In Vivo And *Ex Vivo* Examination Of The Safety Of Oats In Coeliac Disease

A thesis submitted to the University of Dublin
for the Degree of Doctor of Philosophy

by

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Declaration

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Summary

Coeliac disease is a chronic autoimmune disorder of the small intestine characterised by infiltration of intraepithelial lymphocytes, crypt hyperplasia and villous atrophy. It develops in genetically susceptible individuals following exposure to gluten. The only treatment is dietary and all sources of gluten must be permanently removed from the diet. On the basis of studies carried out in the 1930s and 1940s, wheat, barley, rye and oats were deemed to be harmful for coeliac patients and the exclusion of these four grains was the basis of the gluten free diet. However, the true toxicity of oats has more recently been questioned and although a number of studies have indicated oats to be safe for coeliac patients there have also been reports of adverse effects as a result of oats addition to the gluten free diet.

The gluten free diet is difficult to maintain and often high in fat and low in fibre, vitamins and other nutrients. The addition of oats to this diet would have both lifestyle and nutritional benefits. The aim of this thesis was to thoroughly examine the safety of oats in the gluten free diet.

In the first part of this study, a large group of treated and newly diagnosed coeliac patients added pure oats to their gluten free diet for one year. During this time parameters including coeliac serology, symptoms and histology were examined for any signs of disease activation. In addition, the expression of Ki67, CD3 and CD8 were compared between patient biopsies taken at the start and the end of the study, to look for more subtle signs of change. No signs of disease activation were seen, indicating that pure oats can be safely included in the coeliac diet.

In the next part of the study, the IN Cell Analyzer 1000, a system designed for automated analysis of cell-based assays, was adapted for use with tissue sections. This enabled us to attain a numerical measure of the amount of positive staining present on a tissue section. This was used to measure the expression of tTG and SM α-actin in biopsies taken from coeliac patients at the start and end of the oats study, as well biopsies from Marsh grade 0 and Marsh grade 3 coeliac patients. tTG expression is known to be upregulated in active coeliac disease; this was seen in the Marsh grade 3 biopsies but not in the biopsies from the oats study. Likewise, expression of SM α-actin was increased in the Marsh grade 3 biopsies but not in the biopsies from the oats study. This is the first time the expression of tTG and SM α-actin has been assessed in the investigation of oats tolerance in coeliac disease and provides further evidence of a lack of oats toxicity. Additionally, we demonstrated for the first time the co-expression of tTG and SM α-actin in myofibroblasts in the coeliac mucosa. Furthermore, this co-expression was found to be increased in the Marsh grade 3 biopsies compared to the Marsh grade 0 and oats study biopsies.

In the last part of the study, duodenal biopsies from coeliac and control patients were cultured in the presence of PT avenin or PT gliadin. They were then stained for the expression of E-cadherin and cytokeratin 20 and imaged on a confocal microscope. Cytokeratin 20 staining was too inconsistent to draw any conclusions; however, E-cadherin staining showed changes in three out of five coeliac patients following culture with avenin. The concentration of suPAR was measured in culture supernatants and was increased in the same three coeliac patients. In addition, suPAR concentration was significantly increased in all coeliac biopsy supernatants compared to controls, regardless of culture condition. These final results indicate caution may still be necessary when introducing oats to the gluten free diet; however, this is based on the examination of a small number of patients.

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This thesis is dedicated to my parents

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Publications and Communications from this Thesis

Journal Publication

- **S. E. J. Cooper**, N. P. Kennedy, B. M. Mohamed, M. Abuzakouk, J. Dunne, G. Byrne, G. McDonald, A. Davies, C. Edwards, J. Kelly, C. F. Feighery. "Immunological Indicators of Coeliac Disease Activity Are Not Altered by Long-Term Oats Challenge." *Clinical and Experimental Immunology,* 2013 Mar; 171 (3): 313-318.

Book Chapters

- **S. E. J. Cooper**, J. Dunne, B. M. Mohamed, A. Davies, L. Elliott, J. Kelly, C. F. Feighery. "Adaptation of a Cell-Based High Content Screening System for the In-Depth Analysis of Coeliac Biopsy Tissue." In Preparation for the *Methods in Molecular Biology* series.

- **S. E. J. Cooper**, C. F. Feighery. "24 Hour *Ex Vivo* Culture of Coeliac Duodenal Biopsies." In Preparation for the *Methods in Molecular Biology* series.

Poster Presentations

- **S. E. J. Cooper**, N. P. Kennedy, J. Dunne, B. M. Mohamed, C. F. Feighery. "Detailed Analysis of Duodenal Biopsy Tissue in Coeliac Patients Following 1 Year of Oats Ingestion."

    - AOECS International Coeliac Disease Meeting, 2007, Maribor, Slovenia.

    - IMM 10th Annual Meeting, 2007, Institute of Molecular Medicine, St. James's Hospital, Dublin.
        - 2nd Prize in Poster Competition - PhD/MD Category.

- **S. E. J. Cooper**, B. M. Mohamed, R. Comerford, C. F. Feighery. "Detailed Analysis of Duodenal Biopsy Tissue from Coeliac Patients Following 24 Hour Culture with PT Avenin."

    - IMM 16th Annual Meeting, 2013, Institute of Molecular Medicine, St. James's Hospital, Dublin.

Abbreviations

°C	Degrees Celsius
Ag	Antigen
AGA	Anti-gliadin antibody
ANOVA	Analysis of variance
APC	Antigen presenting cell
AU	Arbitrary units
BMI	Body mass index
BSA	Bovine serum albumin
BSPGHAN	British Society of Paediatric Gastroenterology, Hepatology and Nutrition
CB	Citrate buffer
CD	Coeliac disease
CI	Confidence interval
CK20	Cytokeratin 20, keratin 20
CSA	Celiac Sprue Association
DGP	Deamidated gliadin peptide
DH	Dermatitis herpetiformis
dH_2O	Distilled water
E-cadherin	Epithelial-cadherin
EATL	Enteropathy-associated T-cell lymphoma
EC	Epithelial cell
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
EMA	Anti-endomysial antibody
F	Female

Fig	Figure
FITC	Fluorescein isothiocyanate
g	Gram
g	Gravity
GAM	Goat-anti-mouse
GAR	Goat-anti-rabbit
GF	Gluten free
GFD	Gluten free diet
gp	Guinea pig
GPI	Glycosylphosphatidylinositol
GWAS	Genome-wide association studies
h	Hour
H&E	Hematoxylin and eosin
H_2O	Water
HLA	Human leukocyte antigen
HMW	High molecular weight
IEL	Intraepithelial lymphocyte
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IMM	Institute of Molecular Medicine
IMS	Industrial methylated spirit
kg	Kilogram
LMW	Low molecular weight
LP	Lamina propria

LPS	Lipopolysaccharide
M	Male
m	Metre
m	Monoclonal
mg	Milligram
mg/L	Milligram per litre
mg/ml	Milligram per millilitre
MHC	Major histocompatibility complex
min	Minutes
ml	Millilitre
mm	Millimetre
MMP	Matrix metalloproteinase
mol/L	Moles per litre
mRNA	Messenger ribonucleic acid
N	Normal
n	Number
N/A	Not applicable
Neg	Negative
nm	Nanometre
ns	Not significant
O/N	Overnight
OC	Organ culture
OGD	Oesophagogastroduodenoscopy
p	Polyclonal
PBS	Phosphate buffered saline
PCMF	Pericryptal myofibroblast

pg	Picogram
pg/ml	Picogram per millilitre
Pos	Positive
PT	Peptic-tryptic
PWG	Prolamin Working Group
RCD	Refractory coeliac disease
rh	Recombinant human;
RT	Room temperature
SD	Standard deviation
SLE	Systemic lupus erythematosus
SM α-actin	Smooth muscle α-actin
suPAR	Soluble uPAR
TBS	Tris buffered saline
TCD	Treated coeliac disease
TIMP	Tissue inhibitor of metalloproteinases
tTG	Tissue transglutaminase
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
v/v	Volume per volume
W.pos	Weak positive
y	Years
μm	Micrometre
μg/ml	Microgram/millilitre

Chapter 1

General Introduction

1.1 Background

Coeliac disease (CD) is a chronic small intestinal immune-mediated enteropathy that develops in genetically susceptible individuals following exposure to gluten (Jonas F Ludvigsson et al. 2013). The only treatment is lifelong elimination of all sources of gluten from the diet (Koning et al. 2005). Failure to maintain this leads to reactivation of the disease and brings with it the risk of numerous secondary complications (Di Sabatino and Corazza 2009; Alessio Fasano and Catassi 2012).

1.1.1 Symptoms and presentation

Coeliac disease can present in a huge variety of ways. The 'classical' symptoms include diarrhoea, constipation, abdominal pain, weight loss and fatigue (Sollid 2000; Alessio Fasano and Catassi 2012). In babies and children failure to thrive, short stature, diarrhoea and abdominal distension are common symptoms at presentation (Guandalini and Assiri 2014). However, CD has been described as a 'clinical chameleon' as none of these symptoms may be apparent at diagnosis. Instead, secondary symptoms such as anaemia, osteoporosis, other nutritional deficiencies, infertility or dental problems may be the only overt clues to an underlying problem, despite the fact that the mucosa may be severely damaged (Kavimandan et al. 2013; Ertekin, Tozun, and Küçük 2013; Alessio Fasano 2003; A Fasano and Catassi 2001; Assiri et al. 2013).

In addition, many cases of so-called 'silent coeliac disease' are diagnosed when relatives of coeliac patients are screened. In these cases there may be no symptoms of any sort reported although histological analysis may reveal a significantly damaged mucosa. However, following elimination of gluten from the diet, some of these individuals will report a significant improvement in well being and energy levels along with resolution of

minor symptoms such as abdominal discomfort and tiredness which had previously been accepted as normal (McGough and Cummings 2005).

1.1.2 Diagnosis

Serology

In suspected cases of coeliac disease, the first stage of diagnosis is serology. It is important that the patient does not alter their diet at this stage, as removal of gluten from the diet can result in false negative results (Midhagen et al. 2004). One of the earliest serological tests for CD was for immunoglobulin (Ig) A and IgG anti-gliadin antibodies (AGA). These were first described in 1958 and the development of assays to reliably measure levels of AGA in patient serum was a huge step forwards in the diagnosis of CD (Bürgin-Wolff et al. 2002; O'Farrelly et al. 1983). However, with reported sensitivities of 80%-90% and 80% and specificities of 85%-95% and 80% for IgA and IgG AGA respectively, use of these markers has declined in favour of newer, more accurate tests, although it is possible that in very young children AGA remains a more sensitive marker (Table 1.1) (Reddick, Crowell, and Fu 2006; Lagerqvist et al. 2008).

The IgA assay for anti-endomysial antibodies (EMA) was developed in the 1980s and brought with it improved sensitivity and specificity of >90% and >95% respectively (Feighery, Conlon, and Jackson 2006; Reddick, Crowell, and Fu 2006). Its widespread usefulness is somewhat limited as it is labour intensive and qualitative making it relatively expensive and subject to inter-operator variability (van Heel and West 2006; L. A. Harris et al. 2012). In 1997 tissue transglutaminase (tTG) was discovered to be the antigenic target of endomysial antibodies and an enzyme linked immunosorbent assay (ELISA) was developed to measure IgA anti-tTG titres in serum (Dieterich et al. 1997; Dieterich et al.

1998). Sensitivity and specificity of this test is excellent at 95%-98% and 94%-95% respectively (Reddick, Crowell, and Fu 2006). In addition, the test is easy to perform, can be automated and results are quantitative, avoiding inter-operator variability (Dieterich et al. 1998; van Heel and West 2006). The use of EMA and tTG tests together has been shown to provide the most sensitive and specific serological indicator of CD (Hill 2005). Furthermore, the establishment of a gluten free diet (GFD) leads to a gradual decline in both antibodies to normal levels, making them valuable tools in monitoring both mucosal recovery and dietary compliance (Sugai et al. 2010). More recently, deamidated gliadin peptide (DGP) antibodies have emerged as a potential diagnostic target; however, two recent papers have produced conflicting results as to the sensitivity and specificity of both IgA and IgG DGP tests (Giersiepen et al. 2012; L. A. Harris et al. 2012).

Serological Test	Sensitivity	Specificity
IgA tTG	95%-98%	94%-95%
IgA EMA	>90%	>95%
IgA AGA	80%-90%	85%-95%
IgG tTG	40%	95%
IgG EMA	40%	95%
IgG AGA	80%	80%

Table 1.1: Sensitivity and specificity of CD serology tests. IgA = immunoglobulin A; tTG = tissue transglutaminase; EMA = endomysial antibody; AGA = anti-gliadin antibody. (Table adapted from Reddick, Crowell, and Fu 2006)

IgA deficiency is significantly more common among the coeliac population, occurring in about 2% of coeliac patients versus 0.5% of the general population (L. A. Harris et al. 2012). In addition, the prevalence of CD in patients with IgA deficiency is much greater than that of the general population, with estimates of 10%-30%. As most serological assays for CD are IgA-based, patients with CD and IgA deficiency may remain undiagnosed and untreated. Therefore, in cases of suspected CD with negative serology, levels of serum IgA should be checked. If the patient proves to be IgA deficient, IgG-based tests can be carried out. However, the sensitivity of IgG-based assays is considerably lower that of IgA-based assays, at only 40% for both tTG and EMA despite 95% specificity, thus a negative result does not rule out CD in such patients (McGowan, Lyon, and Butzner 2008; Reddick, Crowell, and Fu 2006).

Histology

Following positive serology, an oesophagogastroduodenoscopy (OGD) should be performed. A flattened mucosa may be identifiable visually (Fig. 1.1), however duodenal biopsies must be taken for proper histological diagnosis. Current guidelines indicate histological analysis as the gold standard for the diagnosis of CD, however, as some of the earlier changes seen in CD can also be seen in Crohn's disease, *Helicobacter pylori* gastritis, some parasitic and bacterial infections and other situations, the diagnosis of CD must also take into account the clinical presentation, serology and response to a GFD (Ianiro, Gasbarrini, and Cammarota 2013; Guandalini and Assiri 2014).

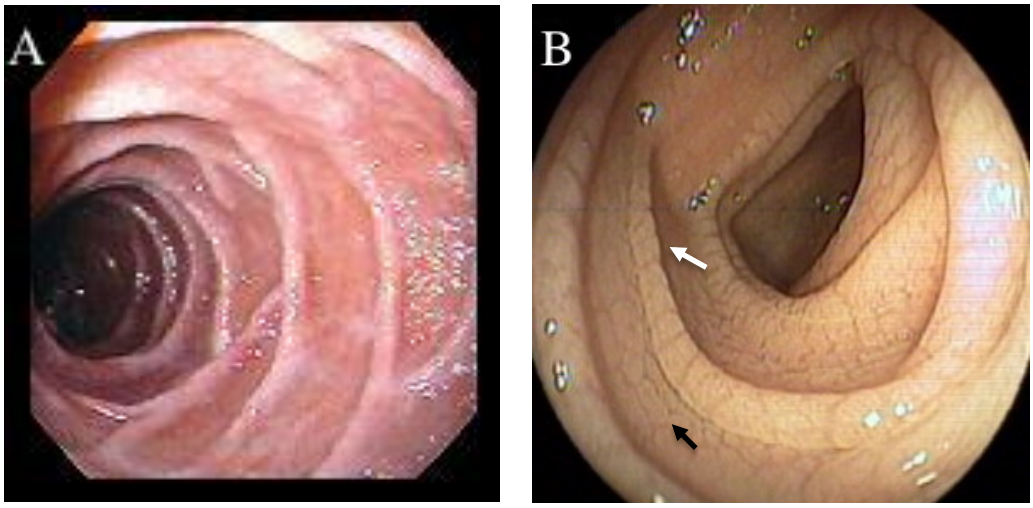


Figure 1.1: Endoscopic views of the duodenum. Examples of normal duodenum (A) and duodenum of a coeliac patient with total villous atrophy, displaying 'mosaic' mucosa (black arrow) and scalloping of duodenal folds (white arrow) (B). (Images from www.endoatlas.com, Copyright © Atlanta South Gastroenterology, P.C. (A) and Ravelli and Villanacci 2012 (B))

A system for describing and classifying the histopathological alterations of CD was developed by Marsh and modified by Oberhuber in the 1990s (Marsh 1990; Marsh and Crowe 1995; Oberhuber, Granditsch, and Vogelsang 1999). The Marsh-Oberhuber classification system categorises biopsies as follows (Fig 1.2):

- Marsh 0: normal mucosa with less than 40 intraepithelial lymphocytes (IEL)/100 epithelial cells (EC).
- Marsh 1: normal villous architecture, normal crypts and an increase in IELs to more than 40 IEL/100 EC.
- Marsh 2: normal villous architecture, crypt hyperplasia and >40 IEL/100 EC.
- Marsh 3 is further subdivided into 3 categories. Common to all categories is crypt hyperplasia and >40 IEL/100 EC. The degree of villous damage distinguishes the subcategories:
    - Marsh 3a: stubby villi
    - Marsh 3b: marked villous atrophy
    - Marsh 3c: total villous atrophy

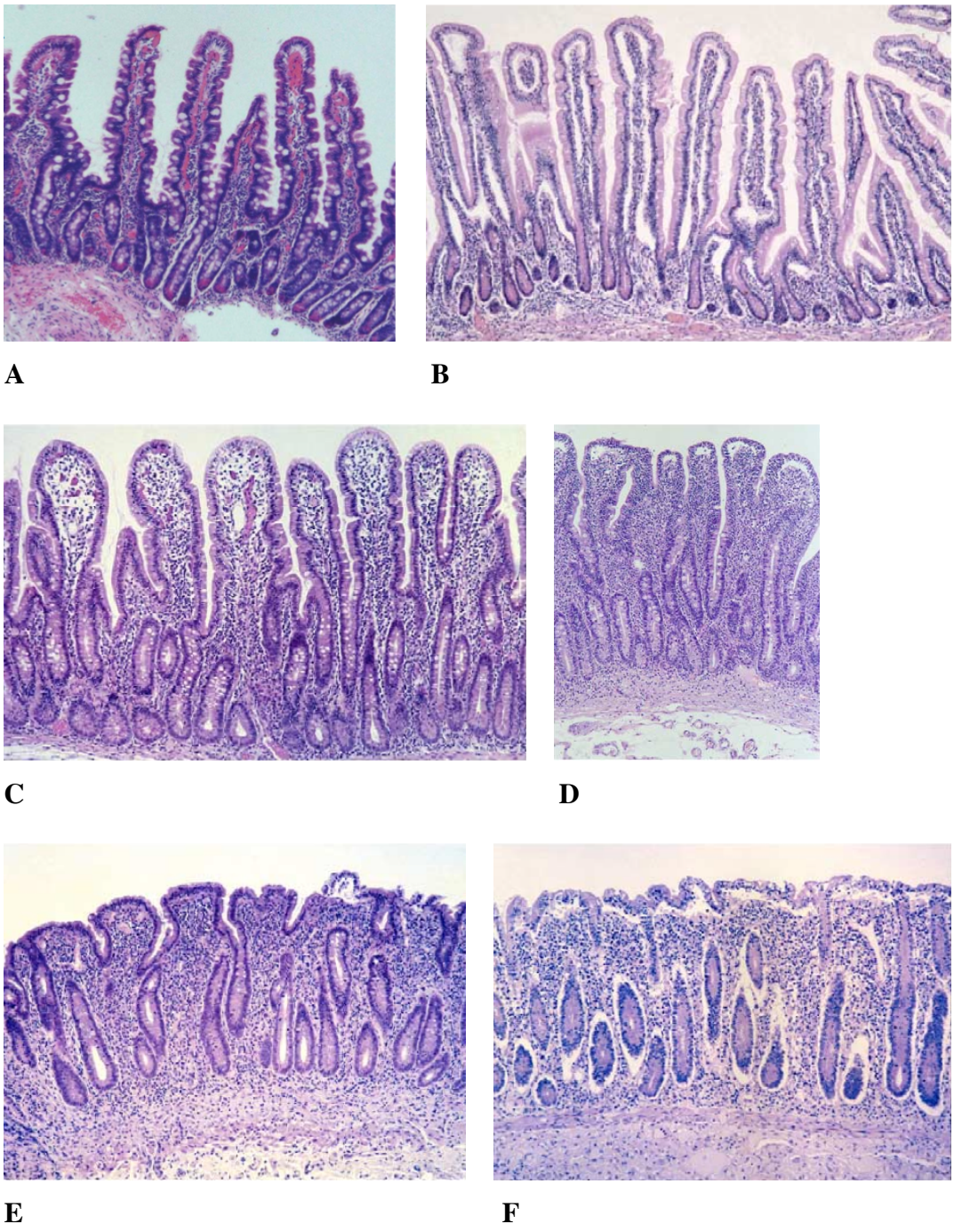


Figure 1.2: The Marsh-Oberhuber classification of histological findings in coeliac disease. Marsh 0 - normal mucosa (A), Marsh 1 - normal mucosa with infiltration of intraepithelial lymphocytes (IELs) (B), Marsh 2 - normal villi with crypt hyperplasia and infiltration of IELs (C), Marsh 3a - stubby villi, crypt hyperplasia and infiltration of IELs (D), Marsh 3b - marked villous atrophy, crypt hyperplasia and infiltration of IELs (E) and Marsh 3c - total villous atrophy, crypt hyperplasia and infiltration of IELs (F). (Images from Ianiro, Gasbarrini, and Cammarota 2013 (A) and Green, Rostami, and Marsh 2005 (B-F))

The Marsh-Oberhuber system considers the upper limit of normal IEL numbers to be 40 IELs per 100 ECs, however, more recently, it has been suggested that this should be lower (Nasseri-Moghaddam et al. 2008). Regardless of this, a Marsh 1 lesion has a positive predictive value of only about 15% and is not enough to make a diagnosis of CD. A Marsh 2 or 3 lesion with positive serology allows a presumptive diagnosis of CD to be made, the resolution of symptoms and serology on a GFD is required to confirm the diagnosis. In the past follow-up biopsies showing normalised histology on a GFD followed by histological relapse after gluten challenge were required to confirm diagnosis; however, this is less commonly done now (Bao, Green, and Bhagat 2012; Schuppan and Zimmer 2013).

1.1.3 Treatment

The only treatment for CD is the complete and permanent elimination of all sources of gluten from the diet. The principal sources of dietary gluten are wheat, barley and rye (Rubio-Tapia et al. 2013). Oats have traditionally been excluded from the GFD also, based on the results of early feeding studies (Ciclitira, Ellis, and Lundin 2005). The true toxicity of oats has been questioned recently; this will be discussed in more detail later.

Once properly established, the GFD is usually very successful in treating CD, resulting in histological and serological normalisation and resolution of symptoms (Rubio-Tapia et al. 2013; Bai et al. 2013). Nonetheless, it is a difficult diet to maintain. Practically, it is challenging to attain complete avoidance of all forms of gluten due to its ubiquitous nature. As well as being present in a wide variety of foods, a less obvious source of accidental gluten ingestion is from non-food items such as lip-glosses, lipsticks and medication. In children, sources may include crayons, paint and Play-doh, as children may put their hands

in their mouths during and after playing with these (L. A. Harris et al. 2012). In addition, gluten free (GF) breads, cereals etc. tend to be much more expensive than similar gluten-containing products, making a gluten free diet costly to maintain (Mulder et al. 2013). Many patients find the diet to be quite unpalatable and lacking in variety (Pietzak 2005). Nutritionally, the GFD can be lacking in B-complex vitamins, vitamin D, fibre, iron, folate, magnesium, zinc and calcium as well as being high in fat (Fric, Gabrovska, and Nevoral 2011).

A small proportion of coeliac patients will fail to respond to a GFD despite strict adherence, this is termed refractory coeliac disease (RCD) and carries an increased risk of secondary complications due to failure of the mucosa to heal (Malamut and Cellier 2013). Although other approaches are being investigated, the GFD remains the only available treatment for CD (Alessio Fasano 2009).

1.1.4 Secondary complications

Failure to adhere the GFD can result in numerous secondary complications, many of which are due to malabsorption of dietary nutrients due to absent or stunted villi. Associated disorders include iron-deficiency anaemia, osteoporosis, infertility, recurrent miscarriage, impaired splenic function, neurological disorders, dermatitis herpetiformis (DH) and deficiencies of micronutrients including folate, B vitamins, vitamin D, copper and zinc (Alessio Fasano and Catassi 2012; Rubio-Tapia et al. 2013; Kennedy and Feighery 2000). In many cases it is these secondary complications that lead to the diagnosis of CD (Green, Rostami, and Marsh 2005). A rare complication of CD is enteropathy-associated T-cell lymphoma (EATL). Although its occurrence is rare, it is extremely serious and the prognosis is very poor with a 2-year survival rate of 15%-20% (Cellier et al. 2000; Di Sabatino and Corazza 2009). Patients with very minor or absent symptoms may be less

likely to adhere to the GFD, however gluten ingestion can cause mucosal changes even if the patient is asymptomatic (Kennedy and Feighery 2000; Murch et al. 2013). The range and potential severity of secondary complications that may arise as a result of non-compliance with the GFD emphasises the need to adhere.

1.1.5 Epidemiology

The known prevalence of CD varies between different populations. In Europe it is one of the most common lifelong disorders and has been shown to affect 1% of the population overall. However, the incidence of CD is variable between different European countries with a large study showing prevalence as high as 2.4% in Finland and as low as 0.3% in Germany (Mustalahti et al. 2010). In Ireland, it is estimated that about 1%-1.5% of the population is affected (Dubé et al. 2005). CD was once considered to be rare in North America with the incidence estimated to be as low as 0.03% in not-at-risk people. It is only relatively recently that these figures have been revised with a large study showing a prevalence of 0.94% in the general population, similar to the average rate in Europe (Fedorak, Switzer, and Bridges 2012; Alessio Fasano et al. 2003). Very few studies have been conducted in Australia and New Zealand with those that have been done reporting a prevalence of 0.4% in Australia and 1.2% in New Zealand (Gujral, Freeman, and Thomson 2012).

CD has long been considered to be most common in Caucasians, with the overall prevalence estimated to be at least 1% (Yuan et al. 2013). However, the highest reported frequency is 5.6% in children in the Sahara and studies have shown it to be a common disorder in Egypt, Tunisia, Iran, Iraq, Saudi Arabia, Kuwait and Jordan (Catassi et al. 1999; A Fasano et al. 2008). In Mexico, a prevalence of 2.7% has been reported, in Brazil and Argentina the frequency is estimated to be 0.11% and 0.6% respectively (J F

Ludvigsson and Green 2011). Although CD is considered to be rare in China, a recent report suggests it may be more common than is appreciated, while in India, where it was also considered to be uncommon, a prevalence of 1.04% has recently been reported (Makharia et al. 2011).

The true epidemiology of CD is hard to estimate due to underdiagnosis. Patients who present with the 'classical' symptoms of CD such as diarrhoea, constipation and abdominal distension are relatively likely to be diagnosed. Those that present with secondary symptoms such as anaemia or osteoporosis only are much less likely to be diagnosed while those with silent CD will only be picked up due to screening, often when a close relative is diagnosed. The iceberg model is frequently used to represent this situation, with the tip of the iceberg, which is above the waterline, representing those with overt CD and those with atypical presentation, silent CD or the potential to develop CD represented by the much larger, submerged and invisible portion of the iceberg (Fig. 1.3) (Scanlon and Murray 2011). Screening studies in western countries have shown that for each case of CD which is diagnosed a further 5 to 10 cases remain undiagnosed, although this has been shown to vary in different countries with an estimated 70% of cases diagnosed in Finland compared to only 5% in North America (A Fasano and Catassi 2001; Reilly and Green 2012).

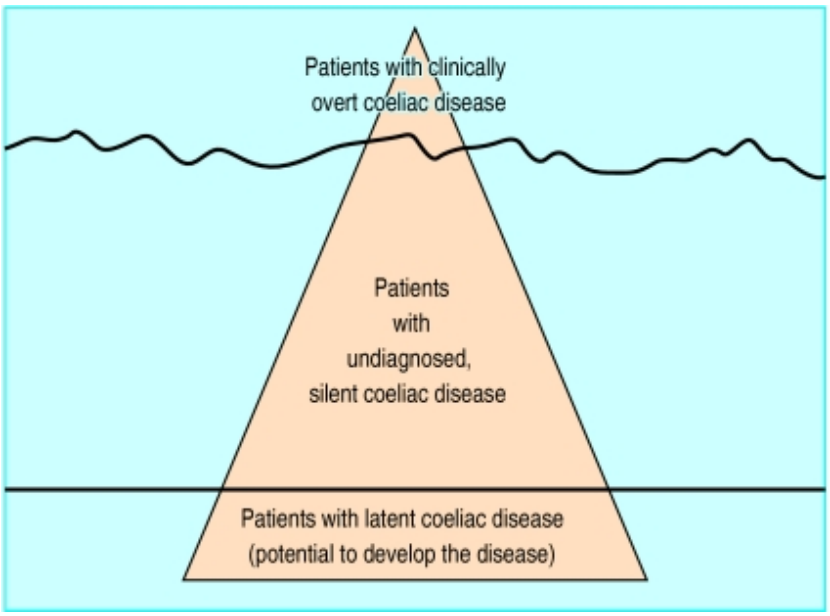


Figure 1.3: The iceberg model of CD. The model depicts the balance between diagnosed coeliac disease (CD) above the waterline versus undiagnosed CD below the waterline. (Image from C Feighery 1999)

1.1.6 Associated conditions

There are several disorders associated with coeliac disease. The most common is type 1 diabetes mellitus with a CD prevalence of about 10% among these patients (Guandalini and Assiri 2014). The frequency of CD is increased to 7% in children with Down syndrome, 6.4% with Turner syndrome and 9.5% with Williams syndrome. Many autoimmune disorders are associated with CD including Sjogren's syndrome, antiphospholipid syndrome, autoimmune thyroid disease, autoimmune liver disease, and Addison's disease. About 30% of adult coeliac patients will have at least one autoimmune disorder versus 3% of the general population (Reilly and Green 2012; Denham and Hill 2013).

1.1.7 Genetics

Coeliac disease is a multifactorial disease and genetics play a pivotal role in its development. The human leukocyte antigen (HLA) class II genes, known as HLA-DQ2 and HLA-DQ8, located in the major histocompatibility complex (MHC) region on chromosome 6p21.3 are the strongest genetic susceptibility factors in coeliac disease. HLA-DQ molecules are responsible for presentation of peptides from outside cells and in coeliac disease HLA-DQ2 and DQ8 are essential for the recognition of gliadin epitopes by antigen presenting cells (APCs). 95% of CD patients are HLA-DQ2 positive and most of the remainder are HLA-DQ8 positive. In a large European study only 0.4% of patients who were diagnosed with CD did not carry HLA-DQ2 or DQ8. This gives HLA typing an extremely high negative predictive value and makes it a valuable tool in ruling out a diagnosis of CD. This is particularly useful in patients with associated conditions such as Down syndrome to avoid the need for regular testing for CD antibodies (Kupfer and Jabri 2012; Monsuur and Wijmenga 2006; Bai et al. 2013; Koning et al. 2005).

HLA-DQ2 and DQ8 are found in 30%-35% of Caucasians but only 2%-5% of carriers will develop CD, emphasising the role of other factors in the disease. Genome-wide association studies (GWAS) have identified 115 genes in non-HLA genetic loci that are associated with increased risk of developing CD. Twenty-eight of these genes are immune-related emphasising the importance of immune dysregulation in CD. Many of these are loci found in other autoimmune diseases and include genes that encode for proteins such as integrins, chemokines, cytokines, proteins associated with signalling pathways and proteins involved in B cell activation, T cell activation, cell adhesion and cell motility. HLA genes are estimated to contribute only 35% of the genetic susceptibility to the development of CD, with the other 65% coming from non-HLA genes; however, the contribution from any

single non-HLA gene is modest. The 4q27 region, which contains IL2 and IL21, has the strongest known non-HLA association with the development of CD but accounts for <1% of genetic risk (Kupfer and Jabri 2012; Scanlon and Murray 2011; Jonas F Ludvigsson et al. 2013).

Familial clustering is stronger in CD than most inflammatory diseases with a multifactorial origin. Nevertheless, studies of monozygotic twins have shown only 75% to 85% concordance, despite an identical genetic make up. This confirms the involvement environmental factors. The timing of an infant's first exposure to gluten has been shown to affect the likelihood of CD development. At present, it is advised that first exposure occurs between 4 and 6 months, preferably in conjunction with breastfeeding. Breastfeeding at the time of first gluten exposure has been found to be the most significant environmental factor in reducing the risk of CD development. Infection may be another important environmental factor in the etiology of CD, potentially increasing intestinal permeability and therefore gluten exposure (Koning et al. 2005; Monsuur and Wijmenga 2006; Scanlon and Murray 2011; Henriksson, Boström, and Wiklund 2013).

1.2 Pathogenesis

1.2.1 Cereals

The earliest evidence of agriculture is in the Middle East and dates back 11,500 years. The development of agriculture lead to a rapid change in the human diet, from that of a hunter-gatherer, surviving mainly on nuts, fruits and some meats, to a diet that was largely dependent on cereals (Gasbarrini et al. 2014; Alessio Fasano 2009). Cereals are members of the Poaceae or grass family, which is divided into the *Pooideae*, *Bambusiodeae*, *Panicoideae* and *Chlorodoideae* subfamilies. Wheat, barley, rye and oats are all members of the *Pooideae* subfamily (Fig. 1.4) (Brown 2012). Cereal grains contain about 70% carbohydrates and 8%-17% proteins. Although the protein content is relatively low when compared to more protein-rich seeds like legumes, cereals are the most important crops in the world, providing over 200 million tonnes of protein for the nutrition of humans and animals annually (Shewry and Halford 2002; Rallabhandi 2012).

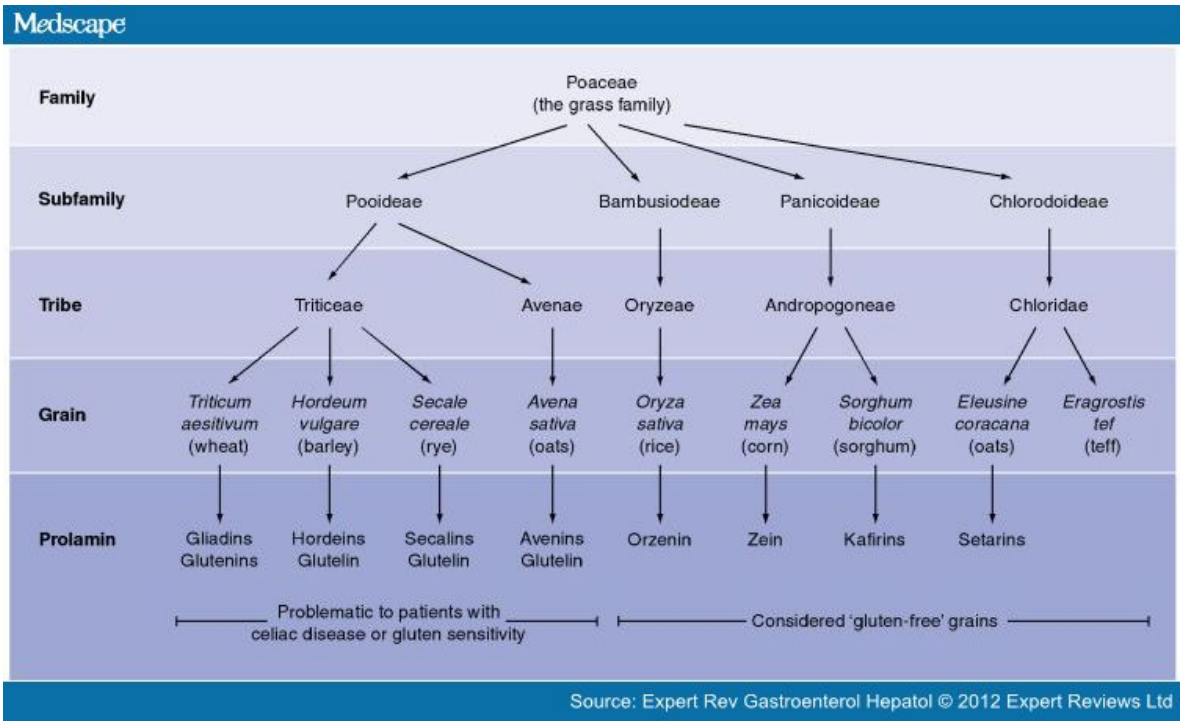


Figure 1.4: Grass family phylogenetic tree. Wheat, barley, rye and oats are all members of the Pooideae subfamily of grasses. (Image from Brown 2012)

1.2.2 Gluten

Wheat grains are composed of three main components which can be separated by milling – the outer husk or bran, the germ or embryo and the endosperm or flour which accounts for 70%-72% of the weight of the grain (Ciclitira, Ellis, and Lundin 2005). Dicke deduced in the 1950s that the injurious element for coeliac patients was contained within the flour (Dicke, Weijers, and Kamer 1953). Further studies elucidated the protein fraction within flour as the causative element (Ellis and Ciclitira 2001). In wheat, gluten accounts for approximately 80% of the protein content (Rallabhandi 2012). Wheat gluten is the rubbery mass left behind when dough is washed to remove starch and water soluble constituents (Wieser 2007). It is one of the most widely used proteins in the food industry, therefore

regular exposure to it usually starts very early in life (Koning 2012; Koning et al. 2005). Its capacity to absorb water, cohesivity, viscosity, elasticity and ability to entrap carbon dioxide make it an essential ingredient in the preparation of a variety of foods including breads, cakes, pasta and noodles. It is also added to food items such as soups, sauces, cooked meats and chips and used as a meat substitute in vegetarian foods (Wieser 2007; Ellis and Ciclitira 2001; Lamacchia et al. 2014; Rallabhandi 2012).

Gluten is a complex mixture of at least 100 proteins. The major components are the alcohol-soluble gliadins and the acid/alkaline soluble glutenins (Koning et al. 2005; Rallabhandi 2012). The gliadins are subdivided into α-, γ-, and ω-gliadins while the glutenins are subdivided into low molecular weight (LMW) and high molecular weight (HMW) glutenins (Koning 2012). Gliadins have long been known to be toxic to coeliac patients and it was thought that they were the sole source of toxicity in wheat; however, there is now some evidence that glutenins may also be involved in the disease process (Rallabhandi 2012; Di Sabatino and Corazza 2009).

Although the term 'gluten' technically refers only to the CD activating elements found in wheat, in the context of CD 'gluten' is used as a general term to describe the trigger of the disease. The prolamins, or alcohol-soluble proteins, found in barley and rye are also capable of activating CD. These are known as hordeins and secalins, respectively. The toxicity of avenins, the equivalent prolamin found in oats, has been the subject of much debate (Kupfer and Jabri 2012; Martucci et al. 2002; Kagnoff 2007; Ellis and Ciclitira 2001).

1.2.3 Epithelial layer

The intestinal epithelium is the largest mucosal surface in the human body, forming an important barrier between the human and the external environment. Gluten has a high glutamine and proline content, making it resistant to complete digestion by gastric, pancreatic and brush border enzymes in the intestine. Under normal conditions, the large, undigested gliadin peptides which gluten is reduced to are unable to cross the epithelium and into the lamina propria (LP); however, in CD a process that is not yet fully understood allows gliadin to make this transition. Possible pathways include transcellular passage, the uptake of peptides by dendritic cell processes that can cross the epithelial cell layer or paracellular passage through a damaged epithelial layer. Paracellular passage via 'leaky' tight junctions between cells is considered most likely. The chemokine receptor CXCR3 has been identified as the receptor that binds gliadin. It is proposed that the binding of gliadin to CXCR3 coincides with the recruitment of the adapter protein MyD88 which triggers epithelial cells to release zonulin, a protein that has been shown to induce tight junction disassembly in intestinal epithelia. The release of zonulin opens up the transepithelial cell junctions and allows gliadin to pass into the LP (Fig. 1.5) (Alessio Fasano and Shea-Donohue 2005; Guandalini and Assiri 2014; Kagnoff 2007; Scanlon and Murray 2011; Rallabhandi 2012; Lammers et al. 2008; Wang et al. 2000).

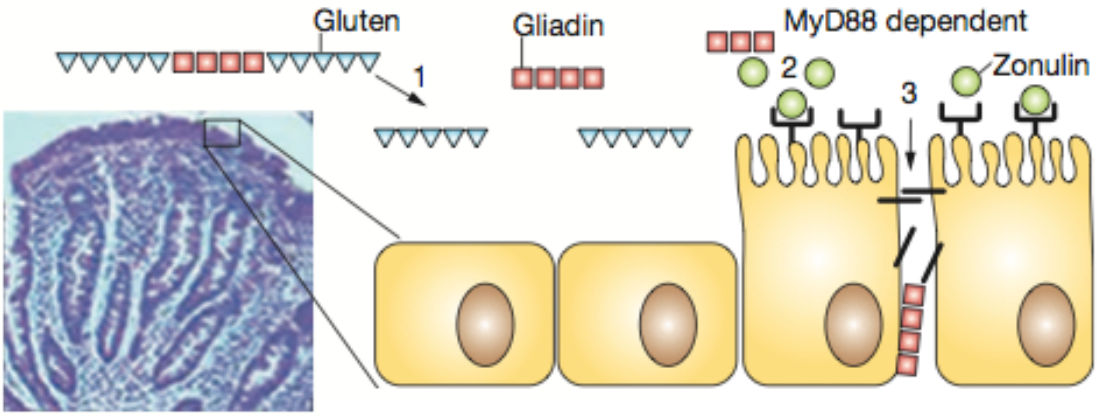


Figure 1.5: Passage of gliadin across the epithelium into the LP. Gluten is incompletely digested leaving large gliadin peptides in the intestinal lumen (1). Gliadin binds to CXCR3 inducing MyD88-dependent release of zonulin (2). Zonulin causes the opening of intercellular tight junctions allowing gliadin to pass into the lamina propria (LP) (3). (Image adapted from Alessio Fasano and Shea-Donohue 2005)

1.2.4 Immune response

Tissue transglutaminase

Tissue transglutaminase is a calcium dependent, ubiquitous enzyme that catalyses the cross-linking of proteins by forming isopeptide bonds between glutamine and lysine residues (Martucci et al. 2002; Kagnoff 2007). It is expressed by almost all cell types although it is usually retained intracellularly in its inactive form and released in response to mechanical or inflammatory stress. tTG is upregulated in apoptosis, angiogenesis, tissue repair, extracellular matrix assembly and cell adhesion. In the small intestine it is expressed just beneath the epithelium, this expression is increased in active CD (Koning et al. 2005; Sollid 2000).

In CD, gliadin crosses the epithelial layer into the LP and immediately encounters tTG. Gliadin has a high content of proline and glutamine residues and an unusually low content of many other amino acids including negatively charged glutamic and aspartic acids (Sollid 2000). Biochemical studies have shown that both DQ2 and DQ8 restricted epitopes have positively charged pockets that preferentially bind peptides containing negatively charged amino acids. Here, tTG plays a pivotal role in the disease process by deamidating gliadin, converting some of the neutral glutamine residues to negatively charged glutamic acid residues (Abadie et al. 2011; McAdam and Sollid 2000; Kagnoff 2007). The resulting deamidated gliadin peptides have greatly increased affinity to the HLA-DQ2/DQ8 molecules on APCs (Guandalini and Assiri 2014).

Adaptive immune response

Coeliac disease is mediated by both the adaptive and the innate immune systems. Once the deamidated gliadin peptides have bound to DQ2 or DQ8 on the APCs they are presented to $CD4^+$ T cells triggering the adaptive response. Activated T cells produce proinflammatory cytokines, some of which drive the activation and clonal expansion of B cells, which in turn produce anti-gliadin and anti-tTG antibodies. The most predominant cytokine produced by the activated T cells is interferon-γ (IFN-γ). This subsequently causes the release of tissue-damaging proteins including matrix metalloproteinases (MMPs). IFN-γ also triggers activation of extracellular tTG, potentially creating a self-upregulating loop for inflammation (Fig. 1.6) (Green and Cellier 2007; Guandalini and Assiri 2014; Alaedini and Green 2005; Di Sabatino and Corazza 2009; McAllister and Kagnoff 2012; Klöck, Diraimondo, and Khosla 2012).

Innate immune response

Gliadin also activates the innate immune system by damaging epithelial cells resulting in increased expression of interleukin-15 (IL-15). This leads to upregulated expression of the natural killer cell marker NKG2D on IELs and its ligand MICA on epithelial cells, resulting in the destruction of epithelial cells (Fig. 1.6) (Green and Cellier 2007; McAllister and Kagnoff 2012).

In both the adaptive and the innate elements of the immune response in CD, gliadin, or one of its equivalent prolamins, is the essential trigger. The complete removal of all sources of these prolamins from the diet usually results in the resolution of all clinical symptoms and repair of mucosal damage (Lamacchia et al. 2014).

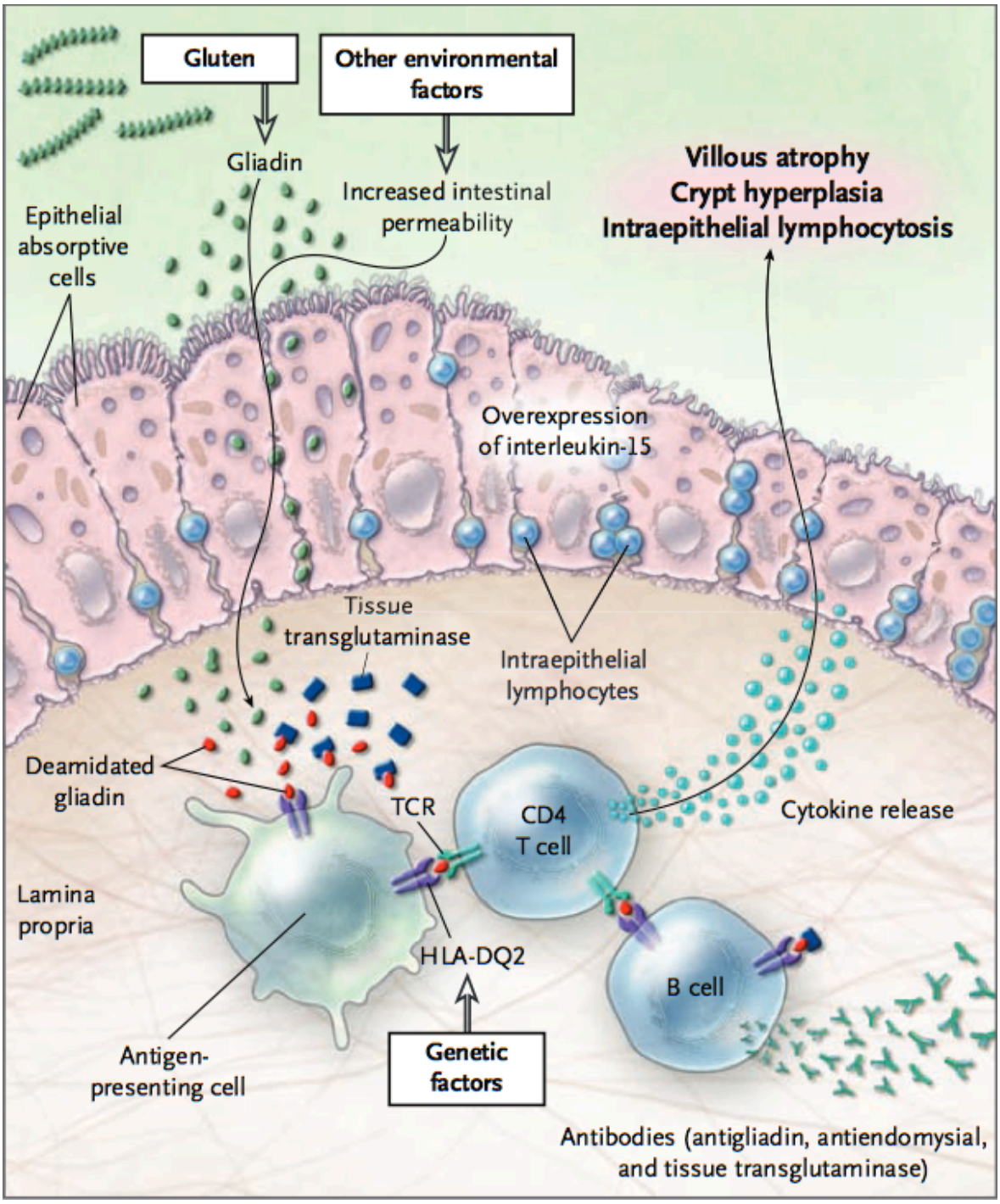
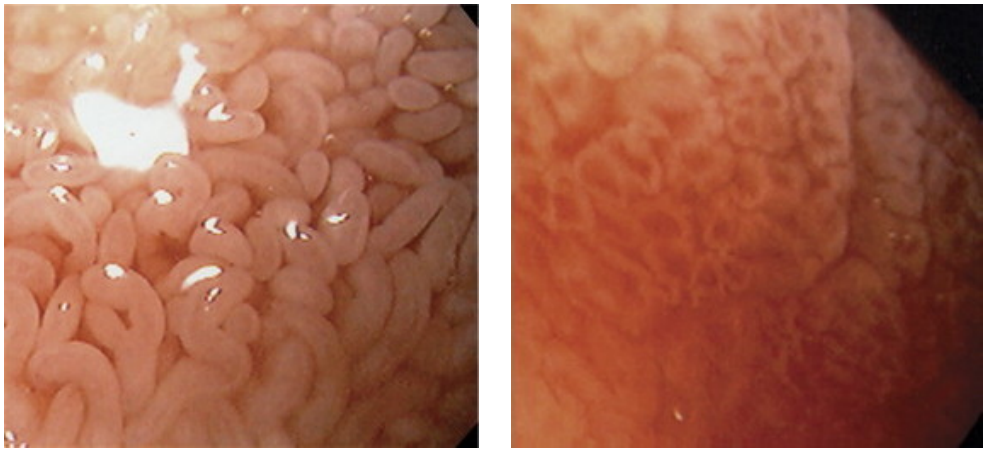


Figure 1.6: Model of CD pathogenesis. Gliadin crosses the epithelial layer into the LP where it is deamidated by tTG. Deamidated gliadin binds to HLA-DQ2/8 on APCs, which subsequently present it to $CD4^+$ T cells triggering cytokine release and activation of B cells and tissue damaging proteins. Gliadin also directly damages epithelial cells causing increased expression of IL-15 resulting in epithelial cell death. (Image adapted from Green and Cellier 2007)

1.2.5 Coeliac lesion

In coeliac disease, the immune response to gluten ultimately results in the transformation of a healthy mucosa, with many villi present, into a flat mucosa, with absence of villi and crypt hyperplasia (Fig. 1.7). The exact mechanism by which this happens remains unknown. Although it has been proven that the rate of epithelial cell proliferation is increased in active CD, it has also been shown that the rate of enterocyte apoptosis is increased and that this correlates with proliferation, thus these two mechanisms appear to balance each other out (Lionetti 2002).



A **B**

Figure 1.7: Magnified views of duodenum during endoscopy. Healthy duodenum with many finger-like villi visible (A) and duodenum with villous atrophy showing flat surface with absence of villi, interspersed with wide circles (B) (Image adapted from Lo et al. 2007)

Cytokines are thought to play an important role in the transformation of the mucosa. The release of IFN-γ by activated T cells leads to the release of tissue-damaging proteins including MMPs. Under normal circumstances, degradation of the extracellular matrix (ECM) by MMPs is balanced by ECM formation by stromal cells. In CD, it is proposed that an imbalance in this process may contribute to the development of villous atrophy. Support for this theory comes from studies demonstrating increased expression of some MMPs in active CD (Sollid 2000; Ciccocioppo et al. 2005). A further study demonstrated a significant increase in expression of MMP-1, -3 and -9 in the mucosa of patients with CD and DH. The increase in expression of all three MMPs was significant, even in patients with only a Marsh grade 1 lesion. Furthermore, the degree of overexpression increased incrementally with each biopsy grade, with the greatest increase in expression of all MMPs seen in mucosae with grade 3 lesions. An increase was also seen in the expression of tissue inhibitor of metalloproteinases (TIMP)-1, possibly indicating an attempt by the usual inhibitory mechanism to limit the degree of damage (Mohamed et al. 2006). Taken together, all of this strongly suggests that MMPs play a very significant role in the tissue remodelling process in CD.

1.3 Oats

1.3.1 History of oats in coeliac disease

Some of the earliest recommendations on the treatment of coeliac disease came from Samuel Gee in 1888. Although he recommended that children with coeliac disease should be given no kind of fruit or vegetables but should be maintained on a diet including raw meat, asses' milk and thinly sliced toasted bread, he did deduce that the best hope of treatment was through diet (Gee 1888). It was not until the 1930s and 1940s that the role of wheat was elucidated when Dicke performed feeding studies, lasting many years, on children with coeliac disease. He observed normalised weight gain and growth on a strict wheat free diet during long-term hospitalisation, followed by periods of stunted growth when the child returned home and the wheat free diet could not be maintained (van Berge-Henegouwen and Mulder 1993). Along with Van de Kamer and Weijers, Dicke concluded that wheat, rye and oats were harmful to coeliac patients while cereals such as maize and rice were safe (Baker and Read 1976).

Over the following 40 years there were very few studies investigating the safety of oats in the GFD. In general, the few studies that did take place were very short term with some lasting only a few days and involved very few patients, sometimes as few as two. Assessment was commonly via measurement of fat excretion although some did look at histology. Results during this time were mixed, with some authors concluding that oats were not harmful to coeliac patients while others reported ill effects from oats exposure (Garsed and Scott 2007; Baker and Read 1976).

1.3.2 Phylogenetics

Oats are phylogenetically more distantly related to wheat than barley and rye. Although all four grains are in the *Pooideae* subfamily of grasses, wheat, barley and rye are all members of the Triticeae tribe while oats belong to the Avenae tribe (Fig. 1.4) (Högberg et al. 2004; Brown 2012). This makes it probable that avenin is less immunogenic than gliadin, hordein and secalin (Koning 2012). Two specific features of avenin offer support to this theory; firstly, avenin contains a lower percentage of proline residues than gliadin, hordein and secalin, and secondly, the positioning of glutamic acid residues on avenin peptides are not optimal for HLA-DQ2 binding (Vader et al. 2002; Vader et al. 2003; Kilmartin et al. 2006). Additionally, avenin represents only 10%-15% of total proteins in oats compared with gliadin in wheat, hordein in barley and secalin in rye which make up 40%-50%, 35%-45% and 30%-50% of total proteins respectively, therefore it is hypothesised that a moderate amount of oats may be tolerable to the majority of coeliac patients (Sadiq Butt et al. 2008).

1.3.3 Benefits of including oats in GFD

The GFD is an extremely challenging diet to maintain due to the presence of gluten in so many foodstuffs (Rubio-Tapia et al. 2013). The establishment of a GFD requires education, dedication, a huge lifestyle change and has been associated with negative emotions including sadness, anger, sacrifice and resignation (Biagetti, Naspi, and Catassi 2013). GF foods are generally expensive and unpalatable. Reported rates for GFD adherence are varied, from less than 50% to over 90%, and appear to depend on a number of factors including age, gender, age at diagnosis and symptoms at diagnosis. Reasons for non-adherence commonly include the poor palatability of GF foods, lack of obvious symptoms following ingestion of gluten and the expense of GF foods (Fric, Gabrovska, and Nevoral 2011; Pietzak 2005).

In addition to all of this, the GFD that is so essential for the mucosal health of coeliac patients is often nutritionally poor, containing high levels of fats and lacking some essential nutrients. Patients strictly adhering to the GFD have been found to have significantly lower intake of nutrients including fibre, iron, folate, calcium, magnesium, B-complex vitamins and vitamin D compared to healthy controls. Furthermore, studies have shown higher than recommended intake of saturated fats and sugars in patients maintaining a strict GFD. The diet has also been shown to be generally imbalanced, with a greater proportion of calories coming from fat and less from carbohydrates. The overall energy intake is often significantly higher also, and this is reflected in the finding of a significantly higher proportion of overweight and obese children among those strictly adhering to the GFD when compared to healthy controls and even when compared to those on a non-strict GFD (Fric, Gabrovska, and Nevoral 2011; Penagini et al. 2013; Kupper 2005).

Oats are a valuable foodstuff, rich in many of the nutrients lacking in the GFD. They are a good source of dietary fibre, iron, magnesium, zinc, thiamine, B-complex vitamins, and protein. Oats also contain β–glucan, a soluble fibre that controls blood glucose and cardiovascular disease. This is of particular significance for coeliac patients due to the association of type 1 diabetes mellitus with CD. Moreover, studies have shown that coeliac patients find the addition of oats to the GFD gives it more variation, better taste, increased satiety and in general increases the palatability of the diet. The fact that oats are generally cheap is also appreciated. These factors mean that oats in the GFD would not only be a valuable source of nutrition but may also help increase levels of compliance with the diet (Sadiq Butt et al. 2008; Fric, Gabrovska, and Nevoral 2011; Penagini et al. 2013; Størsrud, Hulthén, and Lenner 2003).

1.4 Aims of research

Currently there is confusion over the safety of oats in the GFD. This was highlighted in a recent study that showed conflicting advice is being given to CD patients, with 58% of non-expert gastroenterologists recommending that no oats should be consumed in a GFD while 70% of an expert group recommended a moderate consumption of pure oats (Parakkal et al. 2012). Recently issued guidelines from the World Gastroenterology Organisation state that oats are not safe for up to 5% of coeliac patients (Bai et al. 2013). The position taken by the Celiac Sprue Association (CSA) of the United States is that "Oats are not a risk-free choice for those on a gluten-free diet. Products containing oats do not qualify to use the CSA Recognition Seal" (Fric, Gabrovska, and Nevoral 2011). Guidelines issued jointly by the British Society of Paediatric Gastroenterology, Hepatology and Nutrition (BSPGHAN) and Coeliac UK are that about 5% of patients will be sensitive to oats and oats should only be considered in the GFD after clinical resolution has been achieved on a GFD containing no oats. Careful monitoring for any adverse signs or symptoms during reintroduction is also recommended (Murch et al. 2013). The American College of Gastroenterology also strongly advises caution and close monitoring for adverse reaction if introducing oats to the GFD (Rubio-Tapia et al. 2013).

It has been shown that many coeliac patients would like to include oats in their diet but are afraid to do so due to fear of adverse effects or contamination. Some have even introduced oats to their diets and subsequently re-excluded them due to these fears (Markku Peräaho et al. 2004). It is therefore of great clinical significance to determine whether or not oats can be safely included in the GFD.

In light of the conflicting data on the safety of oats in CD, the aim of this thesis was to clarify the effects of oats exposure in celiac patients. In order to achieve this, the objectives of this thesis were:

1. To examine the clinical, serological and histological effects of one year of oats ingestion on a large group of coeliac patients.

2. To adapt the IN Cell Analyzer 1000 system, which is designed for the analysis of cell-based assays, for use with tissue sections and to use this to examine in detail biopsies taken from the coeliac patients prior to and following one year of oats ingestion.

3. To culture biopsies from coeliac patients and control patients in the presence of peptic-tryptic (PT) digests of avenin and gliadin and assess the biopsies and culture supernatants for any mucosal damage or excretion of markers of disease activation.

Chapter 2

Examination of the effects of long-term oats challenge in treated and newly diagnosed coeliac patients

2.1 Introduction

2.1.1 Recent oats feeding studies

In the mid 1990s the first modern studies on the safety of oats in the coeliac diet were published. The approach in these studies was very different to those that preceded them. Janatuinen *et al* recruited 92 patients, some of whom were newly diagnosed with coeliac disease and some who had treated CD, having been on a GFD for at least 12 months and achieved normal or nearly normal villous architecture. 45 patients were randomised to receive oats while the others remained on a standard GFD. Those who were newly diagnosed received oats for 12 months and treated patients received oats for 6 months. Assessment included examination of small bowel morphology, body mass index (BMI), and levels of haemoglobin, serum albumin, iron, calcium and erythrocyte folate. No signs of oat toxicity were found and the authors concluded that a moderate amount of oats could be safely included in the diet of most adult patients with CD (Janatuinen et al. 1995).

Similarly, in the Department of Immunology, St. James's Hospital 10 adult patients with treated CD were recruited to an oats challenge study. These patients added 50 g of oats daily to their GFD for 12 weeks. During this time, the patients were monitored closely with clinical assessments on four occasions including full haematological and biochemical profiles and EMA and AGA testing. Duodenal biopsies were taken prior to and following the oats challenge and, in addition to standard morphological evaluation, IEL counts were performed and enterocyte height was measured. Moreover, patient compliance was recorded in daily diaries and all patients were fully compliant with the study protocol. No evidence of disease activation was seen in any patient in this study (Srinivasan et al. 1996).

Following this, several more studies showed the inclusion of oats in the GFD of both established and newly diagnosed adult and paediatric CD patients to be safe (Hoffenberg et al. 2000; Janatuinen et al. 2002; Srinivasan et al. 2006; Kemppainen et al. 2008; Koskinen et al. 2009; Sey, Parfitt, and Gregor 2011; Kaukinen et al. 2013). However, there have also been reports of adverse reactions to oats, with some investigators suggesting that if oats were taken in sufficient quantity over a longer time period, the cereal might prove to be toxic (Arentz-Hansen et al. 2004; Silano, Di Benedetto, et al. 2007; Silano, Dessì, et al. 2007). In particular, Lundin et al reported a case of a treated patient who, after only a few weeks on pure oats, showed histological deterioration from Marsh Grade 1 to Marsh grade 3A as well as developing dermatitis and positive duodenal biopsy levels of messenger ribonucleic acid (mRNA) for IFN-γ. She resumed a normal GFD without oats and after 12 weeks her mucosa had returned to Marsh grade 1. A subsequent oats challenge had to be terminated prematurely as she became very ill and developed a Marsh grade 3 lesion as well as overt clinical symptoms (Lundin et al. 2003).

A subsequent report described the isolation of avenin-reactive intestinal T cell lines from the above patient and two further patients who developed Marsh grade 3A lesions and clinical symptoms while ingesting oats (Arentz-Hansen et al. 2004). Avenin-reactive T cell lines were also isolated from biopsies of two patients who appeared to tolerate oats. All 5 cell lines were HLA-DQ2 restricted. The authors concluded that avenin-reactive intestinal T cells caused the mucosal inflammation in oats reactive patients. As a consequence of these reports and continuing concerns about the safety of oats, in some recent reviews caution was advised and it was suggested that oats might not be tolerated by all coeliac patients (Haboubi, Taylor, and Jones 2006; Garsed and Scott 2007; Zimmer 2011).

2.1.2 Chapter aims

The aim of this chapter was to perform a detailed examination of a large group of coeliac patients while they included oats in their diet for a significant period of time. To this end we recruited 54 biopsy-proven, diet compliant coeliac patients to take part in a twelve month oats study. During the study, patients recorded their oats intake daily on diary sheets, which were provided, allowing each patient's consumption of oats to be monitored closely. Clinical evaluation of patients was particularly stringent and included maintaining a daily symptom diary with formal clinical examination every three months. Serological responses before oats and during oats ingestion were analysed via EMA testing (Fotoulaki et al. 1999). Anti-tissue transglutaminase antibodies were more recently shown to correlate with mucosal damage and final serum samples were analysed for anti-tTG antibody levels (Vivas et al. 2009). Duodenal biopsy samples taken prior to and following oats ingestion were examined by an experienced histopathologist.

As well as clinical monitoring of patients throughout the trial and routine hematoxylin and eosin (H&E) analysis of pre- and post-oats duodenal biopsies, we also examined for more subtle mucosal changes which could be early indicators of a more gradual damage process. Ki67 identifies a nuclear antigen in all dividing cells and is a widely used marker in the study of cell proliferation (L Maiuri et al. 2001; Srinivasan et al. 2006). Increased levels of $Ki67^{+}$ enterocytes have been shown to be present in areas with villus atrophy but not in normal gut mucosa (L Maiuri et al. 2001). In active coeliac disease, the epithelium is infiltrated by lymphocytes expressing CD3 and CD8. This is one of the first histological changes to occur and hence an appropriate marker to assess when probing for early signs of disease activation (Cellier et al. 2000; Oberhuber 2000). Pre- and post-oats biopsy pairs from 19 patients were stained for these three markers using immunohistochemistry.

2.2 Materials and Methods

2.2.1 Patients

Fifty-four patients with biopsy proven coeliac disease were recruited into the study. Eight patients failed to complete the study and details of the remaining 46 are given in Table 2.1. Thirty-seven of these patients were categorised as having treated coeliac disease, adhering to a gluten free diet for a mean of 10 years. A further 9 patients were more recently diagnosed and six of these started oats within three months of their diagnosis and commencing a gluten free diet.

2.2.2 Ethics

All patients gave informed consent and the St. James's Hospital Ethics Committee approved the study.

2.2.3 Oats

The oats were sourced from Peter Kölln and confirmed to be free from other grains (Srinivasan et al. 1996). Patients were supplied with oats and diary sheets, to log their intake of oats, at each visit. Recipe suggestion sheets were supplied to help patients incorporate oats into their diet. A target oats intake of 50 g per day for one year was planned.

2.2.4 Clinical monitoring

Patients were requested to maintain a symptom diary for the duration of the study and they were clinically assessed on a three monthly basis. Patients were questioned about general

well being and specifically about the development of any of the following symptoms: mouth ulcers, dyspepsia, nausea, vomiting, abdominal discomfort, alteration in bowel habit, diarrhoea, constipation, wind, skin rash or itch. They were also weighed and their body mass index calculated. Blood tests, including haemoglobin level, white cell differential and platelet count were performed on six occasions throughout the study.

2.2.5 Coeliac antibody tests

The endomysial antibody assay was carried out using an indirect immunofluorescence technique as previously described (Daly et al. 2006; C Feighery et al. 1998). Patient serum samples were diluted 1:5 and added to monkey oesophagus slides (Binding Site, England). Fluorescein isothiocyanate (FITC)-labelled rabbit anti-human IgA (DAKO, Denmark) was used to visualise IgA antibodies. A commercial ELISA kit (Celikey, Pharmacia Diagnostics, Sweden) was also used to measure IgA anti-tissue transglutaminase antibodies. As this test was not routinely available at the time of the study, it was performed only on serum samples obtained at study completion.

2.2.6 Routine histology

All patients had a duodenal biopsy taken prior to the start of the oats challenge. The biopsy was taken within a 12-month period prior to commencement of the oats challenge in the majority (78%). In eight patients, with indicators that their disease was in stable remission, a biopsy taken more than one year before the challenge was used as the baseline test. A second biopsy was taken at the end of the study and in 87% of patients this was taken within eight weeks of study completion. Biopsies were sent to Histology for formalin fixation and paraffin embedding. H&E stained duodenal biopsy sections were examined by an experienced histopathologist in a blinded and random manner. Features suggestive of

coeliac disease activity, including villous atrophy, increased intraepithelial lymphocytes, enterocyte nuclear disarray, crypt hyperplasia and increased lamina propria cellular infiltrate, were documented. A Marsh score (Daly et al. 2006; Green, Rostami, and Marsh 2005) was assigned in the following manner: Marsh 0, normal duodenal architecture; Marsh 1, normal duodenal architecture with an increase in intraepithelial lymphocytes; Marsh 2, partial villous atrophy; Marsh 3, total villous atrophy.

	Treated patients	**Recently diagnosed patients**
Numbers	37	9
Sex	14 M, 23 F	2 M, 7 F
Age (years)	Mean 46.7 y (Range 18.7 - 76.8)	Mean 51.2 y (Range 28.3 - 65.1)
Duration of GFD	Mean 9.7 y (Range 1 – 40.1)	Mean 0.4 y (Range 0.1 – 0.9)
EMA at commencement	34 neg, 1 w.pos, 2 pos	3 neg, 2 w.pos, 4 pos
Histology (Marsh Grade)	30 (0,1), 7 (2,3)	2 (1), 7 (2,3)

Table 2.1: Profile of 46 biopsy-proven coeliac patients at commencement of study. M = male; F = female; y = years GFD = gluten free diet; EMA = endomysial antibody; neg = negative; w.pos = weak positive; pos = positive.

2.2.7 Immunohistochemistry

Coating of slides

Prior to use, slides were placed in a slide rack and submerged in a bath of Poly-L-Lysine (Sigma-Aldrich, Ireland) for 10 minutes (min), drained and left to dry at room temperature (RT) overnight. This coats the slides in an adhesive layer and prevents the loss of tissue sections during immunohistochemical staining.

Cutting of biopsies

Prior to cutting, blocks were submerged in Mollifex (VWR, Ireland) for 5 min followed by a few minutes on ice. Sections were cut on a Microm microtome at 4-μm-thickness. They were then transferred to a cold water bath containing a dash of ethanol followed by a 50°C water bath. Labelled, coated slides were used to remove sections from the hot water bath and left to dry on a 50°C hotplate. Slides were then transferred to a slide rack and dried overnight in a 65°C oven.

Staining procedure

Subsequent to an initial period of optimisation, staining was carried out as follows. Sections were dewaxed and rehydrated in two baths of xylene (BDH) followed by two baths of 100% industrial methylated spirit (IMS) (BDH) and one bath each of 95% IMS, distilled water (dH_2O) and tris buffered saline (TBS). Antigen retrieval was performed by placing slides in 0.01M citrate buffer (CB), heating to boiling point in a microwave for 25 min, leaving to cool for 20 min and placing under running tap water (H_2O) for 5 min. Slides were next immersed in 1.5% H_2O_2 in methanol (BDH) for 10 min to block

endogenous peroxidase activity and washed in two baths of TBS. Staining was then performed using the Vectastain Elite Universal ABC Kit (Vector Labs, USA). Sections were incubated with blocking serum for 20 min at RT in a humidity chamber, followed by anti-Ki-67, anti-CD3 or anti-CD8 antibodies (all from Novocastra Labs, UK) for 1 hour (h) at RT in a humidity chamber, further details can be found in Table 2.2. Slides were washed 3 times in TBS/Tween and incubated with biotinylated universal secondary antibody (2° Ab), washed as before and incubated with Vectastain Elite ABC Reagent, both for 30 min at RT in a humidity chamber. Three further washes in TBS/Tween were performed followed by the addition of diaminobenzidine (DAB) substrate solution (Sigma-Aldrich, Ireland) for 10 min at RT and two washes in dH_2O. Slides were then counterstained by being submerged in haematoxylin (Sigma-Aldrich, Ireland) for 1 min, rinsed in tepid running tap H_2O for 1 min and put into cold dH_2O to stop development. Dehydration was carried out using graded alcohols in reverse order to the rehydration process, i.e. one bath of 95% IMS, two baths of 100% IMS and finally two baths of xylene. Slides were mounted using DPX mountant for microscopy (BDH) and a coverslip, left to dry at RT overnight and stored at RT.

Primary Antibodies used in Immunohistopathology							
Antibody	**Clonality**	**Source**	**Ag Retrieval**	**Dilution**	**Stock conc.**	**Incubation**	**Staining**
Ki67	p	Novocastra	Citrate Buffer	1/500 1/1000	-	I h, 25°C	IHC
CD3	m	Novocastra	Citrate Buffer	1/40 1/80	112.5 mg/L	I h, 25°C	IHC
CD8	m	Novocastra	Citrate Buffer	1/40 1/80	28.8 mg/L	I h, 25°C	IHC

Table 2.2: Details of antibodies used in detailed histological analysis of 19 pre- and post-oat biopsies. Ag = antigen; conc. = concentration; p = polyclonal; m = monoclonal; mg/L = milligram per litre; °C = degrees Celsius; IHC = immunohistochemistry.

Assessment

Positive and negative control slides were included in every staining run. Positive control slides containing sections of human tonsil were used to indicate correctly prepared tissues and proper staining technique. Positive staining of the lymphoid nodules indicated a successful staining run. Negative control slides, with duodenal tissue sections that were incubated with PBS instead of the primary antibody, were used to check that staining was specific.

The patient slides were coded and assessed in a blinded manner. In the case of anti-Ki-67 stained sections, the number of positively and negatively stained epithelial cells within 5 fields were counted and the percentage of positive cells calculated; for CD3 and CD8 the number of positively stained cells within 500 cells of the surface epithelial layer were counted and the percentage calculated. All slides were assessed at 40X magnification on an Olympus BX41 microscope; a Miller Ocular eyepiece graticule was used for anti-Ki67 counts.

2.2.8 Statistics

GraphPad Prism was used for statistical analysis of results. The student's paired two-tailed t test was used to compare the percentages of cells positively stained for Ki67, CD3 or CD8 in pre- and post-oats biopsies. P values <0.05 were considered statistically significant.

2.3 Results

2.3.1 Patients who dropped out

Forty-six of the 54 patients who initially enrolled, completed the study. The reasons for withdrawal in the eight patients were: failure to adhere to the study protocol (2 patients); pain post OGD (1 patient); emigration (2 patients); the development of breast cancer (1 patient); no reason given (2 patients). None of these eight patients reported adverse reactions to the oats supplement.

2.3.2 Oats consumption

The 46 patients who completed the study consumed a mean of 286 g oats per week (range 97–513 g) for a median duration of 48 weeks (range 33-58).

2.3.3 Clinical results

Several patients reported mild symptoms such as flatus and abdominal distension with oats addition to their diet but in none were they considered particularly troublesome. Many patients reported improvements in bowel function. No significant change was seen in weights of individuals or the group as a whole while on oats. Weight change for the group as a whole was +0.05 kg (range -12.7 - +7.7 kg). Similarly, for body mass indices no significant change was observed within individuals or for the group, with an overall body mass index change of +0.08 kg/m^2 (range -2.5 - +2.5 kg/m^2). Likewise, haemoglobin level, white cell differential and platelet count remained normal for the duration of the oats challenge.

2.3.4 Coeliac antibody serology

At the commencement of the study, 37 patients had a negative endomysial antibody test (Table 2.1, Fig. 2.1). Repeat testing throughout the study confirmed that almost all patients remained antibody negative with 89% of the total 275 tests in the study giving negative results. Only 16 samples were endomysial antibody positive and these were taken from four newly diagnosed coeliac patients and from two treated patients, one with known poor dietary compliance and one who had not achieved normal histology by the study commencement. At the completion of oats challenge, 44 of the 46 patients had a negative endomysial antibody test (Table 2.3, Fig. 2.1). Tissue transglutaminase autoantibody tests mirrored endomysial antibody results and the same 44 subjects had a negative test (<5 arbitrary units, AU). The two remaining patients were amongst the recently diagnosed sub-group. In one the final EMA test was weakly positive and the anti-tTG level was minimally elevated (5.1 AU) but the histological lesion had improved. The second patient had a positive EMA test, a moderate elevation of anti-tTG antibodies (16.8 AU) and her Marsh grade 3 histology had not changed throughout the study.

2.3.5 Routine histology

Following oats challenge, suitable biopsy material was available for routine histological analysis on 44 of the 46 patients (Table 2.3, Fig. 2.1). In 42 patients (95.5%) the histological lesion had either improved or not changed. Only two patients showed histological disimprovement while ingesting oats (Table 2.3, Fig. 2.1). One of these was a treated patient with known poor dietary compliance as mentioned above. The level of disimprovement in this patient was marginal, from Marsh grade 1 to Marsh grade 2.

The second patient with disimproved histology was also a treated patient and had an interval of 18 months between the pre-oats biopsy and commencement of oats challenge.

While on oats she suffered no significant symptoms, her weight and body mass index were stable and she remained seronegative throughout. The second biopsy was taken after 48 weeks on oats and showed histological disimprovement to a Marsh grade 2 lesion. The patient elected to remain on oats for a further eight months and again suffered no significant symptoms and her weight, body mass index and serology remained stable. A third biopsy was taken after 84 weeks on oats and showed marked histological improvement, with a Marsh grade 1 lesion.

	Treated patients (n = 37)	**Recently Diagnosed patients (n = 9)**
EMA	37 neg	7 neg; 1 pos; 1 w.pos^
tTG	37 neg	7 neg; 2 pos (5.1 and 16.6 AU)^
Histology	33 improved or no change; 2 disimproved; 2 nd	6 improved; 3 no change

Table 2.3: Summary of serology and histology findings after oats challenge. ^In one of these patients, histology had improved; in the second, the Marsh 3 lesion was unchanged. EMA = endomysial antibody; tTG = tissue transglutaminase; n = number of patients; neg = negative; pos = positive; w.pos = weak positive; nd = not done, biopsy unsuitable.

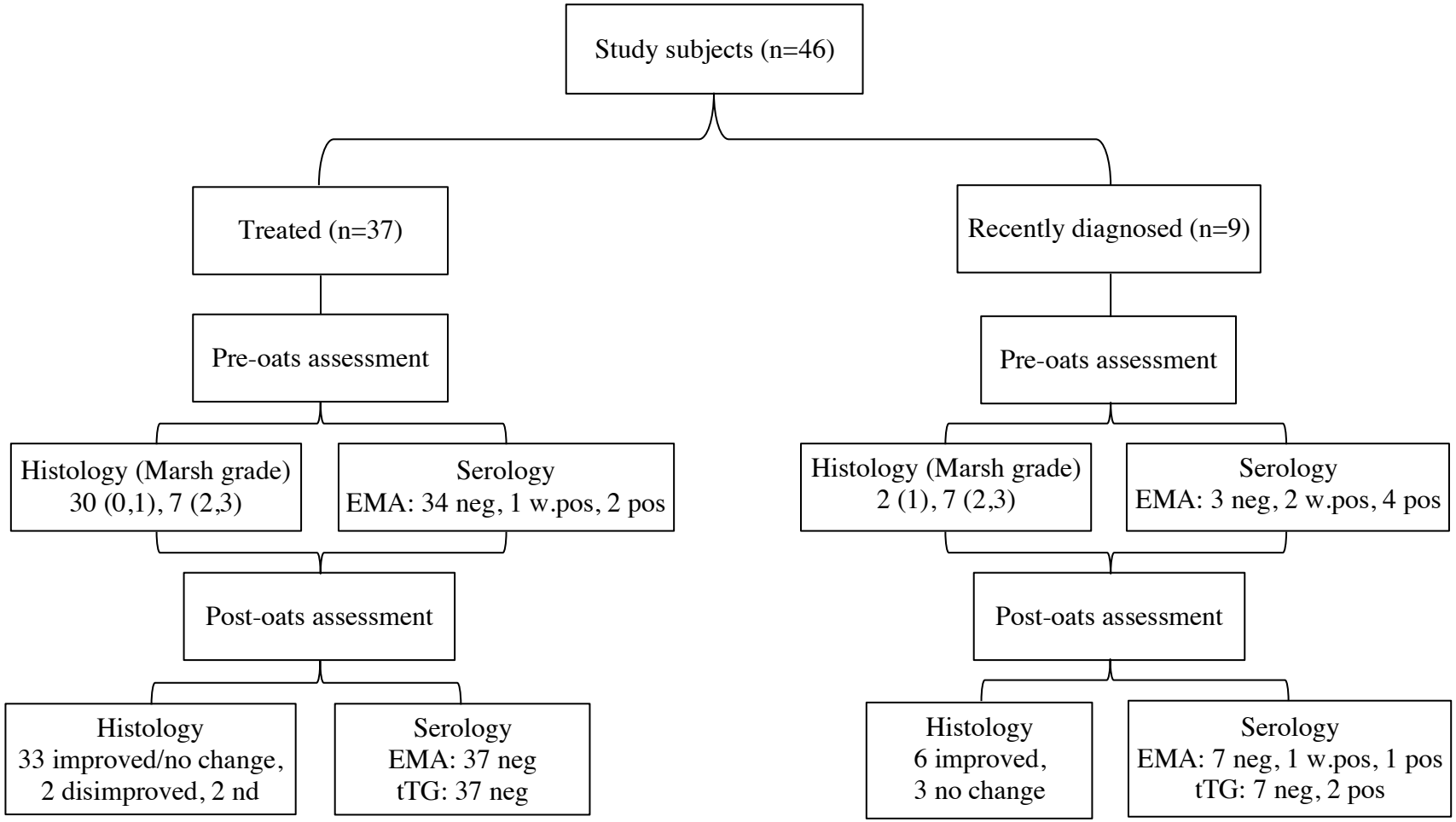


Figure 2.1: Flow chart of serology and routine histology changes during oats challenge study. n = number of patients; EMA = endomysial antibody; neg = negative; w.pos = weak positive; pos = positive; nd = not done, biopsy unsuitable; tTG = tissue transglutaminase.

2.3.6 Immunohistochemistry

A large number of patient biopsies were unsuitable for immunohistochemistry studies. In some cases this was due to poor quality of the biopsy or because all of the biopsy had been used. In many cases the biopsy had been stored in Bouins instead of formalin. Although attempts were made to find a way to stain these biopsies, it proved impossible.

Matched pre- and post-oats biopsy samples from 19 patients were stained for expression of Ki67, CD3 and CD8. The addition of daily oats caused no significant change in the extent of staining for any of these markers (Table 2.4). Positive and negative control slides were included in each run and in all cases showed appropriate staining patterns (Fig. 2.2).

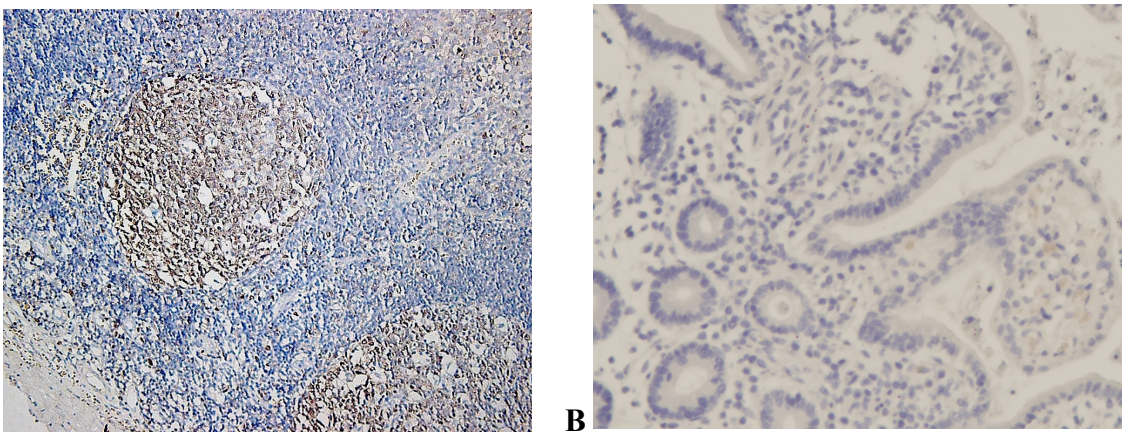
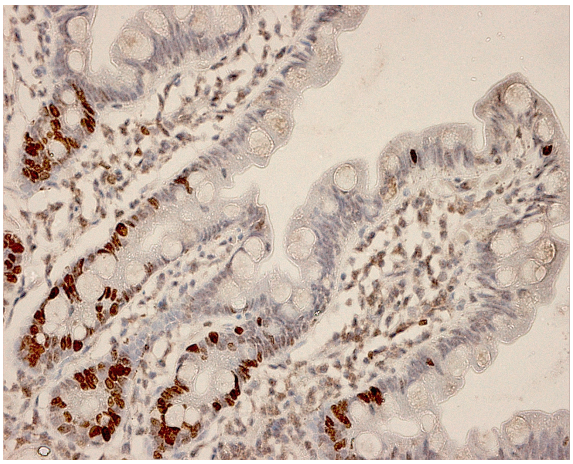


Figure 2.2: Control sections. Representative positive control section of human tonsil showing positive staining (brown) in the lymphoid nodules (A), and representative negative control section of duodenum with absence of positive staining (B).

Ki67

In the case of Ki67, although some patients showed change, the group as a whole remained stable and showed no significant difference in positive staining pre-oats versus post-oats. 53% of enterocytes stained positively before oats and 56% at study end [95% Confidence interval (CI) -5.56 to 11.57] (Fig. 2.3). There was no correlation between any changes seen in Ki67 expression and routine histology results.

A



B

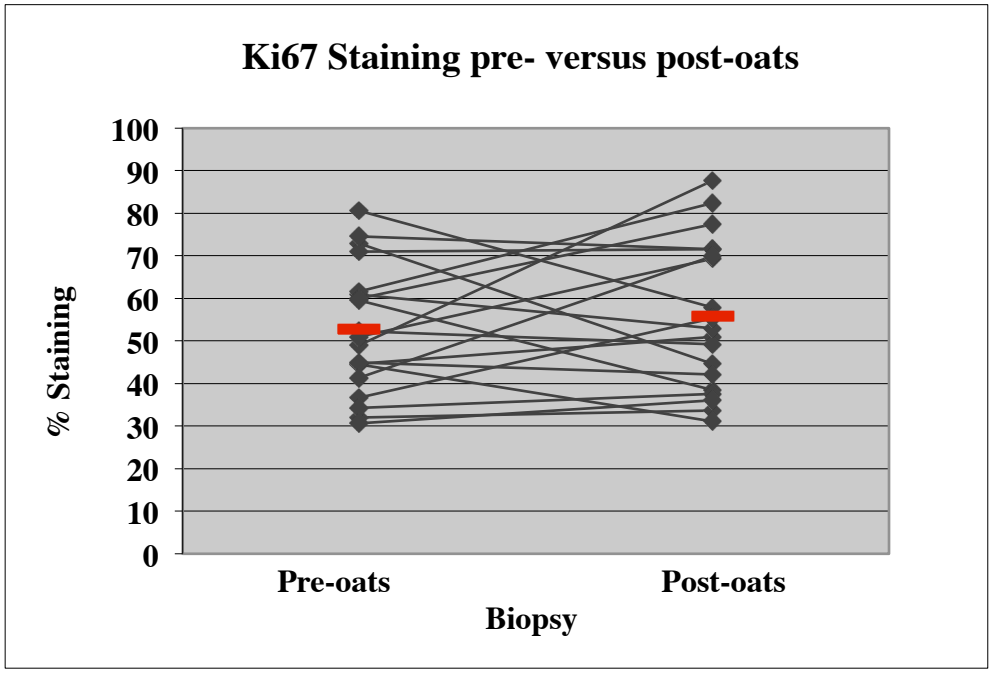
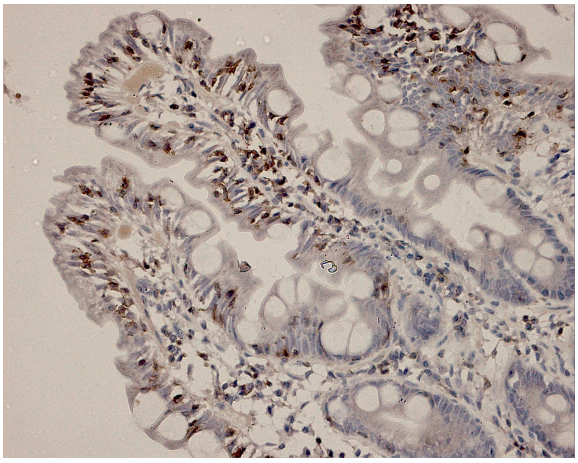


Figure 2.3: Ki67 staining. Ki67 expression (brown) in cryptal epithelial cells of coeliac duodenum (A), individual (black diamond) and mean (red dash) measures of percentage Ki67 staining in coeliac biopsy pairs pre- versus post-oats (B).

CD3

With anti-CD3 staining, although some changes were seen within individual patients, again the group as a whole remained unchanged and no significant difference was seen in staining pre- versus post-oats. The percentage of cells staining with anti-CD3 was 24% before oats and 23% after oats challenge [95% CI -5.89 to 4.25] (Fig. 2.4).

A



B

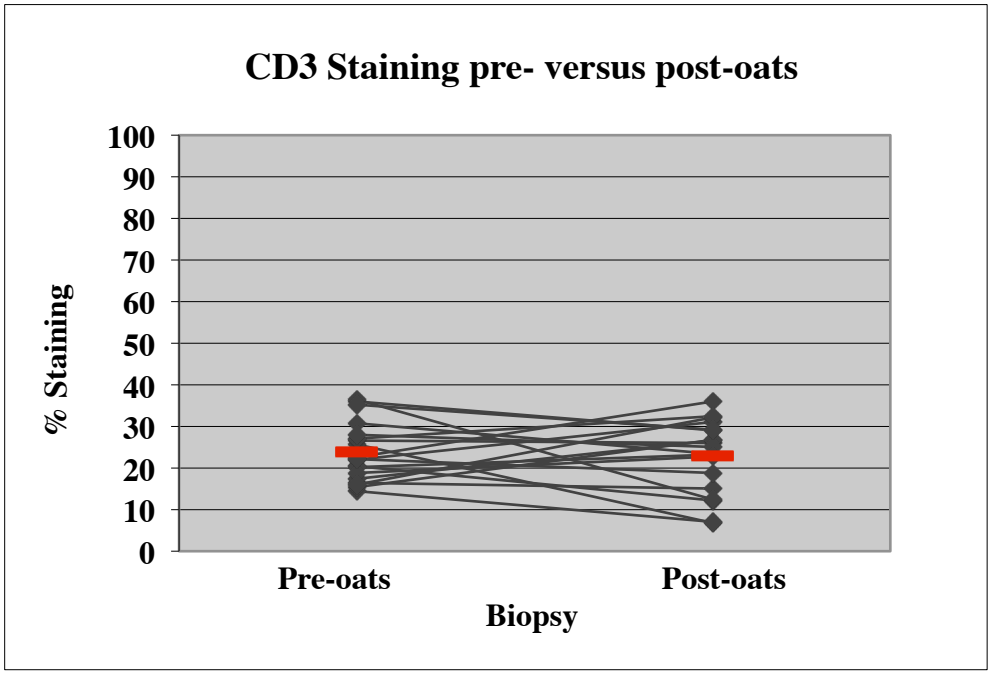
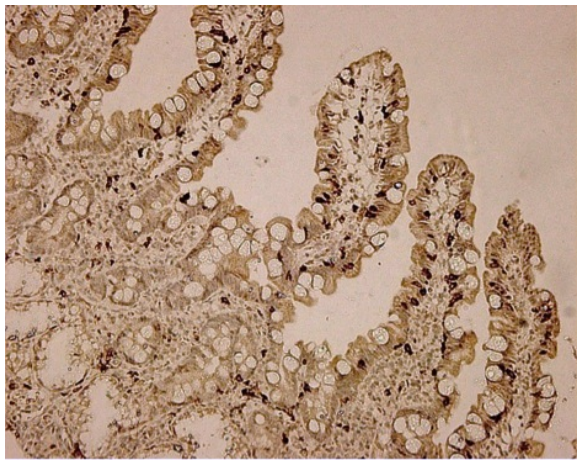


Figure 2.4: CD3 staining. CD3 expression (brown) in the epithelial layer of coeliac duodenum (A), individual (black diamond) and mean (red dash) measures of percentage CD3 staining in coeliac biopsy pairs pre- versus post-oats (B).

CD8

Similarly with anti-CD8 antibody, some patients showed slight change but the group as a whole showed no significant difference in positive staining pre- versus post-oats. The percentage of cells with positive staining before oats was 31% and 28% following oats ingestion [95% CI -10.01 to 4.49] (Fig. 2.5). Patterns of expression of CD3 and CD8 were similar, however, there was no correlation with patterns of Ki67 expression or with routine histology results.

A



B

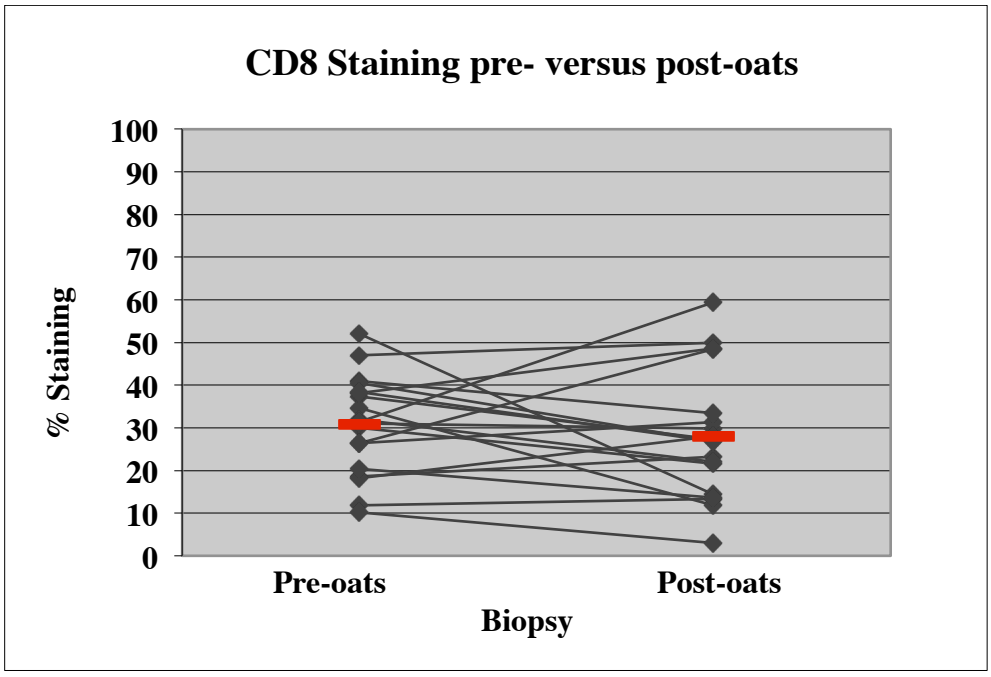


Figure 2.5: CD8 staining. CD8 expression (brown) in the epithelial layer of coeliac duodenum (A), individual (black diamond) and mean (red dash) measures of percentage CD8 staining in coeliac biopsy pairs pre- versus post-oats (B).

	Pre-oats (n = 19)	Post-oats (n = 19)	p-value
Ki67 %	53 ± 15%	56 ± 18%	0.47
CD3 %	24 ± 7%	23 ± 9%	0.74
CD8 %	31 ± 11%	28 ± 15%	0.43

Table 2.4: Results of detailed histological analysis. Pre- and post-oats biopsy samples for 19 patients were stained by immunohistochemistry for expression of Ki67, CD3 and CD8. Results are expressed as the percentage of cells expressing each marker in the cell population analysed ± the standard deviation. n = number of patients.

2.4 Discussion

In this study, 46 patients with CD, adhering to a gluten-free diet, added oats to their daily diet for one year and none experienced significant adverse effects. Detailed clinical, serological and histological evaluations were performed throughout the study and there were no indications of oats ingestion causing disease activation.

2.4.1 Patients who dropped out

As the possibility exists that cases of oat intolerance could be masked by unwell patients dropping out of the study, care was taken to ensure that any patient who dropped out did so for reasons other than oat intolerance. In total 8 patients withdrew before completion of this study. Two patients emigrated after only a few weeks of oats ingestion. One patient was diagnosed with breast cancer and had to be withdrawn. One patient suffered from abdominal pain following the pre-oats OGD procedure and decided to leave the study as a result. Two patients were not compliant with the GFD. Only two patients left without giving a reason. Both had negative serology throughout their participation in the study. Both also had good oats intake and reported virtually no symptoms. There was no indication that either patient left the study due to any ill effects of oats ingestion.

2.4.2 Symptoms

Although many patients reported mild symptoms such as flatus and abdominal distension with inclusion of oats in their diet, this was not a cause for concern. Such symptoms are well documented to be associated with an increase in fibre intake and even non-coeliac individuals have been shown to develop such symptoms with sudden introduction of oats to their diet (Holm et al. 2006; Kaukinen et al. 2013; M Peräaho et al. 2004). Furthermore,

many patients reported improvements in bowel function, a very positive effect of the addition of a source of fibre to the often very fibre-poor GFD (Penagini et al. 2013; Kaukinen et al. 2013).

2.4.3 Serology

EMA serology has been shown to reflect the presence of gluten in the diet of CD patients. It is generally accepted that IgA EMA antibodies are detected in about 90% of untreated coeliac patients, with some reporting this figure to be as high as 100% (Reddick, Crowell, and Fu 2006; Conleth Feighery, Conlon, and Jackson 2006; da Rosa Utiyama et al. 2001). As with all other coeliac-specific antibodies, levels of EMA antibodies gradually decline upon commencement of a GFD, usually disappearing altogether, and are elevated once more during gluten challenge (Midhagen et al. 2004; Bürgin-Wolff et al. 2002). These features are extremely useful in the surveillance of mucosal healing and GFD adherence.

Of the 275 EMA tests performed over the course of this study, 89% were negative, confirming that participants were adhering to their GFD. The fact that these patients were adding oats to their GFD during this time of complete seronegativity supports the hypothesis that the avenin prolamin does not provide a gluten stimulus. As would be expected, a number of newly diagnosed patients were EMA positive during the study and were the source of the majority of the 16 positive and 15 weak positive results. One patient still had positive serology at the end of the study; this was a 45-year-old woman who had been diagnosed only one month prior to the commencement of the study. A second newly diagnosed patient was weakly EMA positive at the completion of the study; however, this represented an improvement from earlier EMA positivity and in addition her histology had improved. These results are not surprising as the elimination of all sources of gluten from the diet is a difficult task requiring the changing of lifetime habits, which some patients

will find harder than others (L. A. Harris et al. 2012). Also, after the establishment of a GFD, clinical improvement can take a long time in adults (Bardella et al. 2007). The other 7 newly diagnosed patients were EMA negative at the study end, showing that inclusion of oats in their GFD did not prevent them from improving serologically. Only two treated patients, one with known poor dietary compliance and one who had not achieved normal histology by the study commencement, had positive EMA tests early in the study and both were negative at the study end. Two further treated patients had weakly positive EMA results early on; one of these admitted dietary lapse and again both were negative by study end.

44 out of 46 patients (96%) were EMA negative at the end of the study. tTG serology for these patients correlated completely with all 44 patients having negative tTG results. This follows patterns seen in previously reported studies where the addition of oats to the gluten free diet did not cause an increase in EMA or tTG serology (Kemppainen et al. 2008; Holm et al. 2006). The two newly diagnosed patients who had a positive and weak positive EMA at study completion were also the only two patients to have a positive tTG result, demonstrating excellent correlation between tTG and EMA.

2.4.4 Routine histology

Although the majority of coeliac patients do show symptomatic and serological improvement upon commencement of a GFD, the histological lesion can be much slower to heal and, especially in adult patients, may never completely recover (Bai et al. 2013). In addition to this, individuals with silent coeliac disease show no overt symptoms of the disease but may have a severely damaged mucosa (Bardella et al. 2007). Hence, the value of histology in patient monitoring cannot be overestimated. Of the 44 patients in our study

who had suitable biopsies, 42 either showed no change or improved histologically while on oats.

In the two remaining subjects, the inflammatory lesion had disimproved. Both were from the treated group. One of these patients subsequently admitted that she was not fully compliant with the GFD. Interestingly, this patient did not report suffering from any symptoms at any time throughout the study. This is in agreement with previous studies which have shown that patients who do not suffer symptoms after dietary lapses are significantly less likely to adhere to the GFD (Pietzak 2005). Nonetheless, her mucosa deteriorated during this time, emphasising the need for dietary compliance even in the absence of symptoms.

The second patient with disimproved histology had a normal biopsy taken 18 months prior to the start of the study. Although she suffered virtually no symptoms with the addition of oats to her diet and remained seronegative throughout, her follow up biopsy showed significant deterioration. The patient chose to remain on oats and a third biopsy taken 8 months later showed that her histology had dramatically improved. It is possible that in the 18 months between the pre-oats biopsy and commencement of oats ingestion there had already been a deterioration in her histology status. Certainly, inclusion of oats in her diet did not cause any significant symptoms or affect her serology. Also, as the patient chose to remain on oats following her second biopsy, she obviously did not associate their addition to her diet with any ill effects. The fact that her histology improved while she remained on oats offers support to the hypothesis that her histological status had already disimproved by the time of commencement of oats.

Six of the 9 newly diagnosed patients who completed the study showed a histological improvement by the end. Four of these 6 patients had a Marsh grade 3 biopsy at study commencement, showing that, even in the case of severe lesions, histological improvement can be achieved on an oats containing GFD. In the remaining three newly diagnosed patients no marked alteration in the features of the biopsy were noted. These were all mature adults (45.8, 49.9 and 59.9 years old), one of whom admitted gluten ingestion and another who failed to achieve seronegativity by the end of the study as discussed previously. These three results are unsurprising as the adult coeliac lesion can be very slow to heal (Monsuur and Wijmenga 2006; Bai et al. 2013). None of the newly diagnosed patients had disimproved histologically at the study end.

2.4.5 Immunohistochemistry

It is possible that any adverse histological changes in response to oats ingestion may be very slow to develop. In order to investigate the possibility of subtle mucosal changes occurring, more detailed analysis was performed on samples from 19 subjects. Although it has been shown that the number of Ki67 positive enterocytes is significantly increased in active CD (L Maiuri et al. 2001; Shalimar et al. 2013), this antigen has received little attention in the investigation of oat tolerance in coeliac patients. Enterocyte apoptosis is also known to be greatly increased in untreated CD and to correlate closely with proliferation (Moss et al. 1996). Consequently, the measurement of Ki67 expression provides not only a measurement of proliferation but also an indication of the level of apoptosis. Furthermore, Ki67 is significantly elevated in patients with only minimal morphological change compared to controls (Settakorn and Leong 2004; Mohamed et al. 2008). In this study, Ki67 expression was examined in pre- and post-oats biopsy pairs from 19 patients and no significant changes were seen, indicating that there was no upregulation

of either proliferation or apoptosis in response to oats. This is in agreement with findings from a previous 3 month oats study in our department (Srinivasan et al. 2006).

An infiltration of IELs provides the earliest and most sensitive mucosal evidence of coeliac disease activation and is therefore the single most important histological feature to probe for, when investigating the possibility of subtle mucosal changes (Oberhuber 2000). The majority of IELs are $CD3^+$ and 70% - 90% are $CD8^+$ (Oberhuber, Granditsch, and Vogelsang 1999), thus these are important markers of IEL infiltration. Settakorn et al found significantly increased numbers of CD3 and CD8 positive IELs in histology-positive biopsies both with and without positive coeliac serology, further demonstrating both the sensitivity of these markers and the importance of histology in diagnosis and patient monitoring (Settakorn and Leong 2004). Increased IEL infiltration following oats addition to the GFD has been reported (M Peräaho et al. 2004); however, our study showed no increase in expression of CD3 or CD8, indicating a lack of disease activation. Similar results have been seen in a number of studies including one by Holm et al who noted that IEL numbers were increased significantly in all CD patients taking part in a gluten challenge but not in those who were challenged with oats (Holm et al. 2006; Kemppainen et al. 2007; Högberg et al. 2004; Srinivasan et al. 1996; Kaukinen et al. 2013). The results of these immunohistochemical studies provide further confirmation of the findings from routine histology.

2.4.6 Comparison with other studies

Our results concur with those of Kemppainen et al, who assessed symptoms, histological status and EMA levels in 32 patients after one year of oats ingestion and concluded that large amounts of pure oats are not harmful to adults with treated CD (Kemppainen et al. 2008). Similarly, Holm et al recruited paediatric patients with both treated and newly

diagnosed CD to a long term oats study, monitored histologically, serologically and clinically, and noted complete recovery in newly diagnosed patients while no effect was seen on established CD patients (Holm et al. 2006). Likewise, a number of other studies have found oats to be well tolerated by both adult and paediatric CD populations (Högberg et al. 2004; Janatuinen et al. 2002; Hoffenberg et al. 2000).

However, our study has some strengths that are lacking elsewhere. The vast majority of studies that included newly diagnosed patients have been conducted on children (Hoffenberg et al. 2000; Högberg et al. 2004; Holm et al. 2006). The adult lesion is known to be much slower to heal following commencement of a GFD and in some cases never heal (Bai et al. 2013). Findings from paediatric studies cannot be automatically inferred to apply to adults. Janatuinen et al included newly diagnosed adult patients in their oats study; however, they excluded patients with severe CD (Janatuinen et al. 1995). In our study, five of the 9 newly diagnosed patients had a Marsh grade 3 biopsy at study commencement and four of these showed histological improvement at the study end. The remaining patient had failed to achieve seronegativity by the study end, as discussed previously, indicating that she had not yet managed to eliminate all sources of gluten from her diet. These results indicate that, even in patients with a severe lesion at diagnosis, mucosal recovery will not be hindered by the presence of pure oats in the diet.

Patient monitoring in our study was particularly stringent, including the use of diary sheets to record oats consumption daily throughout the entire study. This has rarely been done in the past with most studies recording oat intake for only 4 days at a time at particular time points (Kemppainen et al. 2008; Holm et al. 2006; M Peräaho et al. 2004). Two other small studies have reported using daily food diaries for the entire duration of their study, one was a short study carried out in our department involving 10 treated adult patients and the other

involved 10 paediatric patients (Srinivasan et al. 1996; Hoffenberg et al. 2000). Daily recording of oats intake is likely to improve compliance and therefore strengthens the value of our study. In addition, only the previous short study in our department has so far reported measuring Ki67 expression to assess proliferation levels. Despite Ki67 being widely used in the study of proliferation (Lindboe and Torp 2002), no other large oats study has reported its use.

2.4.7 Conclusion

This was a very comprehensive study of a large group of newly diagnosed and treated CD patients as they included oats in their GFD for a significant length of time. A wide range of parameters was monitored including clinical symptoms, serology, routine histology and specific histological markers. Our results showed not only that addition of oats to the GFD did not cause any clinical evidence of disease activation but also that serological and histological improvement occur in the presence of oats, even in newly diagnosed patients with a severe lesion. These results indicate that a moderate amount of pure oats is well tolerated by both treated and newly diagnosed coeliac patients.

Chapter 3

Adaption of the IN Cell Analyzer 1000 system for the analysis of coeliac biopsy tissue

3.1 Introduction

3.1.1 Smooth muscle α-actin

Fibroblasts are spindle-shaped cells found in most types of tissues and characterized by their shape and the expression of vimentin but not smooth muscle α-actin (SM α-actin) or desmin. Under normal circumstances, they play a role in regulating the turnover of the extra cellular matrix. In times of tissue injury, fibroblasts differentiate into myofibroblasts, which participate in wound healing. A defining feature of this transformation is the expression of SM α-actin (Li and Wang 2011).

3.1.2 Co-localisation of tTG and SM α-actin

tTG is known to be expressed in the muscularis mucosa and pericryptal fibroblasts of normal mucosa. This expression is marginally increased in untreated CD (Brusco et al. 1999; Di Sabatino et al. 2012). Studies in our department have shown co-localisation of tTG with SM α-actin in pericryptal and subepithelial elongated fibroblast-like cells. Based on their morphological appearance and expression of SM α-actin, these cells were identified as myofibroblasts, demonstrating for the first time that myofibroblasts co-express two of the major antigenic targets of coeliac autoantibodies. The expression of tTG and SM α-actin by myofibroblasts was found to be increased in patients with untreated CD compared to patients with treated CD and normal control patients (Dunne et al, in preparation). Preliminary staining of biopsies from the oats study patients demonstrated the same pattern of co-localisation of tTG with SM α-actin (Fig. 3.1).

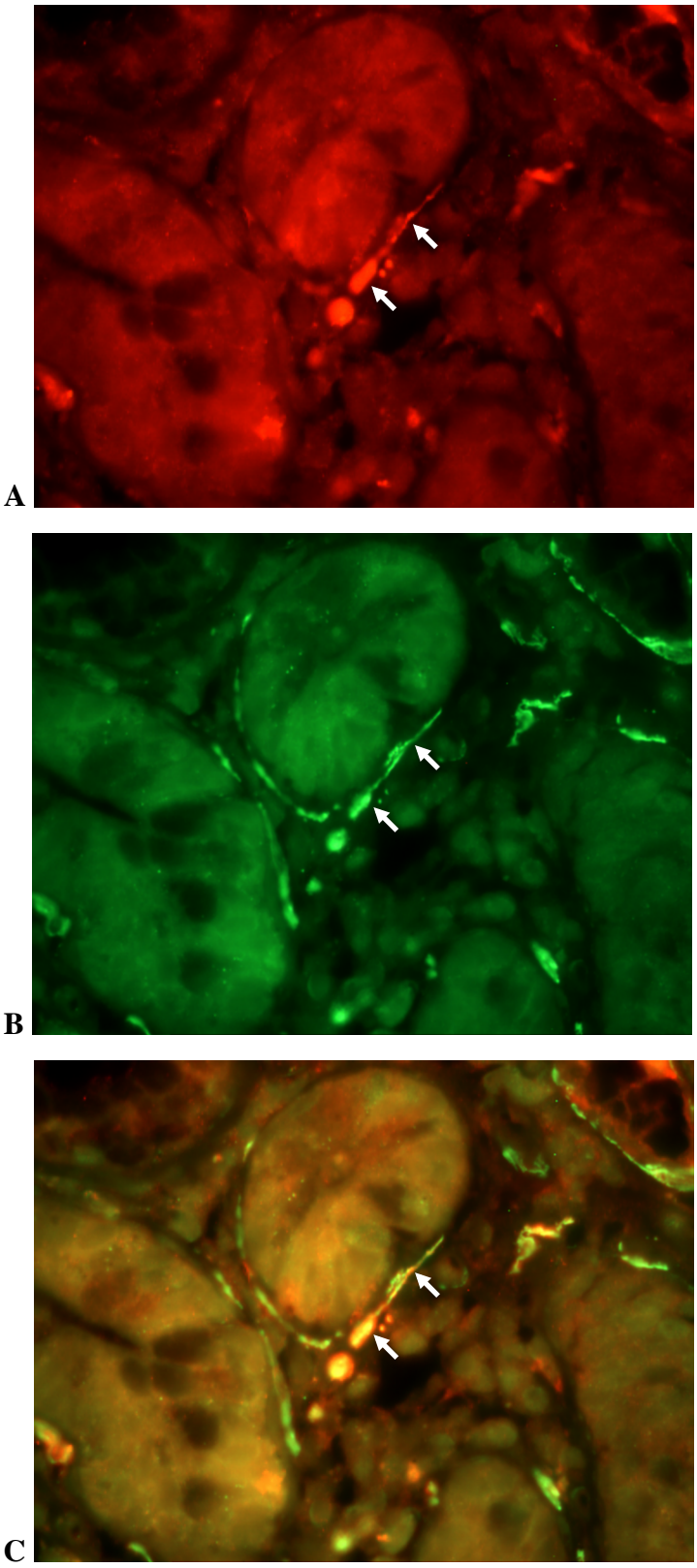


Figure 3.1: Expression of tTG and SM α-actin in coeliac duodenal mucosa. Fluorescent staining demonstrating expression of tTG (A) and SM α-actin (B) by pericryptal myofibroblasts (arrows), an overlay of both images demonstrating co-localisation of tTG with SM α-actin (C).

3.1.3 IN Cell Analyzer 1000

The IN Cell Analyzer 1000 system (GE Healthcare, Little Chalfont, UK) is an advanced microscopy facility that offers fast, automated imaging of live or fixed cells. It is intended for the analysis of cell-based assays, primarily on a microtitre plate, in a streamlined, user-friendly fashion. Using the system's software, protocols can be created to acquire and analyse images from hundreds of multiwell plates in the space of a few hours, with little or no intervention from the user.

3.1.4 IN Cell Analyzer 1000 versus reading on a fluorescence microscope

The traditional method of grading the intensity of fluorescent staining by visual examination on a fluorescence microscope has a number of disadvantages. Visual assessment of the intensity of a fluorescent signal is highly subjective and two independent observers may produce differing results. An individual observer will also be subject to fatigue, especially when examining many specimens, and may produce inconsistent results over time. In addition, only very crude qualitative results i.e. average fluorescence, above or below average fluorescence, can be produced using this method. Furthermore, fluorescently stained specimens fade rapidly, and even more so with examination under a microscope, therefore subsequent analysis at a later date would require the use of more samples and repeated staining.

Using the IN Cell Analyzer 1000 software, images of fluorescently stained cells can be acquired and a numerical measure of the intensity of fluorescent staining can be obtained. This approach delivers a number of advantages over traditional methods. As intensity is being assessed by an automated system, it is completely unbiased and measurements of staining intensity are not affected by the strength of staining in surrounding cells, as can occur with the human eye. A machine will not become tired or suffer from eye strain,

therefore results will be consistent even after many hours of work. The use of this technology advances results from a qualitative to a quantitative form that is reproducible; the same image analysed on different days will produce the same results. Lastly, once an image has been acquired it can be saved permanently, allowing further analysis at any time in the future without the need for repeated staining or use of more samples.

3.1.5 Adaption of IN Cell Analyzer 1000 for use with tissue

The IN Cell Analyzer 1000 is intended for the streamlined analysis of a homogenous population of cells on a plastic plate. The advantages that this system brings to the analysis of cell lines are equally as desirable in the analysis of tissue sections. However, the acquisition and analysis of a tissue section that is a few millimetres long and wide, a few cells thick, contains many different cell types and is fixed between a glass slide and a coverslip is a much bigger challenge than working with individual cells on a plate. The system is designed exclusively for use with cells; therefore there were no guidelines available for adapting it for use with tissue. Consequently, as we attempted to make this transition, the only approach available was trial and error.

3.1.6 Chapter aims

The aims of this chapter were twofold. Firstly, to further examine the oats study patients' biopsies for any signs of damage following long term oats ingestion by assessing expression of tTG and SM α-actin. Secondly, to adapt the IN Cell Analyzer 1000 system for use with tissue to enable more sophisticated and accurate analysis of fluorescent staining than was previously possible.

3.2 Materials & Methods

3.2.1 Immunofluorescent staining

Cutting of biopsies

Biopsies were cut as described in Chapter 2, (Section 2.2.7) with the exception that Thermo Scientific Superfrost Plus electrostatic slides (Fisher Scientific, Ireland) were used. Poly-L-Lysine coated slides autofluoresce and are therefore unsuitable for use in fluorescent staining.

Staining procedure

Sections were dewaxed, rehydrated and antigen retrieved as described in the protocol for immunohistochemical staining in Chapter 2 (Section 2.2.7).

Tissue sections were incubated with antibody to tissue transglutaminase (Roboscreen, Germany), washed twice in phosphate buffered saline (PBS) and then incubated with an Alexafluor^{568}-conjugated secondary goat-anti-rabbit (GAR) antibody (BioSciences, Ireland). Sections were washed as before and incubated with antibody to smooth muscle alpha-actin (Sigma-Aldrich, Ireland) followed by two washes in PBS, a secondary goat-anti-mouse (GAM) FITC antibody (Dako, Denmark) and two more washes in PBS. All primary and secondary antibodies were diluted in a 0.15% solution of bovine serum albumin (BSA) in PBS (v/v) and incubated for 1 h at RT in a humidity chamber. In order to visualize nuclei, sections were finally incubated with Hoechst 33258 (Sigma-Aldrich, Ireland), at a 1 in 1000 dilution in dH_2O, for 5 min at RT in a humidity chamber. Coverslips were mounted using Fluorescence Mounting Medium (Dako, Denmark), left to dry at RT overnight in the dark and stored at 4°C in the dark.

Antibodies used in Immunofluorescent Staining					
Antibody	**Clonality**	**Source**	**Ag Retrieval**	**Dilution**	**Incubation**
tTG	p	Roboscreen	Citrate Buffer	1/100	1 h, RT
Alexafluor^{568} GAR	N/A	BioSciences	N/A	1/200	1 h, RT
SM α-actin	m	Sigma-Aldrich	Citrate Buffer	1/600	1 h, RT
FITC GAM	N/A	Dako	N/A	1/100	1 h, RT

Table 3.1: Details of antibodies used in immunofluorescent analysis of 18 pre- and post-oat biopsies. Ag = antigen; tTG = tissue transglutaminase; p = polyclonal; m = monoclonal, RT = room temperature; GAR = goat-anti-rabbit; GAM = goat-anti-mouse; N/A = not applicable; SM α-actin = smooth muscle α-actin; FITC = fluorescein isothiocyanate.

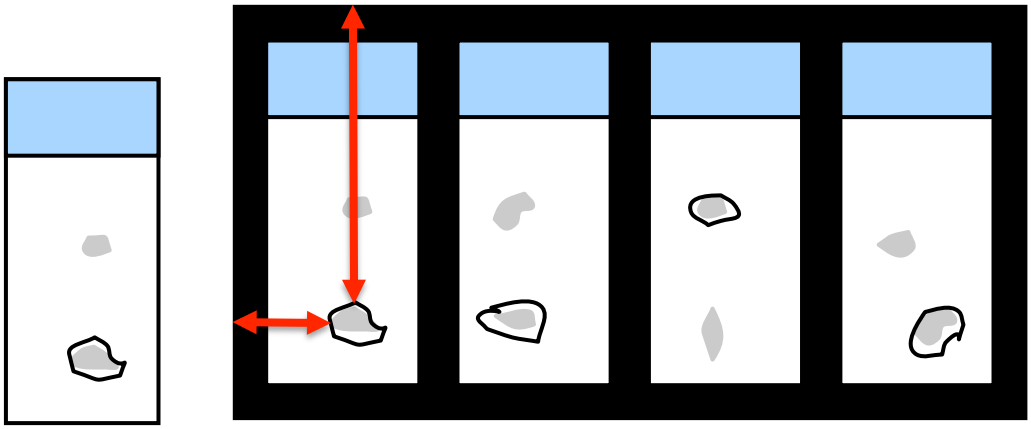
3.2.2 Reading on fluorescence microscope

Prior to acquisition on the IN Cell Analyzer 1000, all slides were viewed on a fluorescence microscope to confirm that staining was successful. Negative control slides, with tissue sections that were incubated in PBS instead of the primary antibody, were used to check that staining was specific.

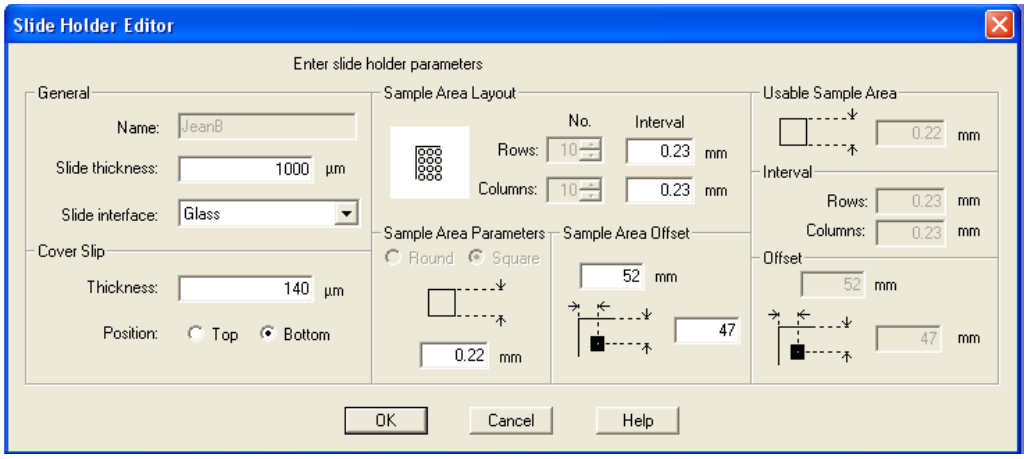
3.2.3 Acquisition on IN Cell Analyzer 1000

Tissue positioning

As the IN Cell Analyzer 1000 was designed for use with cells, a slide holder with the same dimensions as a 96 well plate was available. This was made to hold four slides, with each slide containing wells of cells at regular intervals. It could also be used to hold slides with tissue sections, however, as tissue sections were not at the same regular intervals as wells in a slide, their location had to be manually measured and entered into the software. Tissue sections were very small, therefore accuracy was very important when measuring their location. To help visualise the sections for measuring, the section of interest was circled with a black permanent marker (Fig. 3.2); coloured markers autofluoresced and made the section unreadable. Slides were then placed in the slide holder face down so that analysis would be through the cover slip rather than the entire thickness of the slide. The location of each section from the top left corner of the plate was measured in millimetres. As there is room for the slides to move around within the slide holder it was important that the slides were kept tight to one corner while measuring and transferring to the IN Cell Analyzer 1000. Details such as cover slip thickness, location of section, sample area size required and distance between sample areas were entered into the acquisition software.



A B



C

Figure 3.2: Tissue positioning. Tissue sections were circled with black permanent marker (A), slides were placed face down in the slide holder and the distance in millimetres across and down from the top left corner of the slide holder to each tissue section measured (red arrows) (B), relevant details were entered into the acquisition software for each section (C).

Tissue mapping

The potential acquisition area consisted of a 2.3 mm^2 area, which was divided into 100 squares. Each square was 0.22 mm^2 with a gap of 0.01 mm between squares. As tissue sections were very small, there were usually a lot of squares that did not contain any tissue. Acquisition of images on the IN Cell Analyzer 1000 was very time consuming, up to an hour per section; in addition images used a lot of storage space on the hard drive, therefore it was necessary to identify which squares contained tissue prior to acquisition. To facilitate this, a simple grid was constructed on an excel sheet to represent the potential acquisition area (Fig. 3.3). The grid was composed of a large square divided into ten squares across by ten squares down and labelled 1-10 on the x-axis and A-J on the y-axis so that it mirrored the acquisition grid within the software. The excel sheet also had space to record the individual slide ID and the location of the tissue section as measured in millimetres from the outer edges of the slide holder. Using excitation and emission filters specific for Hoechst nuclear stain (D360/40 - HQ460/40), individual squares were checked for tissue content. Squares containing tissue were marked with a tick and empty squares with an x, until the perimeter of the section was mapped. Using the software, squares containing tissue were selected for acquisition and all unwanted squares eliminated.

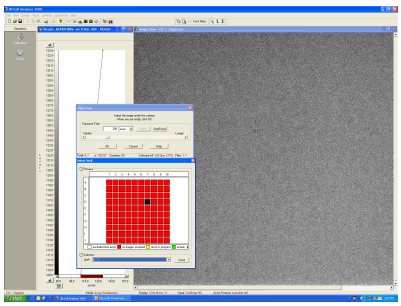
Slide: Sample slide

Across (mm) 52

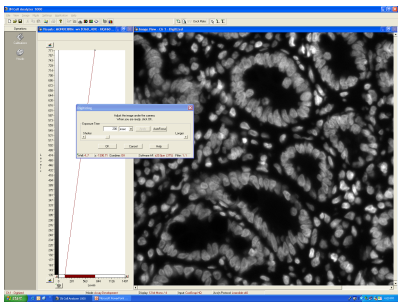
Down (mm) 47

	1	2	3	4	5	6	7	8	9	10
A					✗	✓	✓	✗	✗	
B		✗			✗	✓	✓	✓	✗	
C			✗	✗	✗	✓	✓	✓	✗	
D				✗	✓	✓	✓	✓	✗	
E				✗	✓	✓	✓	✓	✗	
F			✗	✗	✓	✓	✓	✓	✗	
G				✗	✓	✓	✓	✓	✗	
H				✗	✓	✓	✓	✓	✗	
I				✗	✓	✓	✓	✓	✗	
J				✗	✓	✓	✓	✓	✗	

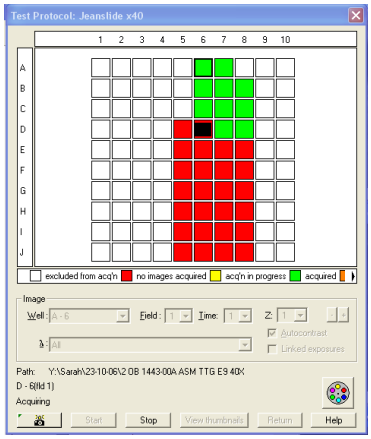
A



B



C



D

Figure 3.3: Tissue mapping. Excel grid showing slide ID, tissue location, empty squares (✗) and squares containing tissue (✓) (A), screenshots taken during tissue mapping showing an empty square (B) and a square with tissue (C), screenshot taken during acquisition showing acquired squares (green), square currently being acquired (black), squares to be acquired (red) and squares without tissue that have been eliminated (blank squares) (D).

Focusing

Visualization of Hoechst nuclear staining was used for the initial focusing and then the 'auto offset' function in the software was used to focus for all three colours. Problems with focusing were overcome by altering the cover slip thickness or exposure time. For additional focus the 'software auto focus' function was activated so that each square was focused individually during acquisition. This increased acquisition time but produced a clearer image.

Acquisition

Using an Acquisition Protocol designed with assistance from staff in the High Content Screening facility at the Institute of Molecular Medicine (IMM), images were acquired from fluorescently stained sections at 40X magnification. Excitation and emission filters were specified for Hoechst (D360/40 - HQ460/40), FITC (S475/20 - HQ535/50) and Alexafluor^{568} (HQ535/50 - HQ620/60) staining. These were designated Wavelength 1, Wavelength 2 and Wavelength 3 respectively. Images were acquired at each individual wavelength in grayscale (Fig. 3.4). In order to create a 24-bit colour composite image, colours had to be assigned to each wavelength, thus Wavelength 1 (Hoechst) was designated blue, Wavelength 2 (FITC) green and Wavelength 3 (Alexafluor^{568}) red. The three images were then fused together by the software to create a merged image with blue nuclei, green SM α-actin, red tTG and also showing areas of co-localisation of SM α-actin and tTG in yellow.

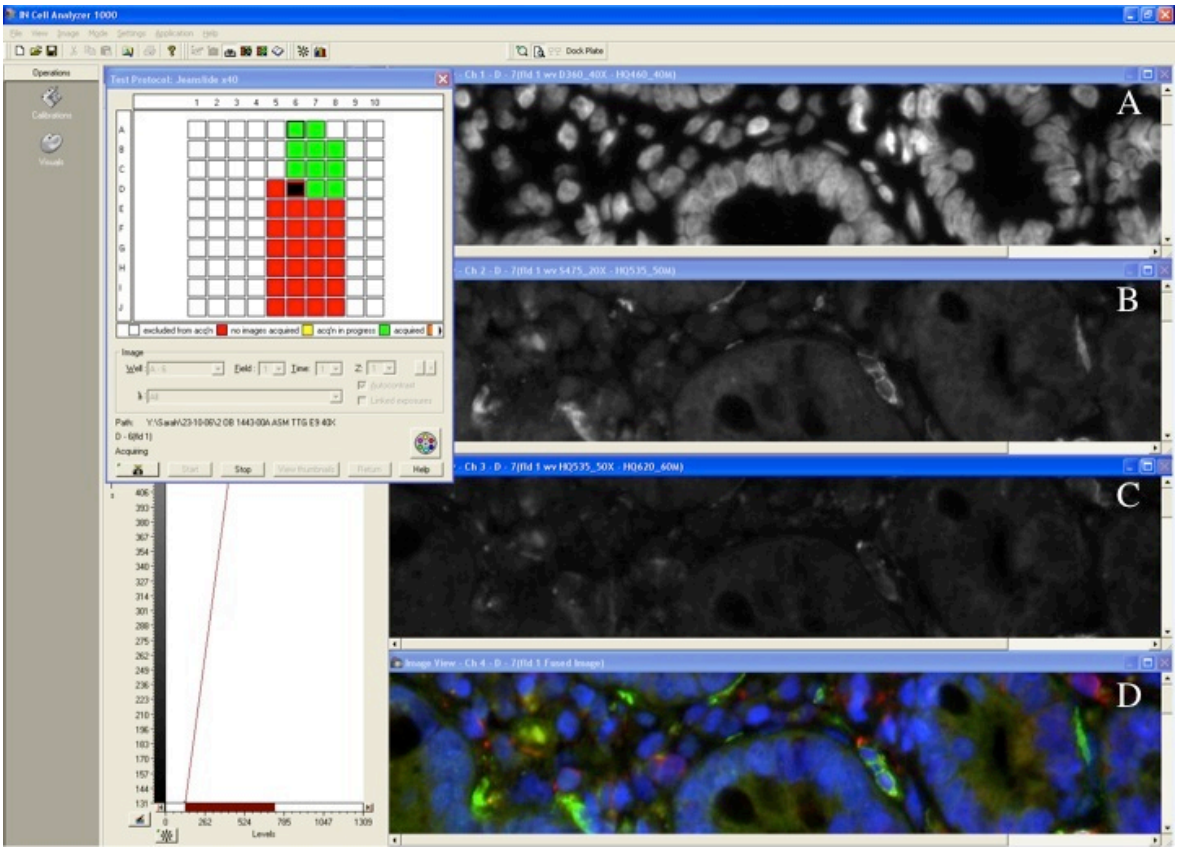


Figure 3.4: Screenshot taken during acquisition of a tissue section on the IN Cell Analyzer 1000. Wavelength 1 (Hoechst) (A), Wavelength 2 (FITC) (B), and Wavelength 3 (Alexafluor^{568}) (C) were all acquired separately in grayscale and merged into 24-bit colour composite image of all three wavelengths (D).

When all selected squares were acquired, an overview of the whole tissue section was produced (Fig. 3.5). Each of the squares within the section could be viewed individually in any of the grayscale channels or as a merged colour composite image and any or all of the squares could be used for analysis.

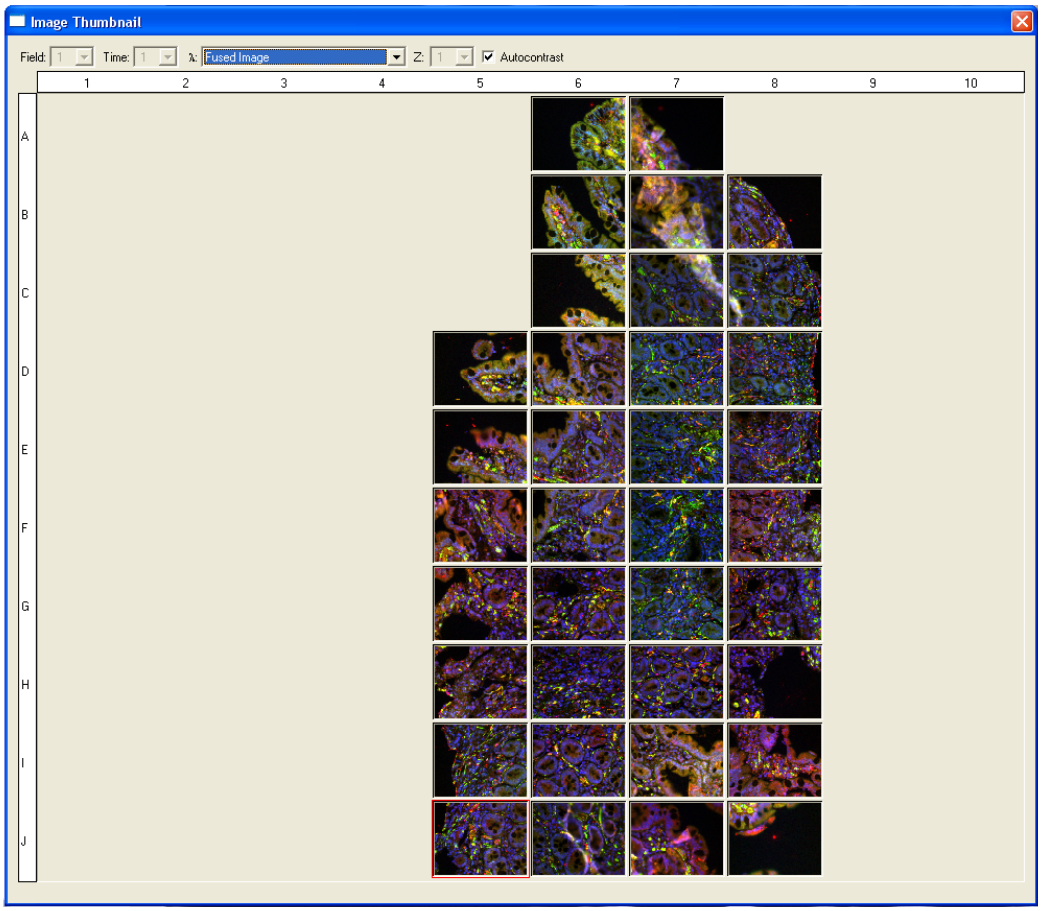


Figure 3.5: Overview of acquired tissue section.

Troubleshooting

Initial attempts to acquire clearly focused images of tissue sections at 40X magnification were unsuccessful. However, following a service of the machine it was possible to acquire clear images at 40X magnification and all sections were reacquired.

All sections were acquired prior to the commencement of analysis. Each biopsy was part of a pair taken from a coeliac patient prior to and following one year of oats ingestion. During analysis it became clear that images from biopsy pairs that were acquired on different days

could not be analysed under the same parameters. Thus, any biopsy pairs that had been acquired on different days had to be reacquired in a single day to make analysis possible.

3.2.4 Analysis

As there were no protocols for analysis of tissue provided in the software, the manufacturer agreed to write a program to fit our requirements. However, the provided program proved unsuitable and ultimately existing protocols, intended for use with cells, had to be adapted and used for tissue analysis.

Intensity of staining

The first approach was to measure the intensity of the fluorescent staining of tTG and SM α-actin in each tissue section. Using the available software on the IN Cell Analyzer 1000, pericryptal myofibroblasts (PCMFs) were selected for analysis based on their elongated morphology and expression of SM α-actin or tTG. The cut-off point, or threshold, for the intensity of staining was altered so that positively stained cells were included for analysis while unstained cells were excluded (Fig. 3.6). Each biopsy pair was analysed at the same cut-off point to ensure each of the pair was examined under the same conditions. If this was not possible, even after re-acquisition, the pair was excluded.

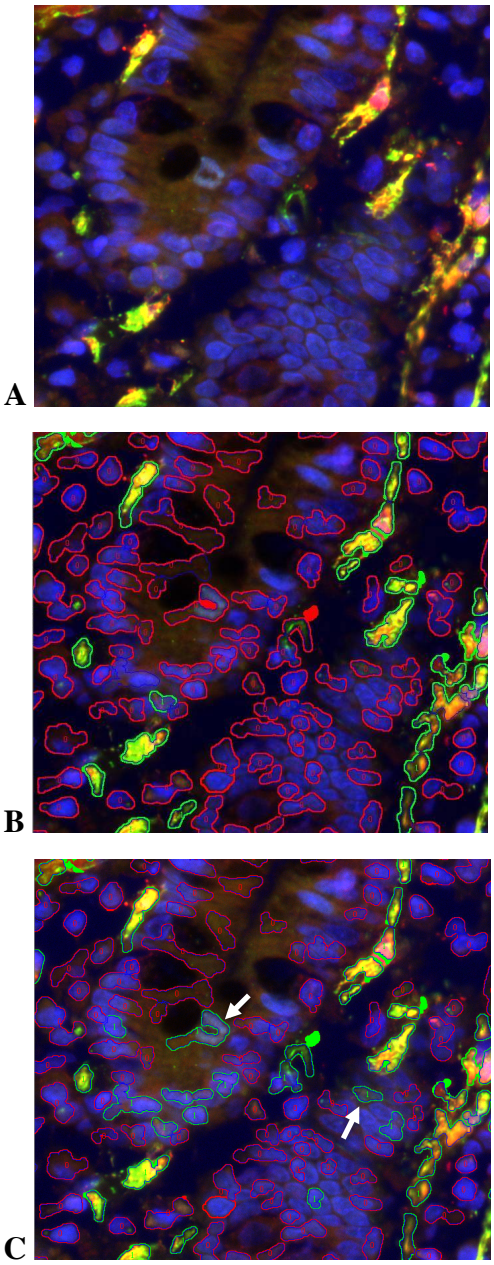
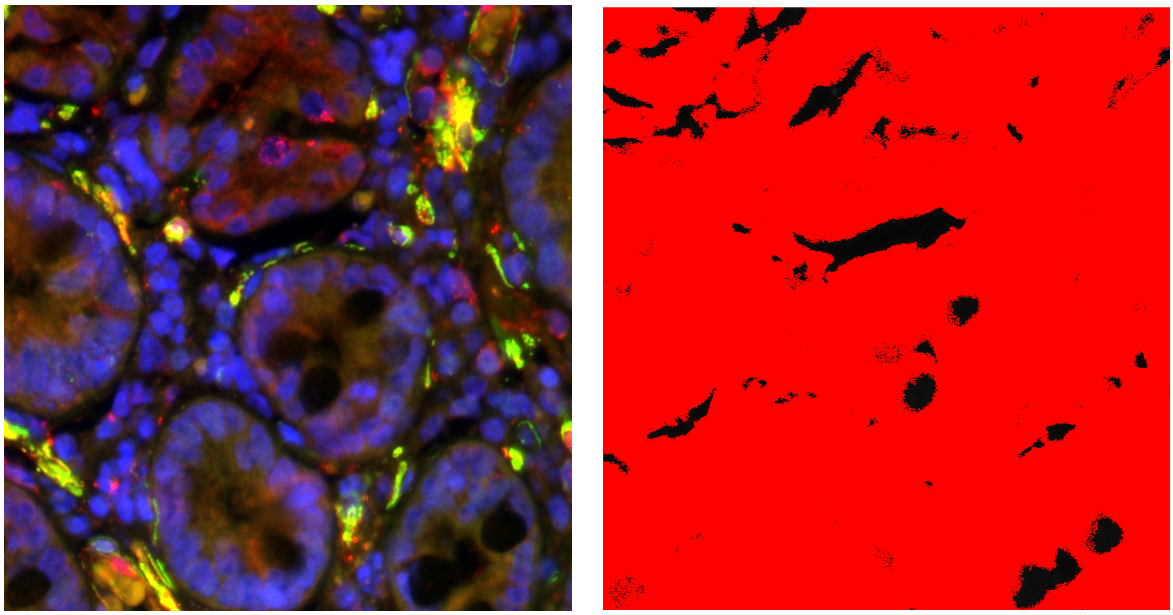


Figure 3.6: Threshold setting. Merged colour composite image from one square of an acquired tissue section showing nuclear (blue), tTG (red) and SM α-actin (green) staining as well as tTG and SM α-actin co-localisation (yellow) (A), using the available software, regions of interest were selected for analysis (green outline) and inappropriate regions, which did not contain positively stained PCMFs, were eliminated (red outline) (B), each image was checked prior to analysis and if any inappropriate regions were selected for analysis (white arrows) the intensity threshold was raised to eliminate them (C); likewise, if any positively stained regions were not selected the intensity threshold was lowered until they were included.

Once the appropriate intensity threshold was established for a biopsy pair, the pair was analysed using the IN Cell Analyzer 1000 software. The intensity of staining in each of the selected regions was calculated by measuring the number of pixels needed to display the degree of staining present and dividing by the area of the region. The results for all regions in a section were then averaged to give an overall measure of the intensity of staining in the tissue section. This was done separately for tTG and SM α-actin expression.

Percentage area positively stained

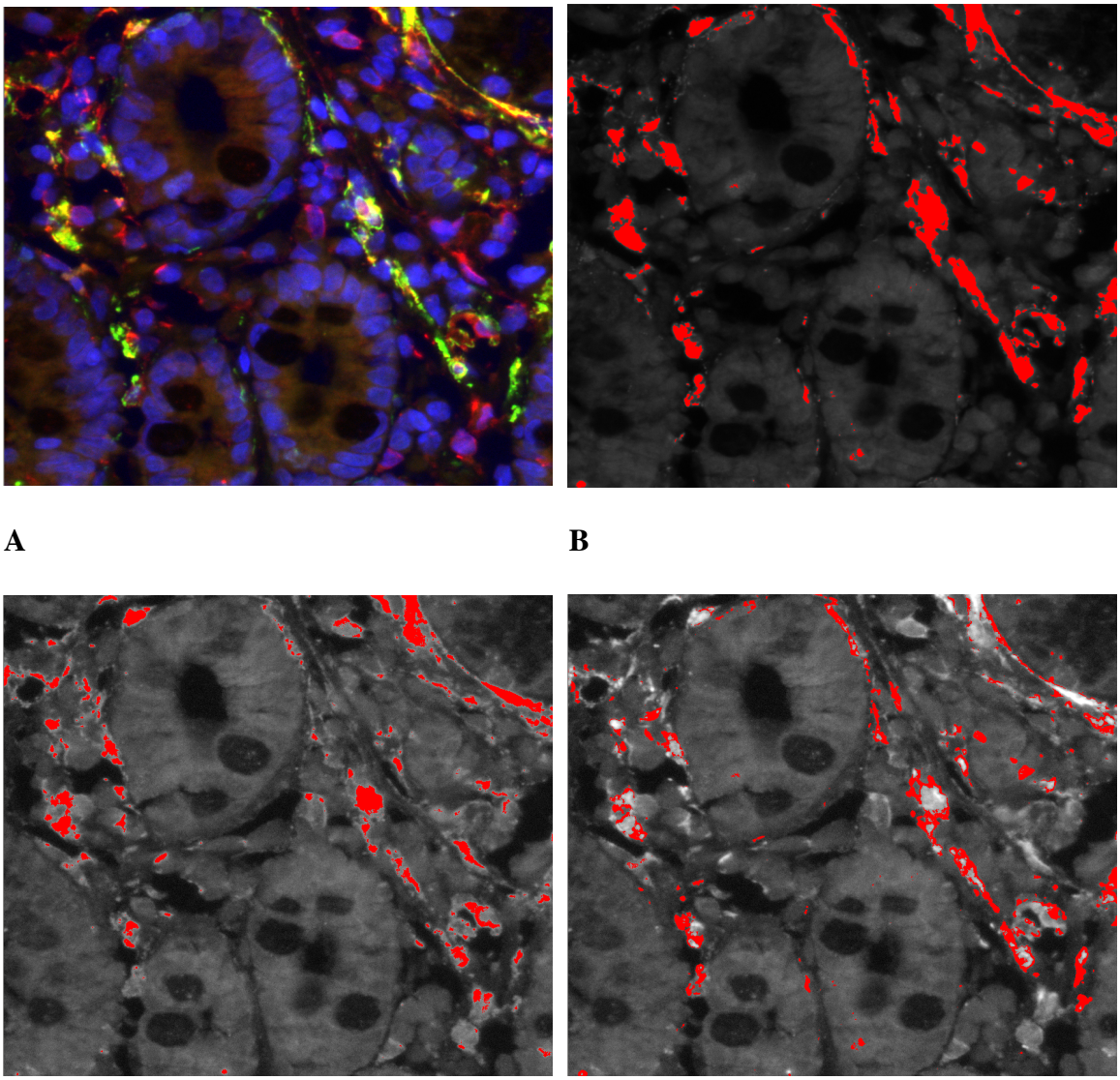
The second approach was the measurement of the percentage area of each tissue section that was positively stained for tTG and SM α-actin. This approach had the added advantage of also allowing the measurement of areas of co-localisation of tTG and SM α-actin. Using an algorithm designed by Connla Edwards in the High Content Screening facility at the IMM, relevant areas were selected on each section. Firstly, the intensity cut off was adjusted so that all areas containing tissue were selected and any gaps in tissue were excluded (Fig. 3.7). This meant that the percentage of positively stained tissue was calculated relevant to the total area of tissue present. As with the previous analysis, each biopsy pair was analysed at the same intensity cut off and if this was not possible the pair was excluded.



A **B**

Figure 3.7: Selection of tissue section area. Merged image from one square of an acquired section (A), selection of all areas containing tissue in the same square (B).

Secondly, the intensity cut off was adjusted individually within the green and the red channels so that only areas expressing SM α-actin and tTG respectively were selected (Fig. 3.8). A threshold of 1.5 X above background was applied. Any section with an intensity cut off of less than 1.5 X above background for either SM α-actin or tTG expression was considered to have an unacceptably high level of background staining and excluded from analysis.



A **B**

C **D**

Figure 3.8: Selection of areas of fluorescent staining. Merged image from one square of an acquired section (A), green channel with areas expressing SM α-actin selected in red (B), red channel with areas expressing tTG selected (C), green channel with areas expressing SM α-actin exclusively selected (D).

Following the identification of the areas containing tissue, areas expressing SM α-actin and areas expressing tTG, the software measured all of these areas within each square and provided a numerical value for each. These values were then used to calculate the percentage of the total tissue area expressing SM α-actin using the following formula:

$$\frac{\text{Area expressing SM } \alpha\text{-actin}}{\text{Total tissue area}} \times \frac{100}{1} = \% \text{ of total tissue area expressing SM } \alpha\text{-actin}$$

The percentage of the total tissue area expressing tTG was calculated in the same manner.

The percentage of the total tissue area co-expressing SM α-actin and tTG was calculated in two stages. After the areas expressing SM α-actin and the areas expressing tTG had been defined as shown in Fig. 3.8 above, the software was able to identify the areas that expressed SM α-actin exclusively. This was achieved by starting with all areas expressing SM α-actin and removing any areas within those that also expressed tTG. Fig. 3.8 illustrates this calculation as follows:

Image B (SM α-actin) – Image C (tTG) = Image D (SM α-actin exclusively)

The software then measured all of the areas expressing SM α-actin exclusively within each square and provided a numerical value.

Once the area expressing SM α-actin exclusively had been calculated, the next step was to use this to calculate the area co-expressing SM α-actin and tTG. This was achieved by subtracting the area expressing SM α-actin exclusively from the area expressing SM α-

actin. Removing all areas that expressed SM α-actin exclusively from all areas that expressed SM α-actin left behind only the areas that co-expressed SM α-actin and tTG. This can be partly illustrated by looking at Fig. 3.8 as follows:

Image B (SM α-actin) – Image D (SM α-actin exclusively) = areas of co-localisation

The numerical value for areas of co-localisation was then used to calculate the percentage of the total tissue area co-expressing SM α-actin and tTG. This was calculated in the same manner as for the percentage of the total tissue area expressing SM α-actin and tTG individually, i.e.:

$$\frac{\text{Area of co-localisation}}{\text{Total tissue area}} \times \frac{100}{1} = \%\text{ of total tissue area co-expressing SM α-actin and tTG}$$

The percentages of the total tissue area expressing tTG and SM α-actin individually as well as the percentage area co-expressing tTG and SM α-actin were calculated for each pre- and post-oats tissue section. Additionally, similarly stained tissue sections from four Marsh grade 0 and three Marsh grade 3 coeliac biopsies were analysed in the same way.

3.2.5 Statistics

GraphPad Prism was used for statistical analysis of results. The student's paired two-tailed t test was used to compare the intensity of tTG and SM α-actin expression in pre- and post-oats biopsies. Analysis of variance (ANOVA) was used to compare the percentage areas expressing tTG, SM α-actin and areas co-expressing both in pre-oats, post-oats, Marsh

grade 0 and Marsh grade 3 coeliac patients. P values <0.05 were considered statistically significant.

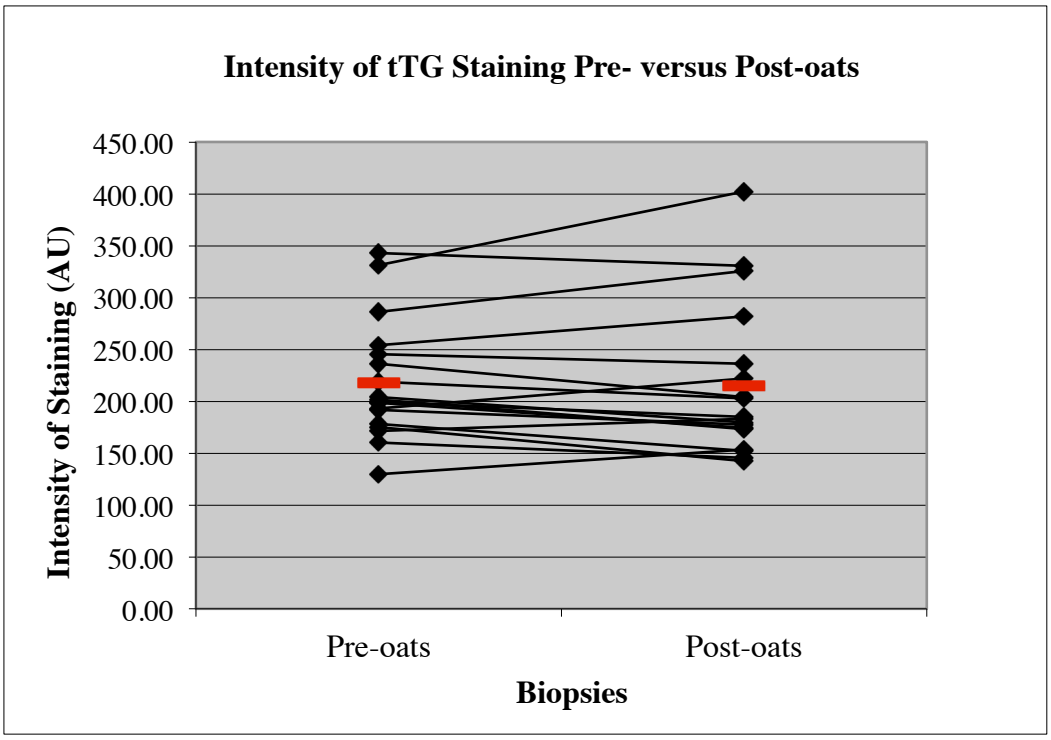
3.3 Results

3.3.1 Intensity of staining

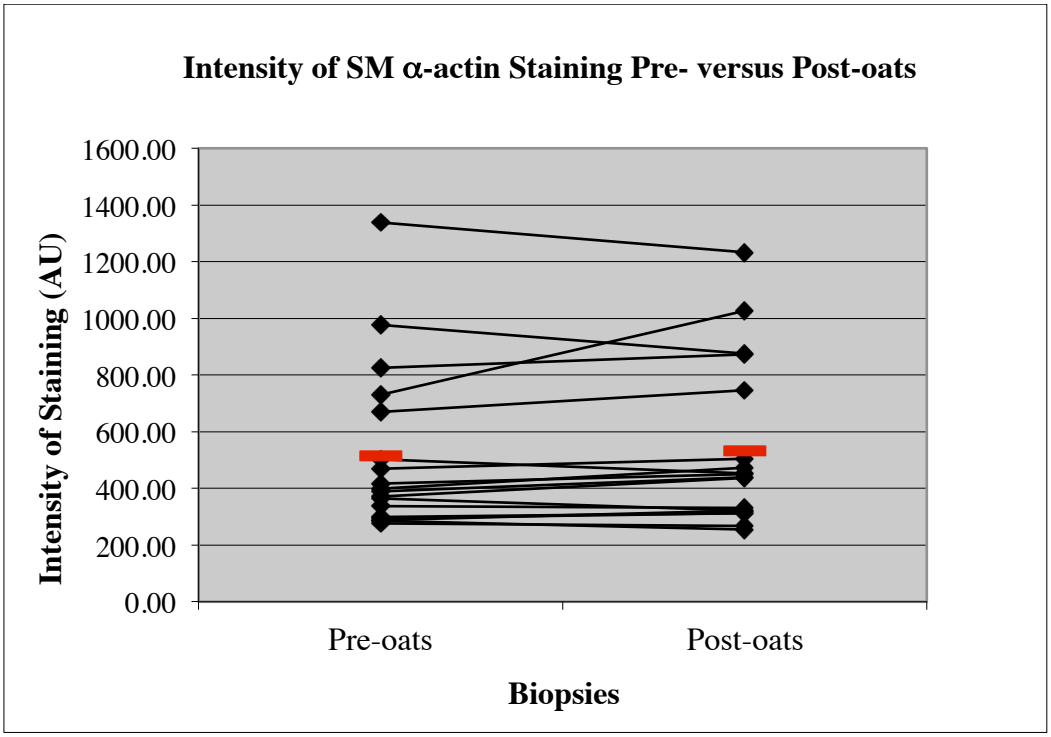
The IN Cell Analyzer 1000 was used to analyze the intensity of fluorescent staining for tTG and SM α-actin of paired sections from 19 patients previously assessed for expression of Ki67, CD3 and CD8. In one of these patients the biopsy pair could not be analysed at the same intensity threshold, even after re-acquisition, and therefore had to be excluded from analysis. Using this technology, numerical results were obtained for intensity of fluorescent staining pre- and post-oats (Table 3.2). While some patients showed slight change, no significant difference in staining intensity of either marker was seen for the group as a whole (Fig. 3.9). The average intensity of tTG expression pre-oats was 218 AU and post-oats was 215 AU [95% CI -17.18 to 12.09], $p = 0.72$, not significant (ns). SM α-actin average intensity pre- versus post-oats was 513 AU versus 535 AU [95% CI -21.20 to 65.77], $p = 0.29$, ns. This follows patterns seen with immunohistochemical staining.

	Analysis	n	Pre-oats	Post-oats	p-value
tTG	Intensity of staining	18	218 ± 57 AU	215 ± 73 AU	0.72
SM α-actin	Intensity of staining	18	513 ± 290 AU	535 ± 289 AU	0.29

Table 3.2: Results of IN Cell analysis. The numerical values for intensity of tTG and SM α-actin staining in pre- and post-oats biopsies were compared using the student's paired two-tailed t test and no significant differences were found. Results are expressed as the mean of each group ± the standard deviation. n = number of patients; tTG = tissue transglutaminase; SM α-actin = smooth muscle α-actin; AU = arbitrary units.



A



B

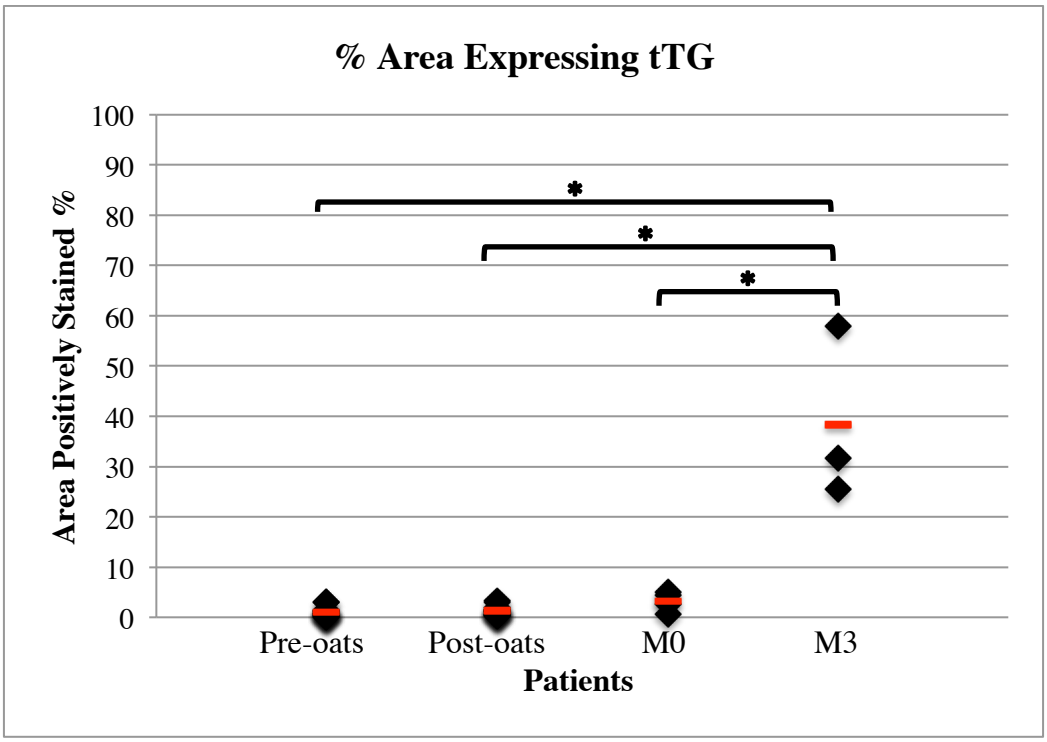
Figure 3.9: Intensity of staining. Individual (black diamond) and mean (red dash) measures of tTG (A) and SM α-actin (B) staining intensity in coeliac biopsy pairs pre- versus post-oats. tTG = tissue transglutaminase; SM α-actin = smooth muscle α-actin; AU = arbitrary units.

3.3.2 Percentage area positively stained

Secondly, the IN Cell Analyzer 1000 was used to measure the percentage of the total area of tTG and SM α-actin expression and co-expression in the 18 biopsy pairs examined for intensity of staining. Two of these patients had to be excluded due to high background staining and or it not being possible to examine the pair at the same intensity cut off. In addition, biopsies from four Marsh grade 0 and three Marsh grade 3 coeliac patients, who did not take part in the oats study, were included in this analysis as controls.

tTG

A one-way ANOVA revealed significant differences in the percentage area of tissue expressing tTG between the groups, [$F(3, 35) = 70.00$, $p<0.0001$]. The means, standard deviations and 95% confidence intervals are presented in Fig. 3.10. Post-hoc Tukey's multiple comparisons tests showed that the Marsh grade 3 tissue sections had significantly higher percentage areas expressing tTG than the pre-oats, post-oats and Marsh grade 0 tissue sections, with a significance level of <0.0001. All other comparisons were not significant.



A

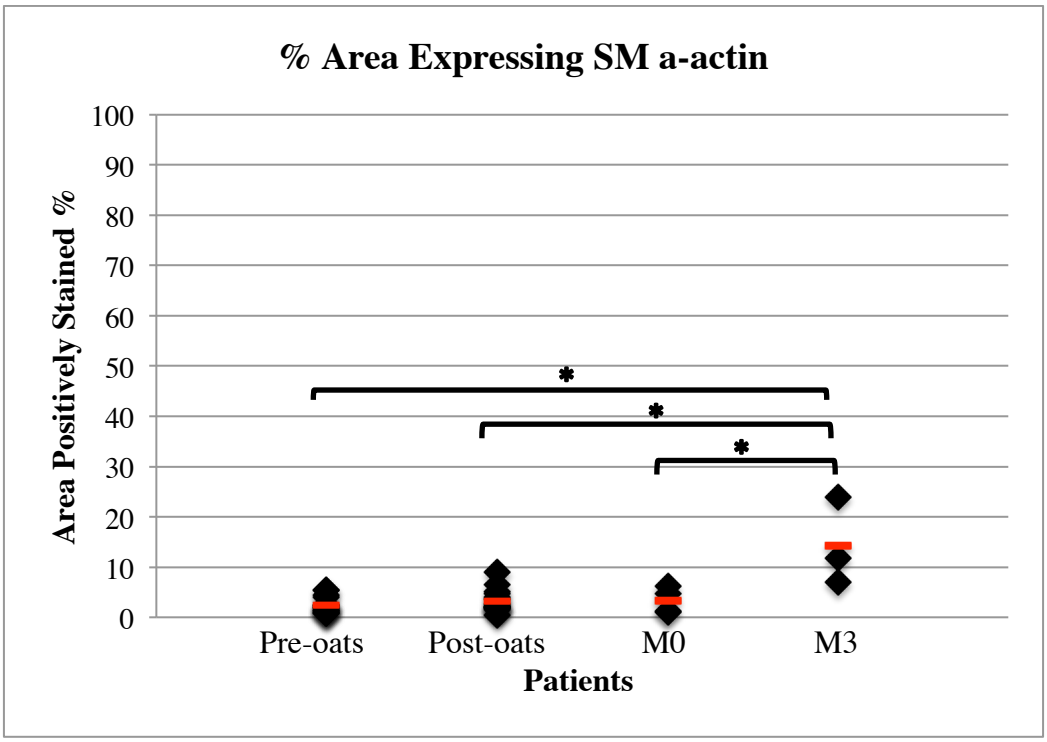
Biopsy type	n	Mean % area expressing tTG	SD	95% CI
Pre-oats	16	1.02	0.88	0.55 – 1.49
Post-oats	16	1.25	0.86	0.79 – 1.7
Marsh grade 0	4	3.14	1.91	0.11 – 6.17
Marsh grade 3	3	38.33	17.32	-4.69 – 81.35

B

Figure 3.10: Percentage tissue area expressing tTG. Individual (black diamond) and mean (red dash) percentage tissue areas expressing tTG in pre-oats, post-oats, Marsh grade 0 and Marsh grade 3 coeliac biopsies (A), means, standard deviations and 95% confidence intervals for area of tTG expression in pre-oats, post-oats, Marsh grade 0 and Marsh grade 3 coeliac biopsies (B). n = number of patients; tTG = tissue transglutaminase; SD = standard deviation; CI = confidence interval; * = significantly different; M0 = Marsh grade 0; M3 = Marsh grade 3.

SM α-actin

A one-way ANOVA showed significant differences in the percentage area expressing SM α-actin in pre-oats, post-oats, Marsh grade 0 and Marsh grade 3 biopsies, [$F(3, 35) = 15.05$, $p<0.0001$]. The means, standard deviations and 95% confidence intervals are shown in Fig. 3.11. Post-hoc comparisons using Tukey's multiple comparisons test revealed significantly higher percentage areas expressing SM α-actin in the Marsh grade 3 tissue sections compared to the pre-oats, post-oats and Marsh grade 0 tissue sections, with a significance level of <0.0001. All other comparisons were not significant.



A

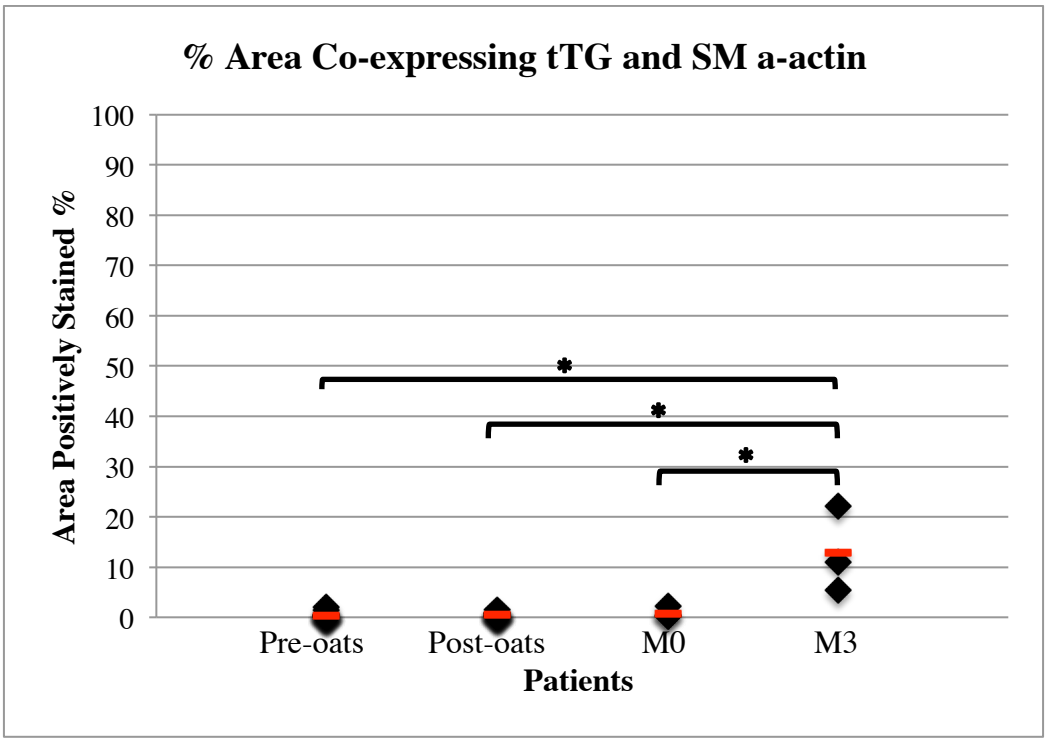
Biopsy type	n	Mean % area expressing SM α-actin	SD	95% CI
Pre-oats	16	2.42	1.55	1.59 – 3.25
Post-oats	16	3.23	2.21	2.06 – 4.41
Marsh grade 0	4	3.28	2.54	-0.76 – 7.31
Marsh grade 3	3	14.23	8.74	-7.48 – 35.93

B

Figure 3.11: Percentage tissue area expressing SM α-actin. Individual (black diamond) and mean (red dash) percentage tissue areas expressing SM α-actin in pre-oats, post-oats, Marsh grade 0 and Marsh grade 3 coeliac biopsies (A), means, standard deviations and 95% confidence intervals for area of SM α-actin expression in pre-oats, post-oats, Marsh grade 0 and Marsh grade 3 coeliac biopsies (B). n = number of patients; SM α-actin = smooth muscle α-actin; SD = standard deviation; CI = confidence interval; * = significantly different; M0 = Marsh grade 0; M3 = Marsh grade 3.

Co-expression of tTG and SM α-actin

Similarly to individual expression of tTG and SM α-actin, the percentage area co-expressing tTG and SM α-actin was found to be significantly different in pre-oats, post-oats, Marsh grade 0 and Marsh grade 3 biopsies using one-way ANOVA testing, [$F(3, 35) = 32.12$, $p<0.0001$]. The means, standard deviations and 95% confidence intervals are given in Fig. 3.12. Likewise, post-hoc Tukey's multiple comparisons tests revealed that Marsh grade 3 tissue sections had significantly higher percentage areas co-expressing tTG and SM α-actin than the pre-oats, post-oats and Marsh grade 0 tissue sections, with a significance level of <0.0001. Again, all other comparisons were not significant.



A

Biopsy type	n	Mean % area co-expressing tTG and SM α-actin	SD	95% CI
Pre-oats	16	0.44	0.53	0.16 – 0.73
Post-oats	16	0.52	0.39	0.31 – 0.73
Marsh grade 0	4	0.80	1.01	-0.81 – 2.41
Marsh grade 3	3	12.82	8.45	-8.17 – 33.81

B

Figure 3.12: Percentage tissue area co-expressing tTG and SM α-actin. Individual (black diamond) and mean (red dash) percentage tissue areas co-expressing tTG and SM α-actin in pre-oats, post-oats, Marsh grade 0 and Marsh grade 3 coeliac biopsies (A), means, standard deviations and 95% confidence intervals for area of tTG and SM α-actin co-expression in pre-oats, post-oats, Marsh grade 0 and Marsh grade 3 coeliac biopsies (B). n = number of patients; tTG = tissue transglutaminase; SM α-actin = smooth muscle α-actin; SD = standard deviation; CI = confidence interval; * = significantly different; M0 = Marsh grade 0; M3 = Marsh grade 3.

Taken together, these results show that the proportion of the total tissue area expressing tTG and SM α-actin is significantly raised in untreated compared to treated coeliac disease. Additionally, the proportion of the total tissue area co-expressing tTG and SM α-actin is significantly increased in untreated versus treated CD. Furthermore, the degree of both individual and co-expression of tTG and SM α-actin in the pre- and post-oats biopsies was very similar to that of the Marsh grade 0 biopsies and significantly different to the expression levels in the Marsh grade 3 biopsies.

3.4 Discussion

3.4.1 IN Cell Analyzer 1000 adaptation

The IN Cell Analyzer 1000 system is designed for the analysis of cell-based assays in a streamlined, user-friendly fashion. In this study we adapted the system and used it to get meaningful results from specific cells within a tissue section on a slide. As the system was not designed for this use and no guidelines were available for this application, a large amount of trial and error was necessary prior to the successful analysis of tissue sections.

The initial approach in the analysis of tTG and SM α-actin expression in the acquired tissue sections was to measure the intensity of staining. This was based on the traditional method of examination on a microscope and visual grading of the intensity of staining as below average, average or above average fluorescence. This analysis showed no significant difference between pre- and post-oats biopsies and was in agreement with patterns seen in previous analysis of Ki67, CD3 and CD8 expression in the same biopsies. However, this method did not provide any measure of what proportion of a tissue section was expressing each marker. Consequently, a marker could be expressed over a much larger area of one tissue section than another, but if the intensity of staining was similar in both, the first method of analysis would find no difference between the two sections. The second method of analysis was to measure the total area of the tissue section and the areas expressing the relevant marker. These figures were used to calculate the percentage area of the tissue section positively stained for each marker, providing a more accurate method of comparing expression between tissue sections.

Under normal circumstances, re-analysis would have required the cutting and staining of more tissue sections from all biopsies. However, one of the advantages of the IN Cell Analyzer 1000 system is that, once acquired, images can be saved indefinitely and reanalysed at any time in the future. Therefore, the sections that had been acquired previously were available for reanalysis and furthermore, similarly stained sections from Marsh grade 0 and Marsh grade 3 coeliac patients were also available for analysis using this methodology. It proved extremely valuable to have images from Marsh grade 0 and Marsh grade 3 biopsies available to provide parameters of tTG and SM α-actin expression in treated and untreated coeliac disease.

The adaptation of the IN Cell Analyzer 1000 allowed us to move from a qualitative, subjective method of analysis using only a general grading system (i.e. average, less than average, above average fluorescence) to a quantitative, objective method of analysis providing numerical results. This provided much more sophisticated and meaningful analysis of the expression of tTG and SM α-actin and made differences in the expression of these markers in untreated and treated CD very clear. It also allowed for the statistical analysis of expression levels in untreated and treated CD as well as pre- and post-oats biopsies. This had proved impossible via visual grading on a microscope. This methodology has the potential to be used for many different markers in many different experimental situations in coeliac disease. It also has the potential to be applied to many different pathologies and tissue types.

3.4.2 tTG and SM α-actin expression

The co-expression of tTG and SM α-actin by myofibroblasts has been previously observed in our department (Dunne et al, in preparation). Tissue transglutaminase plays a key role in coeliac disease and its expression has been reported to be moderately increased in

untreated coeliac biopsy tissue (Brusco et al. 1999; Di Sabatino et al. 2012). Although Brusco et al. reported that tTG expression was only slightly more marked in untreated coeliac mucosa, this was based on immunohistochemical staining and visual inspection only. In this study, using our quantitative technology we obtained a numerical measurement of the extent of tTG expression, this provided a much more sophisticated and reliable method of analysis. We also measured the expression of SM α-actin and the co-expression of tTG and SM α-actin in active and treated CD. For the first time we have shown that there is a statistically significant increase in both the individual expression and the co-expression of tTG and SM α-actin in myofibroblasts in untreated compared to treated coeliac mucosa.

Following the measurement of expression of tTG and SM α-actin in Marsh grade 0 and Marsh grade 3 coeliac biopsies and the establishment of parameters for expression in treated and untreated CD, the values obtained from pre- and post-oats biopsies could be compared not only to each other but also to these parameters. Not only was there no change in tTG or SM α-actin expression pre- versus post-oats but also the values obtained from both sets of biopsies were very similar to those of the Marsh grade 0 biopsies for both markers. These results supported the evidence of the lack of oats toxicity in coeliac patients. Although mucosal tTG expression is known to be increased in active CD, this has previously been ignored in the study of oat tolerance in CD (Gorgun et al. 2009; Almarzooqi, Houston, and Prasad 2013). Koskinen et al evaluated tTG-specific IgA deposits in paediatric jejunal biopsies prior to and following 24 months of oats ingestion and found no significant change in intensity of deposits as assessed by visual grading of staining intensity (Koskinen et al. 2009). Although this study used an entirely different type of analysis, the results are in line with those of our study.

3.4.3 Potential role of myofibroblasts in coeliac disease

In coeliac disease, the exact mechanism by which villi become flattened and crypts elongated is unknown. The co-expression by myofibroblasts of two of the major antigenic targets of coeliac autoantibodies, tTG and SM α-actin, suggests that they may have a role in CD pathogenesis. Fibroblasts and myofibroblasts were in the past regarded as passive connective tissue cells with the sole task of secreting extracellular matrix (Rogler et al. 2001). However, they are now known to secrete a wide spectrum of cytokines, growth factors, chemokines, hormones, neurotransmitters, inflammatory mediators, adhesion proteins and also to express receptors for many of these ligands (Powell et al. 2005). The exact expression profile is specific to the tissue location of fibroblasts and myofibroblasts and they have been shown to play a central role in the pathogenesis of various different pathologies including fibrotic diseases, infections, tumours and autoimmune disorders (Roncoroni et al. 2009). In the lung, myofibroblasts have been shown to play a role in asthma, idiopathic pulmonary fibrosis and cystic fibrosis (Zhang et al. 1996; Zhang, Howarth, and Roche 1996; W. T. Harris et al. 2013). They also have been shown to play a pivotal role in the pathogenesis of heart failure (Van Linthout, Miteva, and Tschöpe 2014).

In the gut, myofibroblasts have a range of functions that includes regulation of epithelial cell proliferation and differentiation, mucosal protection and wound healing, water and electrolyte transport, and extra cellular matrix metabolism. In inflammatory bowel disease, intestinal inflammation and neoplasia, the number of myofibroblasts is increased compared to normal mucosa, indicating that myofibroblasts play an important role in the intestinal immune system (Andoh et al. 2007; Powell et al. 2005). It has been reported that exposure to lipopolysaccharide (LPS), an important component of gram-negative bacteria, induces cell proliferation, collagen synthesis and production of proinflammatory mediators in lamina propria fibroblasts. There is also evidence that, following stimulation with LPS,

lamina propria fibroblasts produce cytokines, including TNFα, that induce barrier dysfunction in epithelial cells (Chakravortty and Kumar 1999). This is of interest since infection in infancy has been linked with an increased risk of CD development (Myléus et al. 2012). Therefore it is possible that infection may stimulate fibroblasts or myofibroblasts to secrete cytokines that cause loss of mucosal integrity and allow gliadin to cross into the lamina propria.

Cytokines, produced by a variety of cells, are known to play a role in the pathogenesis of CD and myofibroblasts are known to be a rich source of cytokines. Furthermore, following stimulation with cytokines, myofibroblasts produce MMP-1 and MMP-3, which are known to be upregulated in active CD and are likely to play a key role in the tissue remodeling process (Powell et al. 2005; Schuppan, Junker, and Barisani 2009; Mohamed et al. 2006). Additionally, increased levels of MMP-1 and MMP-3 mRNA have been found in myofibroblasts during active disease (Daum et al. 1999). Under normal circumstances, a balance between MMP and TIMP production controls the rate of ECM turnover (Andoh et al. 2007). In CD, the disruption of this balance is likely a key factor in the destruction of the mucosa. We have shown here that tTG expression is significantly elevated in myofibroblasts in active CD and tTG is known to be the target of the anti-endomysial antibody response (Dieterich et al. 1997). The possibility exists, therefore, that antibodies targeting tTG expressed in myofibroblasts may disrupt the balance between MMP and TIMP production, contributing to mucosal remodeling. In addition, the targeting of myofibroblasts by autoantibodies may trigger the release of cytokines, some of which may be able to stimulate the myofibroblast in an autocrine manner, as has been demonstrated in myofibroblasts in other organs (Gressner 1998).

3.4.4 Conclusion

In this study we were able to adapt technology designed exclusively for use with cell lines and use it to get meaningful results from tissue sections. The statistically significant differences in values acquired for tTG and SM α-actin expression in treated and untreated coeliac disease, along with the fact that tTG expression is known be increased in active CD, provide validation of the methodology.

The duodenal expression of tTG and SM α-actin by coeliac patients including oats in their diet has not been investigated previously. Here, we have measured the expression of both markers prior to and following one year of oats ingestion and found no significant differences in individual expression or co-expression of either marker pre- versus post-oats. These results provide further evidence of the lack of oats toxicity in coeliac patients.

Myofibroblasts are known to be a source of cytokines, MMPs and TIMPs, all of which play a role in the destruction of the coeliac mucosa. In this study we have demonstrated, for the first time, increased expression and co-expression by myofibroblasts of two of the major antigenic targets of coeliac autoantibodies, tTG and SM α-actin, in active CD. This provides further evidence that myofibroblasts may play a leading role in the pathogenesis of coeliac disease.

Chapter 4

Examination of coeliac biopsies following 24 hours *ex vivo* culture with PT avenin

4.1 Introduction

4.1.1 Organ culture in coeliac disease

Browning and Trier first successfully demonstrated the technique for culturing biopsies from adult human intestinal mucosa in 1969 (Browning and Trier 1969). It was modified from the method used by Trowell in the maintenance of rat organs and remains essentially the same today (Howdle 1984). The basis of the technique is the maintenance of the biopsy on the surface of the medium rather than submerged in it, as would be the case with cell culture, in an oxygen rich environment (Browning and Trier 1969).

Organ culture is a particularly useful tool in coeliac disease research as there is no proper animal model of this disease, although attempts have been made to produce a model in a number of species including mouse, rat, monkey and dog (Marietta, Schuppan, and Murray 2009; Lindfors et al. 2012). Cell lines have also been widely employed in coeliac disease research, however they lack the cell-to-cell interactions that biopsy sections bring (Stoven, Murray, and Marietta 2013).

It has been shown that many features of the coeliac mucosal immune response are reproduced after 24 hours of *ex vivo* gliadin challenge (L Maiuri et al. 1996). Organ culture has been used in coeliac disease research to elucidate immune mechanisms of its progression as well as the contributions of various cytokines (L Maiuri et al. 1996; Luigi Maiuri et al. 2003; Fina et al. 2008). It has demonstrated the involvement of IL-15 in the progression of the disease (L Maiuri et al. 2000). As biopsies contain a wide variety of cell types the issues open to investigation are numerous (Lindfors et al. 2012). Organ culture provides a valuable alternative to more invasive *in vivo* studies and allows control over factors affecting the tissue (Howdle 1984; L Maiuri et al. 2000). Additionally, several

biopsies may be obtained from one patient and cultured under a number of different conditions. This greatly increases the number of experiments that can be carried out per patient and also means that each biopsy will have its own, perfectly matched control.

4.1.2 Organ culture in oats studies

Very few studies of oat tolerance in CD have made use of organ culture. One such study reported an increase in CD25+ lymphocytes in jejunal biopsies cultured with PT avenin while in another a significant increase in IELs was seen in duodenal biopsies cultured similarly (Troncone et al. 1996; Maglio et al. 2011). Conversely, two further studies found no evidence of immune activation in coeliac duodenal biopsies cultured with PT avenin (Picarelli et al. 2001; Kilmartin et al. 2003).

4.1.3 Cytokeratin 20

Cytokeratin 20 (CK20), also known as keratin 20, is a member of the intermediate filament family of cytoskeletal filaments. It is a major keratin in the small intestine, particularly in the duodenum where it is expressed in the cytoplasm of epithelial cells. It is predominantly expressed in the enterocytes and goblet cells of the villi. Expression in the crypts is more patchy, with only a few of the undifferentiated cryptal cells expressing CK20. Under normal circumstances, CK20 plays a significant role in maintaining keratin filament organisation in the intestinal epithelium. Transgenic mice with mutations at a conserved Arg to His site (R80H) show collapse of intermediate filaments in enterocytes of the small intestine. The observation that CK20 expression is increased with differentiation has led to it being widely studied as a marker of differentiation in normal epithelium and cancerous tissue (R Moll, Schiller, and Franke 1990; Wildi et al. 1999; Chan et al. 2009; Roland Moll, Divo, and Langbein 2008; Zhou et al. 2003; Chen and Wang 2004).

4.1.4 E-cadherin

Epithelial-cadherin (E-cadherin) is a transmembrane protein of the adherens junctions of epithelial cells. It is a major mediator of cell-cell adhesion and as such plays a key role in the maintenance of tissue architecture and morphology. It is often down-regulated in colonic adenomas and carcinomas. In addition, reduced expression has been noted in areas of ulceration and in inflammatory bowel disease. It is well known that in untreated CD mucosal integrity is reduced. In line with this, mucosal expression of E-cadherin, as well as levels of E-cadherin mRNA, are reduced in untreated CD and return to normal in treated CD (Barshack et al. 2001; Ciccocioppo et al. 2006; Perry et al. 1999).

4.1.5 Soluble uPAR

Urokinase-type plasminogen activator receptor (uPAR, CD87) is a glycosylphosphatidylinositol (GPI)-linked membrane-bound protein. uPAR is found on a variety of cells including monocytes, activated T-lymphocytes, macrophages, endothelial cells, keratinocytes, fibroblasts, smooth muscle cells, megakaryocytes and some tumour cells. Its expression is elevated in times of stress, injury, inflammation, tissue remodeling and in many cancers. uPAR binds the serine protease urokinase-type plasminogen activator (uPA) which leads to the production of plasmin. This in turn degrades the extracellular matrix either directly or by activating matrix metalloproteinases. uPAR also activates many intracellular signalling pathways. Although the exact mechanisms of uPAR activity are not fully understood, it is known that upon binding with uPA, uPAR is cleaved from its GPI anchor and released into solution. Soluble uPAR (suPAR) is found in blood, serum, plasma, cerebrospinal fluid, urine and culture supernatants in various concentrations

depending on the activation level of the immune system (Thunø, Macho, and Eugen-Olsen 2009; Smith and Marshall 2010; Blasi and Sidenius 2010; Persson and Kjaer 2013).

In healthy individuals suPAR levels are quite stable. Elevated suPAR levels are found in individuals suffering from infections, autoimmune diseases and cancer. In all of these conditions the higher the concentration of suPAR the worse the prognosis of the disease is. However, suPAR levels are not specific to the individual disease but rather reflect the overall level of systemic inflammation and immune activation. suPAR is also a good marker of low grade inflammation and shows promise as a new inflammatory biomarker (Thunø, Macho, and Eugen-Olsen 2009).

4.1.6 Chapter aims

As a follow on to the *in vivo* oats study, the aim of this study was to examine the effects of 24 h of culture with PT avenin or PT gliadin on duodenal biopsies from coeliac and control patients. Following culture, biopsies were examined for any changes in the epithelial cytoskeleton using fluorescent staining and confocal microscopy. Levels of suPAR were measured in the culture supernatants by ELISA to look for any indication of inflammation, immune activation or tissue remodelling.

4.2 Materials and Methods

4.2.1 Prolamins

The prolamin fraction from wheat (gliadin) was a gift from the Working Group on Prolamin Analysis and Toxicity ("PWG-gliadin") and was prepared as described elsewhere (van Eckert et al. 2006). The prolamin fraction from oats (avenin) was a kind gift from Peter Koehler and was produced using the following method. Grains (ca. 200 g) of the German oats cultivar "Scorpion" (harvested in 2009; Nordsaat Saatzucht GmbH, Langenstein, Germany) were dehusked manually and milled on a laboratory mill (type KM 13, Bosch, Munich, Germany). 80 g of oats flour was then defatted with a mixture of *n*-pentane/ethanol (95/5, v/v; 3 x 250 ml) and *n*-pentane (250 ml) by stirring for 30 min and centrifugation at 3550*g* for 15 min @ 20°C. The supernatants were discarded and the defatted flour was dried *in vacuo* in a desiccator for 16 h overnight. 60 g of defatted oats flour was stepwise extracted with buffered salt solution (NaCl 0.4 mol/L / Na_2HPO_4/KH_2PO_4 0.067 mol/L (pH 7.6); 3 x 200 ml) and aqueous ethanol (60%, v/v; 3 x 200 ml) by homogenizing with an "Ultra Turrax" for 5 min at RT and centrifugation at 3550*g* for 30 min, @ 4°C. The three ethanol extracts were combined, concentrated to approximately 450 ml using a vacuum evaporator at 30°C, dialysed for two days against acetic acid (0.01 mol/L) and one day against distilled water and then freeze-dried.

4.2.2 Peptic-tryptic prolamin digests

Avenin or PWG-gliadin (0.5 g material) was suspended in 25 ml dH_2O and the pH was adjusted to 1.8 with HCl (1.0 mol/L) (Frazer et al. 1959). Then, 50 mg of pepsin (no. 7192, Merck, Darmstadt, Germany) was added and stirred for 2 h @ 37°C. After adjusting the pH to 7.8 with NaOH (1.0 mol/L), 50 mg of trypsin (no. 24579, Merck, Darmstadt,

Germany) was added and stirred for another 2 h at 37°C. Finally, the pH was adjusted to 7.0 with HCl (1.0 mol/L), the peptic-tryptic digest was centrifuged at 4000*g* for 20 min @ 20°C, the supernatant was decanted, and the digest was freeze-dried.

4.2.3 Re-suspension of prolamins

In preparation for culture, 5 mg/ml PT (peptic tryptic) gliadin and avenin were re-suspended separately in RPMI-1640 (Bio-Sciences, Ireland) supplemented with 15% heat inactivated, filtered foetal calf serum (Sigma) and a 1% antibiotic/antimycotic solution (Bio-Sciences). 2ml aliquots of re-suspended PT gliadin or re-suspended PT avenin were frozen at -20°C and allowed to defrost and acclimatise to RT prior to use.

4.2.4 Patients

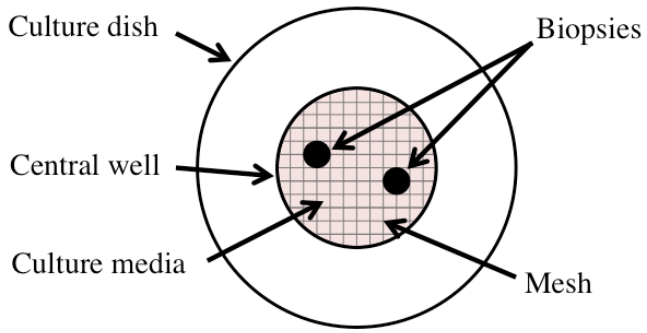
Patients were recruited from the clinical services and the Endoscopy Unit in St. James's Hospital. Six control patients, with clinical details suggestive of a healthy duodenum, were recruited and 8 duodenal biopsies were taken from each. Five treated coeliac patients were recruited and, in addition to 8 duodenal biopsies, blood for tTG analysis was taken from each of these patients. Details of all 11 patients are given in Table 4.2. Two biopsies from each patient were placed directly into formalin as Time 0 controls. The other 6 biopsies were placed in pairs into three containers of sterile RPMI which had been warmed to roughly 37°C.

4.2.5 Ethics

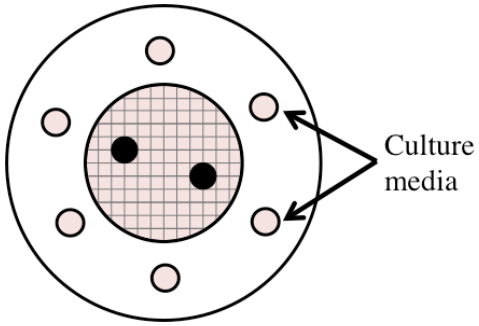
The study was approved by the St. James's Hospital Ethics Committee and all patients gave informed consent.

4.2.6 Organ Culture

Pairs of biopsies collected in tubs of RPMI were orientated villous side up on a nylon mesh filter (Millipore, U.S.A.). 1.5 ml of PT avenin, PT gliadin or RPMI was added to the central well of a culture dish (Thermo Fisher Scientific, Ireland) and the filter with biopsies was placed in the central well so that the biopsies were in contact with the surface of the media (Fig. 4.1). Care was taken to ensure there were no bubbles trapped between the media and the mesh. The remainder of the aliquot of PT avenin, PT gliadin or RPMI was dotted around the outer well of the culture dish to keep the atmosphere humid and prevent the biopsies drying out during incubation. Culture dishes were placed in a modular incubator chamber (Billups-Rothenberg, USA), gassed with 95% O2 / 5% CO2 and placed in a 37°C incubator for 24 h.



A



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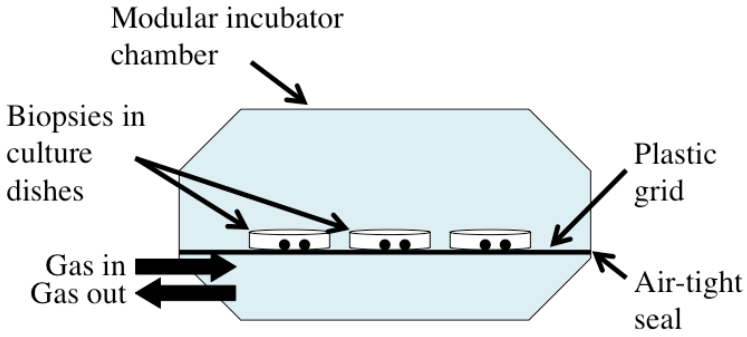


C

Figure 4.1: Organ culture. Biopsies, orientated villous side up, on a nylon mesh, floating on culture media in the central well of a culture dish (A), remainder of the culture media dotted around the central well (B), biopsies in culture dishes in a modular incubator chamber, being gassed with 95% O_2 / 5% CO_2 (C).

Following 24 h of culture, supernatants were aspirated from culture dishes, transferred to microcentrifuge tubes (Thermo Fisher Scientific, Ireland) and stored at -20°C for later analysis. Biopsies were transferred to histology cassettes and immersed in formalin. All 8 biopsies were formalin fixed and paraffin embedded in the Histology Department. Sections were cut from the embedded biopsies as described in Chapter 3 (Section 3.2.1). Cut slides were H&E stained in the Histology Department to check biopsies were orientated correctly in paraffin.

4.2.7 Immunofluorescent staining

Tissue sections were stained as per the procedure described in Chapter 3 (Section 3.2.1). Sections were incubated with a mouse monoclonal antibody to E-cadherin conjugated to Alexafluor^{488} and a rabbit monoclonal antibody to Cytokeratin 20 (Abcam, UK) at 4°C, overnight. Sections were then incubated with an Alexafluor^{568}-conjugated secondary GAR antibody (BioSciences, Ireland) for 1 h at RT. Negative control slides, with tissue sections that were incubated with PBS instead of Cytokeratin 20 and a mouse monoclonal antibody to α-actin conjugated to Alexafluor^{488} instead of E-cadherin conjugated to Alexafluor^{488}, were used to check that staining was specific. Details of all antibodies are given in Table 4.1. All sections were examined and imaged under a confocal microscope (Carl Zeiss MicroImaging GmbH, Germany) at 63X magnification.

Antibodies used in Immunofluorescent Staining						
Antibody	**Clonality**	**Source**	**Ag Retrieval**	**Dilution**	**Stock conc.**	**Incubation**
Cytokeratin 20	m	Abcam	Citrate Buffer	1/100	-	4°C, O/N
Alexafluor^{568} GAR	N/A	BioSciences	N/A	1/200	2 mg/ml	1 h, RT
E-cadherin	m	BD Biosciences	Citrate Buffer	1/20	50 μg/ml	4°C, O/N
α-actin	m	Sigma	Citrate Buffer	1/100	-	1 h, RT

Table 4.1: Details of antibodies used in immunofluorescent analysis organ culture biopsies. Ag = antigen; conc. = concentration; m = monoclonal; O/N = overnight; GAR = goat-anti-rabbit; N/A = not applicable; mg/ml = milligram per millilitre; RT = room temperature; μg/ml = microgram/millilitre.

4.2.8 ELISA

suPAR ELISA was carried out using a Human suPAR Quantikine ELISA kit (R&D Systems, USA). suPAR standard was re-suspended in dH_2O and serial dilutions were used to prepare a range of standards from 4000 – 62.5 pg/ml. Organ culture supernatants were diluted 5-fold in calibrator diluent. Assay diluent was added to each well and all standards and supernatants were added to the plate in duplicate and incubated for 2 h at RT. The plate was then washed 4 times with wash buffer and suPAR conjugate was added to each well and allowed to incubate for 2 h at RT. Following a further 4 washes, substrate solution was added and incubated for 30 min at RT in the dark. Stop solution was added

and the optical density of each well was determined on a microplate reader set to 450 nm. Duplicate readings were averaged and the zero standard subtracted. Using Microsoft Excel, the absorbance value of each standard was plotted against the concentration to create a standard curve. The concentration of each sample was determined from the sample curve and multiplied by 5 as samples were diluted 5-fold.

4.2.9 Statistics

GraphPad Prism was used for statistical analysis of ELISA results. A one-way repeated measures ANOVA was used to compare suPAR expression in coeliac patients across all culture conditions. The student's two-tailed t test was used to compare suPAR expression in control and coeliac patients under individual culture conditions. P values <0.05 were considered statistically significant.

4.3 Results

4.3.1 Patients

Control and treated coeliac patients were recruited from the clinical services and the Endoscopy Unit in St. James's Hospital. The 6 control patients (3M, 3F) were undergoing OGD for a variety of reasons and in all the histology report was of normal duodenal mucosa. The 5 coeliac patients (3M, 2F) were also undergoing OGD for a variety of reasons and in all their most recent histology report was of treated coeliac disease. Although the 5 coeliac patients were selected on the basis of having treated CD, in 2 patients there had been mucosal deterioration since the previous histology report. A further patient was undergoing a Barrett's review and therefore no duodenal biopsies were sent for routine histological examination, however a duodenal biopsy taken less than two years previously showed normal duodenal mucosa. All coeliac patients had negative tTG serology. Details of all patients are given in Table 4.2.

Pt. No.	N/CD	Sex	Age	Indication for OGD	Most recent tTG serology	Histology report
OC1	N	F	36.7	Bowel changes. Family history of colon cancer	-	Normal duodenal mucosa
OC2	N	F	60.1	Acid reflux, epigastric pain	-	Normal duodenal mucosa
OC3	N	M	17.6	Heartburn x52 weeks	-	Normal duodenal mucosa
OC4	N	M	52.7	Dyspepsia	-	Normal duodenal mucosa
OC5	N	F	67.0	Helicobacter pylori	-	Normal duodenal mucosa
OC6	N	M	56.4	Abdominal pain, dyspepsia	-	Normal duodenal mucosa
OC7	CD	M	75.9	Barrett's review	1.1	Not done. Most recent report: normal duodenal mucosa
OC8	CD	F	66.3	Treated CD, recent dyspepsia	1.7	Normal duodenal mucosa
OC9	CD	F	75.2	CD, recent iron deficiency anaemia & diarrhoea	0.6	Duodenal mucosa, some of which is flat & other areas show stubby villi. There are increased numbers of IELs present
OC10	CD	M	52.2	Treated CD, moderate gastritis	1.1	Duodenal mucosa with mild villous blunting associated with crypt hyperplasia. IELs markedly increased. Marsh grade 3a
OC11	CD	M	66.6	Research	2.1	Histology within normal limits, consistent with TCD

Table 4.2: Organ culture patient details. Pt. no. = patient number; N = normal; CD = coeliac disease; OGD = oesophagogastroduodenoscopy; tTG = tissue transglutaminase; OC = organ culture; M = male; F = female; IEL = intraepithelial lymphocyte; TCD = treated coeliac disease.

4.3.2 General effects of culture

The culture process affected all biopsies, regardless of whether they came from a control or a coeliac patient or how they were cultured. This was obvious both with the naked eye and microscopically (Fig. 4.2). At the time of endoscopy, although tiny, biopsies were plump and rounded. Time 0 biopsies were placed directly into formalin and left there for 24 h while the other biopsies were being cultured. After 24 h the Time 0 biopsies still looked nicely plump and rounded. The other biopsies were placed briefly into RPMI and then cultured for 24 h sitting on a mesh filter, which was floating on top of, but not submerged in, culture media. After 24 h these biopsies were visibly flattened and generally difficult to separate from the mesh filter. The effects of this were clear when tissue sections were stained with H&E. Time 0 biopsies had elongated villi, while in all other biopsies the villi were generally much shorter and wider and were often folded over on themselves. Enterocytes were also observed to be much shorter in cultured biopsies compared to Time 0 biopsies.

These changes are likely to be due to a number of factors including the general effects of gravity and the loss of blood supply. In addition, cells may have actively migrated towards the mesh due to the natural tendency of the cells to adhere to a solid surface and in response to the presence of the culture media.

Despite this, the enterocytes of many cultured biopsies looked healthy and, although short, were lined up nicely next to each other and had nicely organised nuclei. This was not the case in all biopsies, with some having disorganised enterocytes and nuclei with enterocytes of extremely short stature.

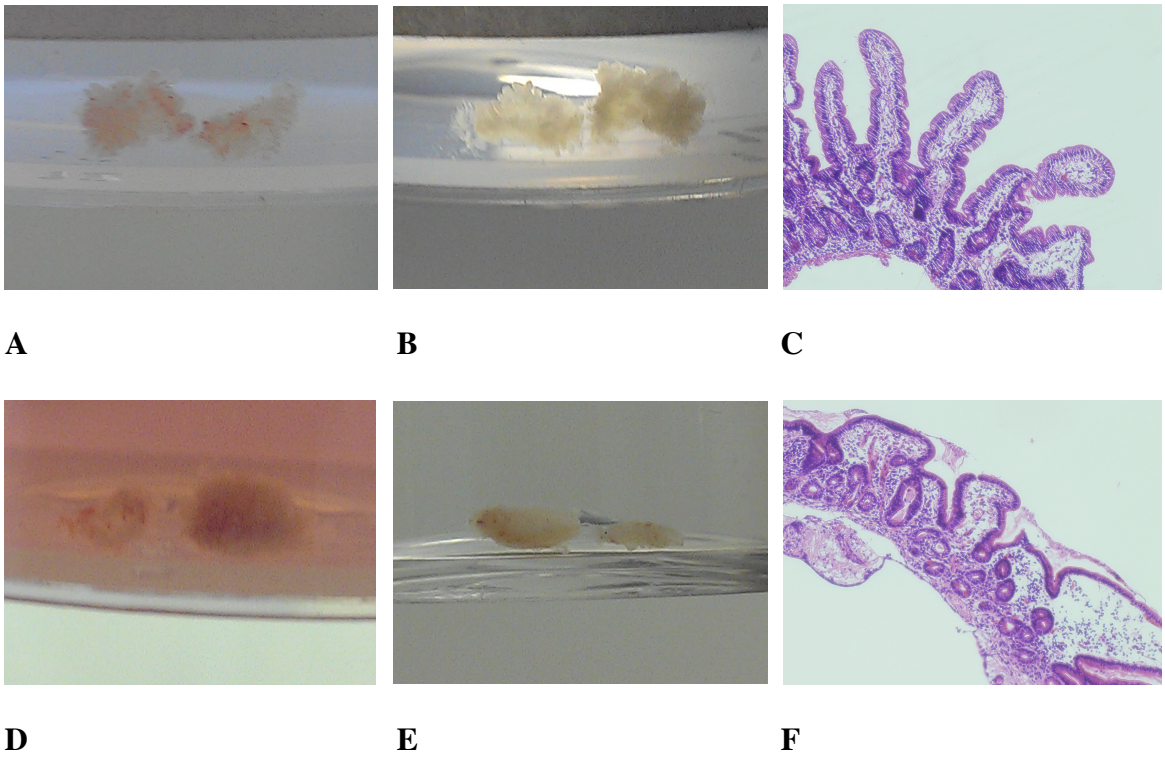


Figure 4.2: Effect of gravity during 24h in culture. Time 0 biopsies from a control patient in formalin shortly after collection (A), the same Time 0 biopsies in formalin 24h later (B), H&E stain of one of the Time 0 biopsies (C), biopsies from the same control patient in RPMI shortly after collection (D), the same biopsies in formalin, following 24h in culture (E), H&E stain of one of the cultured biopsies (F).

4.3.3 Immunofluorescent staining and confocal microscopy

Controls

To confirm visible staining was specific and not simply background fluorescence, a negative control slide was included in each staining run. In place of the directly labelled E-cadherin antibody, a directly labelled α-actin antibody was added to this slide. Like the E-cadherin antibody, the α-actin antibody was a mouse monoclonal antibody, directly conjugated to Alexafluor^{488}. This antibody produced no positive staining along the epithelium (Fig. 4.3). In place of the Cytokeratin 20 primary antibody, PBS was added to this slide, followed by the Alexafluor^{568}-conjugated secondary GAR antibody as usual. This produced only some non-specific background fluorescence.

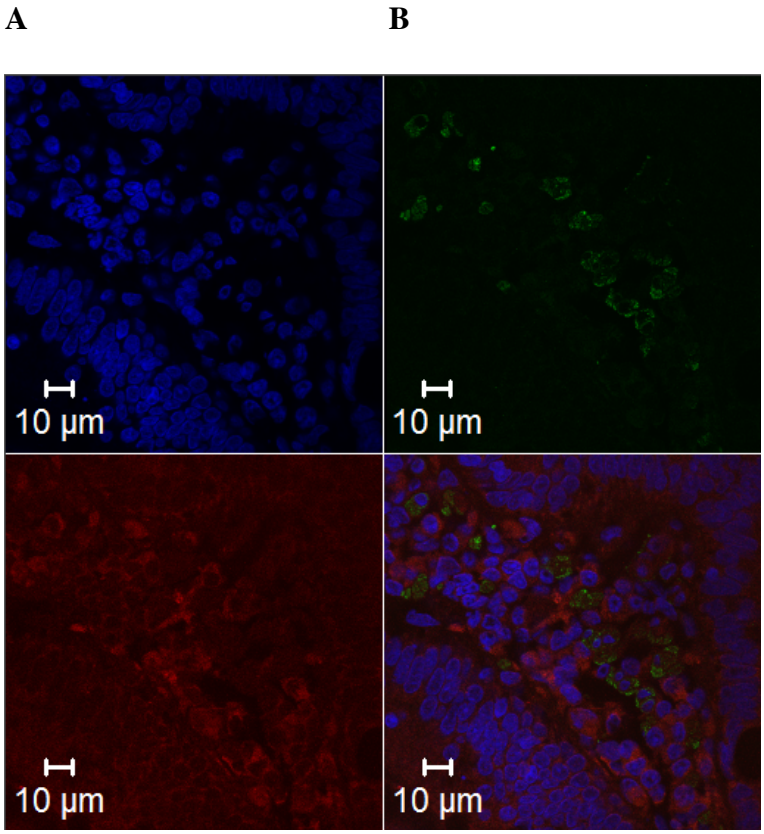
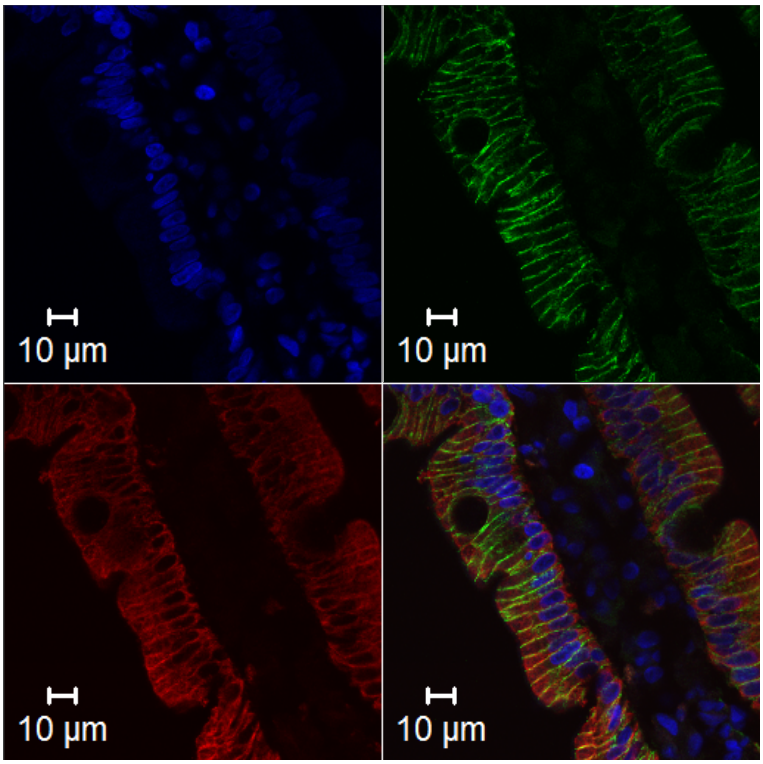


Figure 4.3: Negative controls. Hoechst nuclear stain (A), α-actin mouse monoclonal antibody directly conjugated to Alexafluor^{488} (B), Alexafluor^{568}-conjugated secondary GAR antibody (C), merged image of all 3 channels (D).

Individual staining patterns

In healthy tissue sections, a very orderly pattern of E-cadherin staining was observed (Fig. 4.4). A narrow line of bright green staining was visible between enterocytes, extending the full length of the enterocytes. Cytokeratin 20 staining was observed within enterocytes, encircling the nucleus and extending throughout the entire enterocyte cell.

A **B**

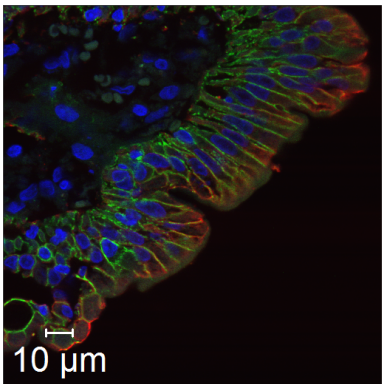


C **D**

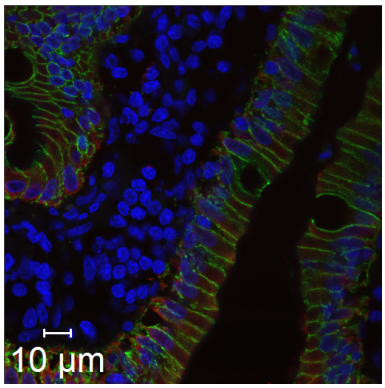
Figure 4.4: Individual staining patterns. Hoechst nuclear stain (A), E-cadherin stain (B), cytokeratin 20 stain (C), merged image of all 3 stains (D).

Control patients

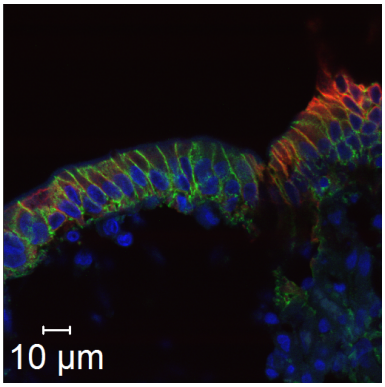
All Time 0 biopsies from control patients displayed healthy, elongated enterocytes (Fig. 4.5 – 4.10). Orderly patterns of E-cadherin staining were present with narrow, generally straight lines of green fluorescence visible between enterocytes. In some biopsies cytokeratin 20 staining was visible throughout the enterocytes, however, in others the quality of staining was not as good. Following 24 h in culture, enterocytes were considerably shorter, regardless of the culture conditions. However, patterns of E-cadherin expression remained similar to that observed at Time 0. Again, cytokeratin 20 staining was good in some biopsies but in others was too inconsistent to draw any conclusions. Nonetheless, enterocytes and nuclei remained organised and no patient showed any signs of a reaction to culture with PT gliadin or PT avenin.



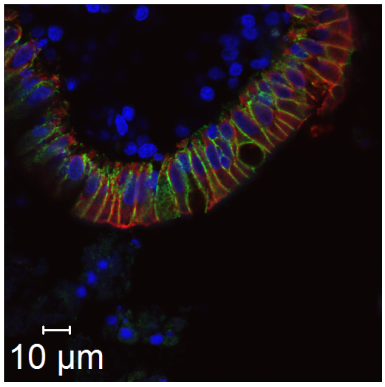
Time 0



RPMI 24 h

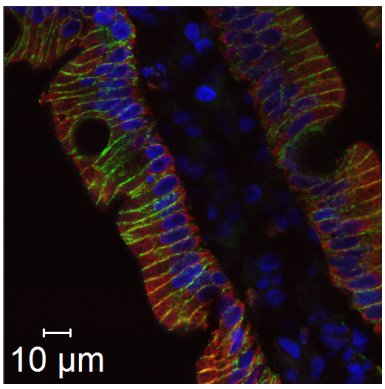


Gliadin 24 h

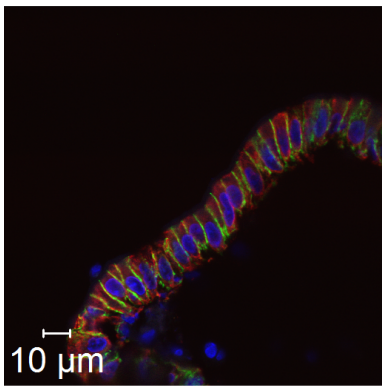


Avenin 24 h

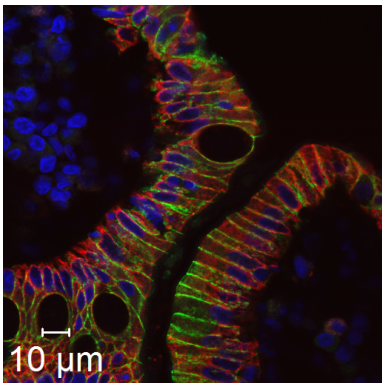
Figure 4.5: Control patient OC1. E-cadherin (green), cytokeratin 20 (red) and Hoechst (blue) staining at Time 0 and after 24 hours in culture with RPMI, PT gliadin or PT avenin.



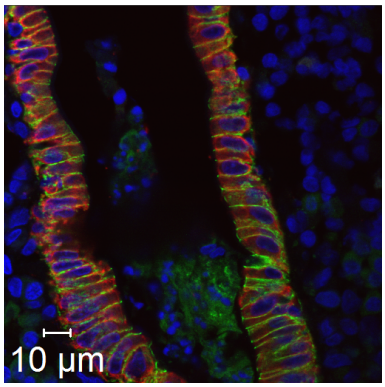
Time 0



RPMI 24 h

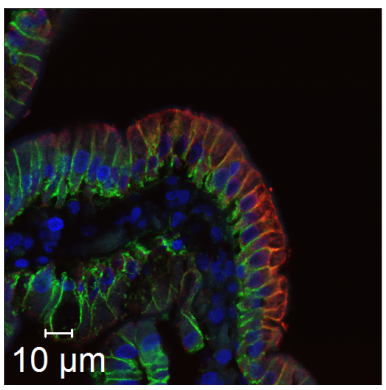


Gliadin 24 h

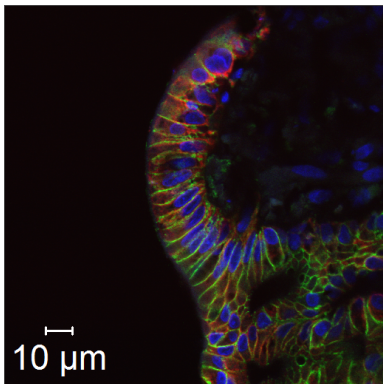


Avenin 24 h

Figure 4.6: Control patient OC2. E-cadherin (green), cytokeratin 20 (red) and Hoechst (blue) staining at Time 0 and after 24 hours in culture with RPMI, PT gliadin or PT avenin.



Time 0



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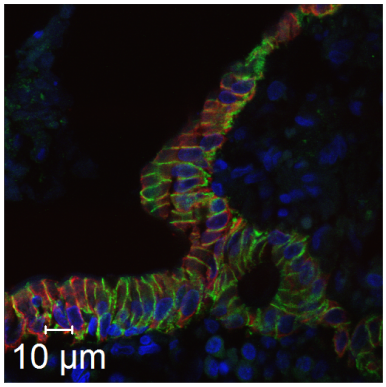
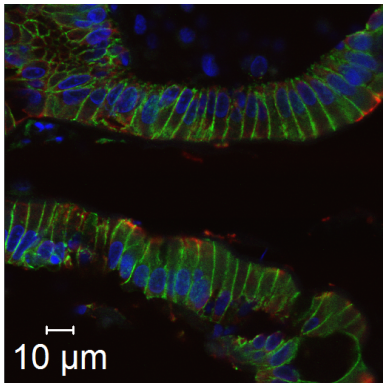
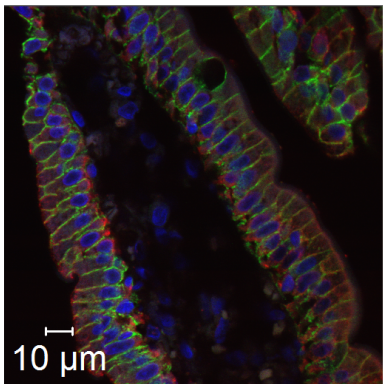


Gliadin 24 h

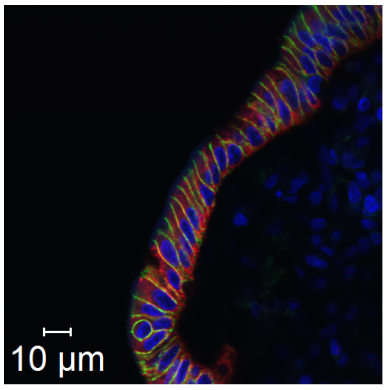


Avenin 24 h

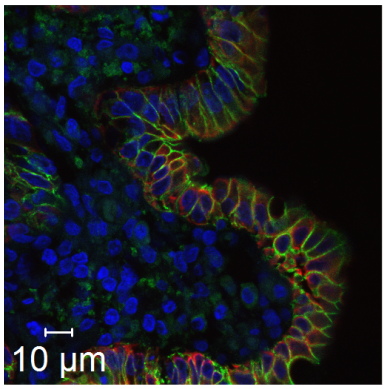
Figure 4.7: Control patient OC3. E-cadherin (green), cytokeratin 20 (red) and Hoechst (blue) staining at Time 0 and after 24 hours in culture with RPMI, PT gliadin or PT avenin.



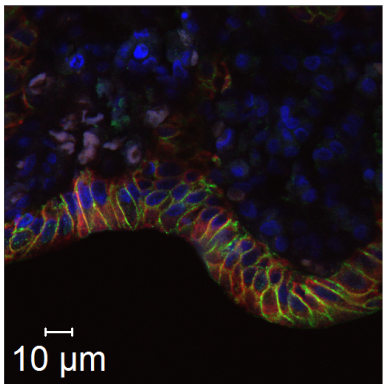
Time 0



RPMI 24 h

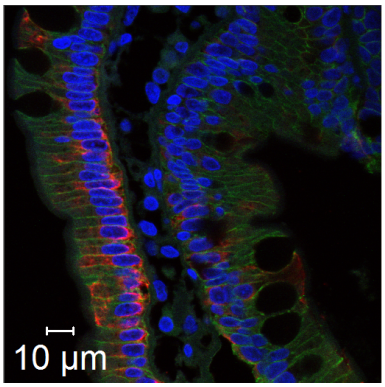


Gliadin 24 h

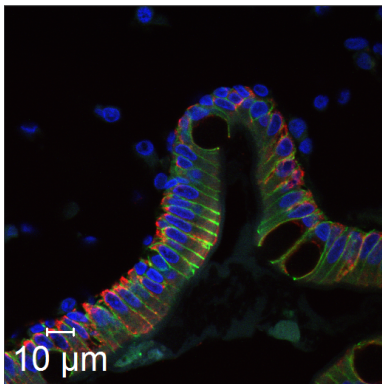


Avenin 24 h

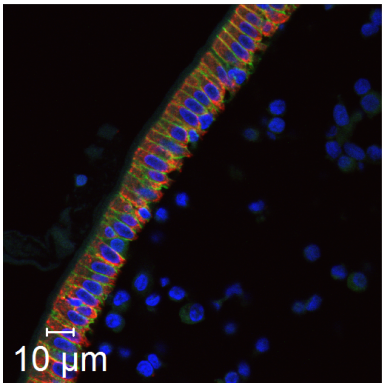
Figure 4.8: Control patient OC4. E-cadherin (green), cytokeratin 20 (red) and Hoechst (blue) staining at Time 0 and after 24 hours in culture with RPMI, PT gliadin or PT avenin.



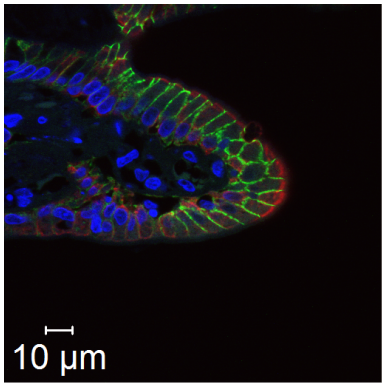
Time 0



RPMI 24 h

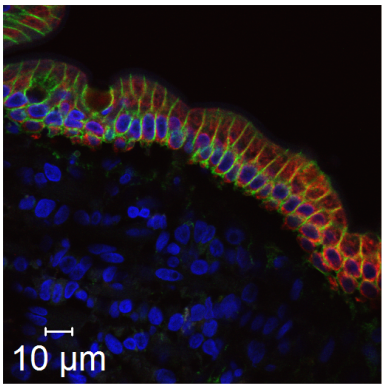


Gliadin 24 h

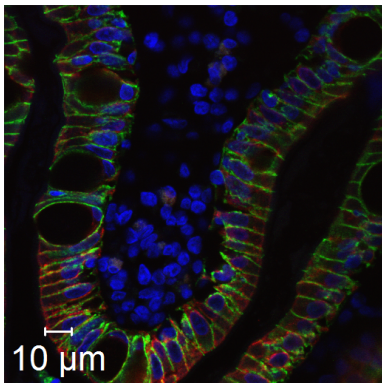


Avenin 24 h

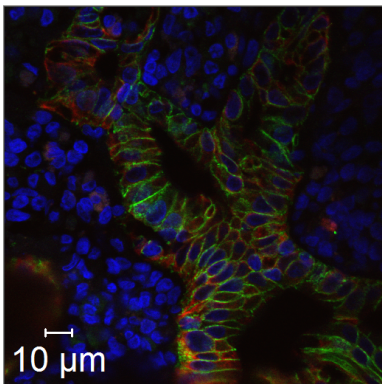
Figure 4.9: Control patient OC5. E-cadherin (green), cytokeratin 20 (red) and Hoechst (blue) staining at Time 0 and after 24 hours in culture with RPMI, PT gliadin or PT avenin.



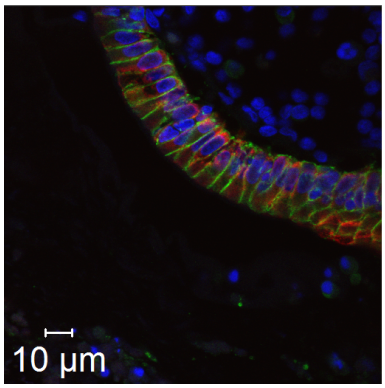
Time 0



RPMI 24 h



Gliadin 24 h



Avenin 24 h

Figure 4.10: Control patient OC6. E-cadherin (green), cytokeratin 20 (red) and Hoechst (blue) staining at Time 0 and after 24 hours in culture with RPMI, PT gliadin or PT avenin.

Coeliac patients

At Time 0, three of the five coeliac patients (OC7, OC8 and OC11) were reported by the Histology Department to have a mucosa consistent with that of treated CD (Fig. 4.11, 4.12, 4.15). These patients displayed elongated, generally organised enterocytes with E-cadherin staining visible between enterocytes. As with the control biopsies, cytokeratin 20 staining was of poor quality. Two patients (OC9 and OC10) were reported by the Histology Department to have increased IELs and villous blunting (Fig. 4.13, 4.14). Nonetheless, these patients displayed elongated enterocytes although these were less organised and fluorescent staining was less well defined.

Following culture with RPMI, all 5 coeliac patients displayed shortened enterocytes, although the degree of shortening varied between patients. Nevertheless, strong E-cadherin staining was still visible between enterocytes. Again, cytokeratin 20 staining was of poor quality.

After 24 h culture in the presence of PT gliadin four out of five patients (OC7, OC9, OC10 and OC11) showed signs of damage. In addition to shortening of enterocytes, E-cadherin staining was less consistent. Although some areas of biopsies still displayed healthy looking enterocytes, in other areas the pattern of E-cadherin expression was altered, with loss of the nice, straight line of green E-cadherin staining between enterocytes. Cytokeratin 20 expression was variable, but as that was the case in all biopsies, this did not provide any additional information.

Twenty-four hours culture in the presence of PT avenin did not appear to have any significant affect on biopsies from two of the 5 coeliac patients (OC8 and OC11). Similarly

to culture with RPMI, enterocytes were shortened but remained generally healthy looking, were nicely arranged next to each other and had good expression of E-cadherin. In contrast, biopsies from three of the coeliac patients (OC7, OC9 and OC10) showed evidence of change in response to culture with PT avenin. Similarly to the majority of biopsies following culture with PT gliadin, there were some healthy looking enterocytes in these biopsies but also areas where enterocytes were less well organised and in particular lacked the consistent expression of E-cadherin between enterocytes that was evident prior to culture.

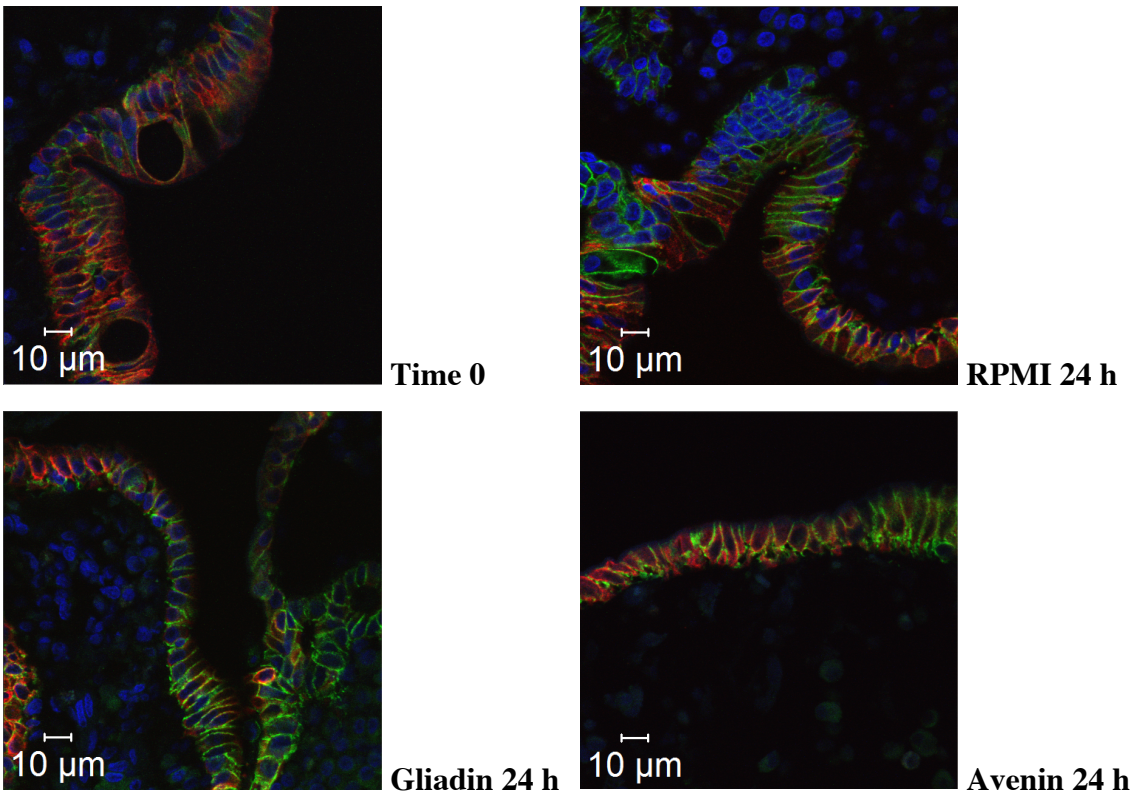
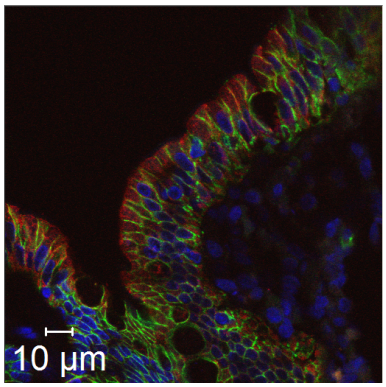
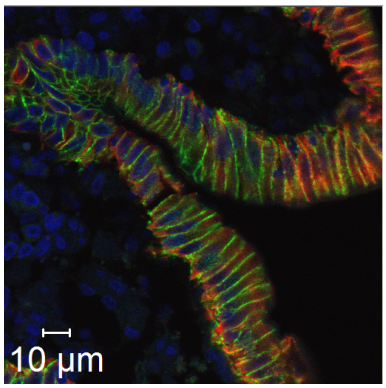


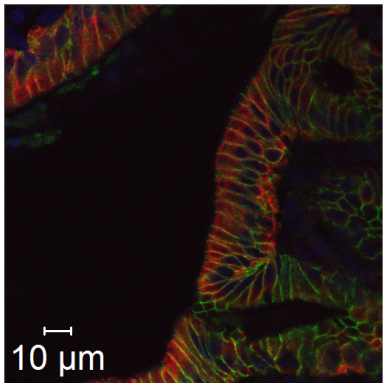
Figure 4.11: Coeliac patient OC7. E-cadherin (green), cytokeratin 20 (red) and Hoechst (blue) staining at Time 0 and after 24 hours in culture with RPMI, PT gliadin or PT avenin.



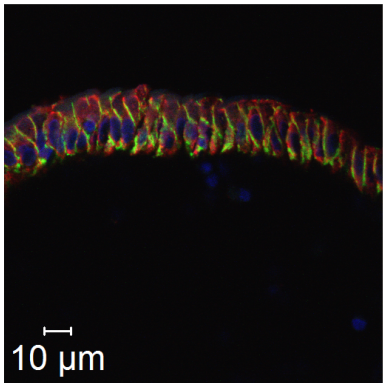
Time 0



RPMI 24 h

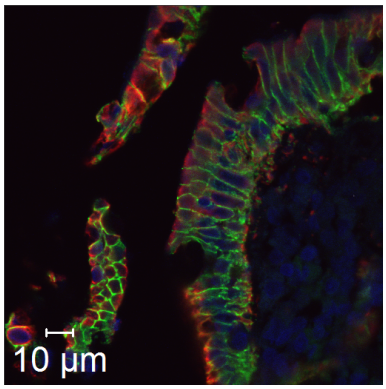


Gliadin 24 h

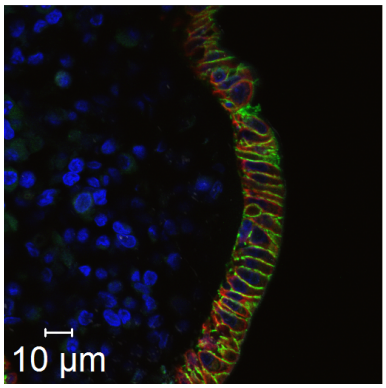


Avenin 24 h

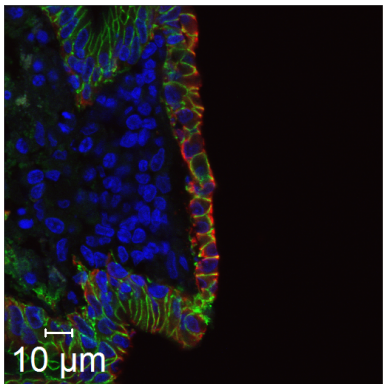
Figure 4.12: Coeliac patient OC8. E-cadherin (green), cytokeratin 20 (red) and Hoechst (blue) staining at Time 0 and after 24 hours in culture with RPMI, PT gliadin or PT avenin.



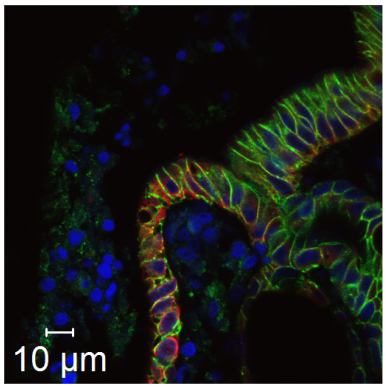
Time 0



RPMI 24 h

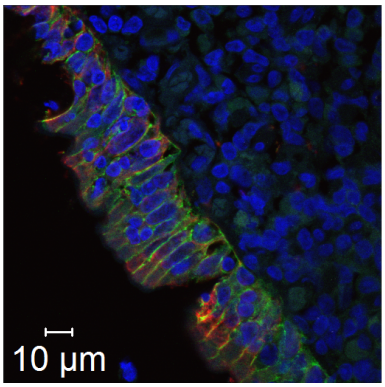


Gliadin 24 h

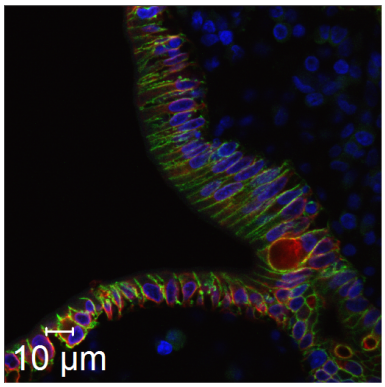


Avenin 24 h

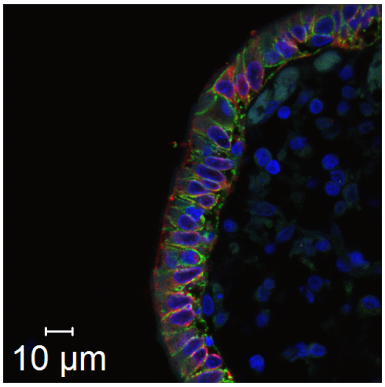
Figure 4.13: Coeliac patient OC9. E-cadherin (green), cytokeratin 20 (red) and Hoechst (blue) staining at Time 0 and after 24 hours in culture with RPMI, PT gliadin or PT avenin.



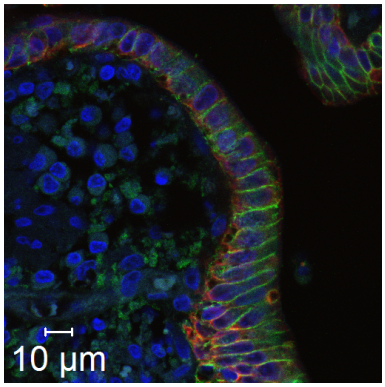
Time 0



RPMI 24 h

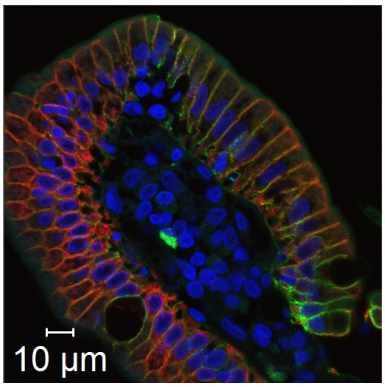


Gliadin 24 h

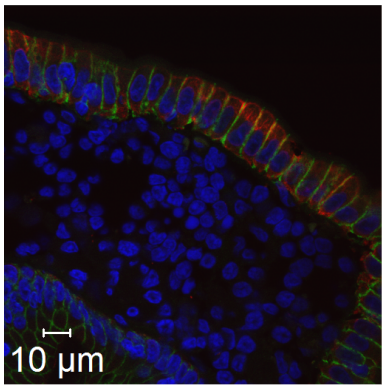


Avenin 24 h

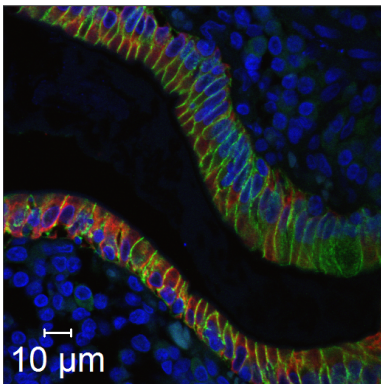
Figure 4.14: Coeliac patient OC10. E-cadherin (green), cytokeratin 20 (red) and Hoechst (blue) staining at Time 0 and after 24 hours in culture with RPMI, PT gliadin or PT avenin.



Time 0



RPMI 24 h



Gliadin 24 h

Avenin 24 h

Figure 4.15: Coeliac patient OC11. E-cadherin (green), cytokeratin 20 (red) and Hoechst (blue) staining at Time 0 and after 24 hours in culture with RPMI, PT gliadin or PT avenin.

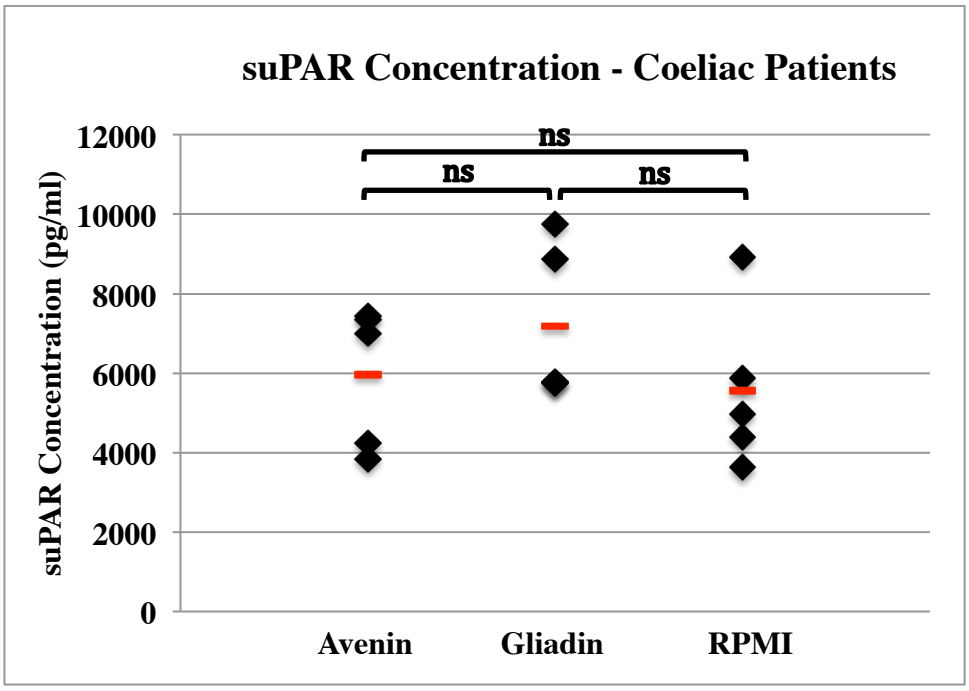
4.3.4 ELISA

The concentration of suPAR was assessed in all organ culture supernatants using ELISA. Individual results are given in Table 4.3.

Patient	Status	Avenin	Gliadin	RPMI
OC1	N	3417	609	1280
OC2	N	3013	2387	2643
OC3	N	2696	5384	2252
OC4	N	4614	3548	4326
OC5	N	4512	2478	1609
OC6	N	3482	3054	1865
OC7	CD	7438	9743	8924
OC8	CD	4244	5779	3627
OC9	CD	6985	5775	5858
OC10	CD	7348	8863	4396
OC11	CD	3832	5746	4973

Table 4.3: Individual suPAR concentrations (pg/ml). ELISA measurement of suPAR concentration in each organ culture supernatant. N = normal, control patient; CD = coeliac patient; suPAR = soluble urokinase-type plasminogen activator receptor; pg/ml = picogram per millilitre.

A one-way repeated measures ANOVA was used to test for significant differences in concentration of suPAR between supernatants from coeliac biopsies cultured with PT avenin, PT gliadin or RPMI. There were no significant differences in concentration of suPAR in coeliac biopsy supernatants under the 3 different culture conditions, [$F(2, 4) = 2.57$, $p = 0.14$] (Fig. 4.16).



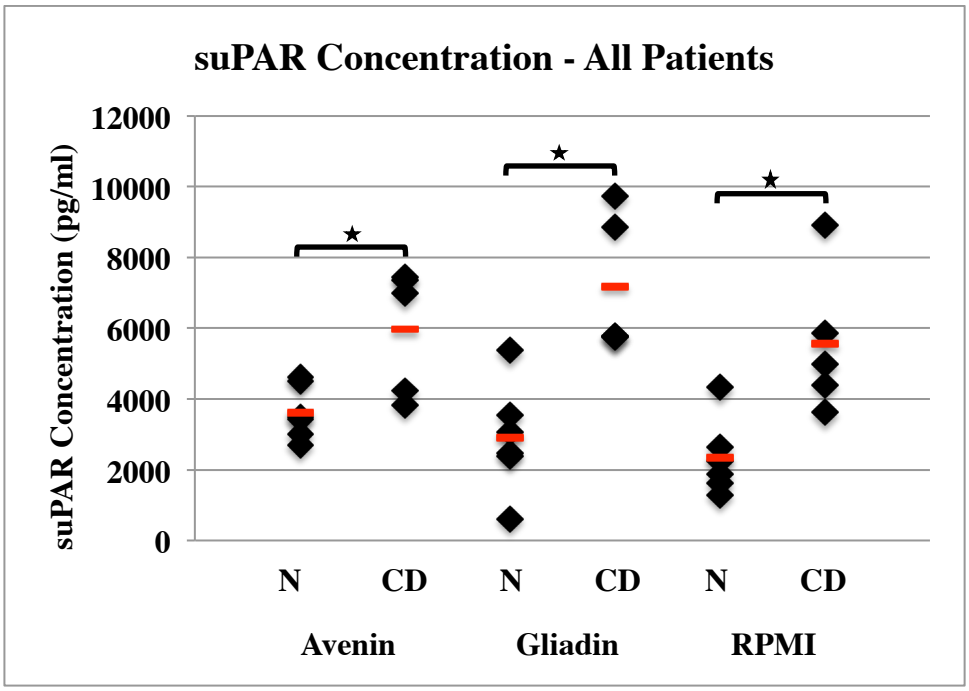
A

Culture condition	suPAR mean concentration (pg/ml)	SD	95% CI
PT avenin	5970	1777	3763 – 8176
PT gliadin	7181	1962	4746 – 9617
RPMI	5555	2052	3007 – 8103

B

Figure 4.16: suPAR concentration in organ culture supernatants. Individual (black diamonds) and mean (red dashes) measures of suPAR concentration in supernatants of coeliac patient biopsies under each culture condition (A), ANOVA statistical analysis of suPAR concentration in supernatants of coeliac patient biopsies under 3 different culture conditions (B). suPAR = soluble urokinase-type plasminogen activator receptor; pg/ml = picogram per millilitre; ns = not significant; SD = standard deviation; CI = confidence interval.

For each culture condition, the student's unpaired two-tailed t test was used to check for differences in suPAR concentration between supernatants from control and coeliac biopsies. Under all culture conditions, mean suPAR concentration was greater in supernatants from coeliac biopsies compared to supernatants from control biopsies (Fig. 4.17). For culture with PT avenin the mean concentration of suPAR in control supernatants was 3622 ± 783 pg/ml and in coeliac supernatants was 5970 ± 1777 pg/ml, $p = 0.017$. Following culture with PT gliadin, the mean concentration of suPAR was 2910 ± 1569 pg/ml in control supernatants and 7181 ± 1962 pg/ml, in coeliac supernatants, $p = 0.003$. Culture with only RPMI also produced significantly different results, with suPAR with concentrations of 2329 ± 1089 pg/ml and 5555 ± 2052 pg/ml in control and coeliac supernatants respectively, $p = 0.009$.



A

Culture condition	Control supernatants	Coeliac supernatants	95% CI	p-value
PT avenin	3622 ± 783 pg/ml	5970 ± 1777 pg/ml	538 to 4156	0.017
PT gliadin	2910 ± 1569 pg/ml	7181 ± 1962 pg/ml	1868 to 6674	0.003
RPMI	2329 ± 1089 pg/ml	5555 ± 2052 pg/ml	1047 to 5405	0.009

B

Figure 4.17: suPAR concentration in organ culture supernatants. Individual (black diamonds) and mean (red dashes) measures of suPAR concentration in culture supernatants of each control and coeliac patient under each culture condition (A), statistical analysis of suPAR concentration by control and coeliac patient biopsies under 3 different culture conditions using the student's two-tailed t test. Results are expressed as the mean of each group ± the standard deviation (B). suPAR = soluble urokinase-type plasminogen activator receptor; pg/ml = picogram per millilitre; * = significantly different; N = normal, control patient; CD = coeliac patient; CI = confidence interval.

4.4 Discussion

4.4.1 Advantages of organ culture

The aim of this study was to expand upon the oats feeding study by culturing duodenal biopsies from treated coeliac and control patients in the presence of PT gliadin or PT avenin. This complemented the oats feeding study by providing a number of features that were not possible in an *in vivo* study. Following one OGD procedure for each patient it was possible to examine the effects of exposure to both gliadin and avenin and to compare these results to uncultured biopsies from the same patient. In an *in vivo* setting this would have necessitated multiple OGD procedures in the same patient. Aside from the ethical issues this would involve, it would be very unattractive and unpleasant for the patient. In addition, as all biopsies were taken at the same time point there was no risk of deterioration of the mucosa between the acquisition of biopsies, as could be the case in an *in vivo* study requiring biopsies to be taken at a number of time points.

It is likely that avenin is less immunogenic than gliadin, hordein and secalin due to oats being less closely related to wheat, barley and rye. In addition, avenin represents only a very small proportion of the proteins present in oats while gliadin, hordein and secalin can account for as much as 50% of the proteins in wheat, barley and rye respectively. Therefore, it may be necessary for coeliac patients to consume a large quantity of oats before a toxic threshold is reached (Garsed and Scott 2007; Koning 2012). The design of this study compensated, to some extent, for these issues. During culture, biopsies were exposed to equal concentrations of PT gliadin or PT avenin. Furthermore, while the average small intestinal transit time is estimated to be between four and five hours and immune activation has been demonstrated in coeliac biopsies after four hours culture with

PT gliadin, in this study biopsies were cultured with PT gliadin or PT avenin for a full 24 hours (Worsøe et al. 2011; Fireman et al. 2007; Kilmartin et al. 2003).

4.4.2 Patients

Every effort was made to recruit patients with clinical details suggestive of a healthy duodenum for the study. Histology reports confirmed that this was the case for all control patients and three of the coeliac patients. Two of the coeliac patients, however, had a damaged duodenal mucosa. This is not overly surprising, as it is known that mucosal damage can be present in the absence of obvious symptoms (McGough and Cummings 2005). In reality this probably provides a good representation of the general coeliac population, as there are likely to be patients that seem well clinically but have some degree of damage to their mucosa. Thus, if oats are deemed safe for treated coeliac patients, unless all patients are scoped prior to the commencement of oats, there will be patients commencing oats without a fully treated mucosa.

4.4.4 Histology

There were a number of difficulties associated with the histological analysis of the cultured biopsies. Following 24 h in culture all biopsies were visibly flattened regardless of culture conditions; this was visible even with the naked eye. As illustrated in Fig. 4.2, this had a significant effect on the morphology of the biopsies, making analysis of the consequences of culture conditions difficult. This may be reflected in the fact that many studies employing organ culture have focused on the analysis of mRNA or culture supernatants (Kilmartin et al. 2003; Picarelli et al. 2001). In those studies that have carried out histological analysis, the effect of gravity on the biopsies is clear (Maglio et al. 2011).

Confocal imaging of sections proved difficult for a number of practical reasons. It was generally difficult to get a clear image that was well focused in all three channels. This was partly due to the fact that the fluorescent staining could have benefitted from some further optimisation if time had allowed. Additionally, at this magnification even minor factors, such as the quantity of mounting medium used, had the potential to affect the focus. Furthermore, great care was taken to get a fully representative image of each tissue section. If sections contained both healthy and damaged enterocytes it was important that the area that was imaged included both. This further limited the areas that could be imaged.

Cytokeratin 20

Cytokeratin 20 is a member of the intermediate filament family of cytoskeletal proteins. Although its expression has not previously been examined in coeliac disease, keratin filaments are known to be important structural stabilisers of epithelial cells (Roland Moll, Divo, and Langbein 2008). Additionally, CK20 is known to be a marker of differentiation in the normal intestinal epithelium (Chan et al. 2009). Under normal circumstances, it is expressed strongly and uniformly throughout the cytoplasm of small intestinal epithelial cells (R Moll, Schiller, and Franke 1990). This pattern of staining was visible in many patient biopsies in this study. Unfortunately, however, staining was too inconsistent even in the control patient biopsies to draw any conclusions on the effect of either avenin or gliadin exposure on the expression of CK20 in the coeliac mucosa. Nonetheless, a significant number of sections displayed the consistent, uniform staining that was expected; therefore further optimisation of this staining would be worthwhile and may yield meaningful results.

E-cadherin

E-cadherin is the main molecular component of the adherens junctions of enterocytes. Adherens junctions are part of the apical junctional complex which controls the cellular polarity and paracellular permeability of enterocytes. In addition to this, adherens junctions are vital for tight junction formation as it is only after adherens junction formation that zonula occludens-1 can migrate apically to form tight junctions. Thus, E-cadherin is of central importance in the formation and maintenance of cell-cell contact in the intestinal epithelium (Ciccocioppo et al. 2006).

In this study, the expression of E-cadherin was examined in biopsies from control and coeliac patients prior to culture and after culture with RPMI or RPMI containing PT gliadin or PT avenin. In control patient biopsies, E-cadherin was consistently expressed between epithelial cells under all circumstances. Although enterocytes were visibly shortened following culture, E-cadherin continued to be expressed strongly between cells and cells were in general nicely organised. When coeliac biopsies had been cultured with PT gliadin the appearances suggested evidence of mucosal damage and there was apparent reduction in E-cadherin expression. This is in keeping with the limited literature on E-cadherin expression in coeliac disease (Perry et al. 1999; Barshack et al. 2001). Interestingly, biopsies from three of the five coeliac patients, OC7, OC9 and OC10, also showed signs of damage and reduced E-cadherin expression following culture with PT avenin. This included the two patients who were reported by histology to have a damaged mucosa. In both of these patients there was good E-cadherin expression at Time 0 and marked changes following culture with PT gliadin or PT avenin. In one, there were some notably shortened epithelial cells following culture with RPMI but, nevertheless, E-cadherin staining was strong. E-cadherin expression has not previously been examined in the coeliac mucosa following exposure to avenin. However, as its expression has been

shown to be reduced in the coeliac mucosa following exposure to gliadin, these results suggest a toxic effect of avenin on these biopsies (Barshack et al. 2001).

Further examination of this situation is warranted. Perry et al measured E-cadherin mRNA expression in active and treated coeliac disease biopsies as well as normal controls and observed reduced levels of E-cadherin mRNA in untreated CD and normal expression in treated CD (Perry et al. 1999). Western blot analysis has demonstrated reduced protein expression of E-cadherin in active CD biopsies and also in human Caco-2 intestinal epithelial cells following culture with PT gliadin (Perry et al. 1999; Sander et al. 2005). In light of these studies, the measurement of protein or mRNA levels of E-cadherin expression in coeliac biopsies following culture with PT avenin would provide further clarification as to whether or not avenin is capable of having a toxic effect on coeliac biopsies.

4.4.5 suPAR

suPAR is a marker of immune system activation. High levels have been correlated with increased mortality risk in infectious diseases such as HIV, tuberculosis, malaria and sepsis. High levels of suPAR have also been correlated with autoimmune diseases including rheumatoid arthritis, type 1 diabetes and type 2 diabetes (Hillman 2011). In type 1 diabetes, the presence of complications, such as cardiovascular disease, has been associated with further elevation of suPAR levels (Theilade et al. 2014). The expression of suPAR in coeliac disease has not previously been examined; however, as it has been demonstrated that suPAR is a general rather than specific marker of inflammation and is a good marker of low-grade inflammation, in this study suPAR levels were measured in the organ culture supernatants (Thunø, Macho, and Eugen-Olsen 2009).

Although the mean suPAR concentration was higher in supernatants from coeliac biopsies cultured with PT gliadin than those cultured with PT avenin or RPMI alone, there was no statistically significant difference between the three conditions. However, when suPAR concentration in control and coeliac biopsy supernatants were compared, for all culture conditions suPAR levels were significantly higher in the supernatants from coeliac biopsies. This may reflect a general state of increased inflammation in coeliac disease. It has previously been shown that enterocyte proliferation is constitutively altered in coeliac disease. Increased proliferation of crypt enterocytes has been demonstrated in patients with untreated CD as well as those with treated or potential CD, when compared to controls. The same study also found increased expression of epidermal growth factor mRNA in both untreated and treated CD (Nanayakkara et al. 2013).

Interestingly, when the individual suPAR concentrations in the supernatants of coeliac biopsies cultured with PT avenin are compared to the E-cadherin results, a pattern emerges. Two patients, OC8 and OC11, had suPAR concentrations similar to that of control patients. The three other patients, OC7, OC9 and OC10, had much higher suPAR concentrations. These are the same three patients that showed evidence of mucosal change and reduced E-cadherin expression in response to culture with PT avenin. It should be emphasised that it is not is not possible to tell whether or not this is proof of a response to culture with avenin from such a small study group. As mentioned before, two of these three patients were reported by histology to have a damaged mucosa. The apparent response to culture with PT avenin in these patients could suggest that patients with untreated coeliac disease might be sensitive to oats.

4.4.6 Conclusion

In this study, biopsies from coeliac and control patients were cultured with RPMI or RPMI containing PT gliadin or PT avenin. This type of study has a number of advantages including the potential to examine the effects of a number of different culture conditions in one patient and the ability to control the amount and duration of prolamin exposure. There are also some disadvantages associated with this model, such as the separation of the biopsy from its natural environment and the physical effect of gravity on the cultured biopsy.

Unfortunately, cytokeratin 20 staining would have required further optimisation in order to provide meaningful results, however, E-cadherin expression indicated a response to culture with PT avenin in three of the five coeliac patients. ELISA testing revealed elevated concentrations of suPAR in the avenin culture supernatants of the same three patients. These results are in contrast with the results from the *in vivo* studies in the previous two chapters which showed no indication of immune activation in response to oats exposure in coeliac patients. Although the results of this study indicate that culture with PT avenin may have a damaging effect on the coeliac mucosa, this is based on the study of a small group of patients and therefore further studies would be necessary in order to determine the significance of these results.

Chapter 5

General Discussion

5.1 Review of results

Although the inclusion of oats in the GFD is quite widely accepted these days there is still some controversy over the safety of this food (Richman 2012; Fric, Gabrovska, and Nevoral 2011). Over the last 20 years there has been a significant number of feeding studies carried out on both newly diagnosed and treated adult and paediatric coeliac populations. The majority of these have concluded that the inclusion of a moderate amount of pure oats in the GFD should be safe for most coeliac patients (Holm et al. 2006; Kemppainen et al. 2008; Sey, Parfitt, and Gregor 2011; Kaukinen et al. 2013). However, a question still remains as to whether some coeliac patients are sensitive to oats (Tuire et al. 2012; Tjellström et al. 2014). Not all coeliac patients are equally sensitive to gliadin; some react violently to the inadvertent ingestion of a small quantity of gluten, while others are able to consume gluten-containing foods occasionally without feeling unwell and as a result are not completely compliant with their GFD (Ciclitira, Ellis, and Lundin 2005; Murray 1999; Pietzak 2005). This spectrum of sensitivity to gliadin could suggest that there might be a minority of coeliac patients, at the most sensitive end of the spectrum, who are also sensitive to oats.

The focus of this study was to use both new and established techniques to look for possible evidence of oats sensitivity. Following one year of oats addition to the GFD of a large group of treated and newly diagnosed coeliac patients, clinical and histological analysis showed no evidence of oats toxicity. We used sensitive immunological markers, including a marker of IEL infiltration and one of epithelial proliferation, and saw no indication of CD activation. We also adapted and validated the IN Cell Analyzer 1000, a system designed for the analysis of cell-based assays, used it to analyse tissue sections from the oats study patients and again saw no evidence of oats toxicity. Some still question the

validity of oats feeding studies on the basis that in many of these studies there were patients who dropped out before study completion (Janatuinen et al. 1995; Högberg et al. 2004; Gatti et al. 2013; Dor and Shanahan 2002). As discussed earlier, in this oats feeding study only two patients left without giving a reason and there was no indication in either patient of an adverse reaction to oats.

We also performed an *ex vivo* organ culture study and it appeared that three out of 5 coeliac patients showed mucosal changes following culture with PT avenin. E-cadherin expression was reduced and enterocytes were less well organised. This is in contrast to the results of the *in vivo* study and raises two important questions: 1) how reliable is the organ culture system in the investigation of coeliac disease? 2) if there was a change to the coeliac mucosa following exposure to oats, how long would this last? A temporary change lasting only a few hours could easily be missed in patients undergoing OGD after a number of hours fasting.

Whether these changes are due to genuine oats sensitivity or issues with the technology is difficult to tell from a small patient sample size. *In vitro* and *ex vivo* models have made significant contributions to our understanding of the pathogenesis of coeliac disease (Marietta, Schuppan, and Murray 2009). However, although organ culture is probably the best of these systems as it incorporates the effects of cell-cell interactions and signalling, it cannot fully replicate the systemic *in vivo* development of CD and the effects of other organs and cell systems (Stoven, Murray, and Marietta 2013). The results of this organ culture study are in contrast to a previous organ culture study performed in this department which showed consistent increase in IFN-γ mRNA in coeliac duodenal biopsies following 4 h culture with PT gliadin, but no increase following 4 h culture with PT avenin (Kilmartin et al. 2003). In the current study, biopsies were cultured in the presence of PT

avenin for 24 hours. If the changes seen in some biopsies were due to oats sensitivity, perhaps this will only occur after excessive exposure to avenin.

Although the majority of studies have concluded that the consumption of pure oats is safe for most coeliac patients, there have been reports of adverse reactions. Most notable was the report by Lundin et al on a patient who developed a Marsh grade 3B duodenal lesion, diarrhoea, itching dermatitis and increased levels of mucosal IFN-γ mRNA after a short-term oats challenge (Lundin et al. 2003). The severity of this patient's reaction is unusual and contrasts with the outcomes of other *in vivo* studies (Kemppainen et al. 2008; Koskinen et al. 2009; Sey, Parfitt, and Gregor 2011). In addition, four other patients in the same study had increased expression of mucosal IFN-γ mRNA. If an increase in IFN-γ mRNA was an indication of oats toxicity histological deterioration would be expected; however, in three of these patients there was no histological change in the intestinal biopsies and one patient actually had improved histology (Lundin et al. 2003). Nonetheless, it is possible that the changes seen at the mRNA level are in line with the changes seen in E-cadherin expression in our organ culture study and that very subtle changes might occur in the coeliac mucosa following oats ingestion.

A subsequent report describing the isolation of avenin-reactive intestinal T cell lines from five out of a group of nine patients with a history of oats exposure concluded that these T cells could cause mucosal inflammation (Arentz-Hansen et al. 2004). However, if these avenin-reactive T cells were a true sign of oats toxicity, more reports of oats intolerant coeliac patients would be expected. This is not the case and this was confirmed in two large retrospective studies which found no evidence of disease activation in treated coeliac patients following long term oats consumption (Guttormsen et al. 2008; Kaukinen et al. 2013).

A key issue concerning the oats debate is to separate the concepts of immunogenicity and toxicity. Sensitivity to gliadin has been demonstrated in a wide range of disease states and in normal subjects and these individuals do not have CD. The AGA assay, for example, was once widely used in CD diagnosis but has been discontinued due to high levels of positivity in healthy people (C Feighery et al. 1998). Autoantibodies to tTG are a much more specific marker of CD; nevertheless, an IgG anti-tTG response has been shown in a significant proportion of non-coeliac patients with various inflammatory diseases (Comerford et al, in press). Likewise, rheumatoid factor is widely distributed in many inflammatory diseases, including systemic lupus erythematosus and inflammatory bowel disease, and yet these patients have no evidence of rheumatoid arthritis (Popescu et al. 2013).

Silano et al tested a number of oat varieties for potential toxicity by culturing peripheral blood mononuclear cells of coeliac children with the corresponding avenins and measuring lymphocyte proliferation and IFN-γ release in the culture medium (Silano, Di Benedetto, et al. 2007). They reported that all varieties were immunogenic with some causing lymphocyte proliferation at levels similar to gliadin; however, if this translated to toxicity of oats it is unlikely that any coeliac patient would be tolerant of oats. Elevated levels of IgA antibodies to avenin have been demonstrated in oats consuming treated coeliac patients, however, similar levels are found in treated CD patients who do not eat oats. Hence, the ingestion of oats is not necessarily associated with increased levels of anti-avenin antibodies in CD patients (Guttormsen et al. 2008).

To conclude, we have demonstrated in this study that the daily ingestion of 40g of oats does not stimulate either a mucosal or a serological response in coeliac patients. However, the results of the organ culture study indicate that caution is still necessary.

5.2 Future studies

In the future, CD3 and CD8 expression could be measured in the organ culture biopsies. As an infiltration of IELs is the first histological change seen in active coeliac disease, this would be particularly useful to help determine if the changes seen in E-cadherin expression in 3 coeliac patients following culture with PT avenin were due to disease activation (Oberhuber 2000). Similarly, as Ki67 expression is known to be significantly elevated in coeliac patients with only minimal morphological changes, measurement of Ki67 expression would provide further clarification (Mohamed et al. 2008).

Elevated expression of the chemokine CXCL10 has previously been demonstrated in inflammatory processes and autoimmunity. More recently, Bondar *et al* demonstrated that CXCL10 is overexpressed in the duodenal mucosa of untreated CD patients and reduced in treated patients. They hypothesised that the CXCR3/CXCL10 axis is activated following the ingestion of gluten and plays an active role in the pathogenesis of CD (Bondar et al. 2014). Similarly, following exposure to gliadin, expression of IL-21 is increased in the duodenal mucosa of coeliac patients where it plays a role in the increased expression of T-bet and production of INF-γ (Fina et al. 2008). Examination of CXCL10 and IL-21 expression in the organ culture biopsies would help to further clarify whether avenin is capable of activating the disease process or not.

As time did not previously allow for the complete optimisation of CK20 staining, in the future, optimisation of this staining would provide further understanding of the effects of avenin or gliadin exposure on the coeliac mucosa. Additionally, increasing patient numbers would strengthen the study and help elucidate if patterns of elevated suPAR expression seen in the supernatants of some coeliac patient biopsies following culture with PT avenin were of significance.

Further directions for this research could include investigating whether some ancient varieties of wheat may be safe for coeliac patients. The safety of candidate wheat varieties could be assessed initially using the organ culture system. Varieties that show no evidence of toxicity in coeliac biopsies could then be trialled in patient feeding studies. An ancient variety of wheat, that would produce bread with a texture and taste similar to that of the bread made from modern varieties of wheat, would be much a healthier and more widely accepted substitute than current gluten free options. Such a variety of bread would have the potential be consumed by non-coeliac and coeliac individuals alike. This would furthermore improve the quality of life for coeliac patients and may mean that individuals with the potential to develop coeliac disease would be less likely to make that progression.

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Appendices

Appendix I

Reagent recipes

Chemicals are from Fisher, BDH, MSD and Sigma-Aldrich.

Tris buffered saline (TBS)

8.77 g	Sodium chloride
6 g	Tris base
1 L	dH_2O

pH 7.4

TBS/tween

1 L	TBS
0.5 ml	Tween 20

Phosphate buffered saline (PBS)

8 g	Sodium chloride
0.2 g	Potassium chloride
1.44 g	Disodium hydrogen phosphate
0.24 g	Potassium dihydrogen phosphate
1 L	dH_2O

pH 7.4

PBS/tween

1 L	PBS
0.5 ml	Tween 20

Citrate buffer

10 mM	Citric acid
1 L	dH_2O

pH 6.0

Appendix II


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Immunological indicators of coeliac disease activity are not altered by long-term oats challenge

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Summary

Coeliac disease is a gluten-sensitive enteropathy that develops in genetically susceptible individuals. The disease exhibits many features of an autoimmune disorder. These include the production of highly specific anti-endomysial autoantibodies directed against the enzyme tissue transglutaminase. It is well accepted that wheat-, barley- and rye-based foods should be excluded in the gluten-free diet. Although several studies report that oats ingestion is safe in this diet, the potential toxicity of oats remains controversial. In the current study, 46 coeliac patients ingested oats for 1 year and were investigated for a potential immunogenic or toxic effect. Stringent clinical monitoring of these patients was performed and none experienced adverse effects, despite ingestion of a mean of 286 g of oats each week. Routine histological analysis of intestinal biopsies showed improvement or no change in 95% of the samples examined. Furthermore, tissue transglutaminase expression in biopsy samples, determined quantitatively using the IN Cell Analyzer, was unchanged. Employing immunohistochemistry, oats ingestion was not associated with changes in intraepithelial lymphocyte numbers or with enterocyte proliferation as assessed by Ki-67 staining. Finally, despite the potential for tissue transglutaminase to interact with oats, neither endomysial nor tissue transglutaminase antibodies were generated in any of the patients throughout the study. To conclude, this study reaffirms the lack of oats immunogenicity and toxicity to coeliac patients. It also suggests that the antigenic stimulus caused by wheat exposure differs fundamentally from that caused by oats.

Keywords: coeliac disease, immunological parameters, oats challenge, tissue transglutaminase

Introduction

Coeliac disease is a gluten-sensitive enteropathy that develops in genetically susceptible individuals [1–3]. The presence of gluten in the diet is essential for coeliac disease to manifest, and a gluten-free diet is a highly effective treatment for this disorder. The traditional diet is based on the exclusion of all wheat protein products and, until recently, barley, rye and oats cereal foods were also excluded. However, unequivocal evidence that these latter three cereals are toxic is sparse. Because oats are related phylogenetically more distantly to the other cereals [4], their true toxicity was particularly questioned. This led to the original oats challenge studies, which reported that oats did not cause activation of coeliac disease [5,6]. More recent studies of paediatric and adult coeliac patients supported these earlier observations [7–11]. However, other investigators reported that oats could activate coeliac disease in some patients, and suggested that if oats were taken in sufficient quantity over a longer time-period the cereal might prove to be toxic [12,13]. Moreover, it was postulated that the mucosal inflammation in oats-reactive patients was caused by oats–avenin activated intestinal T cells [13]. It was demonstrated that the oats reactivity of cell lines was human leucocyte antigen (HLA)-DQ2-restricted and enhanced by deamidation of peptides by tissue transglutaminase. As a consequence of these reports, and continuing concerns about the safety of oats, in two recent reviews caution was

advised and it was suggested that oats might not be tolerated by all coeliac patients [14,15].

In view of the continuing debate about oats toxicity, a group of treated coeliac patients taking 50 g of oats daily for 1 year were investigated in this study. Clinical monitoring of patients was particularly stringent and included maintaining a daily symptom diary with formal clinical evaluation every 3 months. Particular focus was given to immunological events, and these included monitoring the expression of tissue transglutaminase protein in intestinal biopsy tissue; documenting the extent of epithelial infiltration by lymphocytes and enterocyte proliferation in intestinal biopsies; and monitoring levels of endomysial and anti-tissue transglutaminase autoantibodies.

Materials and methods

Patients

Fifty-four patients with biopsy proven coeliac disease were recruited into the study. The oats were sourced from Peter Kölln and confirmed to be free from other grains [6]. A target oats intake of 50 g per day for 1 year was planned. Eight patients failed to complete the study, and details of the remaining 46 are given in Table 1. Thirty-seven of these patients were categorized as having treated coeliac disease, adhering to a gluten-free diet for a mean of 10 years. A further nine patients were diagnosed more recently, and six of these started oats within 3 months of their diagnosis and commencing a gluten-free diet.

Approval for the study was granted by the Hospital Ethics Committee.

Clinical monitoring

Patients were requested to maintain a symptom diary for the duration of the study and were assessed clinically on a 3-monthly basis. Patients were questioned about general wellbeing and specifically about the development of symptoms such as mouth ulcers, dyspepsia, alteration in bowel habit and skin rash or itch. They were also weighed and their body mass index calculated. Blood tests, including endomysial antibody serology, haemoglobin level, white cell differential and platelet count, were performed on six occasions throughout the study.

Coeliac antibody tests

The endomysial antibody assay was carried out using an indirect immunofluorescence technique, as described previously [16]. A commercial enzyme-linked immunosorbent assay (ELISA) kit (Celikey; Pharmacia Diagnostics, Uppsala, Sweden) was used to measure immunoglobulin (Ig)A anti-tissue transglutaminase antibodies, and this test was performed on serum samples obtained at study completion.

Small intestinal histology

All patients had a duodenal biopsy taken prior to the start of the oats challenge. In the majority (78%), the biopsy was taken within a 12-month period prior to commencement of the oats challenge. In eight patients, with indicators that their disease was in stable remission, a biopsy taken more than 1 year before the challenge was used as the baseline test. A second biopsy was taken at the end of the study, and in 87% this was taken within 8 weeks of study completion. The haematoxylin and eosin-stained duodenal biopsy sections were examined by an experienced histopathologist in a blinded and random manner. Features suggestive of coeliac disease activity, including villous atrophy, increased intraepithelial lymphocytes, enterocyte nuclear disarray, crypt hyperplasia and increased lamina propria cellular infiltrate, were documented. A Marsh score [17] was assigned in the following manner: Marsh 0, normal duodenal architecture; Marsh 1, normal duodenal architecture with an increase in intraepithelial lymphocytes; Marsh 2, partial villous atrophy; and Marsh 3, total villous atrophy.

Immunohistochemistry and immunofluorescence

Immunohistochemical staining was carried out on 4-μm-thick formalin-fixed paraffin-embedded tissue sections from 19 patients, as described previously [18]. Following antigen retrieval with citrate buffer, slides were incubated with anti-Ki-67, anti-CD3 or anti-CD8 antibodies (all from Novocastra Laboratories, Newcastle upon Tyne, UK), followed by the avidin–biotin–peroxidase complex detection procedure (Vector Laboratories, Burlingame, CA, USA). Sections were then incubated with diaminobenzidine and counterstained with haemotoxylin (Sigma-Aldrich, Arklow,

Table 1. Details of 46 biopsy-proven coeliac patients.

	Treated patients	Recently diagnosed patients
Number	37	9
Sex	14 male, 23 female	2 male, 7 female
Age (years)	Mean 46·7 (range 18·7–76·8)	Mean 51·2 (range 28·3–65·1)
Duration of GFD (years)	Mean 9·7 (range 1–40·1)	Mean 0·4 (range 0·1–0·9)
EMA at commencement	34 neg, 1 wpos, 2 pos	3 neg, 2 wpos, 4 pos
Histology (Marsh grade)	30 (0,1), 7 (2,3)	2 (1), 7 (2,3)

EMA: endomysial antibody; GFD: gluten-free diet; neg: negative; wpos: weak positive; pos: positive.

Wicklow, Ireland). Finally, the slides were coded and assessed in a blinded manner. In the case of anti-Ki-67-stained sections, the number of positively stained cells within five fields were counted and the percentage of positive cells calculated; for CD3 and CD8 the number of positively stained cells within 500 cells of the surface epithelial layer were counted and the percentage calculated.

In further immunofluorescence studies, tissue sections were incubated with antibody to tissue transglutaminase (Roboscreen, Leipzig, Germany) followed by an AlexaFluor^{568}-conjugated secondary goat anti-rabbit antibody (BioSciences, Dublin, Ireland). Sections were also incubated with antibody to smooth muscle alpha-actin (Sigma-Aldrich) followed by a secondary goat anti-mouse fluorescein isothiocyanate (FITC) antibody (Dako, Glostrup, Denmark). In order to visualize nuclei, slides were then incubated with Hoechst 33258 (Sigma-Aldrich). The extent of antibody labelling in peri-cryptal regions of biopsy pairs was then determined by high-resolution digital fluorescent microscopy followed by advanced image analysis [19] using the IN Cell Analyzer 1000 HCA platform (GE Healthcare, Little Chalfont, UK). Data were expressed as the percentage total area of tissue staining positive for smooth muscle alpha-actin and tissue transglutaminase (with a threshold of ×1·5 above background).

Results

Patient clinical assessments

Forty-six of the 54 patients who enrolled initially completed the study. The reasons for withdrawal in the eight patients were failure to adhere to the study protocol (five patients); emigration (two patients); and the development of breast cancer (one patient). None of these eight patients reported adverse reactions to the oats supplement.

The 46 patients who completed the study consumed a mean of 286 g oats per week (range 97–513 g) for a median duration of 48 weeks (range 33–58). Several patients reported mild symptoms such as flatus and abdominal distension with oats addition to their diet. No significant change was seen in body weights or body mass indices. Similarly, routine laboratory tests remained normal for the duration of the oats challenge.

Small intestinal histology

Following oats challenge, suitable biopsy material was available for routine histological analysis on 44 of the 46 patients (Table 2). In 42 patients (95·5%) the histological lesion had either improved or not changed. Two patients showed histological disimprovement while ingesting oats (Table 2). In one of these, the level of disimprovement was marginal (from Marsh grade 1 to Marsh 2); moreover, both dietetic and serological review suggested that the patient was not fully compliant with the gluten-free diet. The second patient also had a Marsh grade 2 lesion at study completion but then continued to ingest oats for a further 8 months, and a third biopsy taken at this time showed marked histological improvement, with a Marsh grade 1 lesion.

Table 2. Summary of serology and histology findings after oats challenge.

	Treated patients ($n = 37$)	Recently diagnosed patients ($n = 9$)
EMA	37 neg	7 neg; 1 pos, 1 w.pos[†]
tTG antibody	37 neg	7 neg; 2 pos (5·1 and 16·6 AU)[†]
Histology	33 – improved or no change 2 – disimproved*; 2 nd.	6 improved; 3 no change

*Neither patient was fully dietary compliant. [†]In one of these patients, histology had improved; in the second, the Marsh 3 lesion was unchanged. EMA: endomysial antibody; n.d.: not done, biopsy unsuitable; neg: negative; pos: positive; tTG: tissue transglutaminase; w.pos: weak positive.

Coeliac antibody serology

At the commencement of the study, 37 patients had a negative endomysial antibody test (Table 1). Repeat testing throughout the study confirmed that almost all patients remained antibody-negative, with 89% of the total 275 tests in the study giving negative results. Only 16 samples were endomysial antibody-positive and these were sourced from four newly diagnosed coeliac patients and from two treated patients with known poor dietary compliance. At the completion of oats challenge, 44 of the 46 patients had a negative endomysial antibody test. The two patients with positive endomysial antibodies at study end were among the recently diagnosed subgroup (Table 2). Tissue transglutaminase autoantibody tests mirrored endomysial antibody results, and 44 subjects had a negative test (< 5 arbitrary units, AU). The two patients who were endomysial antibody-positive had tissue transglutaminase autoantibody levels that were minimally elevated (5·1 AU) or moderately elevated (16·8 AU).

Immunohistochemistry

Pre- and post-oats biopsy samples in 19 patients were stained for expression of Ki67, CD3 and CD8. The addition of daily oats caused no significant change in the extent of staining for any of these markers (Table 3). In the case of Ki67, 53% of enterocytes stained positively before oats and 56% at study end [95% CI –11·57 to 5·47%]. The percentage of cells staining with anti-CD3 was 24% before and 23% after oats challenge [95% CI –4·25 to 5·89]. Similarly, with anti-CD8 antibody, the percentage of cells with positive staining before oats was 31% and 28% following oats ingestion [95% CI –4·49 to 10·01].

Table 3. Immunohistochemistry and immunofluorescence results.

	Pre-oats ($n = 19$)	Post-oats ($n = 19$)	
Ki-67% ($n = 19$)	53 ± 15%	56 ± 18%	$P = 0{\cdot}47$
CD3% ($n = 19$)	24 ± 7%	23 ± 9%	$P = 0{\cdot}74$
CD8% ($n = 19$)	31 ± 11%	28 ± 15%	$P = 0{\cdot}43$
tTG% ($n = 16$)	1·02 ± 0·88%	1·24 ± 0·86%	$P = 0{\cdot}3741$
SM α-actin% ($n = 16$)	2·42 ± 1·55%	3·23 ± 2·21%	$P = 0{\cdot}1602$

The percentage of enterocytes expressing Ki-67 and percentage of intraepithelial cells expressing CD3 and CD8 before and after oats challenge are shown. The percentage of total tissue area expressing tissue transglutaminase (tTG) and smooth muscle alpha actin (SM α-actin) before and after oats ingestion is also shown.

Quantitative immunofluorescence

To examine further the potential immunogenic or toxic effect caused by oats, pre- and post-oats biopsy sections from 16 patients were stained with anti-tissue transglutaminase and anti-smooth muscle alpha-actin antibodies. Elongated stellate cells in the peri-cryptal region, just beneath the basement membrane, were found to express both proteins. These cells were identified as myofibroblasts, based on their morphological appearance and expression of smooth muscle alpha-actin (Fig. 1).

The extent of antibody labelling in peri-cryptal regions was then determined, employing high-resolution digital fluorescent microscopy followed by image analysis using the IN Cell Analyzer [19]. Expression of tissue transglutaminase before oats was 1·02% and after oats 1·24% [95% CI –0·75 to 0·30], $P = 0{\cdot}3741$, not significant (n.s.). Smooth muscle alpha-actin expression was 2·42% before oats and 3·23% after oats [95% CI –1·99 to 0·36] $P = 0{\cdot}1602$, n.s. (Table 3).

Discussion

In this study, 46 patients with coeliac disease adhering to a gluten-free diet added oats to their daily diet for 1 year, and none experienced significant adverse effects. Detailed clinical, serological and histological evaluations were performed throughout the study and oats ingestion did not cause activation of coeliac disease. Tissue transglutaminase and smooth muscle alpha actin expression in intestinal histological sections were also measured quantitatively and levels were not altered by daily oats ingestion.

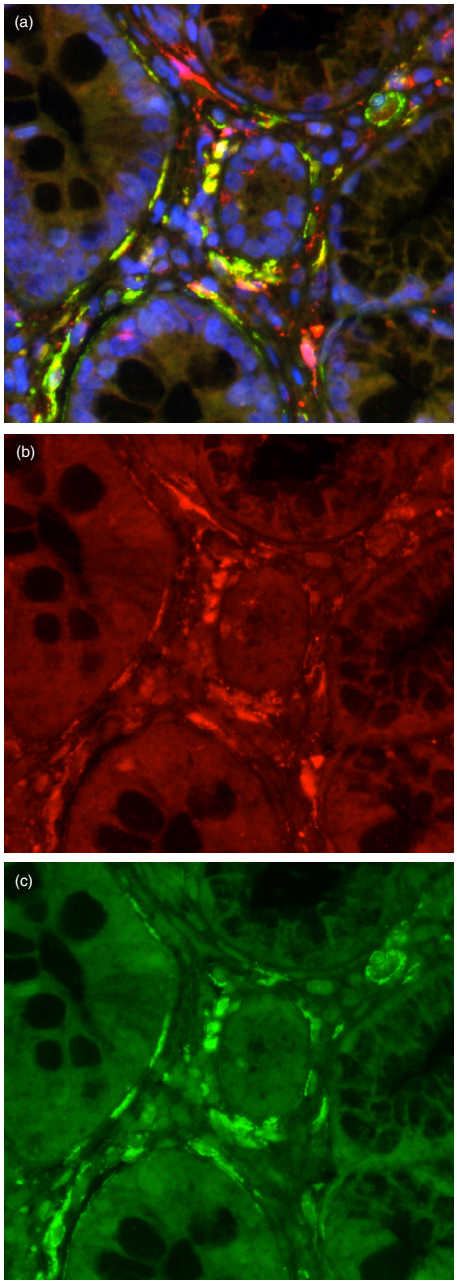

Fig. 1. IN cell analysis: image produced on IN Cell Analyzer 1000 showing nuclear (blue), tissue transglutaminase (red) and smooth muscle alpha-actin (green) staining as well as tissue transglutaminase/smooth muscle alpha-actin co-localization (yellow) (a), images from individual channels showing tissue transglutaminase staining only (b) and smooth muscle alpha-actin staining only (c). The level of tissue transglutaminase and smooth muscle alpha-actin expression was calculated as a percentage of total tissue area.

Routine histological analysis of tissue sections after oats challenge showed histological improvement or no change in 95% of the available samples from 42 of the 44 patients. In the two remaining subjects, the inflammatory lesion had disimproved but, in both, poor adherence to the gluten-free diet was documented and was considered responsible. More detailed analysis was performed on samples from 19 subjects. Whereas infiltration of the epithelium by $CD8^+$ T cells is a classic finding in activation of the coeliac lesion [11,20], in this study oats challenge caused no change in these cell numbers. A similar finding has been made in previous studies [11,21]. Similarly, oats in the diet did not alter the extent of enterocyte Ki67 expression, in keeping with the findings of our earlier 3-month challenge study [22]. In patients with active coeliac disease [23–25], and following gliadin challenge in organ culture experiments [23], increased expression of Ki67 by epithelial cells reflecting increased cell turnover has been reported.

The IN Cell Analyzer, which enables the extent and intensity of fluorescence staining to be documented digitally [19], was used to measure the level of expression of tissue transglutaminase and smooth muscle alpha actin proteins in biopsy tissue. Oats ingestion for 1 year caused no change in the level of expression of either molecule. Smooth muscle alpha actin was expressed primarily in elongated stellate cells, identifying these cells as myofibroblasts [26], and these were found in a peri-cryptal location. Tissue transglutaminase was also expressed strongly in these cells. This concurs with earlier reports of expression of this enzyme in cells with a similar morphology [27]. Increased tissue transglutaminase expression in the coeliac small intestine has been reported previously [27–29], and expression has been shown to decrease with gluten-free diet therapy [28,29]. Several studies have demonstrated that tissue transglutaminase plays a role in inflammation and wound healing [30–33]; nuclear factor (NF)-κB signalling, which is elevated in the coeliac small intestine [34], may mediate its increased expression. In addition to increased expression of this enzyme, we have also observed increased smooth muscle alpha actin expression in active coeliac biopsies in comparison to biopsies from treated subjects or normal controls (manuscript in preparation).

If oats have the potential to activate coeliac disease and behave like other toxic cereals, as has been reported [13], it might be expected that oats ingestion would result in the generation of an anti-endomysial or anti-tissue transglutaminase antibody response. However, despite a mean oats intake of 286 g per week, the patients in this study did not develop these antibodies. Other oats challenge studies have reported a similar finding [8–11]. During this study, only four newly diagnosed coeliac patients and two treated patients with known poor dietary compliance were positive for anti-endomysial antibodies. Samples at the end of the study were also tested for anti-tissue transglutaminase antibody levels and the results concurred with the endomysial antibody tests.

The potential immunogenicity and toxicity of oats to patients with coeliac disease continues to be debated [13–15]. Avenin is the alcohol-soluble protein fraction of oats and contains some homologous sequences with the equivalent fractions in wheat (gliadin), barley (hordein) and rye (secalin) [35]. Moreover, some avenin peptide sequences are susceptible to tissue transglutaminase deamidation and found to induce proliferation of gliadin-reactive T cell lines with production of interferon (IFN)-γ [35]. In the study by Arentz-Hansen *et al.* [13], avenin-reactive T cell lines were generated, with avenin reactivity enhanced by the addition of tissue transglutaminase. Furthermore, the reaction was shown to be HLA-DQ2-restricted. Although the overall properties of avenin, including a lower number of proline residues, make this cereal less immunogenic to coeliac patients, it was argued none the less that in some individuals avenin-reactive T cells could activate coeliac disease.

There are many lines of evidence against oats being toxic in coeliac patients, including the fact that the avenin content of oats is considerably lower than equivalent proteins in wheat, barley and rye [4]. Many clinical studies have found no evidence of oats toxicity and several of these, including this study, have been of extensive duration, lasting a year or longer [10,11,21]. Several of these studies have been on children, and children commonly display rapid evidence of toxicity when given a gluten challenge [11,20]. Even when detailed immunohistological examination was performed on biopsy tissue after oats challenge, no evidence of toxicity was observed, as described here and in other studies [11,22,24].

In conclusion, detailed analysis of the large patient cohort reported in this study reaffirms the lack of oats toxicity to coeliac patients. This conclusion is based on the absence of clinical symptoms, serological changes or histological markers of disease activity during oats consumption. The findings also raise fundamental issues concerning differences in the antigenic stimulus caused by gliadin exposure in coeliac disease, compared with avenin exposure.

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