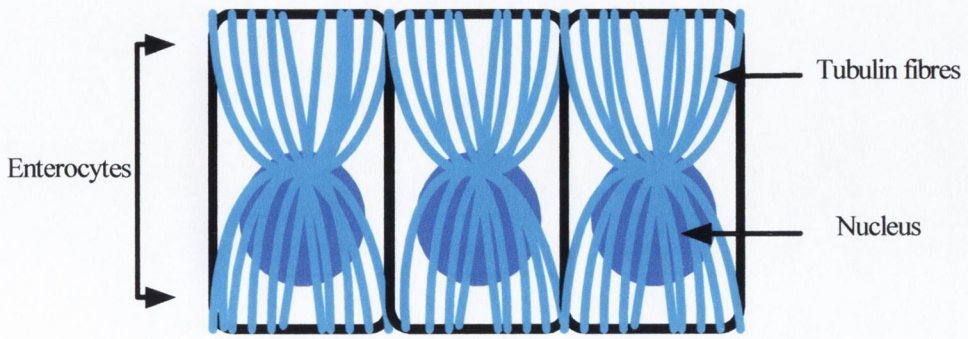


Figure 3.7 (b) The smaller the SD of fibre angle, the more aligned the fibre.

3.2.7.4 Enterocyte tubulin fibre intensity

The Bioapplication reports both the average and total intensity of the output features of the enterocyte tubulin fibre. The fibre mean staining intensity is the approximation number of pixels of the labelled fibre within the enterocytes (i.e. Figure 3.8).

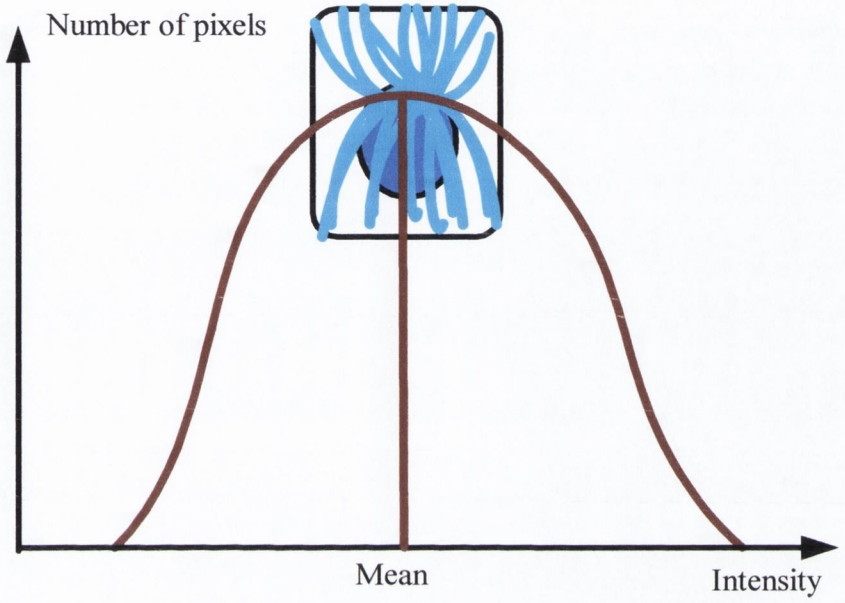


Figure 3.8 The mean number of pixels of a labelled fibre intensity distribution

3.2.7.5 Enterocyte tubulin fibre density

The surface area density is a texture measure which takes into account both the intensity variation and spatial distribution (X and Y) of the pixels (i.e.Figure 3.9). i.e. algorithmic tubulin fibres with uniform high staining intensity will give a lower density than tubulin fibres with lower intensities but with a lot of variability in intensity values between neighbouring matrices.

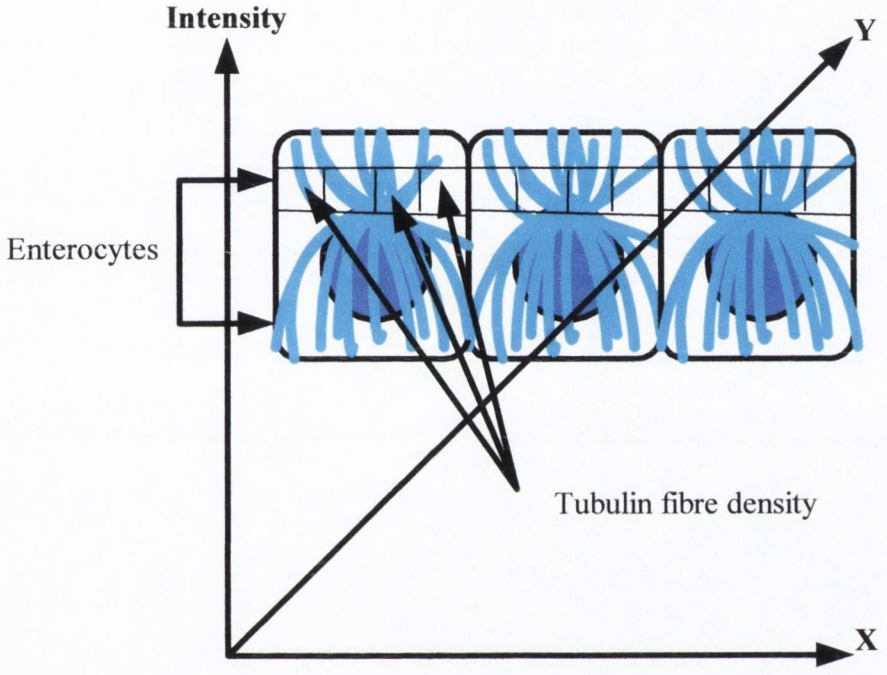


Figure 3.9 Tubulin fibre density

3.2.7 Statistical analysis

The 95% confidence intervals (CI) of the enterocytes structure characterisation and the tubulin fibre profiles for the study group subjects and the three control groups were calculated. The Mann-Whitney U test was then used to assess the statistical significance of differences. A p-value of <0.05 was considered to be statistically significant.

3.3 RESULTS

The approximate measurements of the enterocyte shape were calculated based on the output features from the algorithmic image of the gross pattern of tubulin staining. At least ten cells were examined from each side of the middle third of the villi. The mean and 95% CI of enterocyte measurements and the tubulin fibre profiles are summarised in Table 3.1. Results are expressed as the mean and range in pixels. Likewise, details of nuclear data are summarised in Table 3.1.

3.3.1 Enterocyte length

In the normal small intestine, enterocytes have a columnar shape, and in the study enterocyte length was measured along the medial axis of the cells (Figure 3.10a). In biopsy tissue from normal subjects, the mean enterocyte length was 67 pixels (range 65-74). The enterocyte length was significantly reduced in all the patient groups; in the untreated coeliac patients, mean 53 pixels, $p<0.000$; in treated coeliac patients, mean 56 pixels, $p<0.001$; in the initial biopsy of the study group, mean 57 pixels $p<0.01$; and in the current biopsy in the study group, mean 57 pixels, $p<0.006$). Of interest, within the patient groups, the results were not statistically different (Figure 3.11).

3.3.2 Enterocyte width

Enterocyte width was measured perpendicular to the middle axis of the cell (Figure 3.10). In the normal subjects, the mean enterocyte width was 11 pixels (range 9-12). This was significantly lower than that found in the untreated coeliac patients, mean 14 pixels ($p<0.002$) and the current biopsy in the study group, mean 13 pixels, ($p<0.03$). It was noteworthy that the enterocyte width in the treated coeliac patients and in the initial biopsy of the study group was similar to the normal control subjects (Figure 3.12).

3.3.3 Nuclear shape (roundness)

In the normal enterocyte, the nucleus has an elliptical shape. The measurement of the nuclear shape is based on the perimeter square area of the nucleus (P^2A). A higher nuclear P^2A figure is found in the nucleus of normal columnar epithelial cells, reflecting their elliptical shape (Figure 3.13). As the nuclear shape develops a more rounded shape, the closer the P^2A unit gets to one. This feature

was investigated in this study in the normal subjects and the patient groups. In the biopsies from normal subjects, the mean P^2A value was 1.5 pixels (range 1.4-1.6). The P^2A values were significantly closer to one in all the patient groups, in comparison with the normal subjects; in the untreated patients, mean 1.3 pixels ($p<0.0001$); in the treated coeliac patients mean 1.5 pixels ($p<0.0001$); in the initial biopsy of the study group mean 1.4 pixels ($p<0.001$); and in the current biopsy of the study group mean 1.2 pixels ($p<0.0003$). Of interest, the nuclear P^2A values in the treated coeliac patients were significantly greater than the untreated coeliac patients ($p<0.001$), the initial biopsy of the study group ($p<0.01$) and the current biopsy in the study group ($p<0.0002$; Figure 3.14).

3.3.4 Nuclear length and width ratio (LWR)

The enterocyte nuclear LWR was also examined based on the P^2A. A high nuclear LWR value is found in the nucleus of normal columnar epithelial cells and in the biopsy tissue from the normal subjects, the mean nuclear LWR was 2 pixels (range 1.9-2.2). In comparison with the normal subjects, the nuclear LWR was significantly reduced in the untreated coeliac patients, mean 1.5 pixels ($p<0.0001$); in the initial biopsy of the study group, mean 1.8 pixels ($p<0.01$); and in the current biopsy in the study group, mean 1.5 pixels ($p<0.0005$). In the treated coeliac patients, nuclear LWR was not significantly different from the normal subjects (Figure 3.15). These results suggest that the nuclear shape in both initial and current duodenal section of the study group subjects and the untreated coeliac disease patients have been changed.

3.3.5 Enterocyte tubulin fibre angles

Fibre angle measurement represents the degree of intracellular branching of the tubulin. These measurements were performed based on the algorithmic referenced angles. Normal cells containing radially oriented fibres, such as tubulin fibres radiating from the cell centre, would be expected to have large degree angles. This is what was found in the normal columnar epithelial cells which displayed a high tubulin angle degree, mean 8.6 (range 7.8-9.4). Enterocyte tubulin fibre angles were significantly reduced in all the patient groups, except in the treated coeliac patients; in the untreated coeliac patients, mean angle degree 7.2 ($p<0.010$); in initial biopsy of the study group, mean 6.6 ($p<0.01$); in the current biopsy of the study group, mean 6.4 ($p<0.005$). Furthermore, the treated

coeliac patients showed a significantly higher angle degree, mean 8.3, than the untreated coeliac patients ($p<0.04$) and the current biopsies in the study group ($p<0.02$; Figure 3.16). Based on the algorithmic referenced angles, the tubulin fibres are more aligned to each other in both study group subjects and untreated coeliac disease patients.

3.3.6 Enterocyte tubulin fibre staining intensity

In the tissue from normal subjects, the mean intensity of enterocyte tubulin fibre staining was 174 pixels (range124-220). Staining intensity was significantly greater in all the patient groups except in the treated coeliac patients: in the untreated coeliac patients, the mean intensity was 330 pixels ($p<0.001$); in the initial biopsy in the study group, the mean intensity was 290 pixels ($p<0.04$); in the current biopsy in the study group, the mean intensity was 373 pixels ($p<0.006$). Within these three patient groups, no significant level of intensity staining was found (Table 3). The treated coeliac group, on the other hand, did not show any difference in comparison with the normal control group (Figure 3.17).

3.3.7 Enterocyte tubulin fibre density

The surface area density is a texture measurement which takes into account both the intensity variation and spatial distribution of the pixels within the enterocytes. In enterocytes from normal subjects, the mean tubulin fibre density was 48 pixels (range 43-54). Enterocyte tubulin fibre density was significantly increased in all the patient groups, with the exception of treated coeliac patients: in the untreated coeliac patients, the mean density value was 64 pixels ($p<0.001$); in the initial biopsy of the study group, the mean density was 65 pixels ($p<0.01$); and in the current biopsy of the study group, the mean density was 66 pixels (Figure3.18).

Table 3.1 Characteristics of enterocyte morphology and tubulin profiles in the patient groups.

Measurements	*Normal control group (n=20)*	*SGS (first D2 biopsies) (n=6)*	*SGS (second D2 biopsies) (n6)*	*UTCD group (n=14)*	*TCD group (n=10)*
Enterocyte length	67 pixels (65- 74)	57 pixels (47- 67)	57 pixels (49- 65)	53 pixels (47-59)	56 pixels (65-74)
Enterocyte width	11 pixels (10-12)	11 pixels (9-12)	14 pixels (13-16)	13 pixels (11 -14)	12 pixels (11-13)
Enterocyte nuclear shape (roundness)	1.6 pixels (1.5-1.6)	1.4 pixels (1.3-1.5)	1.2 pixels (1.1-1.3)	1.3 pixels (1.2-1.3)	1.5 pixels (1.4-1.5)
Enterocyte nuclear length width ratio	2 pixels (1.9-2.2)	1.8 pixels (1.6-1.9)	1.5 pixels (1.4-1.7)	1.5 pixels (1.4-1.5)	1.9 pixels (1.7-2)
Enterocyte tubulin fibre angles degree	8.6° (7.8-9.4)	6.6° (5.3-8)	6.4° (5.2-7.5)	7.2° (6.5-7.8)	8.3° (7-9.7)
Enterocyte tubulin fibre staining intensity	174 pixels (124-220)	290 pixels (161-419)	373 pixels (201-545)	330 pixels (249-411)	230 pixels (150-310)
Enterocyte tubulin fibre density	48 pixels (43-54)	65 pixels (52-77)	66 pixels (54-78)	64 pixels (59-69)	59 pixels (49-68)

UTCD: untreated coeliac disease patients; TCD: treated coeliac disease patients; SGS: study group subjects. Results are expressed as mean and 95% confidence intervals.

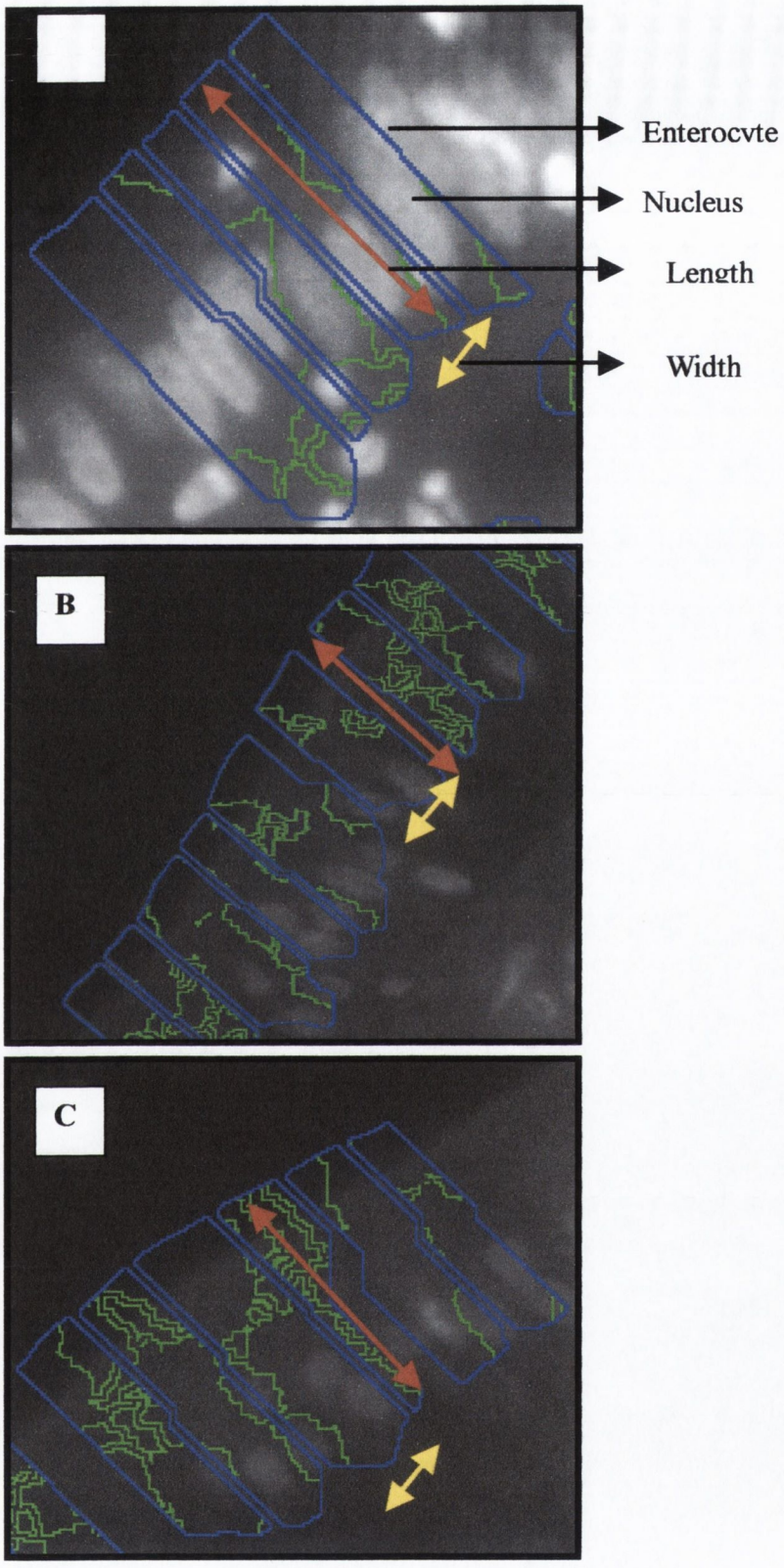


Figure 3.10 Enterocyte length (red) and width (yellow) in small intestinal mucosa of normal controls (A), untreated coeliac disease (B), and study group subjects (initial duodenal biopsy) (C).

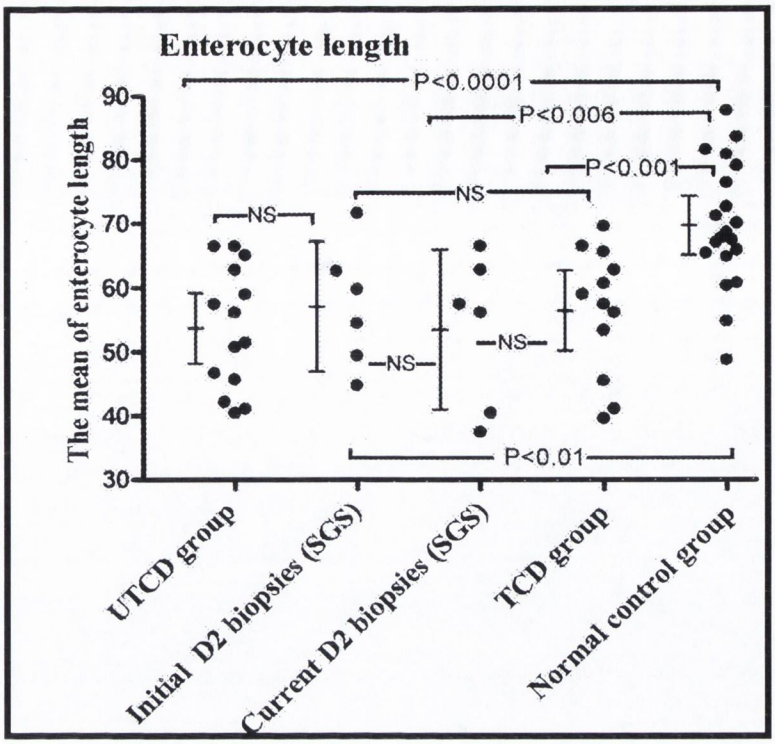


Figure 3.11 The mean of the enterocyte length in the initial and current duodenal section of the study group subjects (SGS), UTCD, TCD patients and the normal control group. Bars represent the mean and 95% CI.

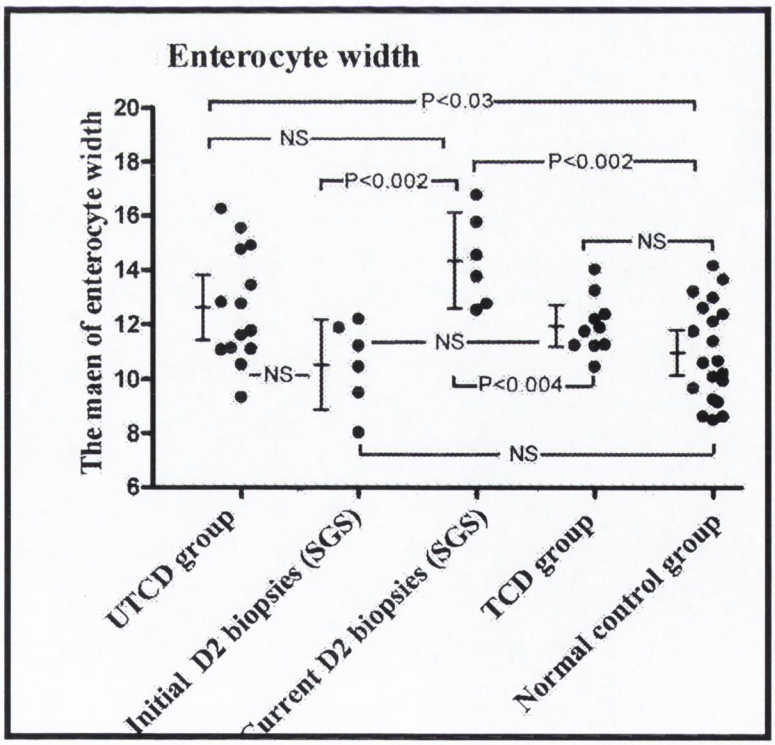


Figure 3.12 The mean of the enterocyte width in the initial and current duodenal section of the SGS, UTCD, TCD patients and the normal control group. Bars represent the mean and 95% CI.

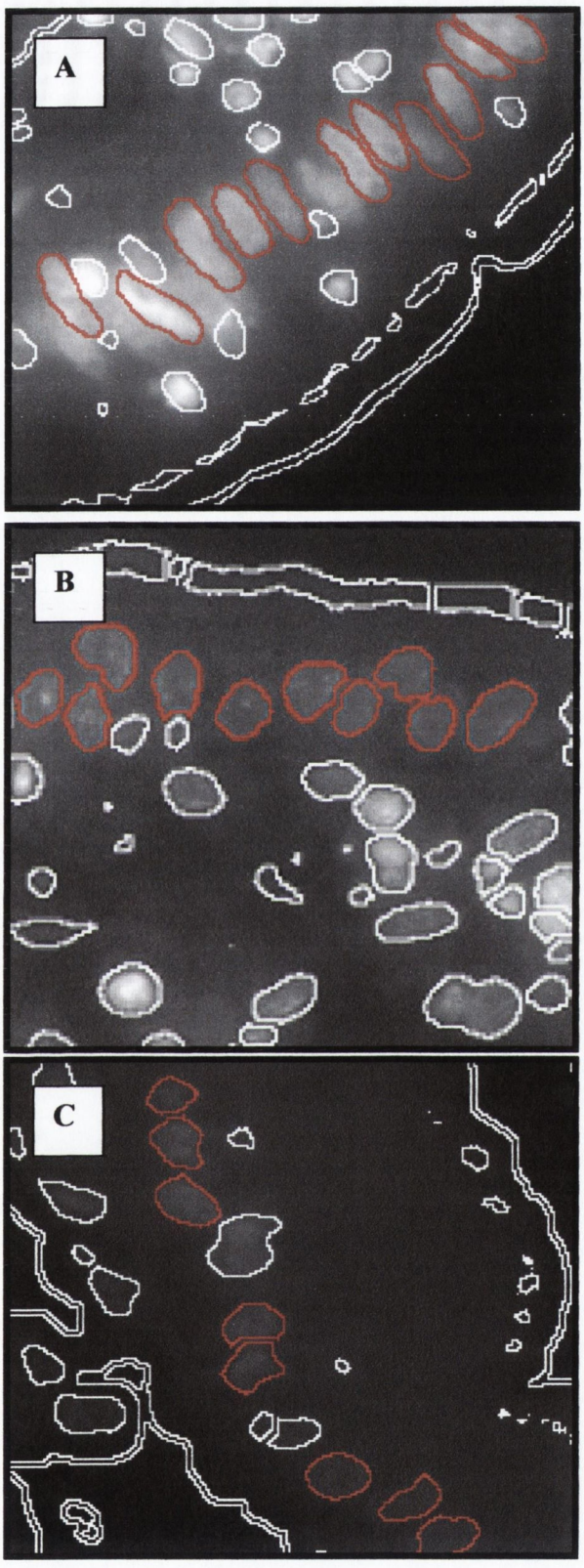


Figure 3.13 The examined enterocytes' nuclei (red) in intestinal mucosa of normal control (A), untreated coeliac disease (B) and study group subjects (initial duodenal biopsy (C).

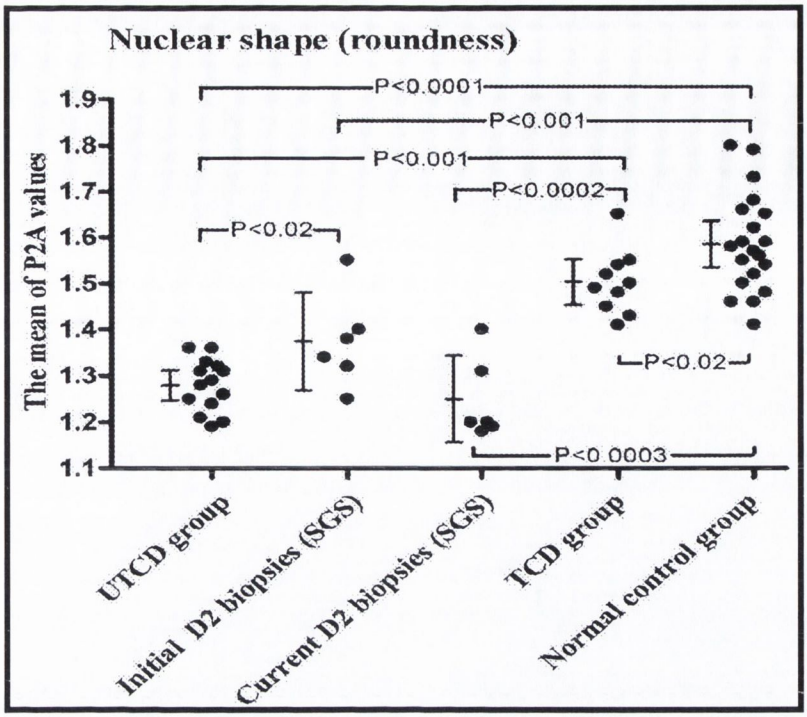


Figure 3.14 The mean of the enterocyte nuclear roundness in the initial and current duodenal section of the SGS, UTCD, TCD patients and the normal control group. Bars represent the mean and 95% CI.

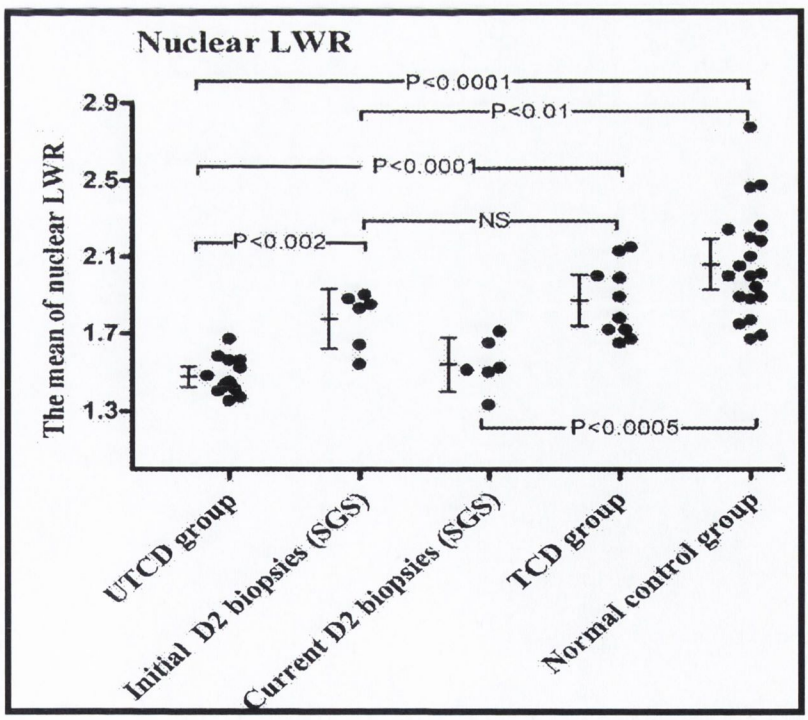


Figure 3.15 The mean of the enterocyte nuclear length width ratio (LWR) in the initial and current duodenal section of the SGS, UTCD, TCD patients and the normal control group. Bars represent the mean and 95% CI.

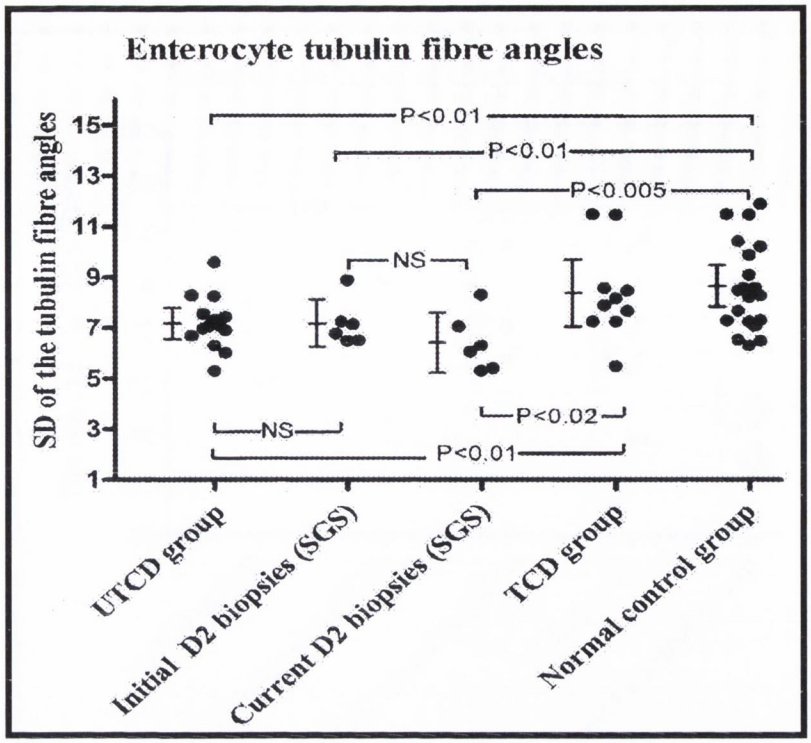


Figure 3.16 The SD of the enterocyte tubulin fibre angles in the initial and current duodenal section of the SGS, UTCD, TCD patients and the normal control group. Bars represent the mean and 95% CI.

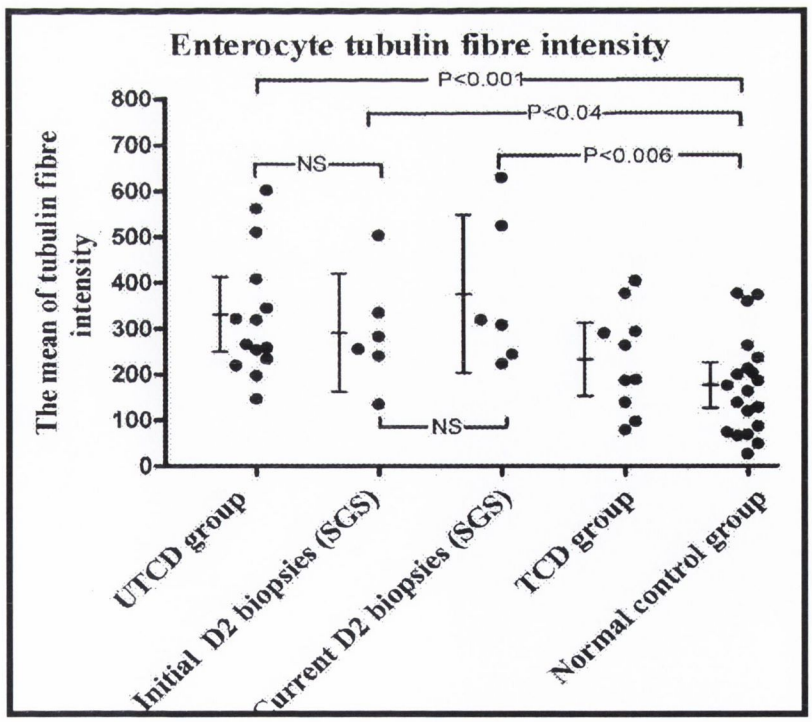


Figure 3.17 The mean of the enterocyte tubulin fibre intensity in the initial and current duodenal section of the SGS, UTCD, TCD patients and the normal control group. Bars represent the mean and 95% CI.

3.4 DISCUSSION

This is the first study to examine in detail enterocyte morphology using the high content analysis (HCA) approach in combination with the morphology explorer bioapplication tools. We were able to use this system to quantify the approximate enterocyte length and width and nuclear shape as well as the tubulin fibre alignments and texture in duodenal sections from a normal control group, patients with positive endomysial antibody test before and after the duodenal lesion had occurred (study group subjects) and coeliac disease patients. We demonstrated significant differences in the enterocyte shape, nuclear shape and tubulin fibre profiles in these groups.

Cytoskeleton proteins are involved in essentially all structure and dynamic aspects of living cell, including maintenance of cell shape, cell movement, cell replication, apoptosis, cell differentiation and cell signalling (Fuchs *et al.*1998). Microtubules are one of the principal protein structures in the eukaryotic cytosol; they are distributed in a radial array and represent the major structural element of the cytoskeleton. As such, they play a central role in maintaining cellular integrity, structure, and transport function (Gilbert *et al.*1991; Allen *et al.* 1985; MacRae *et al.* 1992; Fenteany *et al.* 2004). They also regulate cell morphology, migration, and cell polarity and maintain the plane of cell division (MacRae *et al.*1992, Bershadsky *et al.* 1991; Parczyk *et al.* 1989; Banan *et al.* 1998 Banan *et al.* 1999; Banan *et al.* 2000). Microtubules maintain the overall shape and stability of the plasma membrane (Fenteany *et al.* 2004). These functions are based on the ability of the tubulin subunits to polymerise and the ability to resist depolymerisation.

In this study, using a set of output features of tubulin fibre staining, we analysed the enterocyte shape (length and width) in the normal control group. In addition, we determined the shape of the enterocytes nuclei by measuring the roundness and the length width ratio. Furthermore, the tubulin fibre profiles were also examined in the enterocyte of the normal controls.

In the study group subjects, we demonstrated a significant decrease in enterocyte length in the initial (normal intestinal mucosa) and current duodenal tissue

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In the study group subjects, we demonstrated a significant decrease in enterocyte length in the initial (normal intestinal mucosa) and current duodenal tissue

sections (after the duodenal lesion has occurred), while the increase of the enterocyte width was only evident in the current duodenal biopsies of the study group subjects compared with the normal control group. When we examined the shape of enterocyte nuclei, we found that these cells had rounded nuclei in the study group subjects in comparison with the normal control group where the nuclei had an elliptical shape. We also analysed the texture of enterocyte tubulin fibre in this study and we found significant difference between the study group subjects and the normal controls.

Several studies of cell-based analysis have used the HCA approach in combination with the morphology explorer bioapplication and were able to identify the cell nuclei as a valid object to be morphologically quantified (Ghosh *et al.* 2004; Abraham *et al.* 2004). The nuclear measurements in this study could imply that the enterocyte nuclear properties of the initial duodenal biopsies of the study group subjects have been morphologically altered. This was the case also in the study group subjects after the lesion had developed. It has been demonstrated that the irregular shape of the enterocyte and of their nuclei are features of the coeliac lesion (Magliocca *et al.*1992). Our findings of abnormal enterocyte structure in the study group subjects and coeliac disease patients suggest that these changes might be considered as early features of gut inflammation. Enterocyte function as absorptive cells as well as a physical barrier. Additionally, enterocytes can function as immune cells. Firstly, they express MHC class II proteins (Bland *et al.* 1988) and during inflammation, this expression is extended from just the villous tip to the entire epithelium (Kelly *et al.* 1988). Secondly, enterocytes have been shown to produce their own cytokines, which they secrete in response to injury or infection and to regulate the function of other cells (Stadnyk *et al.*1994).

Tubulin is the major component of the microtubules and these fibres usually radiate outwards from the cell centre to the cytoplasm. However, microtubule orientation differs between cells and reflects their organisation, functions and main routes of trafficking. For example, in epithelial cells, microtubules are aligned along the apico-basal polarity axis of the cell, thus favouring the vesicular trafficking between opposite surface domains (Musch *et al.* 2004). In a study by Gilbert, using laser scanning confocal microscopy, parallel tubulin fibre was

described in colchicine treated Caco-2 cells (Gilbert *et al.*1991). Colchicine has a disruptive effect of microtubules in enterocytes (Pavelka *et al.* 1981). We quantified the tubulin fibre angles and found that these fibres were more aligned to each other in both the initial and the current duodenal tissue sections of the study group subjects. These findings are in agreement with a previous report showing tubulin fibre rearrangement in treated cell culture (NIH 3T3). In this study, an algorithmic application was developed to identify the cytoskeletal fibres and give statistics on these fibre arrangements within a cell (Grove *et al.* Poster presentation, Cellomics meeting, Pittsburgh 2004). Furthermore it has been shown that gluten changes the organisation of the cytoskeleton in cell lines (Intestine 407 cells) (Sjolander *et al.*1988).

The intensity is one of the tubulin fibre features that we quantified using the HCA system. We detected increased levels of tubulin fibre intensity in the initial and current duodenal sections from the study group subjects suggestive of disorganisation of the enterocyte tubulin fibre. The assessment of the tubulin fibre density was also assessed. We showed that there was an increase in tubulin fibre density in the initial and recent duodenal tissue section of the study group subjects. Since the bioapplication tools can measure the uniformity of the tubulin fibre, our finding suggests that the texture of the enterocyte tubulin fibres may be altered in the study group subjects.

In the untreated coeliac disease group, we demonstrated a significant decrease in the enterocyte length and increased width compared with the normal control group. In addition the enterocyte nuclear shape and the tubulin fibre texture were structurally changed compared with the normal controls. Interestingly, in treated coeliac disease patients, the enterocyte length and the nuclear shape were only statistically different in comparison with the normal controls.

The continuity of the epithelium is maintained by tight junctions (zona occulens) and by actin-rich connections (zona adherence and zona occulens), which join the uppermost portions of the cells. It has been found that tubulin is required for the maintenance of adherence junctions, and this may occur via regulation of F-actin in the adherence junction in the epithelial cells (Waterman-Storer *et al.* 2000).

Changes in enterocyte morphology have been reported previously in coeliac disease patients. These changes included nuclear disarray and the development of a cuboidal appearance of enterocytes observed by electron microscopy (Magliocca *et al.*1992). Functional structure and molecular analysis showed that the intercellular junctions between intestinal epithelial cells are abnormal in coeliac disease patients (Schulzke *et al.* 1998; Bjarnason *et al.* 1994; Friis *et al.* 1992; Montalto *et al.* 2002). Coeliac patients have fewer tight junction protein strands (Schulzke *et al.* 1998) and less of the tight junction-associated protein ZO-1 (Montalto *et al.* 2002; Pizzuti *et al.* 2004), pointing to structural alterations at the tight junction. The apical junction protein E-cadherin, required for tight junction formation is reduced in the duodenal epithelium of children with coeliac disease (Barshack *et al.* 2001).

Our findings suggested that the tubulin may have been involved in inducing the enterocyte changes observed in patients with EMA positive test and normal intestinal mucosa. It is possible that the disorganisation of the tubulin structure may affect the adherence of intestinal epithelial cells of the coeliac disease patients. This might explain why these cells are losing their columnar shape in this condition.

In summary, we demonstrated in this study that enterocyte structure could be measured in greater detail using the HCA approach in combination with the morphology explorer bioapplication tools. We demonstrated that the enterocyte shape, nuclei and the tubulin fibre profiles were significantly altered, when we compared the initial and current duodenal tissue sections of the study group subjects and untreated coeliac disease patients with the normal control group and the treated coeliac disease patients. Thus we propose, based on our findings, that the quantification of the enterocyte shape, nuclei, and tubulin fibre of the duodenal section could be used for identification of patients with an early gluten sensitive enteropathy. The morphology explorer bioapplication tool can be used to analyse the enterocytes based on their algorithmic morphological properties, intracellular structures, and the tubulin fibre texture. We showed that enterocytes of the initial duodenal sections from the study group subjects have cellular

morphological changes similar to enterocyte changes detected in the untreated coeliac disease patients.

CHAPTER 4.0

IMMUNOHISTOCHEMICAL CHARACTERISATION OF MATRIX METALLOPROTEINASES -1, -3, -9 AND TIMP-1 IN PATIENTS WITH POSITIVE EMA TEST AND NORMAL DUODENAL MUCOSA AND IN PATIENTS WITH GLUTEN SENSITIVE ENTEROPATHY

4.1 INTRODUCTION

Coeliac disease is a gluten-sensitive disorder characterised by malabsorption and a typical small intestinal histological lesion, marked by considerable tissue remodelling (Maki *et al.* 1997). Similar intestinal lesions are found in dermatitis herpetiformis, another gluten sensitive enteropathy (GSE) (Savilahti *et al.* 1992). The pathogenic mechanisms involved in the tissue damage are not precisely understood, but T-cell reactivity to gluten is likely to play a central role (Sollid *et al.* 2000). Increased production of several cytokines, in particular interferon-γ, has been described in the coeliac mucosa but it is not clear how this leads to the coeliac lesion (Kilmartin *et al.* 2003; Forsberg *et al.* 2002). There is recent interest in the possibility that matrix metalloproteinases (MMPs) might play a pathogenic role in coeliac disease (Daum *et al.* 1999; Salmela *et al.* 2001; Ciccocioppo *et al.* 2005).

MMPs belong to a major family of neutral proteases capable of degrading extracellular matrix and basement membrane components. They participate in the remodelling of normal tissue components and in inflammatory damage in various pathological conditions (Khasigov *et al.* 2001; Nagase et al. 1999; Goetzl *et al.* 1996; Salmela *et al.* 2004; Kirkegaard *et al.* 2004). To date, twenty four different MMPs have been identified: these are divided into four subclasses based on their substrate specificity including collagenases (MMP-1); interstitial collegenases (MMP-8 and -13); stromelysins (MMP-3, -7, -10, -11, -12, and -26); gelatinases (MMP-2 and -9) and membrane type MMPs (de Coignac *et al.* 2000; Grant *et al.* 1999; Welgus *et al.* 1981 and 1990). The proteolytic activity of MMPs is controlled by specific tissue inhibitors of metalloproteinases (TIMPs) (Salmela *et al.* 2002) and non-specific inhibitors, such as α2-macroglobulin (von Lampe *et al.* 2000). TIMPs can bind to the catalytic domain of MMPs in a 1:1 stochiometry to form complexes, thus inhibiting the enzymatic activity of the MMPs (Brew *et al.* 2000). Currently, four different TIMPs have been identified, revealing different tissue and cell type specific expression and regulation patterns (von Lampe *et al.* 2000; Gomez *et al.* 1997). MMPs and TIMPs are produced by various cell population, including myofibroblasts, fibroblasts, and inflammatory cells such as macrophages and lymphocytes (Daum *et al.* 1999; von Lampe *et al.* 2000).

Three studies have investigated mRNA expression of MMPs and TIMPs in coeliac (Daum *et al.* 1999; Ciccocioppo *et al.* 2005) and dermatitis herpetiformis (Salmela *et al.* 2001) intestinal mucosa. Employing *in situ* hybridisation, Daum *et al.* reported increased expression of MMP-1, MMP-3 and the inhibitor TIMP-1 in untreated coeliac disease (Daum *et al.* 1999). Using a similar technique, Salmela *et al.* described elevated expression of MMP-12 in the mucosa of patients with dermatitis herpetiformis, a further gluten sensitive disorder (Salmela *et al.* 2001). In a more recent study, using real-time RT-PCR, increased MMP-1, MMP-12 and TIMP-1 mRNA levels were described in patients with untreated coeliac disease (Ciccocioppo et *al.* 2005).

In this study, using immunohistochemistry, the protein expression of MMP-1, -3, -9 and TIMP-1 was investigated in patients with positive endomysial antibodies and normal duodenal mucosa (study group subjects) and in patients with gluten sensitive enteropathy. The GSE patients were grouped according to the degree of tissue damage, and the findings were compared with those of normal control subjects. In addition, protein expression was correlated with the extent of mucosal damage.

4.2 MATERIALS & METHODS

4.2.1 Patients

4.2.1.1 Study group subjects

Seven patients with positive endomysial antibodies were examined. These patients had normal duodenal biopsies as reported by routine histology. The patients comprised two males with a mean age of 50 years (range, 47-54years) and five females with a mean age of 27 years (range, 20-67 years)

4.2.1.2 Untreated coeliac disease patients

Duodenal biopsies were obtained from ten untreated coeliac disease patients. The duodenal damage ranged from partial villous atrophy and crypt hyperplasia to total villous atrophy. This group consisted of two males with a mean age of 48 years (range, 46-49 years) and eight females with a mean age of 50 years (range, 32-69 years).

4.2.1.3 Treated coeliac disease patients

This group of patients were on a gluten free diet and had moderate duodenal mucosal damage. This group consisted of four males with a mean age of 43 years (range, 28-53 years) and six females, with a mean age of 43 years (range, 25-65 years).

4.2.1.4 Dermatitis herpetiformis patients

Ten dermatitis herpetiformis patients were examined in this study. All had a raised IEL count. Eight had normal villi and two had villous blunting. These patients consisted of eight males with a mean age of 47 years (range, 19-64) and two females, with a mean age of 63 years (range, 63).

4.2.1.5 Normal control group

Ten individuals were screened for evidence of coeliac disease and found to be negative for endomysial and tissue transglutaminase antibodies, they all underwent duodenal biopsy for other upper gastrointestinal disorders but were shown to have normal villi and no increased IEL count. Three males with a mean

age of 51 years (range, 26-73 years) and seven females with a mean age of 56 years (range, 22-82 years) were studied.

4.2.2 Control tissue

The use of controls in any assay is fundamental and in immunohistochemistry it is needed to confirm specificity of the primary monoclonal antibodies. In addition, it is important to ensure that the technique is capable of detecting antigen with minimal background staining. Colonic adenocarcinoma biopsy tissue was used as a positive control for MMPs-1, -3, and -9. These enzymes are important in the initial stage of tumour invasion as they degrade ECM components and they are detected in various levels in tumour cells of stomach cancer (Murray *et al.* 1998). Breast carcinoma biopsy tissue was used as a positive control to detect the expression of TIMP-1 protein. TIMP-1 inhibits the proteolytic invasiveness of tumour cells (Hurskainen *et al.* 1996).

4.2.3 Standardisation of techniques

A guideline was not provided by the manufacturer's antibody specification sheets, as result of which a standardised technique had to be determined for each antibody. Control tissue (colonic adenocarcinoma and Breast carcinoma biopsies) were employed as positive control for this technique. Sections were incubated with MMP-1, -3, -9 and TIMP-1 antibodies at concentrations of 1/5, 1/10, 1/20, 1/25, /150 and 1/100. These antibodies were applied from 30, 40, 60 minutes at room temperature and overnight at 4°C. Based on these studies, concentrations of 1/25 and 1/50 of MMP-1, -3 -9 and 1/20 of TIMP-1 were chosen for this immunoperoxidase technique. The antibody staining time used was overnight for MMP-1, -3 and TIMP-1 and 40 minutes at room temperature for MMP-9.

4.2.4 Immunohistochemistry

Immunohistochemical analysis was carried out on 5μm thick, formalin fixed, paraffin-embedded tissue sections using the avidin-biotin-peroxidase complex detection method (Vector Labs ABC technique, USA). Tissue sections were deparaffinised and heated in a microwave oven for 20 minutes (min) in 0.1 M citrate buffer, (pH 6.0) to retrieve the antigens. Sections were then immersed in 0.05% hydrogen peroxide in 100% methanol for 20 min to block the endogenous

peroxidase activity. After incubation in normal horse serum for 20 min, sections were incubated with the anti-MMP-1, MMP-3, and MMP-9 antibodies (Santa Cruz Biotechnology, Inc. USA) at 1/25 dilution and anti-TIMP-1 antibody (Abcam, Cambridgeshire, UK) at 1/10 dilution in phosphate buffered saline (PBS pH 7.6). Sections were then incubated with biotinylated rabbit anti-mouse IgG (Vector Labs ABC technique, USA) for 30 min, followed by peroxidase-conjugated streptavidin for 30 min. at room temperature. After each antibody application, sections were washed in PBS. PBS and an irrelevant antibody were used as a negative control. Colour was developed using DAB (Sigma) and slides were counterstained with haematoxylin.

4.2.5 Quantification of MMPs and TIMP-1 expression

Using an eyepiece graticule, five fields at high power magnification (x40) (light microscope; Olympus Bx41) were counted in the lamina propria. The number of stained cells was expressed as the percentage of total cells counted in the five fields examined. The slides were coded and read in a blinded fashion.

4.2.6 Statistical analysis

The mean number of lamina propria cells with 95% confidence intervals was calculated. Results were analysed using the non-parametric, Mann-Whitney *U* test. Differences were considered significant when the P-value was less than 0.05.

4.3 RESULTS

4.3.1 Details of MMPs –1, -3, -9 and TIMP-1 expression in the duodenal mucosa of the GSE patients (CD and DH patients)

Duodenal lesions in these patients were classified according to Marsh classification: grade 1: increased intraepithelial lymphocytes counts (n =13); grade 2: increased intraepithelial lymphocytes and villous blunting (n =10); grade 3: total villous atrophy (n = 7). Biopsy sections from ten individuals with normal intestinal mucosa were used as controls: these patients were classified as having a Marsh grade 0 lesion and all had negative coeliac disease antibody serology.

4.3.1.1 MMP-1 expression

Increased numbers of MMP-1 expressing cells were found in the GSE patients compared with the normal controls (Figure 4.1). The mean percentage of lamina propria (LP) cells expressing MMP-1 in patients with grade 1 lesions was 49% (range: 44-54), grade 2 was 51% (range: 46-55%), and in patients with grade 3 was 59% (range: 55-63%) in comparison to the normal control group (mean 22%, range: 19-25%; $p<0.0001$) (table 4.1). With the additional damage to the intestine seen in grade 3 lesions, a further, significant elevation in the expression of MMP-1 was found when compared with grade 1 ($p<0.003$) and grade 2 duodenal tissue damage ($p<0.004$; Figure 4.2).

4.3.1.2 MMP-3 expression

In biopsy specimens from the GSE patients, there was a significant increase in cells positive for MMP-3 per high power field in the LP compared with the normal controls (Figure 4.3). The mean percentage of LP cells expressing MMP-3 in GSE patients with grade 1 duodenal lesions was 51% (range: 47-54%), in grade 2 was 52% (range: 48-55%), and in grade 3 was 59% (range: 55-64 %) compared with the normal control group (mean, 21% (range: 18-24%; $p<0.0001$). Patients with grade 3 duodenal lesions showed statistically significant increase of MMP-3 expression compared with the patients with grade 1 ($p<0.006$) and 2 ($p<0.004$) duodenal lesions (Figure 4.4).

4.3.1.3 MMP-9 expression

Increased MMP-9 expression was detected in duodenal sections from the GSE patients in comparison with the normal control group (Figure 4.5). The mean percentage of LP cells expressing MMP-9 in GSE patients with grade 1 duodenal lesions was 46% (range: 41-50%), grade 2 was 50% (range: 45-55%), and in patients with grade 3 was 61% (range: 55-66%) compared with the normal control group (mean 24%, range: 21-26%; $p<0.0001$). Significantly elevated MMP-9 expression was also seen in patients with grade 3 lesions in comparison with the patients with grade 1 ($p<0.002$) and grade 2 ($p<0.004$) duodenal lesions (Figure 4.6).

4.3.1.4 TIMP-1 expression

The number of cells in the LP expressing TIMP-1 was increased in all GSE patients when compared with the normal controls (Figure 4.7). The mean percentage of LP cells expressing TIMP-1 in GSE patients with grade 1 duodenal lesions was 37% (range: 33-41%), grade 2 was 44% (range: 40-48%), and in patients with grade 3 was 52% (range: 48-55%) in comparison with the normal control group (mean 22%, range: 19-25%; $p<0.0001$). TIMP-1 expression increased in proportion to level of tissue damage, and patients with grade 3 lesions showed a significant elevation in TIMP-1 over grade 1 and 2 lesions ($p<0.0004$ and $p<0.009$ respectively). Thus, the level in TIMP-1 expression largely mirrored that of the MMPs studied (Table 4.1; Figure 4.8).

4.3.2 Details of MMPs –1, -3, -9 and TIMP-1 expression in the duodenal mucosa of the study group subjects

4.3.2.1 MMP-1 expression

Elevated numbers of MMP-1 expressing cells were found in the study group subjects compared with the normal controls (Figure 4.1). The mean percentage of LP cells expressing MMP-1 in the study group subjects was 42% (range: 33-50%) in comparison to the normal control group (mean 22%, range: 19-25%; $p<0.0001$). The level of expression of MMP-1 in the study group subjects was similar to the grade 1 and grade 2 duodenal lesions of GSE patients. The expression of MMP-1 in the study group subjects was significantly less than the GSE patients with grade 3 duodenal lesions ($p<0.007$; Figure 4.2).

4.3.2.2 MMP-3 expression

An increase of LP cells expressing MMP-3 was detected in duodenal sections from the study group subjects in comparison with the normal control group (Figure 4.3). The mean percentage of LP cells expressing MMP-3 in the study group subjects was 44% (range: 37-52%) compared with the normal control group (mean, 21%, range: 18-24%; $p<0.0001$). The study group subjects showed similar percentages of MMP-3 expressing LP cells to the grade 1 and grade 2 lesions of GSE patients. MMP-3 expression was significantly higher in the grade 3 duodenal lesions of GSE patients than the study group subjects ($p<0.002$; Figure 4.4).

4.3.2.3 MMP-9 expression

An increase in MMP-9 expression was detected in duodenal sections from the study group subjects in comparison with the normal control group (Figure 4.5). The mean percentage of LP cells expressing MMP-9 in the study group subjects was 54% (range: 47-61%) compared with the normal control group (mean 24%, range: 21-26%; $p<0.0001$). The study group subjects showed the same level of MMP-9 expression when compared with the GSE patients with grade 2 and grade 3 duodenal lesions. However the study group subjects showed a significantly high level of MMP-9 expression in comparison with the grade 1 lesions of GSE patients ($p<0.01$; Figure 4.6).

4.3.2.4 TIMP-1 expression

The study group subjects showed marked increase in the number of LP cells expressing TIMP-1 compared with the normal controls (Figure 4.7). The mean percentage of LP cells expressing TIMP-1 in the study group subjects was 49% (range: 45-54%) in comparison with the normal control group (mean 22%, range: 19-25%; $p<0.0001$). The study group subjects had similar levels of TIMP-1 expression in comparison with the GSE patients with grade 2 and grade 3 duodenal lesions. The study group subjects had a statistically higher level of this marker when compared with the grade 1 lesions of GSE patients ($p<0.0001$; Figure 4.8).

In summary, all patients with gluten sensitive enteropathy, regardless of the degree of intestinal damage showed a significant increase in the level of

expression of MMP-1, -3, -9 and TIMP-1 in the lamina propria. Furthermore, these levels of expression increased incrementally with the severity of the tissue damage, being most marked in biopsies with a grade 3 lesion. The study group subjects showed a marked increase of these enzymes when compared with the normal controls, even though their intestinal mucosa was structurally reported to be normal.

Table 4.1 Characteristics of MMP-1, -3, -9 and TIMP-1 expression in the study group subjects and patients with gluten sensitive enteropathy (GSE). Results are expressed as the mean percentage and 95% confidence intervals (CI) of MMP-1, -3, -9 and TIMP-1 expression in the study group subjects, GSE patients and the normal controls. Grade 1 = patients with increased intra-epithelial lymphocytes, grade 2 = patients with villous blunting and increased intra-epithelial lymphocytes and grade 3 = patients with total villous atrophy.

Marker	***MMP-1***	***MMP-3***	***MMP-9***	***TIMP-1***
Normal control group (n=10)	22% (19-25%)	21% (18-24%)	24% (21-26%)	22% (19-25%)
Study group subjects (n=7)	42% (33-50%)	44% (37-52%)	54% (47-61%)	49% (45-54%)
Grade 1 (n=13)	49% (44-54%)	51% (47-55%)	46% (41-50%)	37% (33-41%)
Grade 2 (n=10)	51% (46-55%)	52% (48-55%)	50% (45-55%)	44% (40-48%)
Grade 3 (n=7)	59% (55-63%)	59% (55-64%)	61% (55-66%)	52% (48-55%)

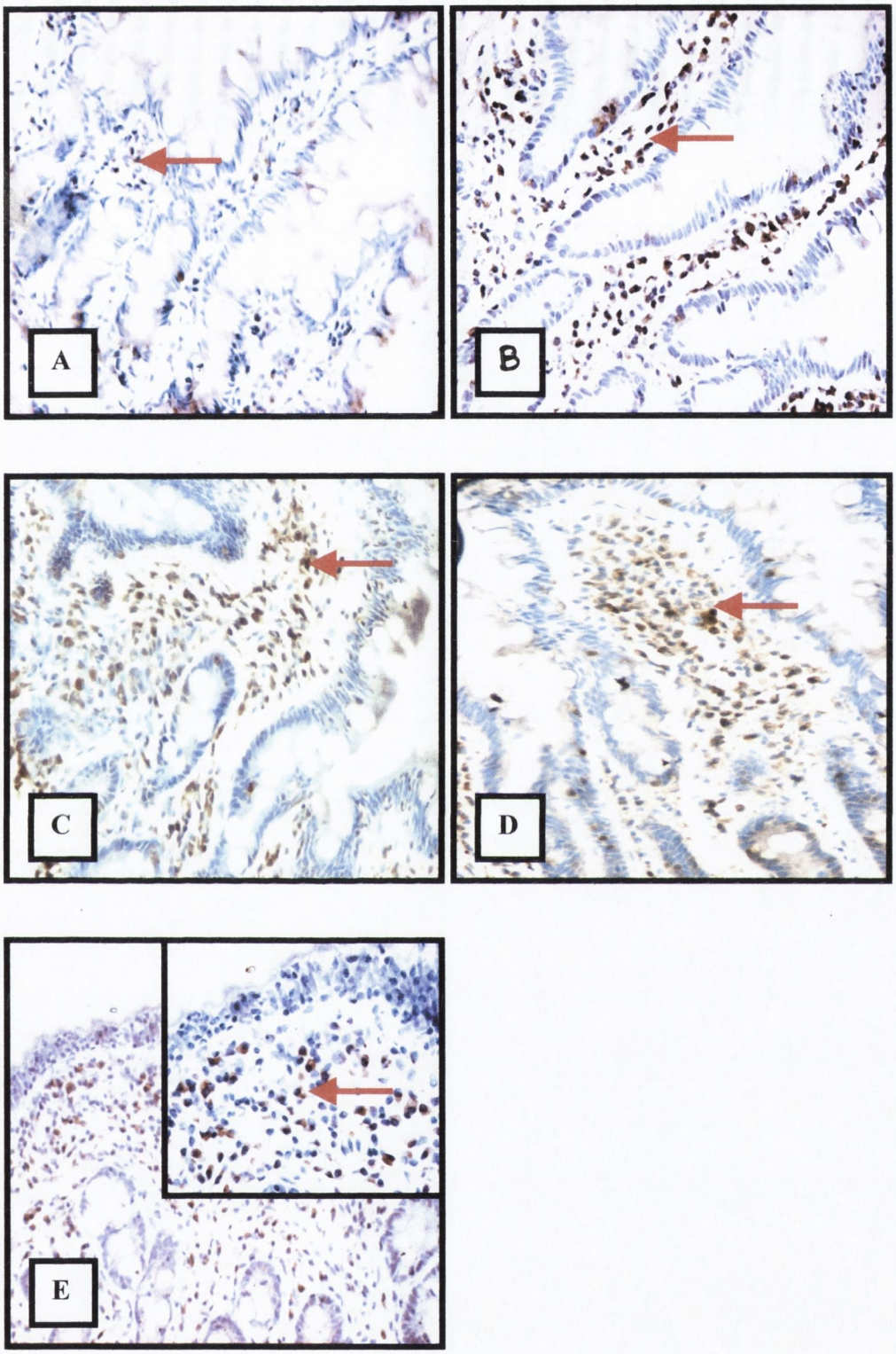


Figure 4.1 Lamina propria cells expressing MMP-1 in the normal control (A), study group subjects (B) and gluten sensitive-enteropathy patients with grade I-III lesions (C-E). Arrows represent the stained lamina propria cells.

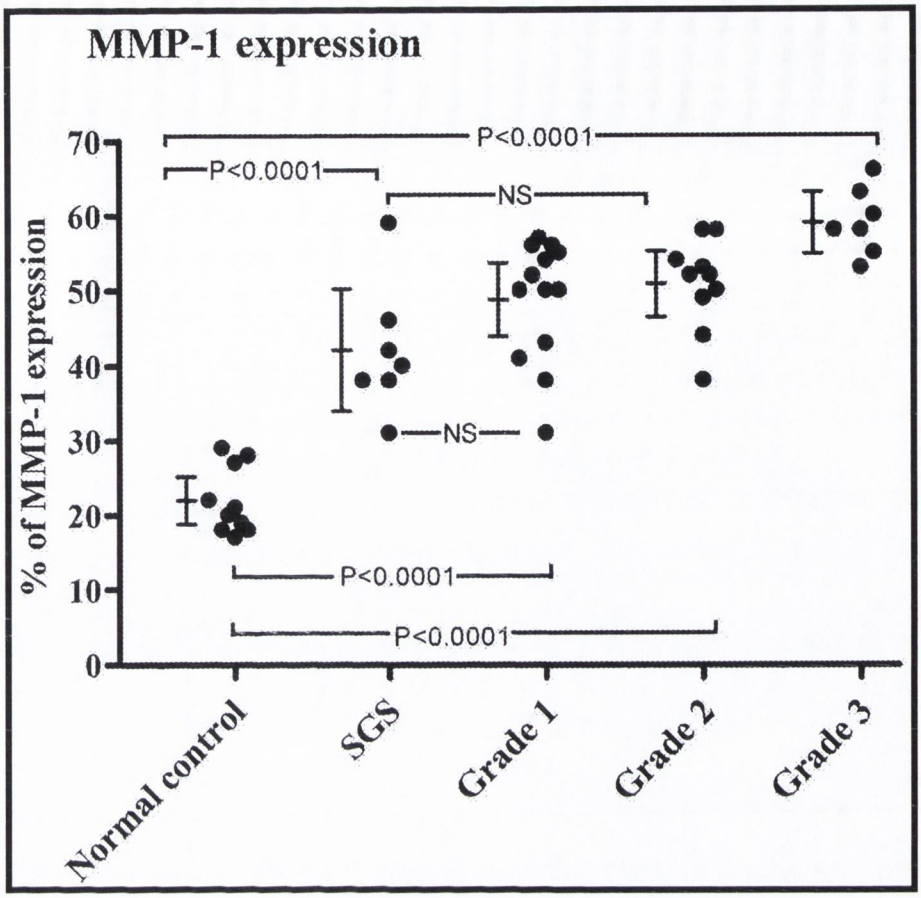


Figure 4.2 Percentages of lamina propria cells expressing MMP-1 in the normal controls, study group subjects (SGS) and gluten sensitive enteropathy (GSE) patients (grade I-III). Differences between the groups were calculated using the Mann-Whitney U test. Bars represent the mean and 95% confidence intervals

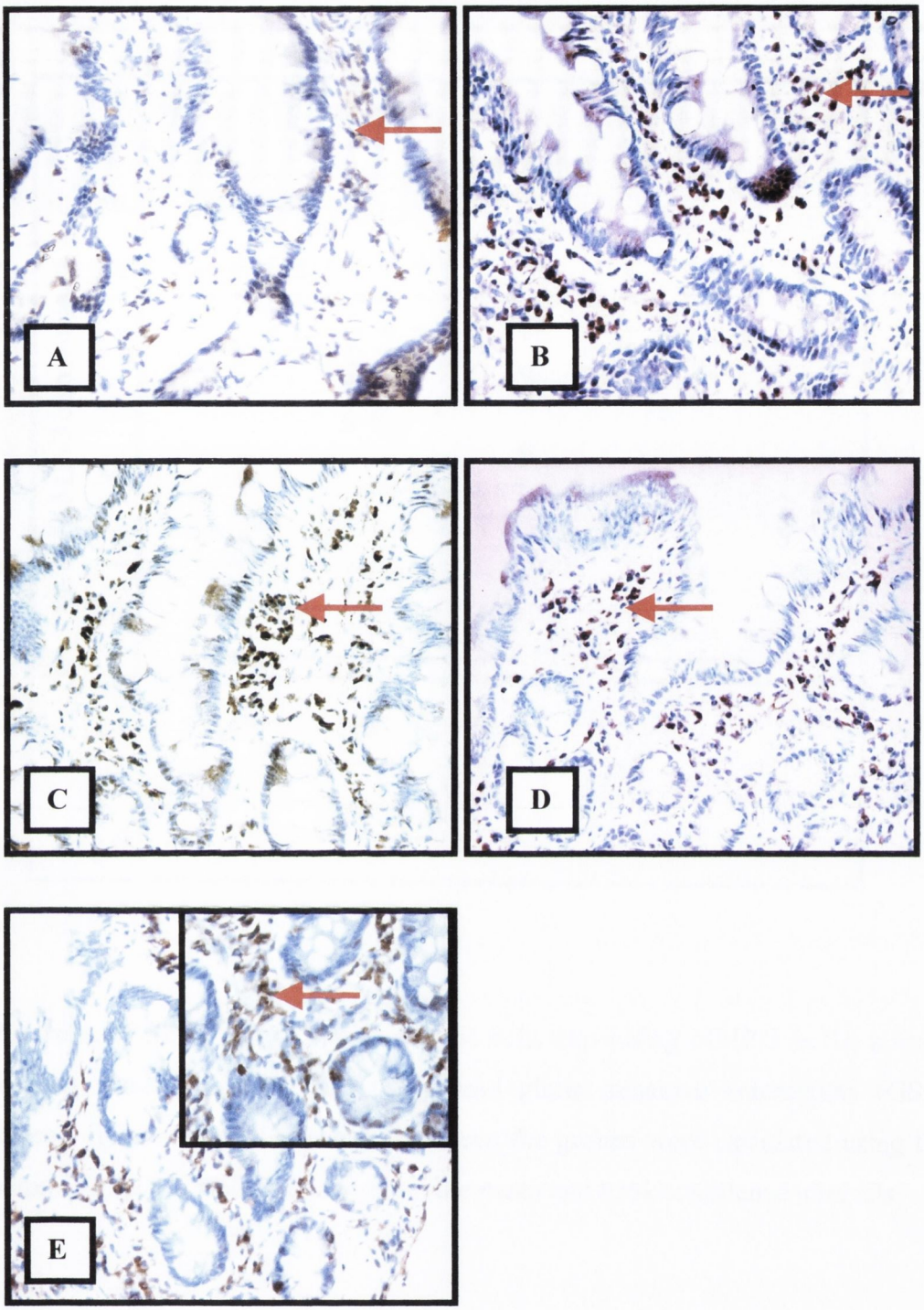


Figure 4.3 Lamina propria cells expressing MMP-3 in the normal control (A), study group subjects (B) and gluten sensitive-enteropathy patients with grade I-III lesions (C-E). Arrows represent the stained lamina propria cells.

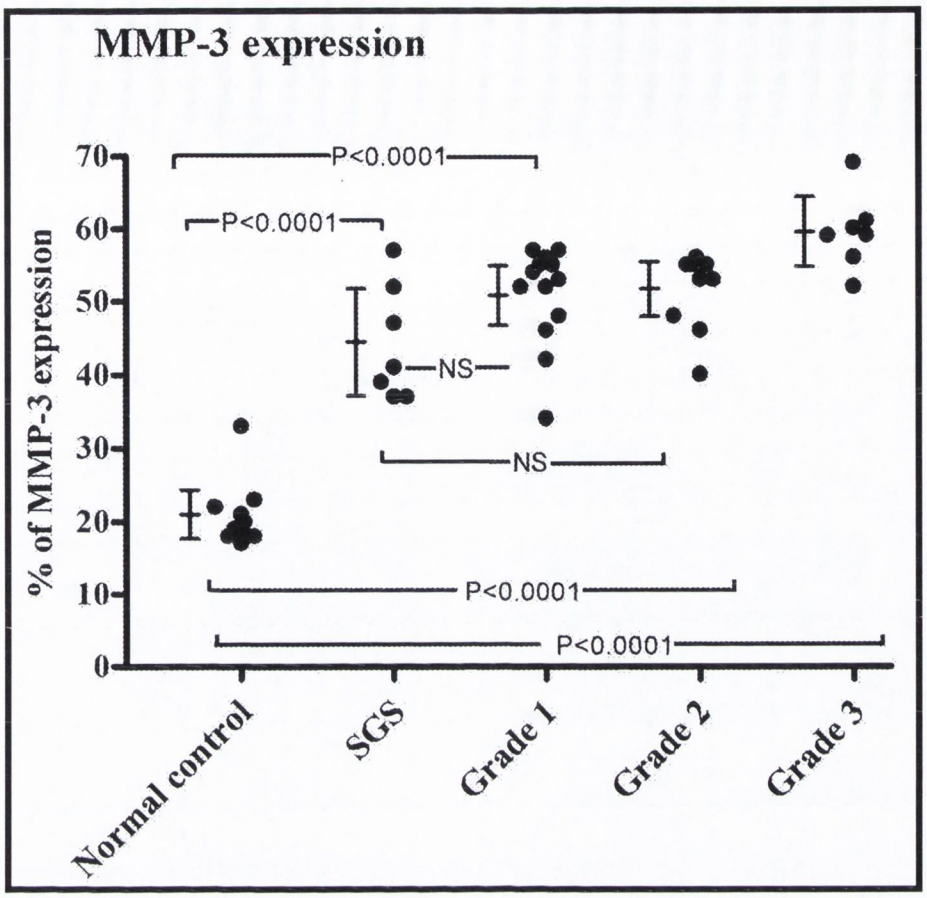


Figure 4.4 Percentages of lamina propria cells expressing MMP-3 in the normal controls, study group subjects (SGS) and gluten sensitive enteropathy (GSE) patients (grade I-III). Differences between the groups were calculated using the Mann-Whitney U test. Bars represent the mean and 95% confidence intervals

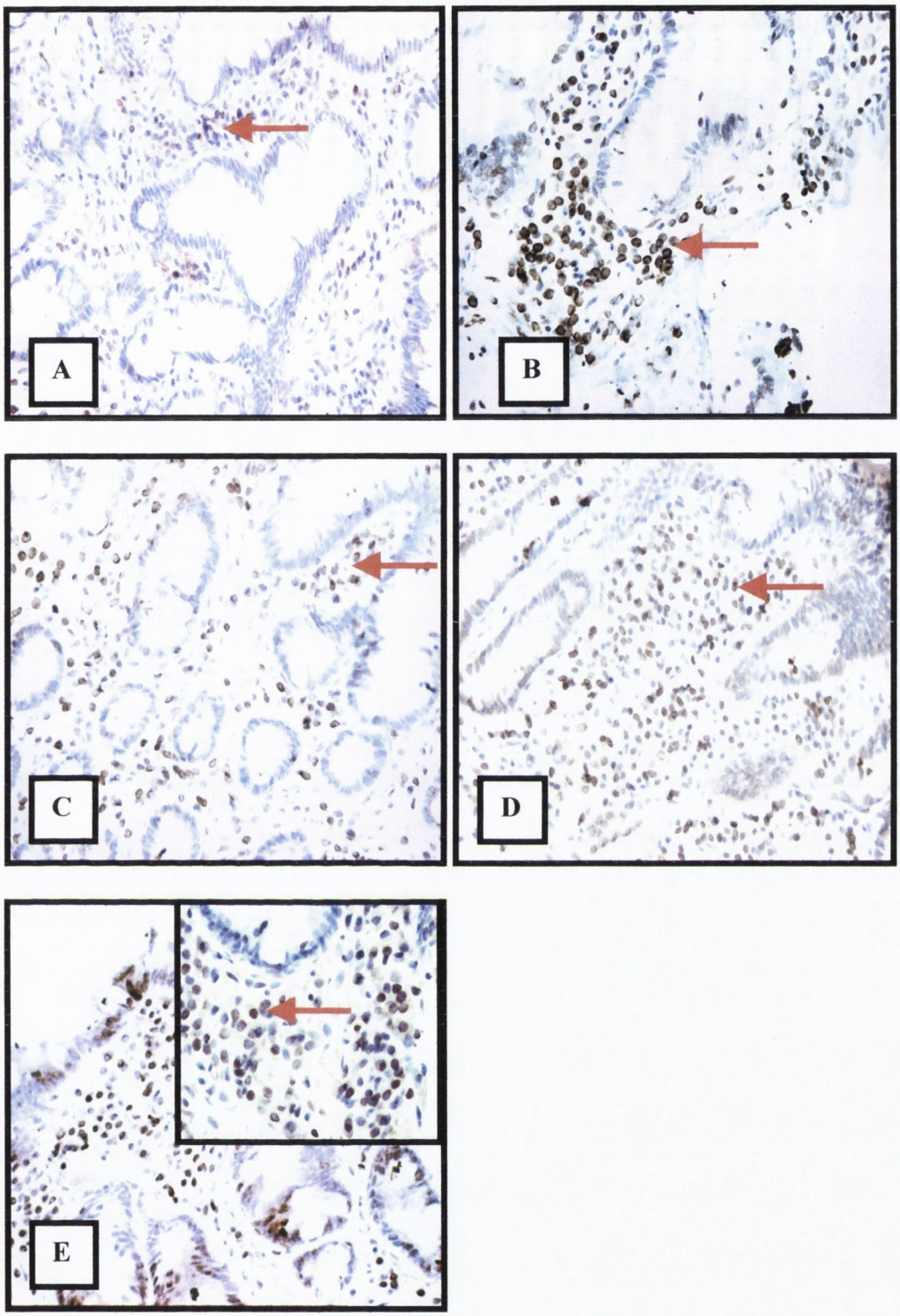


Figure 4.5 Lamina propria cells expressing MMP-9 in the normal control (A), study group subjects (B) and gluten sensitive-enteropathy patients with grade I-III lesions (C-E). Arrows represent the stained lamina propria cells.

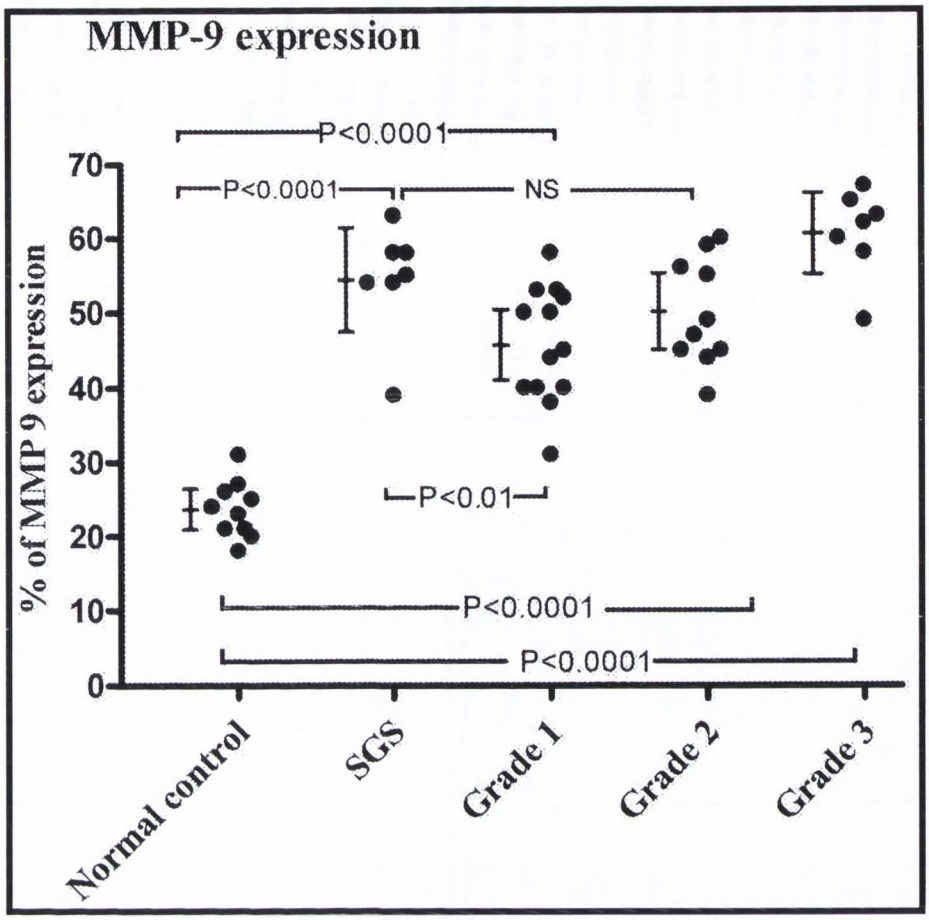


Figure 4.6 Percentages of lamina propria cells expressing MMP-9 in the normal controls, study group subjects (SGS) and gluten sensitive enteropathy (GSE) patients (grade I-III). Differences between the groups were calculated using the Mann-Whitney U test. Bars represent the mean and 95% confidence intervals

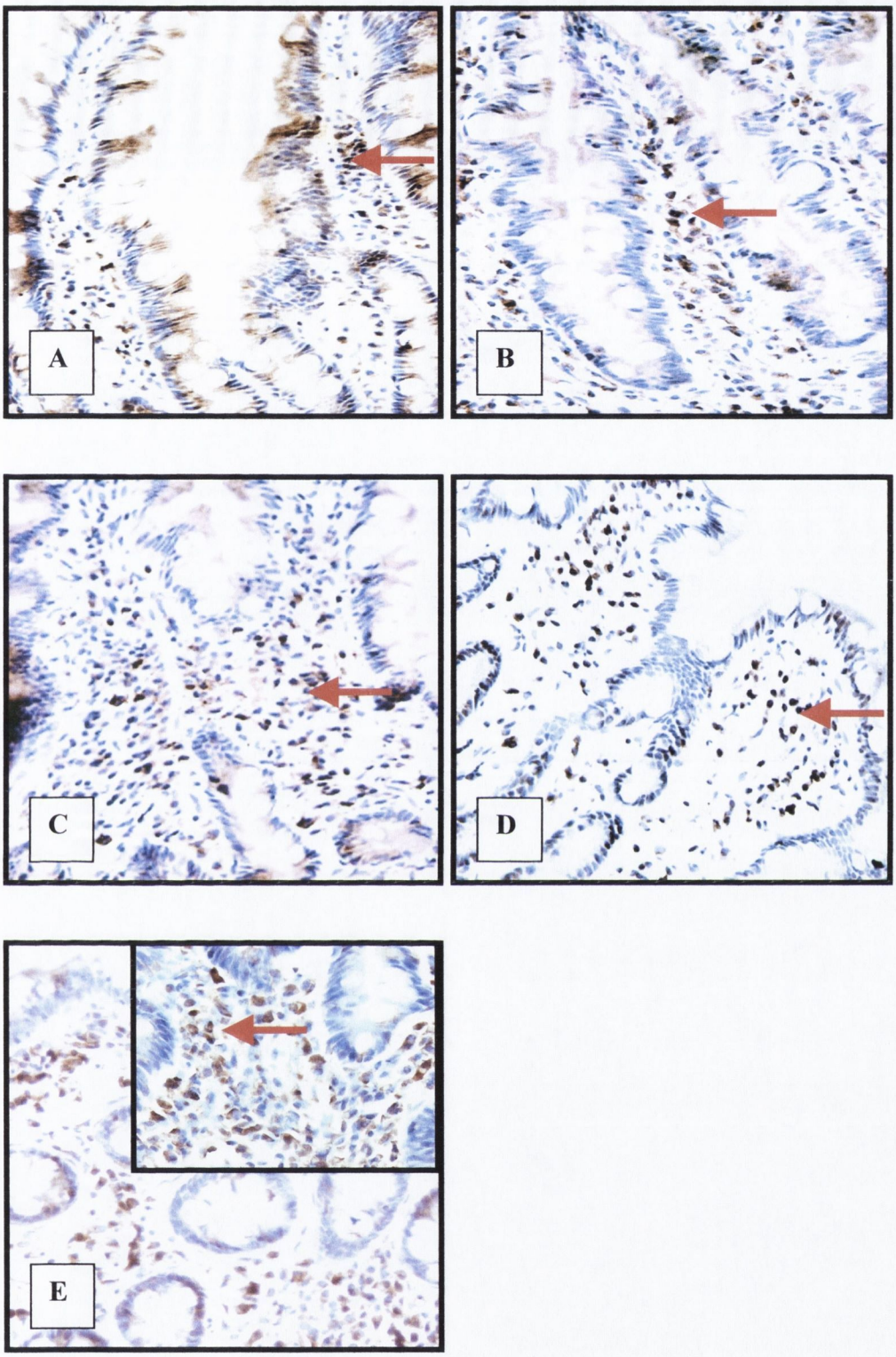


Figure 4.7 Lamina propria cells expressing TIMP-1 in the normal control (A), study group subjects (B) and gluten sensitive-enteropathy patients with grade I-III lesions (C-E). Arrows represent the stained lamina propria cells.

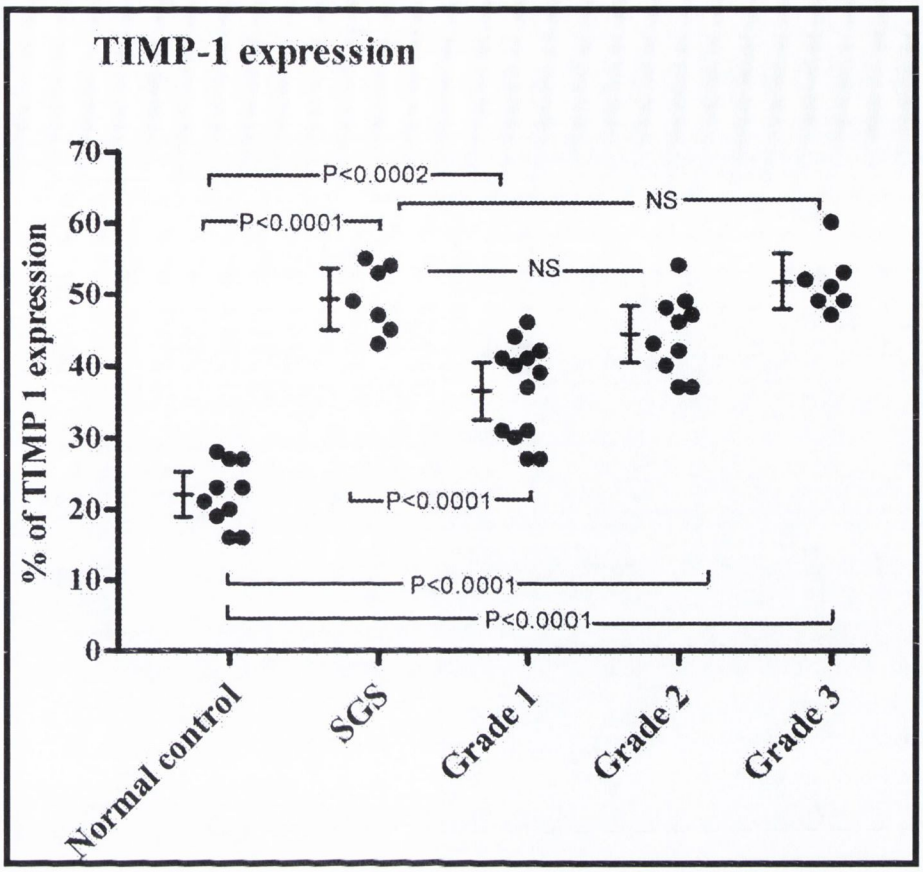


Figure 4.8 Percentages of lamina propria cells expressing TIMP-1 in the normal controls, study group subjects (SGS) and gluten sensitive enteropathy (GSE) patients (grade I-III). Differences between the groups were calculated using the Mann-Whitney U test. Bars represent the mean and 95% confidence intervals

4.4 DISCUSSION

4.4.1 MMPs –1, -3, -9 and TIMP-1 expression and the gluten sensitive enteropathy patients (Coeliac disease and dermatitis herpetiformis)

In this study, the protein expression of the matrix metalloproteinases MMP-1, MMP-3 and MMP-9 and the tissue inhibitor of metalloproteinases-1 (TIMP-1) was investigated in duodenal biopsies from patients with coeliac disease and dermatitis herpetiformis. The patients were grouped according to the degree of mucosal damage. The small intestinal expression of these three metalloproteinases, and of TIMP-1, was significantly increased in all of these patients compared with the normal control group. Furthermore, the expression of these enzymes and the inhibitor mirrored the degree of mucosal damage, with the most marked increase in staining for each of the four proteins observed in patients with Marsh grade 3 lesions.

Interestingly, the expression of all four proteins was also significantly increased in patients with a Marsh 1 lesion. These observations imply that metalloproteinases may be involved in the tissue remodelling seen in the intestinal lesion in gluten sensitive enteropathy. Moreover, the finding of an increased expression of TIMP-1, as the severity of the lesion increased, could represent an involvement of the normal inhibitory mechanism in an attempt to limit the extent of metalloproteinase-mediated damage.

This is the first study to investigate protein expression of metalloproteinases in the intestinal lesion in coeliac and dermatitis herpetiformis tissue. Interestingly, the findings are in broad agreement with three earlier studies that investigated mRNA expression of MMPs and TIMP-1 in coeliac and dermatitis herpetiformis intestinal mucosa (Daum *et al.* 1999; Ciccocioppo *et al.* 2005; Salmela *et al.* 2001). Using *in-situ* hybridisation, Daum *et al.* (1999) reported increased mRNA expression of MMP-1, MMP-3 and TIMP-1 in untreated coeliac disease (Daum *et al.* 1999). Likewise, Salmela *et al.* (2001) described elevated expression of MMP-12 mRNA in the mucosa of patients with dermatitis herpetiformis (Salmela *et al.* 2001). Finally, in a recent study, using real-time RT-PCR, increased mRNA levels of MMP-1, MMP-12 and TIMP-1 were described in patients with untreated coeliac disease (Ciccocioppo *et al.* 2005). However, whereas in these reports

mRNA expression of MMP-3 was either absent or less prominent, in this study equal protein expression of this enzyme was observed (Salmela *et al.* 2001; Daum *et al.* 1999). This highlights the need to examine protein, as well as mRNA expression, of given cell products in order to understand their role in disease processes.

The metalloproteinases, including MMP-1, MMP-3 and MMP-9, can cause degradation of the extracellular matrix and basement membranes and could promote the influx of cells of both the innate (e.g. dendritic cells) and acquired immune system (e.g. T cells) into the lamina propria and epithelial departments (Pender *et al.* 1997; Bailey *et al.* 1994). These events may contribute to the coeliac lesion, including the marked mucosal damage that can develop within hours of *in vivo* gliadin challenge of coeliac patients (Kontakou *et al.* 1995). Evidence in support of this concept is found in a foetal gut model of small intestinal damage, where tissue cultured in the presence of pokeweed mitogen or *Staphylococcus aureus* (Pender *et al.* 1997; Pender *et al.* 1998) showed increased expression of MMP-1 and MMP-3. Moreover, in these models, morphological changes similar to the coeliac lesion developed.

Increased metalloproteinase activity has been implicated in several other inflammatory disorders, such as inflammatory bowel disease, rheumatoid arthritis and may also play a role in malignancy (von Lampe *et al.* 2000; Heuschkel *et al.* 2000; Yoshihara *et al.* 2000; Klimiuk *et al.* 2002; Zhang *et al.* 2003; Murray *et al.*1998). In many of these studies, the findings were based on estimation of mRNA transcripts, using techniques such as *in-situ* hybridisation or PCR (von Lampe *et al.* 2000). Elevated mRNA levels for the three metalloproteinases investigated in this study were reported in inflammatory bowel disease and transcript levels correlated positively with the degree of inflammation in some studies (von Lampe *et al.* 2000; Heuschkel *et al.* 2000; Stallmach *et al.* 2000). Furthermore, MMP protein expression examined by immunohistochemistry was shown to be present extracellularly in areas of mucosal damage in both Crohn's disease and ulcerative colitis (Kirkegaard *et al.* 2004; von Lampe *et al.* 2000). In rheumatoid arthritis, raised levels of metalloproteinase protein were described in

both serum and synovial fluid and found to correlate with clinical indicators of disease activity (Yoshihara *et al.* 2000; Klimiuk *et al.* 2002).

The MMPs are regulated by specific tissue inhibitors of metalloproteinases, or TIMPs (Gomez *et al.* 1997). An imbalance of this system can adversely affect the composition of the intercellular matrix and functions of immunocompetent cells including their adhesion, migration, and differentiation (Gomez *et al.* 1997; Murray *et al.*1998). TIMP-1 is the first member of the TIMP family and is known to form a stable complex with MMP-1, MMP-3 and MMP-9, thereby inhibiting their activity (Brew *et al.* 2000). In this study, although the expression of TIMP-1 mirrored the severity of the histological lesion, it is possible that levels of this inhibitor were insufficient to negate the increased activity of MMPs in coeliac and dermatitis herpetiformis tissue with consequent tissue damage. To support this hypothesis, in patients with grade 3 mucosal lesions it was noted that the number of cells expressing MMP-1, significantly exceeded cells expressing TIMP-1 ($p<0.0002$).

As is the case with metalloproteinases, increased expression of TIMP-1 is reported in various inflammatory conditions, including inflammatory bowel disease, rheumatoid arthritis, and also in malignancy (von Lampe *et al.* 2000; Heuschkel *et al.* 2000; Yoshihara *et al.* 2000; Klimiuk *et al.* 2002; Zhang *et al.* 2003; Murray *et al.*1998). In inflammatory bowel disease tissue, both mRNA transcripts and protein levels of this inhibitor were increased (Kirkegaard *et al.* 2004; von Lampe *et al.* 2000). Raised levels of soluble TIMP-1 have been found in culture supernatants of inflamed biopsies from ulcerative colitis and Crohn's disease patients (Louis *et al.* 2000; Wiercinska-Drapalo *et al.* 2003). Increased soluble TIMP-1 levels were found in synovial fluid (Yoshihara *et al.* 2000) and serum samples of rheumatoid arthritis patients (Klimiuk *et al.* 2002). Likewise, increased tissue transcripts and plasma protein levels of TIMP-1 have been described in patients with gastrointestinal malignancy (Zhang *et al.* 2003; Murray *et al.*1998).

The increased protein expression of MMP-1, MMP-3, MMP-9 and TIMP-1 described in this study was localised to cells widely distributed throughout the LP in patients' biopsies. In the case of MMP-1 and MMP-3, prominent collections of

cells in the subepithelial region were noted. The morphological appearance of these cells was in keeping with cells of both macrophage and lymphocyte lineages. However, the specific identity of cell types was not investigated. In the earlier studies of gluten sensitive enteropathy, RNA transcripts for these proteins were particularly located in fibroblasts, myofibroblasts and macrophages and these cells were principally located in the sub-epithelial region (Daum *et al.* 1999; Salmela *et al.* 2001; Ciccocioppo *et al.* 2005). Cytokines produced by macrophages and T cells including TNF-α, IFN-γ and IL-1 stimulate cellular production of these proteins (Salmela *et al.* 2004). The involvement of TNF was supported by the finding that a p55 TNF receptor fusion protein inhibited MMP-3 production (Pender *et al.* 1998). Overproduction of these cytokines has been reported in coeliac mucosa (Kilmartin *et al.* 2003; Forsberg *et al.* 2002). A further potential T-cell cytokine candidate is IL-17, shown to be upregulated in inflammatory bowel disease and to cause increased secretion of MMP-3 (Bamba *et al.* 2003).

4.4.2 MMPs –1, -3, -9 and TIMP-1 expression and the study group subjects

In this study, we also examined the protein expression of the matrix metalloproteinases MMP-1, MMP-3, MMP-9, and the tissue inhibitor of metalloproteinases-1 (TIMP-1) in patients with positive endomysial antibody (EMA) test and normal duodenal mucosa (study group subjects). These patients showed significantly increased expression of these enzymes in comparison with the normal control group. Interestingly, the level of these MMP-1, -3 -9 and their inhibitor TIMP-1 in the study group subjects was similar to the gluten-sensitive enteropathy (GSE) patients with grade 2 lesions. In the case of MMP-9 and TIMP-1, the study group subjects showed higher expression than the GSE patients with grade 1 lesions.

Although the duodenal mucosa was reported to be structurally normal in the study group subjects, the level of TIMP-1 expression was similar to that in GSE patients with grade 3 lesions. This could support the hypothesis that the protein expression of TIMP-1 might inhibit the involvement of these metalloproteinases in the development of the duodenal lesion of the study group subjects. Furthermore, the inhibition of these enzymes might fail to continue in the

presence of the gluten containing diet intake of these patients. Follow up study of the study group subjects showed that patients with persistent EMA positivity developed intestinal lesions (chapter 2).

Although the study group subjects had structurally normal intestinal mucosa as reported by routine histology, we have found that some of these patients had subtle immunohistological changes including increased IEL subpopulations and crypt cell proliferation (chapter 2). We managed to examine the enterocyte morphological changes and enterocyte tubulin fibre profiles in the duodenal mucosa of these patients (chapter 3). These measurements showed that these patients (study group subjects before the intestinal lesions developed) had changes in enterocyte and tubulin structures suggestive of on going abnormal small intestinal changes.

The increased protein expression of MMP-1, MMP-3, MMP-9 and TIMP-1 described in this study was localised to cells within the lamina propria. The MMP-1 expressing cells were located in the subepithelial region in the duodenal mucosa of the study group subjects, which was similar to the GSE patients. In contrast, MMP-3 prominent collections of LP cells were noted in the intestinal mucosa of the study group subjects, but in the subepithelial region of the GSE patients. This may explain the delay in the development of the intestinal damage in these patients. MMP-3 plays a crucial role in the degradation of the extracellular matrix and basement membranes (Pender *et al.* 1997; Bailey *et al.* 1994). We have found that the LP cells expressing MMP-3 are clustered at the subepithelial region in the grade 3 lesions of GSE patients. The mean percentage of MMP-3 expressing cells was higher than the TIMP-1 expressing LP cells in the grade 3 lesions of GSE patients. In the study group subjects TIMP-1 and MMP-3 expression mirrored each other, however the expression of both these enzymes was upregulated in the study group subjects when compared with normal controls.

Polymorphisms of MMP-1, -3 and -9 genes were not extensively investigated. Study by Mora and colleagues showed that 5A/6A genotype at- 1171 position of the MMP-3 gene is associated with an increased susceptibility for coeliac disease only in male patients (Mora *et al.* 2003). However in a different study examining the polymorphisms of MMP-3 allele 5A or MMP-1 allele 2G in Norwegian and

Swedish populations, no associations were found (Louka *et al.* 2004). Furthermore, there has been no study in the literature examining MMP-9 and TIMP-1 gene polymorphisms (NCBI and OMIM). No single nucleotide polymorphism study of these enzymes has been reported on this subject. However, it possible that other mechanisms including cytokines; such as IFN-γ, TNF-α, IL-1 and IL-17 may have a role in altering the protein expression of these enzymes.

In summary, this is the first study to demonstrate increased protein expression of MMP-1, -3, -9 and the inhibitory molecule TIMP-1 in the intestinal mucosa of patients with positive EMA test and normal intestinal mucosa and in the intestinal mucosa of patients with coeliac disease or dermatitis herpetiformis. Moreover, the level of expression of these enzymes and their inhibitor, although significantly increased even in the study group subjects and grade 1 biopsies, was most significantly elevated in biopsies with the most severe lesion (grade 3), suggesting that these enzymes may play a pathological role in gluten sensitive enteropathy. These findings advance the information reported in earlier studies, in which mRNA transcripts for some of these molecules were shown to be increased in gluten sensitive disorders.

CHAPTER 5.0 GENERAL DISCUSSION

5.0 GENERAL DISCUSSION

The diagnosis of coeliac disease is currently based on small intestinal mucosal biopsy (Walker-Smith *et al.* 1990). No difficulties are encountered when the biopsy shows severe villous atrophy and crypt hyperplasia. In clinical practice, however, the findings may be less straightforward. Diagnostic difficulties arise when the biopsy findings are borderline or normal in patients with potential coeliac disease. These patients are now receiving more attention due to their increasing proportion in the coeliac iceberg. Hence, there seems a need for evaluation of such new cases with this common clinical presentation.

In making an accurate diagnosis of coeliac disease, serologic markers, especially endomysial antibodies and tissue transglutaminase antibodies are very helpful. Several studies have reported the presence of normal villous architecture in patients with positive coeliac serology (Pearce *et al.* 2002; Kaukinen *et al.* 2001; Mino *et al.* 2003; Settakorn *et al.* 2004). Endomysial antibody test (EMA) is a very sensitive and specific predictor of coeliac disease (89% -100% and 100% respectively) (Chorzelski *et al.* 1983; Dieterich, *et al.* 1998; Feighery *et al.*1998; Pearce *et al.* 2002; Collin *et al.* 2005). The EMA test is of particular use in diagnosing symptomatic or potential (pre-clinical) coeliacs since EMA positive individuals with normal duodenal mucosa often later develop a gut lesion (Collin *et al.*1993; James and Scott *et al.* 2000; Dickey *et al.* 2005).

The purpose of this study was to examine and define the histological findings in duodenal biopsies from patients with positive EMA test but apparently normal intestinal mucosa. Detailed immunohistological examination was performed to determine if these patients had subtle changes suggestive of gluten-sensitive enteropathy. In addition follow up of the clinical and laboratory evaluation was carried out of these patients. These patients are referred to throughout the manuscript as "the study group subjects". The findings in the study group subjects were then compared with the untreated and treated coeliac disease patients and a normal control group.

Immunohistochemical studies were performed on the original and the repeat biopsies obtained from the study group subjects, in untreated and treated coeliac

disease patients. The main focus of the study was: to examine the intraepithelial lymphocyte (IEL) population expressing CD2, CD3, CD7, CD8 and CD69 molecules; to measure the crypt cell expression of the Ki67 proliferation marker; to perform detailed examination of enterocyte morphology (including nuclear shape and cytoskeletal structure) and to investigate the expression of matrix metalloproteinases-1, -3, -9 and the of tissue inhibitor of metalloproteinases-1.

5.1 Evidence of increased IELs in the study group.

A significant increase was demonstrated in the number of IELs expressing CD2, CD3, CD7, CD8, and CD69 molecules in the original and in the repeat duodenal biopsies from the study group subjects, when compared with the normal controls. These increased cell counts were similar to those found in treated coeliac patients. It was also demonstrated that there was no difference in the expression of these markers between the original and repeat biopsies from the study group subjects. However, patients with untreated coeliac disease had significantly greater numbers of positively stained IELs than those found in the original biopsies of the study group.

In many patients, the diagnosis of coeliac disease is not accepted, because of the absence of a histological lesion. An increased IEL count may be the only abnormality present in individuals with potential gluten-sensitive enteropathy. Thus, in a proportion of such patients, more detailed histological analysis revealed subtle histological abnormalities such as an increased number of IELs expressing $CD3^+CD8^+$ (Kaukinen *et al.* 2001; Mino *et al.* 2003; Settakorn *et al.* 2004). In keeping with these other studies, raised $CD3^+CD8^+$ IELs in patients with positive AGA, EMA and normal gut mucosa were demonstrated in this study. Taken together the findings of positive coeliac serology and an increase of IELs expressing CD3 and CD8, demonstrate immunohistological abnormalities that occur after gluten exposure, despite the normal microscopic appearance of the intestinal mucosa.

In this study the expression of CD69 was detected on IELs from the duodenal mucosa of the normal controls. This marker was increased in the study group subjects, and in the untreated and treated coeliac patients and interestingly, all the patients groups showed similar percentage increases. This suggests that an

immunological activation process is occurring in the intestinal mucosa of the study group. To date this study is the first to examine the expression of CD69 in small intestinal mucosa.

It was concluded from this set of results that the investigation of small intestinal tissue with monoclonal antibodies to CD2, CD3, CD7 and CD69 is of value in identifying an increased IEL population in patients with gluten-sensitivity. The increase in IELs expressing these molecules could help detect those patients who have positive coeliac disease serology but apparently normal intestinal mucosa.

5.2 Evidence of increased crypt cell proliferation in the study subjects

The assessment of crypt hyperplasia may serve as a marker of minimal inflammatory change in individuals with a positive EMA test but apparently normal small intestinal mucosa. In this study, it was demonstrated that the number of proliferating crypt cells expressing Ki67 was increased in both the original and second duodenal biopsies of the study group compared with the normal control group. An interesting finding was that the level of this marker did not change when comparing the original with the second duodenal biopsies of the study group. This finding is consistent with a recent report showing a raised Ki67 index in individuals with positive coeliac disease serology and normal small intestinal mucosa (Settakorn *et al.* 2004).

Several studies have shown that Ki67 can be used as a sensitive marker for assessing enterocyte proliferation and crypt hyperplasia (Savidge *et al.* 1995; Przemioslo *et al.* 1995; Moss *et al.* 1996). Furthermore, this study demonstrated that the number of crypt cells expressing Ki67 was increased in the untreated coeliac patients compared with the normal control group. In the present study, the staining pattern of proliferating enterocytes in the original duodenal section of the study group was as significantly raised as in patients with untreated coeliac disease.

The alteration in intestinal transport in active coeliac disease may be a result of the immaturity of the villous epithelial cells generated by the hyper-proliferative crypt compartment. Crypt hyperplasia is said to be the first architectural change that occurs during the development of the coeliac intestinal lesion (Oberhuber *et*

*al.*1999). Therefore, it seems likely that the initial event in the pathogenesis of the gut lesion in coeliac disease is the abnormal processes in the crypt. This point argues against the theory that the activation of T-cells directly leads to villous atrophy and that this is the main explanation for the structural alterations in the intestinal mucosa. However, T cells may indirectly cause crypt hyperplasia and T-cells, stimulated with mitogens, have been shown to cause crypt cell proliferation (Ferreira *et al.* 1990).

Enterocyte proliferation has been shown to be induced by cytokines such as interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β) and IL-15 (Kraeger *et al.* 1991; McGhee *et al.* 1993; Lionetti *et al.* 1999; Maiuri *et al.* 2000). In coeliac disease, IL-15 has been shown to be involved in the generation of villous atrophy, by favouring the selective expansion of IELs, particularly TCR$\gamma\delta^+$ and CD8$^+$ TCR$\alpha\beta^+$ lymphocytes, and in promoting the appearance of T cell clonal proliferation in refractory coeliac disease (Maiuri *et al.* 2000; Mention *et al.* 2003; Hüe *et al.* 2004; Jabri *et al.* 2000). It has been shown that blocking IL-15 or its receptor prevents both tissue damage and lymphomagenesis that occur in coeliac disease (Di Sabatino *et al.* 2006).

Of interest, in this study both activation of gut T cells and increased enterocyte proliferation were detected in the original study group patients suggesting that both might be equally involved in causing mucosal damage in coeliac disease patients. Furthermore, this study demonstrated that patients with positive coeliac antibodies, increased IELs and increased crypt cell proliferation, went on to subsequently develop a duodenal lesion compatible with that seen in typical coeliac disease.

5.3 Enterocyte morphological changes detected by Cellomics

The aim of this study was to learn more about the enterocyte structure and whether this gives insight to the early mucosal response in patients with gluten-sensitive enteropathy. This study examined in great detail the enterocyte morphology in normal individuals, in the six study group subjects before and after the intestinal lesion had developed and in coeliac disease patients, using the high content analysis approach (Cellomics). This study showed using this advanced

technology, that the internal structure of the normal enterocyte could be visualised and studied.

The enterocyte morphology from normal individuals was examined and the findings were used to observe the changes of these cells from the study group subjects and coeliac disease patients. It was demonstrated that the enterocyte length was reduced in the initial and in the repeat duodenal biopsies of the study group subjects, whilst the enterocyte width was markedly increased only in the repeat duodenal biopsies of these patients compared with the normal control group. It was also found that the enterocyte tubulin texture was significantly rearranged in all the patient groups versus the normal controls. Moreover, enterocytes from the study group subjects and untreated coeliac patients had rounded nuclei compared with the normal controls, in whom the nuclei had an oval shape.

Based on the above information enterocytes clearly appear to be cuboidal in coeliac disease patients. It has been demonstrated in previous reports that the irregular shape of the enterocyte and their nuclei are features of the coeliac lesion (Magliocca *et al.*1992). Their finding and the finding from the present study suggest that enterocytes might contribute to the early features of gut inflammation. Enterocytes can function as immune cells and it has been found that these cells express MHC class II proteins (Bland *et al.* 1988) and during inflammation, this expression is extended from the villous tip to the entire epithelium (Kelly *et al.* 1988).

The demonstration of tubulin rearrangements in this study is in agreement with a pervious study where an algorithmic application was developed to identify the cytoskeletal fibres and give statistics to these fibre arrangements within a cell (Grove *et al.* Poster presentation, Cellomics meeting, Pittsburgh 2004). Furthermore, it has been shown that gluten changes the organisation of the cytoskeleton in a cell line (Intestine 407 cells) (Sjolander *et al.*1988).

The nuclear measurements in this study indicate that the enterocyte nuclear profile in the initial duodenal biopsies of the study group subjects was morphologically changed. This was also the case in the study group after the lesion had developed

and in untreated coeliac disease patients. These observations suggest that the tubulin rearrangement has an influence on the enterocytes and on their nuclear shape in patients with potential and/or untreated coeliac disease. This is in keeping with the fact that cytoskeleton proteins are involved in the maintenance of cell shape, movement, replication, apoptosis, differentiation and intracellular signalling (Fuchs *et al.* 1998; Banan *et al.* 1999; Fenteany *et al.* 2004).

5.4 The intestinal protein expression of matrix metalloproteinases in coeliac disease and dermatitis herpetiformis (gluten-sensitive enteropathy) patients.

Using immunohistochemistry, the protein expression of matrix metalloproteinases (MMP-1, -3, -9) and their physiological inhibitor, tissue inhibitor metalloproteinases 1 (TIMP-1) was examined in duodenal biopsies. In addition to patients with untreated coeliac disease, treated coeliac disease and the study group subjects, tissue from patients with dermatitis herpetiformis was also studied. Duodenal lesions in these patients were classified based on routine histology, according to the Marsh classification: grade 1: increased IELs counts (n =13); grade 2: increased intra-epithelial lymphocytes and villous blunting (n =10); grade 3: total villous atrophy (n= 7).

In this study, it was demonstrated for the first time that the protein expression of MMP-1, -3, -9 and their inhibitor TIMP-1 was significantly increased in the GSE patients and the study group subjects compared with the normal control group. These findings are in agreement with three earlier studies that investigated mRNA expression of MMPs and TIMP-1 in coeliac and dermatitis herpetiformis intestinal mucosa (Daum *et al.* 1999; Ciccocioppo *et al.* 2005; Salmela *et al.* 2001). The finding of this study suggests that these enzymes might be involved in the disease process in patients with GSE. Furthermore, this analysis could help distinguish patients with an early gluten-sensitivity, particularly in patients with positive coeliac disease serology but apparently without intestinal inflammation. The increased TIMP-1 expression, which paralleled the increase in MMP-1, -3 and -9 in patients with GSE, suggests a crucial role of TIMP-1 in controlling these enzymes.

In the normal small intestine, extracellular matrix (ECM) formation by stromal cells balances ECM degradation mediated by matrix metalloproteinases (Birkedal

et al. 1993). These enzymes play important roles in foetal development, tissue remodelling, and angiogenesis (Matrisian *et al.* 1990; Werb *et al.* 1999; Khasigov *et al.* 2001; Salmela *et al.* 2004). It has been shown that increased synthesis and functional activity of these matrix degrading enzymes was related to tissue injury in a number of disease states (Kirkegaard *et al.* 2004; von Lampe *et al.* 2000).

There is recent interest in the possibility that matrix metalloproteinases might play a pathogenic role in coeliac disease. The rapid destruction of the villous architecture that can take place within a few hours of challenging coeliac disease patients with gliadin *in vivo* may be due to degradation of interstitial collagens by enzymes such as MMP-3 (Kontakou *et al.* 1995; Pender *et al.* 1998). This is in keeping with this current study in which MMP-3 enzyme was significantly increased in the lamina propria of duodenal sections in patients with GSE patients and in the study group subjects.

The findings of TIMP-1 protein expression in this study were in agreement with a previous study showing increased TIMP-1 mRNA expression in coeliac disease patients (Daum *et al.* 1999). In contrast to the findings of Daum *et al.* who reported high TIMP-1 mRNA expression in coeliac disease patients on a gluten free diet (Daum *et al.* 1999), the present work showed that TIMP-1 expression was higher in patients with grade 3 duodenal lesions compared with grade 1 and 2. This finding suggests that when these enzymes are induced their physiological inhibitor is also induced and their expression is correlated with the severity of the intestinal damage. Furthermore, it appears that cells expressing TIMP-1 are induced by the same biological factors that induce the expression of MMP-1, -3 and -9 in GSE patients.

In conclusion MMP-1, -3, -9 and their inhibitor TIMP-1 were significantly increased in GSE and the study group subjects compared with the normal controls. In addition the expression of these enzymes correlated with the degree of mucosal damage. Furthermore, even patients with a grade 1 lesion had higher expression of TIMP-1 compared with the normal control group. This could suggest that TIMP-1 promotes ECM remodelling in these patients.

5.5 Conclusions

The studies reported in this thesis demonstrated that the small intestine of a group of patients with positive coeliac serology but apparently normal histology had subtle changes indicative of early immunological activation. These changes included increased expression of CD2, CD3, CD7, CD8 and CD69 on the IELs in duodenal biopsies from these patients. Increased numbers of positive Ki67 crypt cells were also present. In addition, the enterocyte morphological changes and the increased expression of MMPs suggest that these patients have entered an early inflammatory process. Moreover, the follow-up results in these patients demonstrated that this initial immunological response helped develop an intestinal lesion over time. Based on these results, it was proposed that immunohistochemical characterisation of crypt cells and lymphocyte subpopulations in the small intestinal mucosa may be used as a tool to identify patients with an early coeliac lesion, particularly in those with positive coeliac disease serology. These findings also highlight the importance of the follow-up of patients with persistent positive coeliac serology.

This is the first study to examine the enterocyte to such a high level of morphological detail in duodenal sections from normal individuals, as well as in patients. Moreover, the finding that the enterocytes from the duodenal sections of the study group subjects, before an overt lesion developed had subtle morphological changes suggests that these abnormalities were present from the start. This is supported by the finding of increased IELs markers and the upregulation of crypt cell proliferation that were reported (chapter two) and by the increase of MMP-1, -3, -9 and TIMP-1 expression (chapter four). It was also found that there was tubulin rearrangement in the enterocytes of the study group and coeliac disease patients. From this study, it can be concluded that the results obtained by the high content analysis system are likely to be important and can reveal alterations in enterocyte nuclei and tubulin fibres, which may be caused by gluten-sensitivity.

In this study, it was demonstrated for the first time that the protein expression of MMP-1, -3, -9 and the inhibitory molecule TIMP-1 were markedly increased in the intestinal mucosa of patients with a positive EMA test (and apparently normal intestinal mucosa) and in the intestinal mucosa of patients with coeliac disease or

dermatitis herpetiformis. Moreover, the level of expression of these enzymes and their inhibitor, although significantly increased even in the study group subjects and patients with grade 1 lesion, was most significantly elevated in biopsies with the most severe lesion (grade 3), suggesting that these enzymes may play a pathological role in gluten-sensitive enteropathy.

5.6 The key findings of the thesis

The key findings of this thesis are as follows:

1. The presence of endomysial antibodies in a patient's serum, despite the presence of normal routine intestine histology findings indicates the likelihood of gluten-sensitive disease.
2. Even though an increase in intraepithelial lymphocyte numbers is not coeliac specific, their up-regulation is important in identifying patients who have immunological activation in their small intestinal mucosa.
3. The increased expression of the matrix metalloproteinases and their inhibitor reveal that it is possible that these enzymes play a pathogenic role in gluten-sensitive enteropathy patients.
4. Using the high content analysis the enterocyte height and width can be quantified as well as it is nuclear shape. These measurements can detect patients with subtle immunological changes in their duodenal tissue.

5.7 Future work

Based on the results of this thesis, it is obvious that individuals identified by the presence of endomysial antibodies have evidence of early gluten-sensitivity in their tissue. In chapter two of this thesis, the main features detected in these patients were increased mucosal expression of CD3, CD8, CD7 and CD69 on the intraepithelial lymphocytes as well as Ki67 in the crypt cells. Concerning the increase of T lymphocytes at the surface epithelium in these patients, future work should be directed towards the morphological and physiological behaviour of these cells in the presence of gluten

Recently, it was reported that IL-15 appears to be central to coeliac disease, and probably inflammatory bowel disease pathogenesis (Maiuri *et al.* 2000; Mention *et al.* 2003). In coeliac disease, IL-15 induces crypt cell proliferation and is involved in the generation of villous atrophy, in favouring the selective expansion of IELs, particularly TCR$\gamma\delta^+$ and $CD8^+$ TCR$\alpha\beta^+$ lymphocytes, and in promoting the appearance of T cell clonal proliferation in refractory coeliac disease (Maiuri *et al.* 2000; Mention *et al.* 2003; Hüe *et al.* 2004; Jabri *et al.* 2000). Since IL-15 over-expression is associated with intestinal damage and blocking it and its receptor prevents this damage in the coeliac disease patients, it would be of great interest to examine IL-15 expression in individuals with positive endomysial antibodies and normal intestinal mucosa.

High content analysis was used to quantify the morphological characterisation of the enterocytes in the duodenal mucosa of coeliac subjects. Thus, it is worthwhile expanding the work to examine T-lymphocytes from the gut mucosa using this technology.

With the finding of increased expression of matrix metalloproteinases -1, -3, -9 and tissue inhibitor of metalloproteinases-1 in the intestinal mucosa of the coeliac subjects, future work should be directed towards identifying the cells that express these enzymes in intestine organ culture in the presence or the absence of gliadin.

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APPENDICES

APPENDIX I-REAGENTS

I-IMMUNOPEROXIDASE

BUFFERS AND SOLUTIONS USED IN THE EXPERMENTS

10 X Phosphate buffered saline (PBS) (stock solution)

80g NaCl

2g KCl

11.5g $Na_2 HPO_4$

2g KH_2PO_4

Make up to 1 litre with distilled water

Store at - 4^0C

Adjust pH to 7.4

1X PBS (working solution)

10X PBS solution was diluted 1:10 with distilled water.

Tween added preferably after dilution (0.5ml/l)

10X TRIS buffered saline (TBS) (stock solution)

6.055g TRIS

17.532g NaCl

Make up to 1 litre with distilled water

Store at - 4^0C

Adjust pH to 7.6

1X TBS (working solution)

10X TBS solution was diluted 1:10 with distilled water.

Tween added preferably after dilution (0.5ml/l)

0.001M EDTA

3.72 g/L

Make up to 1 litre with distilled water

Adjust pH to 8.0

0.01M Citrate buffer

21.05 g/L CB

Make up to 1 litre with distilled water

Store at - 4^0C

Blocking solution

30% H_2O_2

Methanol

Distilled water (DW)

Working solution

Required concentration of H_2O_2 in methanol or DW was prepared.

DAB substrate 3,3-diaminobenzidine tetrahydrochloride dihydrate

II-IMMUNOFLUORECENT

10 X Phosphate buffered saline (PBS) (stock solution)

80g NaCl

2g KCl

11.5g Na_2HPO_4

2g KH_2PO_4

Make up to 1 litre with distilled water

Store at - 4^0C

Adjust pH to 7.4

1X PBS (working solution)

10X PBS solution was diluted 1:10 with distilled water.

Dilution buffer

1XPBS
0.05 30% ml Bovine Serum Albumin (BSA)
0.01 ml Sodium Azid (NaN3)

0.01M Citrate buffer

21.05 g/L CB

Make up to 1 litre with distilled water

Store at - 4^0C

APPENDIX II

MONOCLONAL POLYCLONAL ANTIBODIES

i-The used antibodies, their source and specificity in chapter two of this thesis

Antibodies	*Source*	*Clone.*	*Specificity*
Anti-CD2	Novocastra UK	NCL-CD2-271	Human antigen (LFA-2)
Anti-CD3	Novocastra UK	NCL-CD3-PS1	Human T cell antigen receptor complex
Anti-CD7	Novocastra UK	NCL-CD7-272	Marker for early T cell lineage
Anti-CD8	Novocastra UK	NCL-CD8-4B11	Cytotoxic cells
Anti-CD69	Novocastra UK	NCL-CD69	Human CD69 antigen (activation markers)
Anti-Ki-67	Novocastra UK	NCL-Ki67p	Human ki67nuclear antigen (Proliferation marker)

ii-The used monoclonal antibodies in chapter four

Structure	Nucleus	Microtubules	Secondary antibody
Protein		Tubulin	
Antibody	1μM Hoeschst 33258	α- tubulin	Alexa flour
Clone		B-5-1-2	
Source	Sigma, St Louis, MO	Sigma, St Louis, MO	Molecular Probes, Eugene, OR
Dilution	1/500	1/100	1/500

iii-The used monoclonal antibodies, their source and specificity in chapter three of this thesis

Antibodies	Clone	Source	Specificity
Anti-MMP-1	(3B6): sc-21731	Santa Cruz Biotechnology, Inc. USA	Human MMP-1
Anti-MMP-3	(1B4): sc-21732	Santa Cruz Biotechnology, Inc. USA	Human MMP-3
Anti-MMP-9	(2C3): sc-21733	Santa Cruz Biotechnology, Inc. USA	Human MMP-9
Anti-TIMP-1	ab 1827	Abcam, Cambridgeshire, UK	Mouse TIMP-1

STANDARDISATION

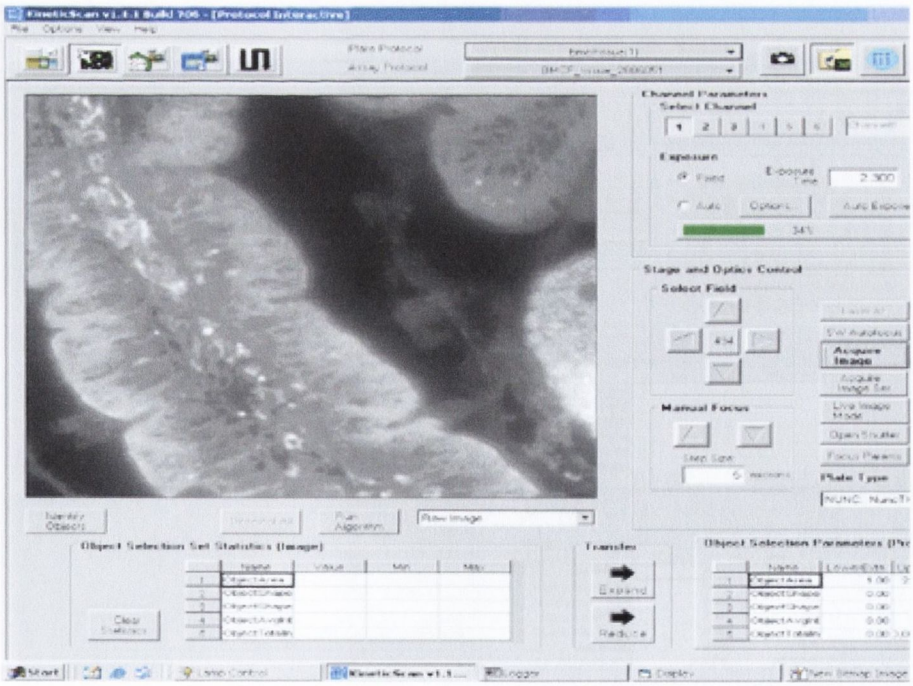
Standardised technique for immunohistochemical staining of MMPs –1, -3, -9 and TIMP-1 markers.

MMP-1,-3	1:25/1:50	Overnight at 4^0C	Citrate buffer	40 mins after Ag retrieval	0.5% in DW
TIMP-1	1:10/1:20	Overnight at 4^0C	Citrate buffer	10 mins after Ag retrieval	0.5% in methanol
MMP-9	1:25/1:50	40 min at RT	Citrate buffer	40 mins prior Ag retrieval	0.1% in methanol

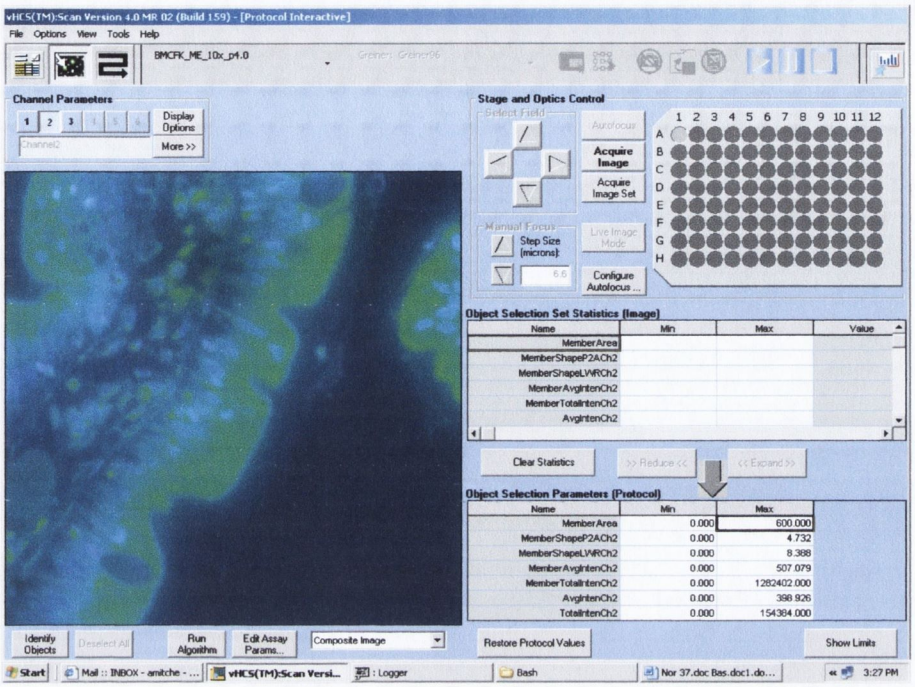
APPENDIX III



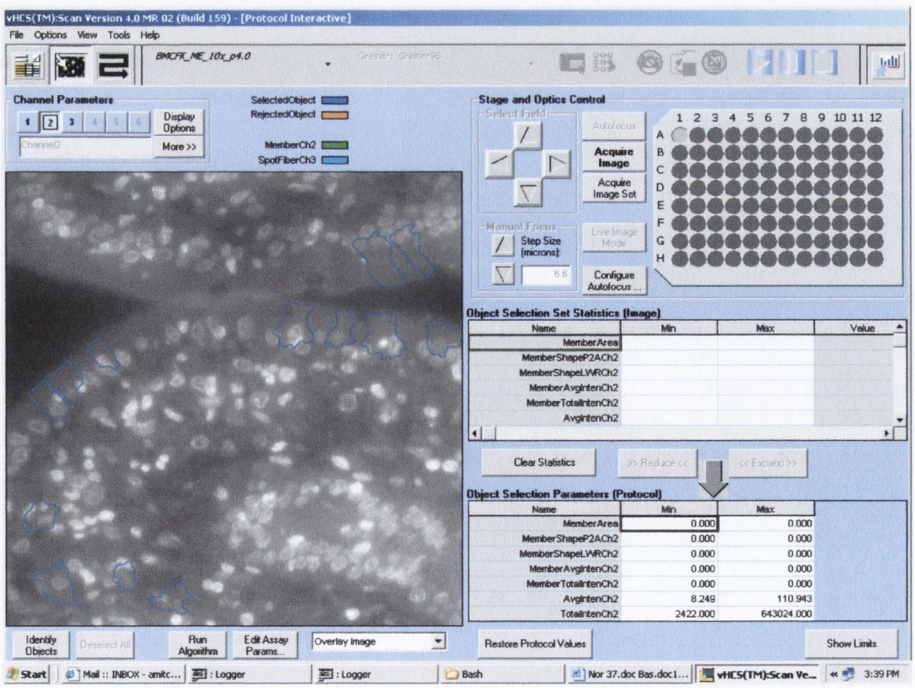
i-Cellomics® Kineticscan



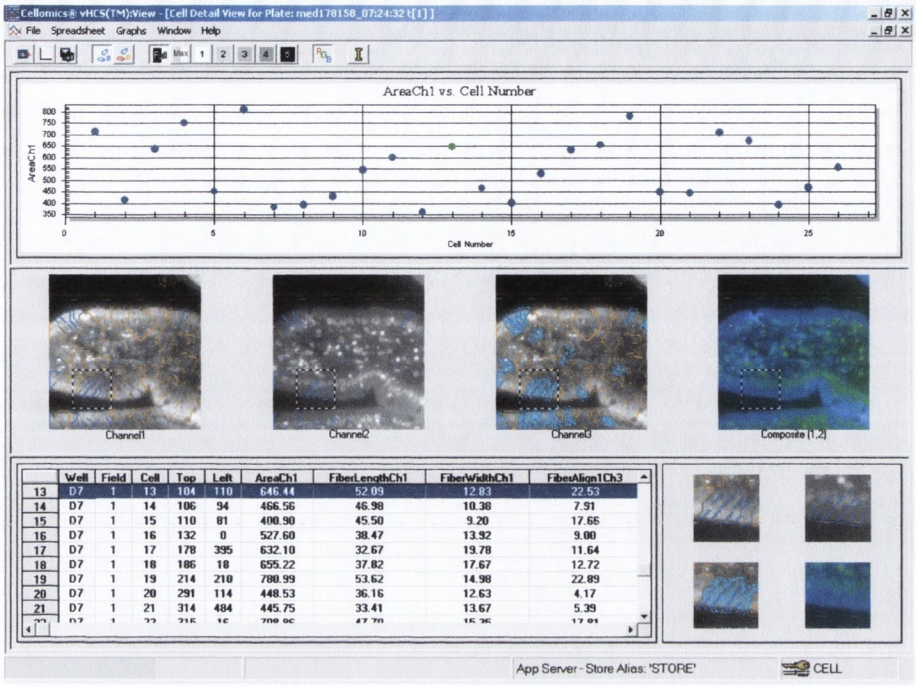
ii- Scanned duodenal section using Cellomics® Kineticscan



iii-Identified duodenal section using Virtual scan

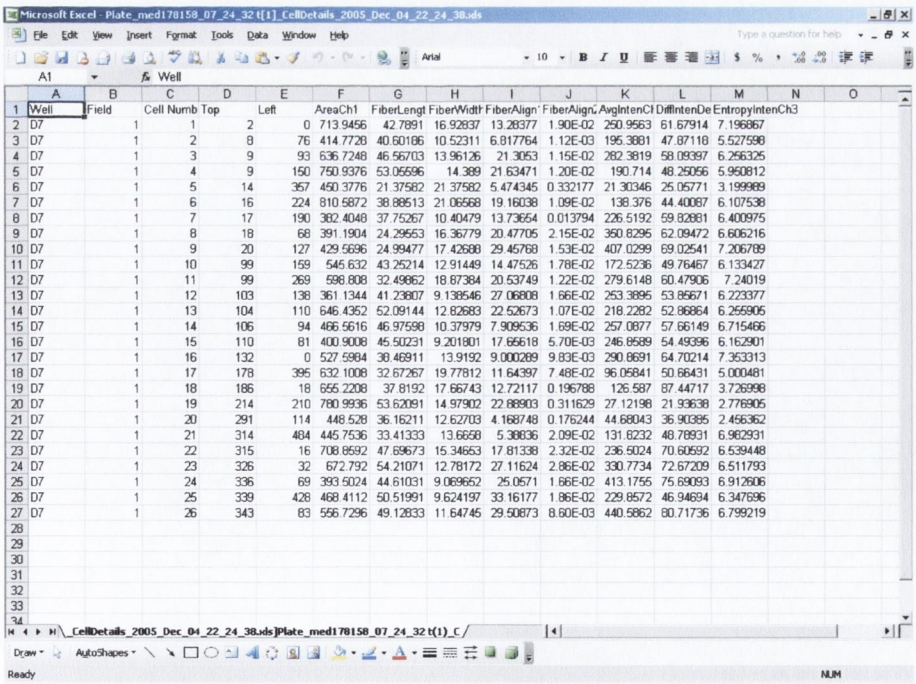


iv-Individualised enterocytes using the Bioapplication tools (assay parameters)



	Well	Field	Cell	Top	Left	AreaCh1	FiberLengthCh1	FiberWidthCh1	FiberAlign1Ch3
13	D7	1	13	104	110	646.44	52.09	12.83	22.53
14	D7	1	14	106	94	466.56	46.98	10.38	7.91
15	D7	1	15	110	81	400.90	45.50	9.20	17.66
16	D7	1	16	132	0	527.60	38.47	13.92	9.00
17	D7	1	17	178	395	632.10	32.67	19.78	11.64
18	D7	1	18	186	18	655.22	37.82	17.67	12.72
19	D7	1	19	214	210	780.99	53.62	14.98	22.89
20	D7	1	20	291	114	448.53	36.16	12.63	4.17
21	D7	1	21	314	484	445.75	33.41	13.67	5.39

v-Data was generated using Virtual scan



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	Well	Field	Cell Numb	Top	Left	AreaCh1	FiberLengt	FiberWidth	FiberAlign	FiberAlign	AvgIntenCl	DiffIntenDe	EntropyIntenCh3		
2	D7	1	1	2	0	713.9456	42.7891	16.92837	13.28377	1.90E-02	250.9563	61.67914	7.196867		
3	D7	1	2	8	76	414.7726	40.60186	10.52311	6.817764	1.12E-03	195.3881	47.87118	5.527598		
4	D7	1	3	9	93	636.7248	46.56703	13.96126	21.3053	1.15E-02	282.3819	58.09397	6.256325		
5	D7	1	4	9	150	750.9376	53.05596	14.389	21.63471	1.20E-02	190.714	48.25056	5.960812		
6	D7	1	5	14	357	450.3776	21.37582	21.37582	5.474345	0.332177	21.30346	25.05771	3.199989		
7	D7	1	6	16	224	810.5872	38.88513	21.06568	19.16038	1.09E-02	138.376	44.40087	6.107538		
8	D7	1	7	17	190	382.4048	37.75267	10.40479	13.73654	0.013794	226.5192	59.82881	6.400975		
9	D7	1	8	18	68	391.1904	24.29553	16.36779	20.47705	2.15E-02	360.8295	62.09472	6.606216		
10	D7	1	9	20	127	429.5696	24.99477	17.42688	29.45768	1.53E-02	407.0299	69.02541	7.206789		
11	D7	1	10	99	159	545.632	43.25214	12.91449	14.47526	1.78E-02	172.5236	49.76467	6.133427		
12	D7	1	11	99	269	598.808	32.49862	18.87384	20.53749	1.22E-02	279.6148	60.47906	7.24019		
13	D7	1	12	103	138	361.1344	41.23807	9.138546	27.06808	1.66E-02	253.3895	53.85671	6.223377		
14	D7	1	13	104	110	646.4352	52.09144	12.82683	22.52673	1.07E-02	218.2282	52.86864	6.265905		
15	D7	1	14	106	94	466.5616	46.97598	10.37979	7.909536	1.69E-02	257.0877	57.66149	6.715466		
16	D7	1	15	110	81	400.9008	45.50231	9.201801	17.66618	5.70E-03	246.8589	54.49396	6.162901		
17	D7	1	16	132	0	527.5984	38.46911	13.9192	9.000269	9.83E-03	290.8691	64.70214	7.363313		
18	D7	1	17	178	395	632.1008	32.67267	19.77812	11.64397	7.48E-02	96.05841	50.66431	5.000481		
19	D7	1	18	186	18	655.2208	37.8192	17.66743	12.72117	0.196788	126.587	87.44717	3.726998		
20	D7	1	19	214	210	780.9936	53.62091	14.97902	22.88903	0.311629	27.12198	21.93638	2.776905		
21	D7	1	20	291	114	448.528	36.16211	12.62703	4.168748	0.176244	44.68043	36.90385	2.466362		
22	D7	1	21	314	484	445.7536	33.41333	13.6658	5.38836	2.09E-02	131.8232	48.78931	6.962931		
23	D7	1	22	315	16	708.8592	47.69673	15.34653	17.81338	2.32E-02	236.5024	70.60592	6.539448		
24	D7	1	23	326	32	672.792	54.21071	12.78172	27.11624	2.86E-02	330.7734	72.67209	6.511793		
25	D7	1	24	336	69	393.5024	44.61031	9.069652	25.0571	1.66E-02	413.1755	75.69093	6.912606		
26	D7	1	25	339	428	468.4112	50.51991	9.624197	33.16177	1.86E-02	229.8572	46.94694	6.347696		
27	D7	1	26	343	83	556.7296	49.12833	11.64745	29.50873	8.60E-03	440.5862	80.71736	6.799219		

vi-Data exported to Microsoft Excel sheet