Dipeptidyl peptidases in gastrointestinal health and disease

by

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Thesis
Submitted to Flinders University
for the degree of

Doctor of Philosophy
College of Medicine and Public Health
10th December 2018

Declaration

I certify that this thesis does not incorporate, without acknowledgement, any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material published or written by another person except where due reference is made in the text.

Simone Jaenisch July 20th 2018

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Acknowledgements

This thesis would not have been possible without the financial support I received in the form of an Australian Postgraduate Award/Research Training Scheme scholarship from Flinders University and the Australian Government. Research is a team sport and this thesis would not have been possible without the continued support of my supervisors, laboratory and Department colleagues, friends and my family. I would like to acknowledge the initial two years of my candidature that was spent at the University of South Australia and the Basil Hetzel Institute. I would like to thank both institutes and their staff for their support during the first two years of my candidature and their contributions made to my research training. I would also like to thank both institutes and staff for being so supportive of my decision to transition to Flinders University for the final years of my candidature.

First and foremost, I wish to acknowledge my primary supervisor, mentor and friend Dr Roger Yazbeck for the opportunity to learn from him and study within his laboratory for the past four years. His continued guidance, encouragement, expertise and friendship have been invaluable throughout my PhD and played a significant role in helping me overcome major challenges throughout my PhD. He has nurtured my love of research and knowledge and I am very appreciative of the training and experience I acquired throughout my candidature. I will very much miss working in your laboratory. I extend my gratitude to my associate supervisor, Associate Professor Catherine Abbott for the opportunity to carry out many of my experiments in her laboratory. Her experimental knowledge and advice in the laboratory setting was very valuable and I thank her for her continued support throughout my project. I would also like to thank my associate supervisor Professor David Watson for welcoming me to his laboratory and for his support and advice throughout my candidature.

I wish to thank the members of the Upper Gastrointestinal Research Laboratory (Isabell Bastian, Tingting Wang, Tayna Irvine, George Mayne and Chris Re'vell) headed by Dr Damian Hussey with whom our laboratory is strongly affiliated. The support from the Upper GI laboratory persons significantly eased the transition when I arrived at Flinders University from the Basil Hetzel Institute. Isabell and Tingting, your knowledge of hospital equipment and facilities, laboratory expertise and willingness to assist me throughout my candidature has been incredible and was always very much appreciated. I wish to thank Dr Damian Hussey for his suggestions and willingness to offer alternatives when equipment have been out of order was very much appreciated and saved me a great deal of stress. I also thank Dr Hussey for his willingness to piggyback with ethics applications to assist us in the collection of patient samples.

I would like to acknowledge the Basil Hetzel Institute Gastroenterology Department (Dr James Fon, Dr Ian Roberts-Thomson, Wendy Uylaki) for the kind donation of bio-banked IBD tissue samples analysed in Chapter 2. I would also like to thank Wendy Uylaki for taking the time during the early stages of my candidature to train me in qPCR related methods, giving me the confidence to trouble-shoot and perform these experiments later.

I would like to acknowledge the contributions from the Flinders Medical Centre Gastroenterology & Hepatology Department and Professor Peter Bampton to Chapters 3 and Chapter 4. I thank Professor Bampton for his kind donation of the extensive bio-banked plasma samples that formed a major component of Chapter 3. I also extend my gratitude to Ruth Prosser for her assistance in obtaining clinical information relating to these samples for my analysis. I thank the contributions of Professor Bampton, Ruth Prosser and Helen Lewis regarding the submission of human ethics to the Southern Adelaide Clinical HREC. Ruth Prosser, Helen Lewis and Libby Bambacas were instrumental in recruiting patients to the study

(Chapter 4) and I am very grateful for their assistance. I would also like to thank the Flinders Medical Centre Gastroenterologists; Dr Sam Edwards, Dr Laurie Chittie & Dr Reme Mountifield who were involved in the study for their contributions to the recruitment of patients and the collection of tissue and blood samples (Chapter 4).

I would like to acknowledge the wonderful work of our collaborator Professor Mark Gorrell and his laboratory staff at the Centenary Institute at the University of Sydney for performing the FAP enzyme activity assays on our plasma samples described in Chapter 4.

I thank Professor Antonio Ferrante, head of the Immunopathology Department at the Women's and Children's Hospital in Adelaide and his laboratory staff for the opportunity to learn and conduct immune function assays within his laboratory (Appendix 6.9). Their support and understanding was amazing and I hope that this work can be explored further.

I would like to express my sincerest gratitude to Michelle Squire. Your friendship, mentorship and the time you spent helping me with experiments throughout my research training was very much appreciated. I would also like to thank her for allowing me to present the ^{13}C-based *in vitro* data to Chapter 2 of this thesis. Including this data helped to reinforce the potential of DPP4 as a biomarker for gastrointestinal diseases.

To my fellow research students and friends, Michelle Squire, Arif Malik, Dr Steven Due, Ashley Hocking and Michaela Johnson, your support, advice and friendship throughout my candidature has been invaluable and helped me to overcome the many challenges that arose during my candidature. Arif I cannot thank you enough for your extensive advice on qPCR and your willingness to share laboratory consumables. Steve you're an absolute inspiration. Your

ability to manage clinical work, surgical training, laboratory research and a young family is commendable. Your friendship, advice and support has been nothing short of exceptional.

Finally, I would like to thank my family for their support and patience over the last four years. I thank my parent's for providing me with an environment that was supportive of my aspirations and goals and for doing everything within their power to support me and help me succeed, I love you both very much. To my partner Jay, I thank you for your patience and understanding while I completed my studies. You've been my rock throughout my candidature, thank you for always listening to my complaints and for always giving me perspective.

Publications & presentations

Publications arising during candidature:

Jaenisch, S. Squire, M., Butler, R., Yazbeck, R. *In vitro* development and validation of a non-invasive (13)C-stable isotope assay for ornithine decarboxylase. *Journal of Breath Research*. 10 (2) 2016.

Yazbeck, R., **Jaenisch, SE.,** Watson, DI. From blood to breath: New horizons for esophageal cancer biomarkers. *World Journal of Gastroenterology.* 22 (46) 2016.

Yazbeck, R., **Jaenisch, SE.,** Abbott, CAA. Potential disease biomarkers: dipeptidyl peptidase 4 and fibroblast activation protein. *Protoplasma*. 2017.

Manuscripts derived from this thesis in preparation for publication:

Jaenisch, SE., Abbott, CA., Yazbeck, R. 'Recent insights into the DPP4 inhibitors and inflammatory disease'. (Derived from Chapter intended submission to Trends in Pharmaceutical Science)

Jaenisch, SE., Abbott, CA., Bampton, P., Butler, RN., Yazbeck, R. 'Differential dipeptidyl peptidase expression and activity in Crohn's Disease and Ulcerative Colitis'. (Derived from Chapter 3, intended submission to World Journal of Gastroenterology)

Jaenisch, SE., Abbott, CA., Bampton, P., Gorrell, MD., Yazbeck, R., 'Dipeptidyl peptidases during colorectal inflammation of IBD'. (Derived from Chapter 4, intended submission Journal of Crohn's and Colitis)

Yazbeck, R., **Jaenisch, SE**., Squire, M., Abbott, CA., Brooks, DA., Parkinson-Lawrence, E., Butler, RN. 'Development of a (13)C stable isotope assay for dipeptidyl peptidase-4 enzyme activity'. (Derived from Appendix 6.1)

Presentations relating to candidature:

Basil Hetzel Institute, Seminar Series October 2014, **Oral presentation**

University of South Australia, School of Pharmacy and Medical Sciences, Research Symposium April 2015, **Oral presentation**

Women's and Children's Hospital, Immunopathology Department, Research Seminar Series October 2015, **Oral presentation**

Australian Society for Medical Research (ASMR) National Scientific Meeting, Adelaide, November 2015, **Poster Presentation**

Australian Society for Medical Research (ASMR) Local Scientific Meeting, June 2016, **Oral Presentation**

United European Gastroenterology Week, Vienna, Austria, October 2016, **Poster Presentation**

Abbreviations

Arbitrary units	AU
C reactive protein	CRP
Crohn's Disease	CD
Dextran sodium sulphate	DSS
Dipeptidyl peptidase	DPP
Fibroblast activation protein	FAP
Glucagon like peptide	GLP
Inflammatory bowel disease	IBD
Interferon	IFN
Interleukin	IL
Interquartile range	IQR
Multiple sclerosis	MS
Mononuclear cells	MNCs
Nucleotide-binding oligomerization domain containing 2	NOD2
Phorbol myristate acetate	PMA
Polymorphonuclear cells	PMNs
Polymerase chain reaction	PCR
Prolyl endopeptidase	PREP
Reactive oxygen species	ROS
Rheumatoid arthritis	RA
T helper	Th
Toll like receptor	TLR
T regulatory cells	T regs
Trinitrobenzene sulfonic acid	TNBS
Tumour necrosis factor-α	TNF-α
Ulcerative colitis	UC

Abstract

Current methods for diagnosis and monitoring of gastrointestinal health and disease are highly invasive, expensive and represent a major burden to patients, the economy and the health care system. New serological, tissue and non-invasive biomarkers that can better reflect and/or predict the physiological changes that occur in the gastrointestinal tract during disease would reduce the burden of disease and improve disease management. Dipeptidyl peptidases (DPPs) have roles in gastrointestinal health and immunity and have been proposed as potential biomarkers for gastrointestinal conditions including inflammatory bowel disease (IBD) and Barrett's oesophagus. This thesis aimed to better understand the roles of DPPs in gastrointestinal health and disease and explore their potential as disease biomarkers by characterising the expression and activity profiles of DPPs during health and gastrointestinal disease.

The mRNA expression and enzyme activity profiles of DPP enzymes throughout the gastrointestinal tract was initially characterised in mucosal biopsies from healthy donors. Low levels of DPP9 mRNA were found throughout the gastrointestinal tract. In comparison, higher levels of DPP8 mRNA was found in the gastrointestinal tissues, which may suggest a more prominent role for DPP8 in gastrointestinal health. Low but detectable levels of DPP4 mRNA was found in colonic and gastric tissues, though the highest levels of DPP4 mRNA were found within ileal tissues. Consistent with this, high levels of DPP4 enzyme activity were found within the ileum. In comparison, DPP8/9 enzyme activity was found at more consistent levels in the different gastrointestinal tissues.

Initial analysis of enzyme activity levels during oesophageal and ileal disease identified differences in DPP4 enzyme activity. Higher levels of DPP4 enzyme activity were found in biopsies taken from lesions of Barrett's oesophagus compared to oesophageal tissue from

healthy individuals. Though in a patient with Crohn's Disease (CD), lower levels of DPP4 enzyme activity were found within affected ileal tissue compared to matched inactive tissue. These results may suggest utility of mucosal DPP4 enzyme activity as a biomarker of gastrointestinal health and integrity throughout the gastrointestinal tract, in various disease paradigms.

Breath testing represents a novel, non-invasive technique that has clinical applications in diagnosis and monitoring of different conditions. Our laboratory previously developed and validated a ^{13}C-based non-invasive assay to quantify mucosal DPP4 enzyme activity *in vitro*. Application of this novel ^{13}C-DPP4 assay in oesophageal cancer cell lines found differential $^{13}CO_2$ levels that approximately corresponded to the DPP4 enzyme activity levels detected using the colorimetric assay, suggesting that the ^{13}C-DPP4 assay can selectively detect mucosal DPP4 activity. While further validation studies are necessary, a ^{13}C-DPP4 breath test that can detect and quantify DPP4 enzyme activity throughout the gastrointestinal tract, could have clinical applications in the diagnosis and monitoring of Barrett's oesophagus and IBD.

The utility of DPPs as plasma and tissue biomarkers in human IBD was investigated further in two separate observational studies (Chapter 3 & 4). Lower levels of plasma DPP enzyme activity were found in patients with IBD, compared to non-IBD individuals. Further analysis also found that plasma DPP enzyme activity and plasma FAP enzyme activity were lower in patients with evidence of active inflammation, which may suggest utility for plasma DPP enzyme activity in the diagnosis and monitoring of IBD.

Differences in DPP mRNA expression and DPP enzyme activity were also found within the colorectal tissue of IBD patients. Higher levels of DPP4, FAP and DPP8 mRNA were found in the colorectal tissues from patients with IBD compared to controls. Increased DPP enzyme

activity was also found in inflamed colorectal tissues compared to matched inactive tissue from IBD patients, suggesting that changes to DPP mRNA expression and enzyme activity may be associated with gastrointestinal damage or inflammation.

In summary, the significant original contributions to knowledge achieved in this thesis are firstly, an addition to the current understanding of DPP mRNA expression and enzyme activities in gastrointestinal health and disease. Secondly, this thesis provides further clinically relevant evidence for the usefulness of DPPs as biomarkers to diagnose and monitor premalignant and inflammatory conditions. The ability for DPP4 activity to be detected using a non-invasive ^{13}C-based approach is novel and enhances the potential clinical utility of DPP4. It is envisaged that future studies may be able to develop and validate the ^{13}C-DPP4 assay that will provide accurate real-time information relating to DPP4 enzyme activity and expression in the gastrointestinal tract that can be used to diagnose and monitor disease states.

Thesis outline and structure

The overall aim of this thesis was to broaden the understanding of dipeptidyl peptidases (DPP) during gastrointestinal health and disease. The specific aims of this thesis were to:

1. Characterise the mRNA expression and enzyme activity profiles of the dipeptidyl peptidase enzymes throughout healthy gastrointestinal tract and evaluate the clinical potential of DPP4 as a gastrointestinal marker (Chapter 2)
2. Explore the potential roles of dipeptidyl peptidase enzymes in gastrointestinal health and disease by examining the expression and activity profiles in gastrointestinal disease (Chapter 2, 3 & 4)
3. Characterise plasma DPP activity during inflammatory bowel disease, exploring disease specific factors that may be associated with plasma DPP activity (Chapter 3 & 4)

This thesis is presented as five separate chapters and an appendix. Experimental Chapters 2, 3 and 4 each include a separate introduction, methods, results and discussion section. This was done to help with future preparation of chapters as manuscripts for publication. Due to the similarity of studies detailed in Chapter 3 and Chapter 4, some literature is discussed repeatedly throughout this thesis. For consistency and ease of reading, references are presented in Chapter 7 in the Harvard Author Date format. Separate references as required are included as necessary for the Appendix.

Chapter 1 includes two reviews. The first is a review of the current understanding of inflammatory bowel disease, including clinical features, disease management, the multifaceted nature of its pathogenesis and the need for clinical biomarkers that can better monitor and predict disease behaviour.

Dipeptidyl peptidase 4 has been proposed as a biomarker in a number of disease paradigms and may have utility in IBD. This second component of Chapter 1 broadly describes dipeptidyl peptidases and what is known about their roles in gastrointestinal health and inflammation, and their potential as biomarkers.

Chapter 2 aimed to further the understanding of DPP mRNA expression and activity throughout the healthy gastrointestinal tract and its utility as a biomarker of disease. This chapter characterises the mRNA expression of DPP enzymes and DPP activity along the gastrointestinal tract in clinical samples derived from patients undergoing endoscopic procedures at Flinders Medical Centre, Adelaide. This chapter also describes DPP enzyme activity in diseased oesophageal tissue and ileal tissue that is not presented elsewhere in this thesis. Exploring the clinical potential of mucosal DPP4 activity as a biomarker of gastrointestinal disease and/or integrity, this Chapter also describes the quantitation of DPP4 activity using a novel non-invasive ^{13}C-based assay *in vitro*.

Chapter 3 is a retrospective observational study that aimed to characterise plasma DPP activity and DPP mRNA expression within tissue samples from IBD patients. Plasma and tissue samples analysed in this Chapter were non-matched bio-banked samples from Flinders Medical Centre, Gastroenterology Department and the Queen Elizabeth Hospital respectively. The addition of Sitagliptin, a selective DPP4 inhibitor to DPP enzyme assays, allowed for further characterisation of plasma DPP activity into DPP4 and residual activity that identified further differences between CD and UC patients. Limitations associated with the retrospective nature of this study highlighted the need for more comprehensive characterisation of DPPs during IBD and was the basis for the study detailed in Chapter 4.

Chapter 4 details the results from a prospective observational study, a collaboration with the Gastroenterology and Hepatology Department at Flinders Medical Centre. This study builds upon the results presented in Chapter 3, providing a more comprehensive analysis of DPP enzymes during IBD. To guide recruitment and ensure statistical significance, power calculations were performed by a statistician using plasma DPP activity as the primary endpoint (n=30/group). Despite recruitment efforts between 2015 - 2017, the final sample sizes were lower than required per the power calculation. The plasma findings presented here, contrasts that of Chapter 3, though the low sample size and differences in clinical disease activity and severity likely contributed to the inconsistencies between these studies. However, the thorough collection of biopsy material and clinical information from IBD and non-IBD patients in this study added significant value to this study. Consequently, this is the first study to comprehensively characterise both DPP mRNA expression and DPP enzyme activity in colorectal IBD, highlighting changes to DPP mRNA expression and DPP enzyme activity that have not been previously reported in human IBD.

Chapter 5 summarises and discusses the final conclusions of this thesis. It also describes the potential future directions of this work particularly what further knowledge is required before DPPs can be utilized as clinical biomarkers.

Additional experimental and validation data that was collected during my candidature, though is not presented in the main body of this thesis is presented within the **Appendix** (Chapter 6)**.** The original aims of this thesis included characterising the role of DPPs during neutrophil responses in IBD patients. Preliminary data using neutrophils isolated from healthy donors, was collected throughout 2015-2016 at the Women's and Children's Hospital and is presented in Appendix 6.9. However, due to technical difficulties associated with the lifespan of neutrophils and delays with blood processing, this work could not be completed in the IBD

patients recruited in Chapter 4. Although this data is still preliminary, it does suggest there could be specific roles for DPP enzymes in neutrophil responses *in vitro*.

Also included in the Appendix is a draft manuscript entitled '*Development of a ^{13}C stable isotope assay for Dipeptidyl Peptidase-4 Enzyme Activity'* on which I am listed as second author. Throughout my candidature I had a significant role in planning and data collection for this manuscript, assisting with *in vitro* ^{13}C-based assays and cell-culture. I also performed all colorimetric DPP4 enzyme activity assays and qPCR experiments relating to this manuscript, the corresponding data analysis and edited draft versions of this manuscript. This manuscript describes the initial development and validation of the ^{13}C-DPP4 *in vitro* assay that led to its application in Chapter 2 of this thesis.

References are included throughout the Appendix where relevant. Separate reference lists have been included for the draft manuscript (Chapter 6.1) and the preliminary neutrophil data (Chapter 6.9). For other sections Chapter 6.2-6.8, relevant references are included as footnotes.

Chapter 1. Introduction

Inflammatory Bowel Disease (IBD) is a chronic inflammatory condition that affects the gastrointestinal tract. Despite the high prevalence of IBD worldwide its pathogenesis is poorly understood, though is thought to involve a combination of Western lifestyle factors and host genetics. The unpredictable nature of disease flares contributes to the highly debilitating nature of this disease. C reactive protein and faecal calprotectin remain the most extensively used biomarkers of inflammation for IBD despite the limited clinical information they provide. Non-invasive biomarkers that can accurately predict disease course and outcomes are required and could lead to earlier clinical decisions and improved disease management. Dipeptidyl peptidase (DPP)-4 is a serine protease that has roles in gastrointestinal health and inflammation, and has been identified as a potential therapeutic target and biomarker for inflammation during IBD. Chapter one provides an overview of the current understanding of IBD and discusses what is known about DPPs in gastrointestinal health and IBD.

1.1 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic, debilitating condition that affects the gastrointestinal tract, and is characterised by relapsing symptoms of abdominal pain, diarrhoea, rectal bleeding, fatigue and weight loss (Strober et al., 2007, Wallace et al., 2014). IBD is classified into two main subtypes, Crohn's Disease (CD) and Ulcerative colitis (UC), each with specific aetiology and disease pathogenesis. The majority of patients with IBD are diagnosed between the ages of 15-30 (Hanauer, 2006, Wilson et al., 2010), though up to 25% of cases are diagnosed in patients under the age of 18 (Benchimol et al., 2011, Muller et al., 2013, Wilson et al., 2010), with adult onset and paediatric onset distinct in pathogenesis and clinical presentation (Quezada et al., 2013, Turunen et al., 2006).

IBD is a lifelong condition and in most cases requires continuous management to maintain remission (Mowat et al., 2011). The nature of the disease flares and symptoms represents a significant burden to patients physically, emotionally and financially (Lonnfors et al., 2014, Turner and Griffiths, 2011, Devlen et al., 2014, Andrews et al., 2010). Extra-intestinal manifestations are common in IBD patients, particularly during periods of active flares and can affect any organ system (Levine and Burakoff, 2011). Most common complications involve the musculoskeletal system and require further management (Atzeni et al., 2014, Levine and Burakoff, 2011), further compromising the quality of life of IBD patients.

The incidence of IBD has risen dramatically worldwide over the last 50 years (Gasparetto and Guariso, 2013, Molodecky et al., 2012, Norgard et al., 2014), with global incidence estimated at 396 per 100,000 (Wallace et al., 2014). Incidence is greatest in Canada, the USA, Europe, Australia, New Zealand and the Middle East (Molodecky et al., 2012, Vegh et al., 2014), with prevalence in western societies estimated to be as high as 1/1000 (Gasparetto and Guariso,

2013). In Australia the incidence of IBD is 30.3 per 100,000 (Vegh et al., 2014) with disease prevalence estimated at 74,955 in 2013, and predicted to rise significantly over the next decade (PricewaterhouseCoopers, 2013). The rising incidence of IBD, combined with current disease management strategies and increased hospitalization are associated with significant economic burden, with annual costs to the Australian government estimated in excess of $2.7 billion (Access Economics Pty., 2007, PricewaterhouseCoopers, 2013). Better understanding of disease aetiology and pathology will lead to the identification of new clinical management strategies that will improve patient outcomes and reduce the economic burden of disease.

1.1.1 Crohn's Disease

CD can occur along the entire length of the gastrointestinal tract; however, it most commonly affects the ileum and/or colon (Sauer and Kugathasan, 2009). It is characterised by discontinuous transmural inflammatory lesions, non-ceasating granuloma formation and fibrotic complications (Bouma and Strober, 2003, Strober et al., 2007, Xavier and Podolsky, 2007). Classification of disease based on the location and disease behaviour (i.e. non-stricturing, non-penetrating) (Laass et al., 2014) is important in understanding likely disease course and suitable treatment options (Mowat et al., 2011). Extracellular matrix deposition as a result of the transmural inflammation can lead to thickening of the bowel wall (Latella et al., 2014), producing clinically relevant complications including stricture and bowel obstruction (Rieder and Fiocchi, 2008) that require surgical intervention to manage (Rieder and Fiocchi, 2009).

1.1.2 Ulcerative Colitis

Ulcerative colitis (UC), unlike CD that can affect any region of the gastrointestinal tract, is localised to the rectum and colon and is characterised by continuous, superficial mucosal

inflammation (Bouma and Strober, 2003, Conrad et al., 2014). Diagnosis is based on endoscopic and histological findings of superficial mucosal inflammation and bloody diarrhoea that is negative for microbial pathogens (Conrad et al., 2014, Mowat et al., 2011). Characterising the extent of colitis in UC patients is also important to create a more complete clinical picture leading to better disease management (Conrad et al., 2014).

It is estimated that up to 50% of UC patients will experience active disease in any year, with a proportion of patients suffering from continual disease relapses (Mowat et al., 2011). Severe disease episodes in UC are also associated with a small risk of mortality due to complications including bowel perforation, toxic megacolon and infectious complications (Turner and Griffiths, 2011).

1.1.4 Current treatment options

Cure for IBD is rare though can be achieved through total abdominal colectomy and end ileostomy in patients with UC, though not those with CD (Hwang et al 2008). Ultimately the majority of patients with IBD will require continual medical support for their condition (PricewaterhouseCoopers, 2013). A number of therapeutic agents are currently used for the management of IBD (Table 1.1) that vary in their mechanisms of action and suitability for long-term disease management. Biological agents, infliximab, adalimumab and golimumab, that target tumour necrosis factor-α (TNF-α) have shown considerable clinical efficacy for the treatment of both CD and UC (Cohen et al., 2014). Biological agents are administered intravenously or subcutaneously and require frequent clinic visits for treatment infusion (Buisson et al., 2013, Amiot and Peyrin-Biroulet, 2015). Despite the efficacy of IBD therapies, side effects ranging from mild toxic effects such as nausea and headaches, through to more severe or life-threatening toxicities including bone marrow suppression and cancer have been reported, and may necessitate frequent monitoring to identify toxicities early (Carter et al.,

2004b, Gisbert et al., 2007, Scott and Osterman, 2013, Marchioni Beery and Kane, 2014). A meta-analysis that included thirty studies across 20 different countries identified decreasing rates of surgery amongst both CD and UC patients within 10 years of initial diagnosis (Frolkis et al., 2013), and may be suggestive of improvements to the management of IBD. Despite this, surgical intervention is required in most IBD patients at some point in their lifetime due to disease complications or poorly managed disease (Hwang and Varma, 2008), highlighting the need for improved disease management strategies.

Characterising disease location, severity and complications is important for the appropriate management of IBD; however, current endoscopic technologies commonly used to evaluate the state of disease are invasive, costly and limited in the clinical information they provide (Sands, 2015). The ability to predict disease relapse and disease behaviour (Sands, 2015, Liverani et al., 2016) could lead to earlier clinical decisions and improved management of IBD. Blood and faecal markers including c reactive protein (CRP) and faecal calprotectin can help to differentiate between IBD and irritable bowel syndrome (Sands, 2015) and may have applications as part of a larger panel of biomarkers to predict disease relapse, complications and poor disease outcomes (Sands, 2015, Liverani et al., 2016). Despite the promise of CRP and faecal calprotectin, these markers are subject to a number of limitations, these biomarkers are typically only considered alongside other analytes and clinical information (Mowat et al., 2011, Lewis, 2011). There is a need to identify and validate new biomarkers that can accurately reflect and predict disease course in IBD, limiting the necessity of invasive procedures like endoscopy and improving the overall management of this disease. One potential plasma biomarker of disease activity is dipeptidyl peptidase 4, which will be discussed in the later sections of this review.

Table 1.1 Current therapeutic options for the management of IBD.

Treatment	Indications	Administration	Side effects	References
5-aminosalicylates *e.g. Sulfasalazine, Mesalazine*	First line therapy (mild disease) Maintenance therapy for UC	Oral or rectal enema	Nausea, headache, pain, diarrhoea and nephrotoxicy	(Carter et al., 2004b, Marchioni Beery and Kane, 2014, Scott and Osterman, 2013)
Corticosteroids *e.g. Prednisolone, Prednisone, Budesonide, Hydrocortisone,*	Short-term therapy for moderate-severe disease flares. Suitable for CD & UC. Not suitable as maintenance therapy.	Oral or intravenous	Cosmetic effects, diabetes, glaucoma, cataracts and psychiatric complications	(Scott and Osterman, 2013, Mowat et al., 2011, Carter et al., 2004b)
Thiopurines *e.g. Azathioprine, 6-mercaptopurine*	Effective for induction and maintenance of remission in CD Effective maintenance therapy in UC	Oral	Allergic reactions, bone marrow toxicity, hepatotoxicity	(Rogler and Sandborn, 2013, Mowat et al., 2011, Fraser et al., 2002)
Antibiotics *e.g. Metronidazole, ciproflaxin*	Abscess & bacterial overgrowth in CD	Oral or intravenous	Peripheral neuropathy and tendon weakness	(Mowat et al., 2011, Nitzan et al., 2016)

Methotrexate	Second line therapy for CD –suitable for induction or maintenance	Oral, subcutaneous or intramuscular	Gastrointestinal toxicity, hepatotoxicity, opportunistic infections	(Mowat et al., 2011)
Biological agents *e.g. Infliximab, Adalimumab*	Suitable for both induction and maintenance therapy in CD & UC	Intravenous, subcutaneous	Infections, neutralizing antibodies, malignancy	(Cohen et al., 2014, Sandborn et al., 2014, Panaccione et al., 2008)
Surgery	In CD surgery is warranted to treat disease complications (stricture, fistula) & poor symptom management. In UC, poor medical management can warrant surgical resection. UC patients are also at risk of complications such as toxic megacolon (bowel perforation) that warrant surgery. Elective preventative surgery to reduce colorectal cancer risk is also performed in both UC and CD	NA	Short bowel syndrome, malabsorption	(Hwang, 2008, Casillas and Delaney, 2005)

1.2 Pathogenesis of IBD

Despite enormous advances in the knowledge and understanding of IBD, the complex aetiology and the large network of interacting factors remains obscure. Several environmental and host factors have been identified in relation to IBD and are thought to contribute to the dysregulated intestinal immune response that characterises IBD.

1.2.1 Environment and lifestyle

The gastrointestinal tract is a complex network of enterocytes, goblet cells, endocrine cells, immune cells, enzymes and transporter proteins that act as a barrier between the external lumen contents that includes microbes and food-stuffs, and the internal tissue environment (Kurashima et al., 2013). In this unique niche, the intestinal immune system must remain tolerant of the external environment and normal antigens, with loss of tolerance associated with IBD (Duchmann et al., 1995). Immune education or priming through exposure to specific microorganisms during early life is critical for the development of a functioning immune system (Thomas et al., 2017). The hygiene hypothesis first described by Strachan, is now widely supported by epidemiological data, suggesting that the increasing incidence of allergy and autoimmune-type disease is related to reduced exposure to infectious agents (Okada et al., 2010, Lambrecht and Hammad, 2017) as a direct result of improvements in hygiene, sanitation and living conditions. A systematic meta-analysis analysis of 29 studies identified inverse correlations between lifestyle factors, including pet/animal exposure and sharing a house or bed, and the development of IBD (Cholapranee and Ananthakrishnan, 2016). It is thought that these factors may be associated with a lower level of hygiene leading to increased microbial exposure that is protective against allergic and inflammatory disease (Cholapranee and Ananthakrishnan, 2016).

Rates of parasitic infection have been shown to inversely correlate with the frequency of allergic and autoimmune-type diseases (Caraballo, 2018). Helminth infections are associated with Th2-type reactions and immune regulation (Sipahi and Baptista, 2017), with studies in mice and pigs finding *Ascaris lumbricoides* infection has immunosuppressive effects (Caraballo, 2018). However, clinical trials examining the effect of therapeutic infection with *Trichuris suis* and *Necator americanus* in IBD reported no improvements to the clinical responses of patients (Sipahi and Baptista, 2017). The balance between disease and the immunosuppressive nature of helminth infections is complex, and further understanding of these interactions is required before these infections can be used therapeutically.

In 1913, *Mycobacterium avium paratuberculosis* (MAP) was suggested as a specific causative agent of IBD, primarily due to similarities between IBD and the bovine infection, Johne's disease (Khan et al., 2011, Chacon et al., 2004, Naser et al., 2004). It was proposed that humans became infected with MAP after consuming contaminated milk (Chacon et al., 2004). Various techniques have been used to identify MAP within the tissue and blood of IBD patients, including ELISA (Tanaka et al., 1991), immunohistochemistry (Tanaka et al., 1991), PCR (Ricanek et al., 2010) and bacterial culture techniques (Collins et al., 2000). However, the identification of MAP in IBD varies considerably, with studies also identifying MAP within control patients as well as IBD patients (Collins et al., 2000, Juste et al., 2009, Naser et al., 2004, Ricanek et al., 2010). The high positivity of MAP in non-IBD patients may suggest that disease only manifests in genetically predisposed individuals (Juste et al., 2009). The importance of MAP infection in the pathogenesis of IBD remains largely unknown, some studies have suggested antibiotics targeting MAP may represent an effective therapeutic for IBD associated with MAP (Alcedo et al., 2016), though further trials are necessary to demonstrate the utility of MAP eradication in IBD.

Cigarette and tobacco smoking are known major risk factors for several chronic diseases (Rom et al., 2013). However, in IBD, cigarette smoking has paradoxical effects, contributing to the pathogenesis of CD, whilst having almost therapeutic effects in UC (Birrenbach and Bocker, 2004, Lindberg et al., 1988, Mahid et al., 2006, Regueiro et al., 2005). Smoking impairs a number of immune and regulatory functions in the gut including cytokine production, phagocytic functions and antioxidant capacities, which may contribute to the development of IBD (Birrenbach and Bocker, 2004, Sher et al., 1999). Nicotine, one of the main components of cigarettes, mediates its effects through the nicotine acetyl choline receptors that are expressed throughout the gastrointestinal tract (Orr-Urtreger et al., 2005). Mice lacking various subunits of the receptor exhibit altered gastrointestinal function and experience more severe colitis compared to controls during the dextran sodium sulphate (DSS) model (Orr-Urtreger et al., 2005), suggesting an important role for the receptors in mediating normal gastrointestinal function and inflammation. Previous work has suggested that nicotine administered transdermally through nicotine patches, in addition to conventional IBD medications (corticosteroids, mesalazine), improves clinical symptoms in UC patients (Pullan et al., 1994). This is consistent with findings that smokers are protected against the development of UC (Lindberg et al., 1988, Mahid et al., 2006). However, the role of cigarette smoking in CD appears to be more complex, with past-smokers remaining at greater risk of developing CD (Lindberg et al., 1988, Mahid et al., 2006). Furthermore, current and former smokers experience more severe disease and lower quality of life compared to CD patients who had never smoked (Quezada et al., 2013), which may suggest that smoking has long-term effects on the immune system and intestinal health that can contribute to IBD.

Psycho-social factors have been implicated in a number of gastrointestinal disorders, including the onset and exacerbation of IBD (Mayer, 2000). Psychological stress disrupts the gut-brain axis and can alter a number of gastrointestinal functions including secretion, motility, gut

permeability and mucosal blood flow that may contribute to the pathophysiology of gastrointestinal conditions and their broad symptoms (Konturek et al., 2011). In patients with gastrointestinal diseases including IBD, perceived stress was found to positively correlate with depressive scores, fatigue and pain (Edman et al., 2017). A systematic review that included 15 studies also found positive associations between stress, depression and anxiety, and pain in patients with IBD (Sweeney et al., 2018). Given the association between psychological factors and gastrointestinal disease, psychological therapies including cognitive behavioural therapy, hypnosis and mindfulness have been proposed to assist in the management of irritable bowel syndrome and more recently IBD (Ballou and Keefer, 2017). Improvements to quality of life in IBD patients have been associated with psychological therapy, with some evidence of improvements to inflammatory responses and disease activity (Ballou and Keefer, 2017). Although the role of psycho-social factors in the pathogenesis of IBD are not fully understood, the addition of psychological interventions to current pharmacological therapies may lead to improvements in patient quality of life by enhancing resilience and coping strategies (Ballou and Keefer, 2017).

Diet has been strongly associated with the pathogenesis of IBD and can be manipulated to promote gastrointestinal health. Previous work has identified Western diets, in particular, increased consumption of saturated fats, mono and disaccharides, as well as meat and protein, as potential IBD risk factors (Hou et al., 2011, Chapman-Kiddell et al., 2010). In contrast, increased intake of fruit (more than four times a day) or dietary fibre (more than 22.1g/day) reduced the risk of developing CD by 40% and 85% respectively, though had no effect on the risk of UC (Hou et al., 2011). Dietary fibre, or non-digestible carbohydrates that are derived from fruits and vegetables (Buttriss and Stokes, 2008) are particularly important for gastrointestinal health, aiding bowel movements (Brotherton et al., 2014), promoting intestinal barrier function and mucosal immunity (Kim et al., 2013, Rose et al., 2007). Consequently,

dietary fibre consumption may protect against the development of IBD and play a role in the management of IBD gastrointestinal symptoms.

The consumption of fish oil has also been shown to reduce clinical symptoms in IBD patients (Belluzzi et al., 2000) and may be related to the anti-inflammatory effects associated with omega 3 polyunsaturated fatty acids (Calder, 2006). Despite the major roles of dietary components in gastrointestinal health and disease, advice to IBD patients is to self-monitor, identify potential food triggers and avoid these, particularly during disease flares (Aleksandrova et al., 2017). Dietary interventions and adherence to specific diets such as the specific carbohydrate diet or the low fermentable oligosaccharides, disaccharides, monosaccharides and polyol diet has been associated with improvements to gastrointestinal symptoms in IBD patients (Aleksandrova et al., 2017, Hou et al., 2014); however, further work is required to better understand the role of these diets in IBD before these restrictive diets are widely recommended as part of routine therapy. The multitude of environmental factors contributing to the pathogenesis of IBD underlines the need for a holistic approach to the management of IBD.

1.2.3 The intestinal microbiome in IBD

The intestinal microbiome is a vast population of microorganisms that exist in symbiosis with the host with broad roles in immunity, metabolism and gastrointestinal health (Lynch and Pedersen, 2016). Western lifestyle factors and behaviours can alter the microbiome, referred to as a microbial dysbiosis and is associated with poor gastrointestinal health (Dupaul-Chicoine et al., 2013, Simon and Gorbach, 1986) and predisposition to the development of chronic inflammatory diseases such as IBD (Kuhn and Stappenbeck, 2013). Initial colonization of the gastrointestinal tract begins at birth and plays a significant role in the resultant microbiome (Walker and Lawley, 2013, Sekirov et al., 2010). The composition of the intestinal microbiome

is thought to be highly individual and dependent on host factors including genetics (Bonder et al., 2016) and diet (David et al., 2014). Despite the inter-variability in microbiome composition, the overall functions broadly include digestion, immunity, vitamin synthesis and competitive inhibition of pathogens (Lynch and Pedersen, 2016). Premature birth, caesarean deliveries and reductions in breast feeding are associated with delayed colonization and microbial dysbiosis (Walker and Lawley, 2013), with early delivery and preterm birth weight associated with the development of IBD later in life (Sonntag et al., 2007). Antibiotics can also alter the composition of the intestinal microbiome and use of certain antibiotics including penicillins, in paediatric populations has been associated with an increased risk of developing IBD (Kronman et al., 2012).

IBD is in part, characterised by a microbial dysbiosis with reductions to microbial diversity (Chassaing and Darfeuille-Michaud, 2011, Damman et al., 2012, Duchmann et al., 1995). Lower numbers of beneficial anti-inflammatory microbes and increased numbers of pro-inflammatory bacteria are found in the mucosa of IBD patients and may contribute to the pathogenesis of disease (Chassaing and Darfeuille-Michaud, 2011) through altered immunoregulatory functions and the production of short-chain fatty acids (Morgan et al., 2012). Using 16S Ribosomal DNA sequencing of faecal (Ma et al., 2018) and mucosal samples (Nishino et al., 2018, Walker et al., 2011), studies have identified distinct changes to the composition of the microbiome in patients with IBD. Patients with IBD commonly have reduced proportions of species belonging to *Bacteriodetes* and *Firmicutes* (Ma et al., 2018, Nishino et al., 2018, Walker et al., 2011). Ma et al. also found that the relative proportion of *Bacteriodetes* inversely correlated with clinical disease activity indices in patients, suggesting relationships between disease activity and microbial composition. Invasive *Escherichia coli* has been reported in 36.4% of ileal specimens from CD patients (Chassaing and Darfeuille-Michaud, 2011) and up to 10 times more bacterial penetrance within the mucosal layer has

been identified in IBD patients compared to controls (Chassaing and Darfeuille-Michaud, 2011), which may suggest that the closer proximity of the microbiota to the underlying mucosal tissue could have a role in the pathogenesis of IBD through stimulation of the immune response.

Preclinical studies have demonstrated the necessity of luminal bacterial antigens for the initiation and propagation of intestinal inflammation. Germ-free conditions have been shown to prevent colitis in numerous models of colitis (Sellon et al., 1998, Parkes et al., 1998) including the transgenic Tgε26 model of colitis that is characterised by significant T cell driven inflammation (Veltkamp et al., 2001). Using specific pathogen free mice, Veltkamp *et al.* reported severe colitis in the Tgε26 model that were driven by CD4+ T cell responses to the normal enteric bacteria (Veltkamp et al., 2001). A separate study found that preventative or therapeutic treatment with broad spectrum antibiotics, vancomycin-imipenem, significantly reduced DSS colitis (Rath et al., 2001), suggesting prominent roles for bacterial antigens in driving intestinal inflammation.

The prominent roles of the intestinal microbiome in the pathogenesis of IBD led has identified the microbiome as a potential point of therapeutic intervention. Manipulation through dietary changes, pre and probiotics as well as faecal transplantation represent potentially useful treatments for re-establishing the microbial population the is altered in IBD patients (Basson et al., 2017). The use of faecal transplantation is highly effective for the management of *Clostridium difficile* infection, though indications for its use in IBD are less clear (Weingarden and Vaughn, 2017). Synthetic stool, or purified intestinal bacteria has been proposed as an alternative to conventional faecal transplant (Petrof et al., 2013) and could have applications in IBD; however, further validation of this method is required.

1.2.4 Genetic susceptibility to IBD

Genetic predisposition is a critical determinant of IBD pathogenesis. Over 90 genetic susceptibility loci have been identified for IBD (Barrett et al., 2008, Franke et al., 2010, Graham and Xavier, 2013, Kaser and Blumberg, 2011), many of which are implicated in immune functions and regulation (Table 1.2) (Mokry et al., 2014), highlighting the importance of dysregulated immune responses in the pathogenesis of IBD. It is estimated that up to 40% of CD patients across Europe and North America carry one of the major NOD2 variants (Henckaerts and Vermeire, 2007), highlighting the major contribution of NOD2 to IBD risk. The remainder of this section will focus on the role of NOD2 in the pathogenesis of IBD.

Table 1.2 Genetic mutations in immune-relevant loci associated with IBD.

Gene	Chromosome location	Protein	Function	Reference
NOD2	16q12	Pattern recognition receptor composed of 2 caspase binding domains, a nucleotide binding domain and leucine rich repeat domain	Recognition of bacterial muramyl peptides and activation of NF-κβ	(van Heel et al., 2005, Hugot et al., 2001)
TLR4	9q32-33	Type 1 transmembrane protein	Recognition of lipopolysaccharide from gram negative bacteria	(Brand et al., 2005, Franchimont et al., 2004)
TLR2	4q35	Type 1 transmembrane protein	Recognition of bacterial peptides	(Hong et al., 2007)
TLR5	1q41-1q42	Type 1 transmembrane protein	Recognition of bacterial flagellin	(Sheridan et al., 2013)
CD14	5q22-q32	Glycosylphosphatidyl inositol-anchored receptor	Receptor for lipopolysaccharide, initiation of inflammatory cascade	(Wang et al., 2012b)
NF-κβ	4q24	105kDa non-DNA binding cytoplasmic protein, 50kDa DNA binding protein	Transcription factor involved in immune responses	(Zou et al., 2011)
IL23R	1q31.1	Cytokine receptor	Immune regulation; activation of CD4+ T cells	(Chua et al., 2012, Franke et al., 2010)

IRGM	5q33.1	Immunity-related GTPase	Mediates IFN-regulated resistance to intracellular bacteria and protozoa, autophagy	(Glas et al., 2013, Massey and Parkes, 2007)
ATG16L1	2q37	Atg16L protein, forms part of the Atg12-Atg5-Atg16L complex	Autophagy	(Hampe et al., 2007)
CASP9	1p36	Cysteine protease	Cell apoptosis	(Guo et al., 2011)
MST1	3p21	Macrophage stimulating 1 (serum protein)	Cell migration, proliferation, adhesion, apoptosis and cytokine secretion	(Franke et al., 2010, Hauser et al., 2012)

Nucleotide-binding oligomerization domain containing 2 (NOD2) was identified as a susceptibility gene for IBD in 2001 (Hugot et al., 2001, Ogura et al., 2001). *NOD2,* also referred to as *CARD15,* is localised to chromosome 16 and encodes a 1040 amino acid protein, composed of two caspase recruitment domains (CARDs), a nucleotide binding domain and leucine rich repeats (Lesage et al., 2002, Ogura et al., 2001, Yao, 2013). NOD2 is a pattern recognition receptor expressed within the cytosol of immune cells and intestinal epithelial cells (Yao, 2013) that recognises bacterial muramyl dipeptide, and initiates the inflammatory response through the activation of NF-κβ (Henckaerts and Vermeire, 2007, Watanabe et al., 2006). Consequently, NOD2 signalling is required for normal immune responses in the intestine towards commensal and pathogenic bacteria. Loss of NOD2 is associated with reduced antimicrobial abilities and increased pathogenic bacterial load in the intestine (Kim et al., 2011, Petnicki-Ocwieja et al., 2009), suggesting that NOD2 mutations may contribute to the microbial dysbiosis observed in IBD.

Over 118 different *NOD2* mutations have been identified that convey differential susceptibility to IBD (Yao, 2013), with three major mutations accounting for 80% of *NOD2* genetic variants (van Heel et al., 2005). NOD2 mutations in IBD patients have been associated with defective dendritic cell responses towards the bacterial component, muramyl dipeptide (Kramer et al., 2006), reduced defensin-5 secretion from paneth cells (Wehkamp et al., 2004) and enhanced phagocytic activity of activated monocytes from NOD2 variants with IBD (Wolfkamp et al., 2014).

NOD2 has also been implicated in toll like receptor (TLR) signalling pathways, with co-stimulation of NOD2 and TLR2 using bacterial peptidoglycan associated with a reduced pro-inflammatory response (Watanabe et al., 2006, Wehkamp et al., 2004). However, the absence of NOD2 creates a pro-inflammatory environment and stimulates the production of interferon

(IFN)-γ producing T cells due to dysregulated TLR2 responses (Watanabe et al., 2006). Antigen presenting cells derived from NOD2$^{-/-}$ mice produce increased IL-12 in response to TLR2 stimulation, increasing the differentiation of CD4+ T cells into IFN-γ producing T cells (Watanabe et al., 2006). NOD2$^{-/-}$ mice were also more susceptible to the development of colitis induced by *E. coli* expressing oval albumin (Watanabe et al., 2006), suggesting that in the absence of NOD2, inflammatory responses are exacerbated in NOD2$^{-/-}$ mutants by a lack of control of TLR2 signalling. Prior stimulation of NOD2 with muramyl dipeptide *in vitro* is associated with reduced secretion of pro-inflammatory mediators (Hedl et al., 2007), though this effect is lost in NOD2 homozygous mutant. Mutations of the NOD2 gene have clear implications for the risk of developing IBD; however, the research to date indicates that the pathogenesis of IBD is a complex process mediated by interactions with both environmental and genetic factors.

1.3 The Immunopathogenesis of IBD

1.3.1 Innate immunity

The intestinal immune system is composed of several defence mechanisms that protect against pathogens. These defences limit the exposure of the immune system to the luminal bacteria and generate inflammatory reactions to clear invading pathogens (Danese, 2011, Wallace et al., 2014). In IBD, activation of the inflammatory cascade is dysregulated and persistent, leading to chronic inflammation (Bouma and Strober, 2003).

Physical barriers represent the first line of defence against infectious agents and noxious stimuli. The epithelial layer lining the intestinal lumen is selectively permeable, allowing the passage of nutrients whilst preventing the translocation of luminal bacteria. Intestinal goblet cells secrete glycosylated mucins that arrange into a dense mucous layer, rich in antimicrobial

secretions from paneth cells and intestinal epithelial cells (Dupaul-Chicoine et al., 2013, Yamaguchi et al., 2009). During IBD, mucus and antimicrobial production is reduced, and epithelial tight junctions are disrupted, increasing epithelial permeability (Al-Sadi et al., 2014, McGuckin et al., 2009, Pastorelli et al., 2013, Petit et al., 2012, Teshima et al., 2012). A study by Noth *et al.* reported that a single infliximab infusion (5mg/kg) produced a clinical response in CD patients within 7 days, corresponding to improvements in intestinal epithelial paracellular permeability compared to pre-treatment as indicated by lactulose and mannitol sugar uptake (Noth et al., 2012). Increased intestinal permeability has also been reported in healthy first-degree relatives of IBD patients (Teshima et al., 2012) and in association with NOD2 mutations (D'Inca et al., 2006). Changes to intestinal permeability are a characteristic feature of IBD and may contribute to both disease onset and disease severity. A greater understanding of how intestinal permeability interpolates with IBD will lead to a better understanding of disease pathogenesis.

The cells of the innate immune system form a vital part of the hosts defence, identifying antigens via pattern recognition receptors that recognise the conserved molecular patterns of pathogens (Pastorelli et al., 2013). In 1988, toll like receptors (TLRs) were identified in humans, for which there are 10 subtypes that differentially recognise various bacterial components, including lipopolysaccharide, peptidoglycan, lipoproteins and CpG unmethylated DNA (Takeda et al., 2003). Stimulation of TLRs with their specific ligands causes activation of the NF-κβ pathway through MyD88 (Takeda et al., 2003). In the healthy intestine, TLR expression in monocytes/macrophages, dendritic cells and intestinal epithelial cells is down-regulated, associated with tolerance towards TLR ligands (Fukata and Arditi, 2013). In IBD, mucosal expression of TLR4 and TLR2 is up-regulated (Cario and Podolsky, 2000, Walsh et al., 2013), contributing to the lack of tolerance towards luminal bacterial antigens in IBD patients. TLR4 activation also prevents epithelial migration and increases apoptosis *in vitro*

(Gribar et al., 2008), suggesting that TLR4 activation may alter the integrity of the epithelium and contribute to the dysfunctional immune response that characterises IBD (Figure 1.1).

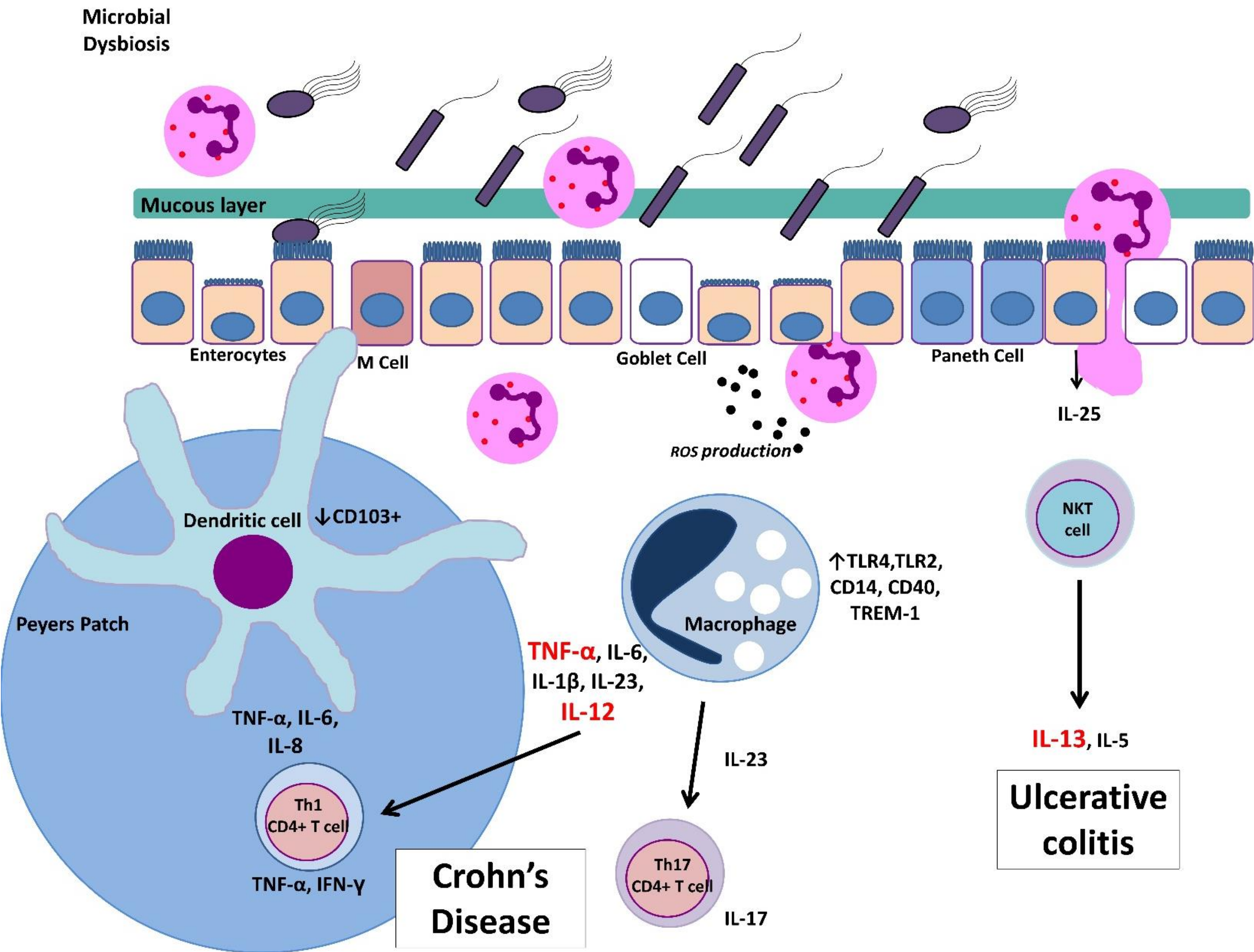


Figure 1.1 Dysregulated immune responses in Crohn's Disease and Ulcerative Colitis. Immune tolerance is lost in human IBD resulting in excessive inflammatory infiltrates and immune responses generated towards luminal antigens. The epithelial and mucous layers are impaired, assisting the translocation of luminal bacteria into the tissue and the stimulation of the immune response. Neutrophil infiltration is a significant feature and forms the basis of mucosal ulcers. The resident dendritic cells and macrophages are also activated and hyper-responsive to stimuli, creating a pro-inflammatory environment and initiating CD4+ T cell responses.

Neutrophils are the first responders to sites of tissue damage and pathogenic invasion. Following initial antigenic exposure, resident macrophages and epithelial cells secrete various chemotactic factors, leading to neutrophil attraction and extravasation from the blood to the tissue site (Fournier and Parkos, 2012, Szabady and McCormick, 2013). Neutrophils then act to clear invading pathogens by phagocytosis and the release of toxic reactive oxygen species (ROS) and cytotoxic enzymes. This response is further amplified by secretion of cytokines and chemokines that recruit and activate other immune cells such as macrophages and other neutrophils (Kumar and Sharma, 2010). Following the acute phase of inflammation, neutrophils become apoptotic and are engulfed by macrophages, preventing non-specific tissue damage as a result of their cytotoxic enzymes (Jaillon et al., 2013).

Neutrophils are the main constituent of mucosal ulcers and crypt abscesses, and represent a significant feature of the gross histology of IBD (Xavier and Podolsky, 2007). The transepithelial migration of neutrophils and generation of ROS contribute to the enhanced permeability of the mucosal layers in IBD (Brazil et al., 2013). Blocking the migration of neutrophils using antibodies directed against adhesion molecules, such as vascular cell adhesion molecule, significantly improves clinical disease parameters and quality of life in patients with CD (Brazil et al., 2013). Neutrophils isolated from patients with IBD also have a number of functional defects such as altered production of ROS (Keshavarzian et al., 1997, Maor et al., 2008, Nielsen et al., 1994, Somasundaram et al., 2013, Verspaget et al., 1988), impaired superoxide dismutase activity (Kruidenier et al., 2003, Maor et al., 2008, Verspaget et al., 1988) and delayed apoptosis (Brannigan et al., 2000, Somasundaram et al., 2013), which allows neutrophils to persist in inflammatory lesions and exacerbate inflammation (Grisham and Granger, 1988). Preclinical evidence in animal models is conflicting, suggesting that blocking neutrophil activation and recruitment prior to colitis induction can suppress the intestinal damage in mice caused by trinitrobenzene sulfonic acid (TNBS); however, it was

unable to alter colitis severity after TNBS colitis induction (Wallace et al., 1998). Neutrophils are major players in host defences against invading microbes, though better understanding of neutrophil responses and their contribution to gastrointestinal inflammation may lead to the identification of inflammatory pathways and molecular changes that represent suitable targets for the treatment of IBD.

Resident intestinal macrophages form a vital part of the mucosal immune system, clearing pathogens, whilst also maintaining an anti-inflammatory environment. In healthy intestine, resident macrophages are tolerant of luminal bacteria, partially due to the down-regulation of TLR2 and TLR4, and the co-stimulatory molecule CD14 (Sanders et al., 2014). Intestinal macrophages create an anti-inflammatory environment by secreting anti-inflammatory mediators, IL-10 and retinoic acid, that stimulate the production of T regs, a CD4+ subtype with roles in suppressing immune responses (Bain and Mowat, 2014, Denning et al., 2007). In contrast, during IBD intestinal macrophages are hyper-responsive, up-regulating co-stimulatory molecules CD14, CD40, TREM-1, activation molecules CD163 and CD68, and pattern recognition receptors TLR2 and TLR4 (Demetter et al., 2005, Franze et al., 2013, Kamada et al., 2008, Perminow et al., 2009, Schenk et al., 2007). These pro-inflammatory macrophages are associated with the spontaneous secretion of pro-inflammatory cytokines, as well as loss of tolerance and hyper-responsiveness towards commensal bacteria (Franze et al., 2013, Reinecker et al., 1993, Kamada et al., 2008, Palmer et al., 2009, Schenk et al., 2007). IBD derived intestinal macrophages secrete pro-inflammatory cytokines IL-12, TNF-α that promote the production of Th1/Th17 CD4+ T cells (Figure 1.1). Th1 cells produce large quantities of IFN-γ that further activate macrophages, exacerbating this inflammatory cycle and contribute to disease severity (Sanchez-Muñoz, 2008).

Dendritic cells are antigen presenting cells that reside in the lamina propria, with roles in priming of naïve CD4+ T cells (Banchereau and Steinman, 1998). There are two main types of dendritic cells plasmacytoid and myeloid, that differ in their expression of cell surface receptors and TLRs (Hostmann et al., 2012). IBD patients with active disease have lower numbers of dendritic cells in the peripheral blood, compared with healthy patients and those with inactive disease, with a high correlation between numbers of peripheral blood plasmacytoid dendritic cells and disease activity (Baumgart et al., 2005). Circulating plasmacytoid dendritic cells from patients with active disease have increased expression of maturation markers compared with healthy controls (Baumgart et al., 2005, Hostmann et al., 2012), correlating with the enhanced production of pro-inflammatory cytokines (Baumgart et al., 2005). Despite this, circulating dendritic cells from UC patients have reduced ability to stimulate T cell responses, an effect that is ameliorated when the UC-dendritic cells are exposed to probiotic bacteria (Mann et al., 2013). In murine colitis there is increased total numbers of dendritic cells in the inflamed mucosa, and lower numbers of CD103+ dendritic cells that are responsible for the production of T regs and IL-10 (Laffont et al., 2010, Strauch et al., 2010). CD103+ lamina propria dendritic cells from mice with colitis also have impaired tolerogenic capacity, instead promoting IFN-γ producing T cells (Laffont et al., 2010), suggesting that during mucosal inflammation, normal dendritic cells responses are altered and may play a role in initiating or aggravating inflammation.

The innate immune system including the intestinal epithelium play a major role in maintaining mucosal immunity and initiating the inflammatory responses from CD4+ T cells that characterises inflammation in IBD. The following section will describe the major roles of the adaptive immune system, specifically CD4+ T cell responses in IBD.

1.3.2 Adaptive immunity

The CD4+ T helper subtype are most strongly implicated in IBD due to the prominent cytokine profiles observed in the disease. Naïve CD4+ T cells differentiate into effector subtypes Th1, Th17, Th2 or T regs depending on cytokine signals received from the surrounding cellular environment (Zenewicz et al., 2009). Historically, CD has been associated with a Th1/Th17 profile (Brand, 2009) while UC has been described as a Th2 type disease (Sartor, 2006) and highlights distinct immunological differences between these two gastrointestinal diseases.

Th1-type responses are identified by the predominance of Th1 cytokines, IFN-γ, IL-6 and IL-12 (Parronchi et al., 1997) and are normally involved in cell mediated defences and delayed hypersensitivity reactions (Singh et al., 1999), as well as in autoimmune and inflammatory conditions like CD (Niessner and Volk, 1995) and multiple sclerosis (Leung et al., 2010). Blocking the IL-6 signalling pathway induced T cell apoptosis *in vitro* and also reduced colitis severity in three separate animal models of colitis (SCID, IL-10$^{-/--}$ and TNBS) that are characterised by aberrant Th1 responses (Atreya et al., 2000). A separate study by Neurath *et al.* previously reported the importance of IL-12 signalling in the generation of Th1 polarizing responses in TNBS-induced colitis in mice, reporting that IL-12 neutralization significantly improved wasting disease and reduced IFN-γ production in lamina propria T cells (Neurath et al., 1995). Clinical trials using biological therapies to target IL-12 have reported improvements to clinical response and remission in CD (Mannon et al., 2004, MacDonald et al., 2016) further highlighting the roles of IL-12 pathways in intestinal inflammation and the importance of targeting this cytokine pathway in CD.

Th17 cells are involved in the normal mucosal defences, as well as the pathogenesis of autoimmune conditions, inflammatory disease and neutrophil responses, and are characterised

by the production of IL-17 and TNF-α these cells are (Miossec and Kolls, 2012). Unlike other CD4+ T helper cells, Th17 cells demonstrate a high degree of plasticity in response to environmental changes such as microbial exposure (Ivanov et al., 2009, Brand, 2009, Raza et al., 2012) and cytokine environments (Stockinger and Omenetti, 2017). Inflamed tissues from IBD patients, particularly CD patients, contain high levels of Th17 cells and IL-17 (Bengsch et al., 2012, Seiderer et al., 2008), however, the specific involvement of these cells in the pathogenesis of IBD remain largely unclear (Stockinger and Omenetti, 2017). On one hand, specific targeting of the Th17 derived cytokine, IL-17A exacerbates disease activity in CD patients (Symons et al., 2012), while targeting dual Th17/Th1 pathways through the neutralization of IL-23 has shown promising results in trials with patients with CD (Cohen et al., 2014). The dichotomy of Th17 cells is still being understood, identifying conditions that promote Th17 pro-inflammatory and regulatory functions is vital and will lead to a greater ability to target these responses in IBD.

Th2 cytokine responses play a major role in defences against parasitic infections, however, aberrant Th2 responses have also been implicated in asthma, allergic disease and UC (Jovanovic et al., 2014). The source of Th2 cytokines in UC was assumed to be the Th2 CD4+ T cells; however, studies suggest a subset of innate lymphoid cells, designated natural killer T cells which are dominant producers of IL-13 in human and experimental models of UC (Sartor, 2006, Fuss and Strober, 2008, Fuss et al., 2004, Heller et al., 2002) may also be involved in the pathogenesis of UC. The oxazolone model of colitis mimics human UC both in the gross pathology and immunopathology and therefore is a good model for investigating the cellular pathology of UC (Heller et al., 2002). Neutralizing IL-25, a potent inducer of IL-13, improved clinical parameters in mice with oxazolone induced colitis, and reduced Th2 cytokines and NKT cells (Camelo et al., 2012). IL-13 also stimulates the phosphorylation of the transcription factor STAT6 that is enhanced in affected tissue from paediatric UC patients (Rosen et al.,

2011). Genetic knock down of STAT6 significantly protected mice against oxazolone induced colitis, preventing significant changes to Th2-type cytokines IL-33, IL-4, IL-5, IL-13, IFN-γ and IL-17 (Rosen et al., 2013). Moreover, in a separate study, the neutralization of both IL-4 and IL-13 significantly reduced disease activity in the oxazolone model of colitis (Kasaian et al., 2014). A separate pilot study of 16 UC patients investigated the effect of treatment with IFN-β-1a, reported that in addition to alleviation of clinical symptoms, the production of IL-13 by mucosal mononuclear cells was altered, with significant reductions seen only in patients with clinical responses (Mannon et al., 2011). A separate study by Lee *et al.* reported that treatment with IFN-α in mice enhanced the T reg population and suppressed colitis induced by T cell transfer (Lee et al., 2012). T regs often identified by the expression of FoxP3 are involved in the regulation of CD4+ responses through the secretion of TGF-β and IL-10 (Himmel et al., 2008) with roles in the prevention of autoimmune and inflammatory disease. In IBD, the ratio of T regs to other CD4+ subtypes is skewed, leading to dysregulated and uncontrolled CD4+ responses (Eastaff-Leung et al., 2010). Promotion of the T reg population using IL-33, reduced disease severity during TNBS colitis in mice (Duan et al., 2012) suggesting that promotion of the T reg population could be an effective treatment for intestinal inflammation.

The current evidence suggests that IBD is a complex, multifactorial disease, where lifestyle factors and genetics play a role in driving inflammation and the loss of immune tolerance to the intestinal microbiome. While the exact cause of IBD may vary between patients, the clinical symptoms and burden associated with this disease is common to all patients suffering with IBD. There is a need to develop and validation new biomarkers that can help to guide clinical decisions providing accurate information about clinical responses and disease behaviour to improve the overall management and outcome for patients with IBD. Dipeptidyl peptidase (DPP)-4 is a serine protease that has roles in immunity, gastrointestinal health implicated as both a biomarker of disease and a potential therapeutic target in IBD. The following section

will review DPP4 and the other DPP enzymes and the associations between these enzymes and IBD.

1.4 Dipeptidyl Peptidases

The DPP4 S9b protease family consists of four structurally homologous serine proteases: DPP4, fibroblast activation protein (FAP), DPP8 and DPP9. The family of proteases have similar enzyme activities, cleaving N-terminal dipeptides from peptides such as chemokines, growth factors and neuropeptides, with a proline or alanine residue in the penultimate position (i.e. X-Pro or X-Ala) (Gorrell, 2005, Sulda et al., 2010, Yazbeck et al., 2009a). DPPs along with few other proline specific peptidases, can hydrolyse proline peptide bonds (Mentlein, 1988, Walter et al., 1980, Vanhoof et al., 1995). This activity is unique as the large rigid pyrrolidine ring of proline, induces conformational strain on the peptide α-helix, creating a kink (Vanhoof et al., 1995). This conformational change prevents the activity of many proteases and also contributes to the specific activity of DPP4 which is restricted by the position of the *trans* proline residue (Vanhoof et al., 1995). The abundance of proline residues in biological molecules highlights the importance of DPP enzymes in the digestion of proline rich peptides (Table 1.3). DPP2 (EC 3.4.14.2) and prolyl endopeptidase (PREP; EC 3.4.21.26) also share this post-proline cleaving activity and are SC Clan serine proteases; however, they lack structural homology to DPP4 related enzymes (Venalainen et al., 2004) and are classed to the S28 and the S9A family of serine proteases, respectively (Wilson and Abbott, 2012, Yazbeck et al., 2009a). The DPP enzymes have differential substrate preferences, expression profiles and physiological roles; however, all may be implicated in IBD.

1.4.1 Dipeptidyl peptidase 4

DPP4 is the most-well characterised protease of the S9b family, cleaving a number of biologically relevant molecules that have led to its widespread investigation in areas including inflammation, metabolism, glucose homeostasis, malignancy as well as cardiovascular and gastrointestinal health. The cellular and soluble expression patterns of DPP4, combined with the specificity of DPP4 enzyme activity has identified a number of potential *in vivo* substrates, while kinetic studies conducted *in vitro* have helped to delineate the most likely DPP4 *in vivo* substrates (Lambeir et al., 2001b, Lambeir et al., 2002), many of which have roles in gastrointestinal health or immunity and are important in intestinal inflammation and IBD (Table 1.3). Glucagon-like peptide-2 (GLP-2) has attracted significant interest as therapeutic targets for IBD and is discussed further below.

DPP4 was first identified in 1966 in rat liver and kidney homogenates using the chromogenic substrate glycyl-DL-prolyl-β-napthylamide, where it was first referred to as dipeptide napthylamidase (Hopsu-Havu and Glenner, 1966). DPP4 protein has since been identified on the apical surface of endothelial, epithelial cells and activated lymphocytes, with abundant expression found in the lungs, kidney and mature villus enterocytes of the small intestine (Yazbeck et al., 2009a, Gorrell, 2005) where DPP4 has a key role in protein assimilation (Tiruppathi et al., 1993). An enzymatically active soluble/serum form of DPP4 has also been identified (Durinx et al., 2000, Iwaki-Egawa et al., 1998, Yu et al., 2011) that is thought to result from the enzymatic shedding of membranous DPP4 from various cell types including adipocytes, smooth muscle cells (Rohrborn et al., 2014) and specialized T lymphocytes (Nargis et al., 2017, Casrouge et al., 2018). A separate study by Chowdhury *et al.* found that treatment of preadipocytes with insulin, stimulated shedding of DPP4 protein into the cell culture medium, with the authors suggesting that DPP4 expression, activity and shedding may

be regulated in part by a negative feed-back loop that includes glucose regulatory hormones and other gastrointestinal hormones that can be cleaved by DPP4 (Chowdhury et al., 2016). Roles for soluble DPP4 have also been proposed in immune stimulation (Ikeda et al., 2013, Lee et al., 2016); however, the mechanisms regulating plasma DPP4 and its physiological roles remain largely unclear.

Table 1.3 DPP4 substrates have roles in IBD. **ND = not defined.*

Substrate	Significance in IBD	Effect of DPP4 cleavage	Reaction kinetics			References
			k_m	k_{cat}	k_{cat}/k_m	
Eotaxin (CCL11)	Increased in IBD plasma of IBD patients & participates in inflammation in DSS models	Reduced chemotactic potential	25 ± 6	1.9 ± 0.3	0.08 ± 0.01	(Lambeir et al., 2001b, Mir et al., 2002, Waddell et al., 2011, Forssmann et al., 2008)
IP-10 (CXCL10)	Potential therapeutic target, studies investigating anti-IP-10 therapy	Reduced chemotactic potential	4 ± 1	1.8 ± 0.2	0.5 ± 1	(Lambeir et al., 2001b, Sandborn et al., 2017, Proost et al., 2001)
ITAC (CXCL11)	Chemokine, theoretical roles in IBD	Reduced chemotactic potential	9 ± 2	10 ± 1	1.2 ± 0.1	(Lambeir et al., 2001b, Proost et al., 2001)

RANTES (CCL5)	Major driver of chronic colitis in murine model	Altered receptor specificity	1 ± 0.3	0.04 ± 0.004	0.04 ± 0.01	(Lambeir et al., 2001b, Ajuebor et al., 2001, Oravecz et al., 1997)
Stromal-cell derived factor (SDF-1, CXCL12)	Major chemokine involved in leukocyte Th1 cell trafficking	Reduced chemotactic potential	2 ± 1	12 ± 1	5 ± 2	(Lambeir et al., 2001b, Werner et al., 2013, Proost et al., 1998, Shioda et al., 1998)
Glucagon like peptide 1 (GLP-1)	Unchanged in plasma of IBD patients. Roles in IBD unclear.	Inactivation	36 ± 7	7.1 ± 0.5	0.2 ± 0.07	(Lambeir et al., 2002, Trejo-Vazquez et al., 2018, Hartmann et al., 2000a, Mentlein, 1999, Mentlein et al., 1993b)
Glucagon like peptide 2 (GLP-2)	Potential therapeutic target. GLP-2 anti-inflammatory properties and enhances intestinal repair	Inactivation	36 ± 6	0.87 ± 0.06	0.024 ± 0.01	(Lambeir et al., 2002, Drucker et al., 1999, Hartmann et al., 2000a, Mentlein, 1999)

Neuropeptide-Y (NPY)	Regulates immune responses. Deletion of NPY reduces colonic inflammation in murine models	Altered receptor specificity	52 ± 8	40 ± 3	$7.6x10^5 ± 5x10^4$	(Lambeir et al., 2001a, Hassani et al., 2005, Chandrasekharan et al., 2008, Mentlein et al., 1993a, Frerker et al., 2007)
Substance P	Increased in IBD and animal models	Inactivation	ND*	ND*	ND*	(Grouzmann et al., 2002, Margolis and Gershon, 2009, Mentlein et al., 1993a, Yazbeck et al., 2009a)

Cellular DPP4 expression profiles are determined in part by the cell type and the tissue location. Studies have found that fibroblasts isolated from different tissues exhibit differential expression profiles of DPP4 (Nemoto et al., 1999). Meanwhile, within the intestinal epithelium, up to 7-fold higher concentration of DPP4 mRNA are found on the surface of mature villous cells compared to those within the crypt (Darmoul et al., 1991) and may suggest that tissue and specific environmental factors (such as exposure to dietary components) may be involved in the regulation of DPP4 expression. Transcriptional regulation of DPP4 has been proposed with the identification of a 5' flanking sequence of the DPP4 gene (Bohm et al., 1995) that may serve as a binding site for various transcription factors including NF-κβ, AP2, c-myc, butyrate responsive elements, and hepatic nuclear factor-1 (Bohm et al., 1995).

DPP4 expression and activity is altered in response to changes in glucose concentrations, hypoxia and inflammation. Separate *in vitro* studies have found that the DPP4 mRNA expression and enzyme activity is inversely related to exogenous glucose concentrations within the media (Das et al., 2014, Gu et al., 2008). The transcription factor, hepatic nuclear factor-1 was also altered in response to the high glucose concentrations, paralleling the changes to DPP4 mRNA, providing a potential mechanism for the regulation of DPP4 in response to glucose (Gu et al., 2008). Previous work from Nemoto *et al.* found that treatment of primary fibroblasts in culture with pro-inflammatory stimulants including, various cytokines (IL-1α, TNF-α or IFN-γ), LPS or phorbol 12-myrisate 13-acetate upregulated DPP4 mRNA, protein and enzyme activity in a time dependent manner (Nemoto et al., 1999). Changes to DPP4 expression and activity in response to proinflammatory stimuli were also found to be different in fibroblasts isolated from gingiva compared to periodontal ligaments, suggesting that tissue specific factors are also involved in the regulation of DPP4 expression in response to inflammatory stimuli (Nemoto et al., 1999). A study by Chowdhury et al. found that hypoxia (1% pO_2) reduces the expression of DPP4 protein on the surface of adipocytes *in vitro* (Chowdhury et al., 2016).

Understanding the physiological stimuli and conditions that alter DPP4 expression is important and may provide insights into the roles of DPP4 during disease and whether DPP4 represents a suitable marker or therapeutic target.

1.5 DPP4 in gastrointestinal health, damage and inflammation; potential biomarker or therapeutic target

DPP4 is expressed throughout the length of the gastrointestinal tract, with the highest expression found in association with the brush boarder of the ileum (Darmoul et al., 1994, Triadou et al., 1983). In comparison, very low levels of DPP4 expression and activity have been reported in the colon, oesophagus and stomach (Sahara et al., 1983a, Goscinski et al., 2008c). The spatial differences in DPP4 expression and activity led authors to suggest that DPP4 is involved in the specific digestive functions of the small bowel (Triadou et al., 1983). Tiruppathi *et al.* demonstrated specific roles for intestinal DPP4 in the hydrolysis and assimilation of proline rich peptides, finding that animals lacking enzymatically active DPP4 were unable to maintain weight when fed proline rich diets (Tiruppathi et al., 1993). Studies in pigs have also shown that small intestinal DPP4 activity is halved during acute gastrointestinal damage induced by weaning (Montagne et al., 2007), consequently DPP4 may represent a useful marker of intestinal integrity.

A hallmark of gastrointestinal diseases and damage is loss or damage to gastrointestinal mucosal structures that can lead to malabsorption and nutritional deficiencies. DPP4 is highly expressed in the small intestine and is altered during intestinal damage and has been identified as a potential marker of small intestinal mucosal damage and/or integrity (Detel et al., 2007, Ziegler et al., 2003). In a single case study of drug induced villous atrophy, Ziegler *et al.* reported alteration to DPP4 mRNA expression, with lower levels during peak damage while DPP4 expression recovered considerably in association with improvements to gastrointestinal structure and integrity (Ziegler et al., 2003). Separate work in coeliac disease initially focussed on the roles of DPP enzymes in disease pathogenesis. In coeliac disease, small intestinal damage results from an inability to digest the proline rich gliadin peptides (Hausch et al., 2002)

found in barley and rye. DPP4 and DPP4-like enzyme, PREP, have been shown to metabolise gliadin peptides (Matysiak-Budnik et al., 2005, Kozakova et al., 1998) suggesting involvement of these enzymes in the pathogenesis of coeliac disease. Early work found DPP4 enzyme activity was reduced in intestinal tissue from patients with coeliac disease during both acute disease and remission (Sjostrom et al., 1981, Smith and Phillips, 1990), which might suggest DPP4 is contributing to disease pathogenesis. An inverse correlation between small intestinal DPP4 activity and the degree of mucosal lesions was reported in patients with coeliac disease and malabsorption syndromes, with the authors suggesting that intestinal DPP4 represents a marker of intestinal damage (Detel et al., 2007). The dramatic loss of DPP4 expression/activity that occurs widely in small intestinal disease and damage suggests that loss of DPP4 may be a useful marker of small intestinal damage.

DPP4 has been identified as a potential biomarker of numerous inflammatory and autoimmune type diseases (Table 1.4) (Yazbeck et al., 2017) with reports finding that plasma DPP activity inversely correlates with markers of disease and inflammation. A study by Hildebrandt *et al.* found that fasting plasma DPP4 activity levels were approximately 27% lower in patients with IBD compared to healthy controls, and inversely correlated with disease severity markers in CD and UC (Hildebrandt et al., 2001). The authors also identified negative correlations between plasma DPP4 activity and other plasma inflammatory measures including CRP and orosomucoid, suggesting that plasma DPP4 activity could be useful for monitoring disease activity in IBD patients (Hildebrandt et al., 2001). Plasma DPP4 activity was also unchanged between CD and UC patients despite the differing cytokine profiles present in each disease (Hildebrandt et al., 2001). A separate study by the same group, investigated parameters that might be useful for predicting disease course in IBD (Rose et al., 2002). In contrast to the previous study, plasma DPP4 activity was not significantly different in patients with active compared to inactive CD (Rose et al., 2002), which may have been related to the lower patient

numbers in this study compared to that of the Hildebrandt *et al.* study. In the study by Rose *et al.* a number of clinical differences within the patient cohort are identified including medications, previous surgeries and complications (fistula); however, whether these clinical factors contribute to altered plasma DPP4 activity is unclear.

Due to the significant variation in disease severity and behaviour in IBD, Xiao *et al.* limited their investigation to patients with active disease that required hospitalization, suggesting their cohort may have been more uniform than previously discussed studies, containing only patients with severe disease phenotypes (Xiao et al., 2000). Despite the small sample size, Xiao *et al.* found plasma DPP4 activity was reduced by approximately 70% in patients with IBD compared to controls and was associated with an increase in the proportion of circulating intact GLP-$2_{(1-33)}$. A separate study by Moran *et al.* also confined analysis to CD patients with active disease, finding plasma DPP4 protein expression was 1.5 times lower those with CD compared to control patients. Consistent with other studies, Moran *et al.* also found plasma DPP4 protein was negatively associated with CRP; however, did not correlate with CDAI in these patients (Moran et al., 2012). Despite that variation in patient cohorts, these studies suggest utility for plasma DPP activity and plasma DPP4 protein as a biomarker of disease activity in IBD that warrants further characterisation.

The protein expression of DPP4 within affected tissue from IBD patients has been previously evaluated by Moran *et al.* Consistent with other studies characterising DPP4 in small intestinal disease, the authors found DPP4 protein was 2.7-fold lower in active ileal tissue from CD patients compared with controls (Moran et al., 2012). The expression and activity of DPP4 during colonic IBD and how this is altered from normal tissue has yet to be characterised.

Table 1.4 DPP4 enzyme activity and protein expression during inflammatory disease.

Conditions	Soluble		Tissue		References
	Activity	Protein	Activity	Protein	
IBD	↓	↓	-	↓	(Hildebrandt et al., 2001, Xiao et al., 2000, Moran et al., 2012)
Rheumatoid Arthritis	↓	↓	-	-	(Cordero et al., 2001, Hagihara et al., 1987)
Systemic Lupus Erythematosus	↓		-	-	(Hagihara et al., 1987)
Multiple Sclerosis	↓	↓	-	-	(Tejera-Alhambra et al., 2014)
Psoriasis	-	-	↑	↑	(van Lingen et al., 2008)
Asthma	-	↑	-	-	(Lun et al., 2007)
Arthritis	↓	↓	-	-	(Busso et al., 2005)

Combined variable immunodeficiency	-	↑	-	-	(Mahmoudi et al., 2013)
Depression	↓		-	-	(Elgun et al., 1999, Maes et al., 1997)
Hepatitis C infection	↑	↑			(Ragab et al., 2013)
Non-alcoholic steatohepatitis	↑	-	-	-	(Balaban et al., 2007)
Tonsillar Hypertrophy	↑	-	-	-	(Stankovic et al., 2008)
Recurrent Tonsillitis	↓	-	-	-	(Stankovic et al., 2008)
Chronic Rhino sinusitis	-	-	↓	-	(Grouzmann et al., 2002)
Bronchial Inflammation	-	-	↓	-	(Landis et al., 2008)
Chronic Obstructive Pulmonary Disease	↓	-	-	-	(Somborac-Bacura et al., 2012)

Myocardial infarction	-	-	↓	↓	(Krijnen et al., 2012)
Heart Failure	↑	-	-	-	(dos Santos et al., 2013)
Coeliac Disease	↑	-	↓	-	(Detel et al., 2007)

Soluble refers to serum/plasma, ↑ increased, ↓ decreased, relative to control, - indicates data not available

DPP4 also represents a unique therapeutic target for the management of gastrointestinal conditions due to its ability to cleave the intestinotrophic hormone, GLP-2 (Estall and Drucker, 2006). GLP-2 promotes intestinal growth and repair, though has a short biological half-life as a result of DPP4 mediated cleavage (Drucker, 2003, Ivory et al., 2008). Administration of supraphysiological concentrations of GLP-2 reduces disease severity and mortality in animal models of colitis (Wu et al., 2015a, L'Heureux and Brubaker, 2003) and necrolizing enterocolitis (Nakame et al., 2016). In patients, GLP-2 degradation resistant analogue, teduglutide, has shown efficacy in the management of short-bowel syndrome (Jeppesen et al., 2012) and active CD (Buchman et al., 2010), however, the injectable nature combined with concerns regarding increased risk of neoplasia (Ring et al., 2018) have somewhat dampened research efforts in this area. Orally available DPP4 inhibitors have also been investigated as a therapeutic strategy for intestinal diseases due to their ability to extend the half-life of GLP-2. Simultaneous administration of the DPP4 inhibitor, valine-pyrolidide and GLP-2, prolonged the half-life of biologically active GLP-2, enhancing its intestinotrophic effects in rats (Hartmann et al., 2000b).

Studies utilizing DPP inhibitors in experimental models of colitis (Yazbeck et al., 2010b, Mimura et al., 2013) and short bowel syndrome (Okawada et al., 2011), have reported reduced disease severity and improved intestinal functions that correlate with increased circulating GLP-2 concentrations. In a separate study, mice with DSS colitis treated with the DPP4 inhibitor, Lys[Z(NO_2)]-pyrrolidide in combination with an aminopeptidase inhibitor, had improved clinical disease parameters and reduced inflammatory infiltrates (Bank et al., 2006), as well as increased gene expression of TGF-β and *foxp3* that is associated with T regs (Bank et al., 2006). Modified T reg populations and reduced MPO activity were also reported in mice treated with non-selective DPP inhibitors Ile-Thia and Ile-Pyrr-(2-CN)*TFA during DSS

colitis (Yazbeck et al., 2010b), suggesting potential roles for DPP enzymes in regulating immune responses involved in the pathogenesis of colitis.

Inhibitors utilized by Yazbeck *et al.* and Bank *et al.* were non-selective, and effects could have been mediated through DPP enzymes other than DPP4 (Lankas et al., 2005). To determine the potential involvement of DPP4 and the other DPP enzymes in colonic inflammation of DSS colitis, Yazbeck *et al.* also characterised the mRNA expression and enzyme activity of DPPs within the affected tissues. Despite not reaching statistical significance, colonic mRNA expression of DPP4, FAP, and DPP9 was lower in diseased animals, while the mRNA expression of DPP8 and DPP2 was significantly enhanced in colonic tissue from diseased animals (Yazbeck et al., 2010b). Consistent with this, the authors found enzyme activity attributed to DPP4 appeared to decrease while activity attributable to DPP8/9 and DPP2 increased (Yazbeck et al., 2010b). In contrast, a separate study by Sakanaka *et al.* found that DPP4 activity was increased in the colonic mucosa in mice with DSS colitis compared to disease free controls. The authors also found that FAP mRNA was higher within the damaged mucosal tissue from animals with DSS colitis compared to controls (Sakanaka et al., 2015). Despite the contrasting results from Yazbeck *et al.* and Sakanaka *et al.* these studies suggest that the mRNA expression and enzyme activity of DPPs is altered during colonic inflammation. These studies suggest that other DPP enzymes may be involved in colonic inflammation and suggest the need to characterise all DPP enzymes in human IBD. Understanding whether these changes are associated with damage, such as during ileal disease, or reflect the high levels of infiltrating immune cells and inflammation present in IBD lesions will assist in determining the utility of DPPs in IBD and whether they represent a biomarker or therapeutic target.

Despite the promising results produced by DPP inhibitor studies, genetic knock-down of DPP4 does not protect animals against experimental colitis (Baticic et al., 2011, Detel et al., 2016,

Yazbeck et al., 2010b); however, a number of immunological changes have been reported in $DPP4^{-/-}$ mice. DPP4 null animals were found to have constitutively higher levels of circulating vasoactive intestinal peptide, neuropeptide Y, IL-6 and IL-10 compared to wild type animals (Baticic et al., 2011). Colonic IL-10 levels were also enhanced in $DPP4^{-/-}$ animals, with the authors suggesting that DPP4 may be involved in the regulation of IL-10 during inflammation (Baticic et al., 2011). $DPP4^{-/-}$ animals also have constitutively higher proportions of circulating CD8+ and natural killer cells compared to wild type animals (Detel et al., 2016), providing further evidence that DPP4 is involved in immune responses. During colitis $DPP4^{-/-}$ mice were found to have enhanced colonic MPO activity and increased expression of NF-κβ in epithelial cells compared to wild type animals (Detel et al., 2016), which could suggest regulatory roles for DPP4 in mediating the inflammatory response in the intestinal epithelium.

1.6 DPP4 and DPP inhibitors modulate immune functions; implications for IBD

DPP4 was first identified as a drug target for the management of type-2 diabetes in 1996 (Gorrell et al., 2001) due to its capacity to cleave the insulinotropic hormone, GLP-$1_{7\text{-}36}$ (Hansen et al., 1999, Mentlein et al., 1993b). GLP-1 is secreted by the L cells of the distal small-intestine and colon and simulates insulin secretion (Deacon et al., 1998). Due to its degradation by DPP4, GLP-1 within the plasma has a rapid half-life of less than 1 minute (Hansen et al., 1999, Deacon et al., 1996). Preclinical studies using non-selective inhibitors such as valine-pyrrolidide and P32/98, found that DPP inhibition could extend the biological activity of GLP-1, and subsequently alter glucose and insulin responses (Deacon et al., 1998, Pospisilik et al., 2002). To date a number of selective DPP4 inhibitors have been approved for the clinical management of hyperglycaemia in type-2 diabetes (Table 1.5).

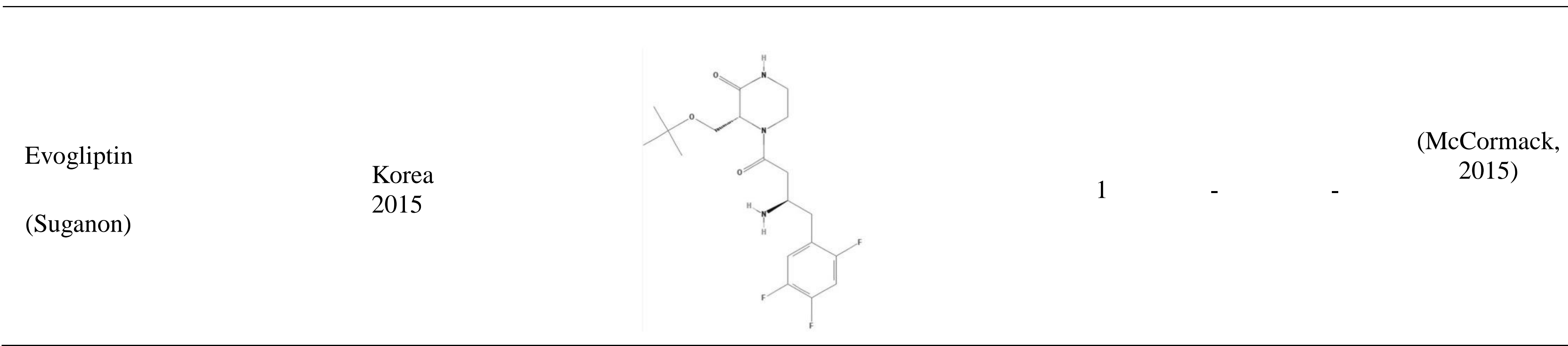
Table 1.5 DPP4 inhibitors approved for the management of type-2 diabetes.

Inhibitor	Company	Approval	Chemical Structures	IC50 (nmol/L)			References
				DPP4	DPP8	DPP9	
Sitagliptin (Januvia)	Merck & Company	FDA/US 2006		18	$>1.x10^{4}$	$>1.x10^{4}$	(Kim et al., 2005)
Vildagliptin (Galvus)	Novartis	EU 2007		3.5	$9x10^{3}$	-	(Brandt et al., 2005, Kirby et al., 2010)

Saxagliptin (Onglyza)	Bristol-Myers Squibb & Astra Zeneca	FDA/US 2009		4	-	-	(Wang et al., 2012a)
Linagliptin (Tradjenta)	Eli Lilly & Company and Boehringer Ingelheim	FDA/US 2011		1	$4x10^4$	$>1x10^4$	(Thomas et al., 2008)
Gemigliptin	LG Life Sciences	Korea 2012		10	$2.7x10^5$	$2.3x10^4$	(Kim et al., 2016)

Anagliptin (Suiny)	Sanwa Kagaku	Japan 2012		3.3	$8.5x10^4$	$5.6x10^4$	(Watanabe et al., 2015)
Teneliglipti n (Tenelia)	Mitsubishi Tanabe Pharma	Japan 2012		0.37	260	540	(Yoshida et al., 2012)
Alogliptin (Nesina)	Takeda	FDA/US 2013		7	$>1x10^5$	$>1x10^5$	(Kirby et al., 2010)

Imigliptin	Xuanzhu Pharma Co Ltd	China 2014		9	>1x10^{5}	>1x10^{5}	(Shu et al., 2014)
Trelagliptin		Japan 2015		1.3	-	-	(McKeage, 2015)
Omarigliptin (MK-3102)	Merck & Co	Japan 2015		1.6	>6.7x10^{4}	>6.7x10^{4}	(Biftu et al., 2014)

Evogliptin (Suganon)	Korea 2015		1	-	-	(McCormack, 2015)

Chemical structures adapted from National Centre for Biotechnology Information (NCBI). PubChem Compound Database

DPP4 inhibitors are a highly safe and effective treatment for the management of hyperglycaemia in type-2 diabetes (Amori et al., 2007, Williams-Herman et al., 2008). Few studies have identified severe adverse reactions including acute pancreatitis (Elashoff et al., 2011), arthralgia (Men et al., 2017), cardiovascular complications (Scirica et al., 2013, White et al., 2013) and more recently IBD (Abrahami et al., 2018) in association with DPP4 inhibitors use. However, it is unknown why certain patients receiving DPP4 inhibitors are predisposed to these reactions. In contrast, beneficial off target effects have also been reported in patients receiving DPP4 inhibitor therapy. Clinical (Read et al., 2010, Barbieri et al., 2013, McCormick et al., 2014) and pre-clinical studies (Matsubara et al., 2012, Akita et al., 2015, Vittone et al., 2012) suggest that DPP4 inhibition significantly improves cardiovascular function, reducing atherosclerotic plaque burden and vascular dysfunction, suggesting that DPP4 inhibitors are highly beneficial for vascular health. A separate population-based cohort study on insurance claims lodged between 2005 and 2012, found that DPP4 inhibitors may also reduce the incidence of autoimmune conditions, including rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, psoriasis, psoriatic arthritis and IBD (Kim et al., 2015). Analysis of 243,153 eligible patients found the combined risk of autoimmune conditions was reduced in patients receiving DPP4 inhibitor therapy compared to patients receiving non-DPP4 therapy (relative risk 2.99 vs 3.88) (Kim et al., 2015). Specific roles for DPP4 in the immune response have been reported (Klemann et al., 2016, Waumans et al., 2015) and together may suggest further involvement of DPP4 in IBD as a driver of inflammation and a potential therapeutic target.

DPP4 is differentially expressed on the surface of immune cells, where it is also referred to as the cell surface antigen, CD26 (De Meester et al., 1992). CD26 expressing CD4+ T cells are involved in specific T helper functions and enhanced responses to recall antigens (Morimoto et al 1989). CD26 is only weakly expressed by naïve T cells, though is upregulated *in vitro* during activation by mitogens such as phytohemagglutinin (Cordero et al., 1997). Co-stimulation with the pro-inflammatory cytokine, IL-12 that is involved in the differentiation of Th1 CD4+ cells, has been reported to further enhance CD26 expression (Cordero et al., 1997, Salgado et al., 2000) and may suggest a role for CD26 in Th1 driven immune responses. In line with this, CD26 expression has been identified on the surface of specific CD4+ T cell subpopulations, with highest expression reported on Th17 cells that are involved in driving inflammation during IBD (Bengsch et al., 2012, Jiang et al., 2014), and the lowest expression on T regulatory cells, implicated in the regulation or suppression of immune responses (Salgado et al., 2012).

CD26 expressed on CD4+ T cells contributes to T cell activation through interactions with membrane proteins such as CD45 (Torimoto et al., 1991) and caveolin-1 (Ohnuma et al., 2008) though studies using DPP4 inhibitors have also highlighted roles for DPP4 enzyme activity in T cell responses (Tanaka et al., 1997, Adams et al., 2004). CD26 expressing T cells are thought to contribute to the pathogenesis of diseases such as rheumatoid arthritis and have been shown to correlate with disease activity (Muscat et al., 1994). In experimental models of arthritis, the subcutaneous injection of rats with non-selective DPP4 inhibitors including Ala-Boro-Pro or Lys(Z(NO_2))-thiazolidide prevented joint inflammation and changes to body weight associated with arthritis induction (Tanaka et al., 1997). The authors also found that treatment of lymphocytes with Lys[Z(NO_2)]-thiazolidide significantly reduced mitogen and antigen induced proliferation *in vitro*, however, when repeated in lymphocytes isolated from DPP4 deficient animals, identical lymphocyte responses were observed (Tanaka et al., 1997),

suggesting that inhibition may not be mediated by DPP4. A separate study by Steinbrecher *et al.* investigating the roles of DPP4 in an experimental model of autoimmune encephalitis found DPP4 inhibition using Lys[Z(NO2)]-pyrrolidide delayed the onset of disease compared to control animals (Steinbrecher et al., 2001), and may suggest that DPP4 inhibition is beneficial and can reduce disease severity in T cell driven inflammation.

As discussed in Chapter 1.3, neutrophil infiltration is a significant feature of IBD. Studies in various animal models of inflammation have shown that neutrophil recruitment is reduced by DPP4 inhibition, suggesting roles for DPP4 in neutrophil recruitment. In a murine model of diabetic wound healing, linagliptin treatment reduced neutrophil infiltration, and pro-inflammatory markers, cyclooxygenase-2 and macrophage inhibitory protein-2, while enhancing re-epithelization of wounded tissue (Schurmann et al., 2012). A separate study investigated the effect of DPP4 inhibition in a murine model of ischemia-reperfusion injury induced lung graft failure (Jungraithmayr et al., 2010) where enhanced neutrophil recruitment and oxidation contribute to the loss of graft function. Treatment of lung-allografts with DPP4 inhibitor, AB192, prior to surgical transplant surgery improved graft ultrastructure and reduced graft neutrophil infiltration compared to untreated controls (Jungraithmayr et al., 2010). The authors also found that DPP4 inhibition enhanced VIP within lung tissue (Jungraithmayr et al., 2010), suggesting that VIP may be involved in neutrophil recruitment during airway inflammation.

Enzymatically active DPP4 may also act as a neutrophil chemo-repellent. In an *in vitro* chemotaxis system, unstimulated and TNF-α stimulated human derived peripheral blood neutrophils were found to migrate away from recombinant DPP4 in a dose dependant manner (Herlihy et al., 2013). DPP inhibitors, Diprotin A and DPPI 1c hydrochloride when added to the chemotactic system alone showed no chemoattractant abilities. When the inhibitors were

added in combination with the recombinant DPP4 gradient, the chemo-repulsive activity of DPP4 was significantly attenuated suggesting that the ability of DPP4 to act as a chemo-repellent is dependent on its enzymatic activity (Herlihy et al., 2013). In a separate study of lung inflammation, Herlihy *et al.* found that treating mice with aerosolized recombinant DPP4 reduces neutrophil infiltration to the lungs induced by bleomycin, though no changes to other inflammatory cell types were observed (Herlihy et al., 2013). The authors replicated these results in a separate study of collagen-induced-arthritis, reporting that the injection of recombinant DPP4 to the joint reduced neutrophil and macrophage infiltration, improving joint inflammation and disease severity (Herlihy et al., 2015). The authors hypothesised that the gradient of DPP4 between tissues and serum is important for the normal recruitment of immune cells, and that the addition of recombinant DPP4 to tissues may alter this gradient, affecting immune cell recruitment (Herlihy et al., 2015). DPP4's ability to directly modify immune cell infiltration remains unclear, and further studies should investigate the physiological relevance of this phenomenon *in vivo*.

ROS production is a major neutrophil function and can contribute to tissue damage in IBD (Fournier and Parkos, 2012). Kroller-Schon *et al.* examined the effect of the DPP4 inhibitor, linagliptin, on the *in vitro* reactive oxygen species production of human neutrophils. Linagliptin dose-dependently reduced ROS production in lipopolysaccharide-stimulated neutrophils as determined by chemiluminescence (Kroller-Schon et al., 2012). Other DPP4 inhibitors, sitagliptin, vildagliptin, alogliptin and saxagliptin; however, had little effect on *in vitro* ROS production from human neutrophils (Kroller-Schon et al., 2012). Linagliptin also reduced the adhesion of lipopolysaccharide-activated human neutrophils to an endothelial monolayer *in vitro* (Kroller-Schon et al., 2012). Kroller-schon and colleagues speculate that the observed differences between linagliptin and other DPP4 inhibitors may be attributable to differences in their chemical structures. This work may suggest that linagliptin can be used to reduce

neutrophil adhesion, recruitment and ROS production which could have applications in chronic inflammatory conditions like IBD.

Macrophage responses are also altered within the mucosa of IBD patients. In a murine model of atherosclerosis, treatment with alogliptin significantly reduced macrophage content within plaques (Shah et al., 2011). Using flow cytometry, the authors reported reduced numbers of pro-inflammatory M1 macrophages ($CD11b^{+}Ly6C^{high}$), while macrophages expressing anti-inflammatory M2 markers, CD163 and Ym-1, were increased within the adipose tissue of inhibitor treated mice, suggesting that DPP4 inhibition may also modulate macrophage differentiation (Shah et al., 2011). Using an *in vitro* cellular transmigration system, Shah *et al.* confirmed that treatment with either alogliptin or sitagliptin reduced cellular migration of the monocytic cell line, THP-1, towards the chemokine, CCL-2. Furthermore, in an *in vivo* setting, injection with enzymatically active DPP4 was shown to stimulate the migration of labelled-monocytes into the aorta, a finding that was prevented by treatment with sitagliptin (Shah et al., 2011), suggesting that DPP4 may also be involved in macrophage responses.

Many studies previously utilized non-selective DPP inhibitors, in both *in vivo* and *in vitro* models, consequently, some of the effects originally attributed to DPP4 may be mediated through other DPP related enzymes. Furthermore, common methods for the detection of DPP activity are not selective for DPP4, and may reflect activity from other enzymes including FAP, DPP8 and DPP9 (Aertgeerts et al., 2005, Ajami et al., 2004, Abbott et al., 2000). Other DPP enzymes are also altered during various disease states described below, suggesting the importance of appropriately characterising DPP4 in addition to other DPPs in biomarker investigations to appropriately identify suitable biomarker candidates.

1.7 Other DPP enzymes

1.7.1 Fibroblast Activation Protein

Unlike DPP4 that is highly expressed on the surface of endothelial, epithelial and immune cells, the expression of highly related enzyme, FAP, is limited in normal tissues, instead associated with sites of wound healing, fibrosis and cancer (Gorrell, 2005). FAP also referred to as seprase, shares 52% of its amino acid identity with DPP4 (O'Brien and O'Connor, 2008), increasing to 70% between the amino acids that form the catalytic site in the α/β hydrolase domain (Kelly, 2005). Despite the high similarity between DPP4 and FAP, selective (Kim et al., 2016) and non-selective DPP inhibitors (Lankas et al., 2005) are less likely to inhibit FAP compared to other DPP enzymes, such as DPP8 and DPP9. FAP has DPP4-like exopeptidase activity, with the ability to cleave N-terminal post-proline bonds from substrates such as Ala-Pro- and Gly-Pro; however, its affinity for these peptide bonds is less than that of DPP4 (Aertgeerts et al., 2005). In addition to its DPP4-like activity, FAP unlike DPP4 and other DPP enzymes, exhibits collagenase or gelatinase activity, with type-1 collagen a major substrate for FAP (Aggarwal et al., 2008, Kelly, 1999, Pineiro-Sanchez et al., 1997). Consistent with its substrate preference, FAP is an integral membrane protein that is expressed on the apical

surface of activated fibroblasts that are present in areas of fibrosis, wound healing and cancer (Levy et al., 2002, Yazbeck et al., 2017, Levy et al., 1999). FAP is implicated in inflammatory and fibrotic conditions, with expression is induced by pro-inflammatory cytokines (Milner et al., 2006) and fibrotic factors such as TGF-β (Chen et al., 2009) and may have relevance to IBD that is characterised by relapsing-remitting inflammation and fibrotic complications.

Specific roles for FAP have been proposed in the pathogenesis of cartilage degradation of rheumatoid arthritis (Ospelt et al., 2010, Waldele et al., 2015). FAP is highly expressed on the surface of synovial fibroblasts in rheumatoid arthritis and is localized along the protruding membrane of the fibroblasts (Ospelt et al., 2010, Waldele et al., 2015). Studies from Ospelt *et al.* and Waldele *et al.* used different models of arthritis and blocked FAP in different ways, using an enzyme inhibitor and selective deletion respectively, though both would suggest that blocking or inhibiting FAP would produce little clinical benefit in this setting. While FAP may not represent a suitable therapeutic target, a study by Laverman *et al.* has described the use of specifically labelled FAP antibodies to assist radiological imaging techniques to assess the degree of joint inflammation (Laverman et al., 2015). Using radiological imaging techniques, the authors reported significant accumulation of anti-FAP antibodies in the affected joints of mice that strongly correlated with the degree of inflammation (Laverman et al., 2015), with very little background staining. Assessing FAP expression using these techniques could assist in evaluating the degree of inflammation and monitoring the effectiveness of treatments (Laverman et al., 2015). FAP expression and activity is also up-regulated in liver fibrosis (Levy et al., 1999, Levy et al., 2002, Wang et al., 2005), idiopathic pulmonary fibrosis (Acharya et al., 2006) and in association with strictures in CD patients (Rieder et al., 2011), suggesting potential utility of monitoring FAP expression in IBD that requires further evaluation.

Like DPP4, a circulating form of FAP also exists that has been previously referred to as anti-plasmin cleaving enzyme (Lee et al., 2006). Keane *et al.* developed and validated an assay for the specific detection of FAP enzyme activity in tissue and body fluids. Consequently, FAP enzyme activity has been quantified in human and primate plasma (Keane et al., 2013) and in various primate organs, where the authors reported high activity levels in healthy skin samples, which may suggest roles for FAP in some normal physiological conditions (Keane et al., 2013). Using this assay, studies have identified elevated FAP plasma activity in patients with liver cirrhosis (Williams et al., 2015, Uitte de Willige et al., 2013) and have suggested that plasma FAP (referred to as cFAP by others) could assist in differentiating patients according to their risk of clinically relevant liver fibrosis (Williams et al., 2015). Further work by Uitte de Willige and colleagues found that plasma FAP activity declines significantly following liver transplantation (Uitte de Willige et al., 2017) and suggests that plasma FAP activity may be a useful measure for predicting or monitoring fibrotic disease and highlights the need for further work to characterise the relationship between plasma FAP, tissue FAP expression and current clinical markers.

1.7.2 Dipeptidyl peptidase 8/9

Dipeptidyl peptidase 8 (DPP8) and 9 (DPP9) are cytoplasmic monomeric proteins that also exhibit DPP-like activities, cleaving N-terminal post-proline bonds (Bjelke et al., 2006), cleaving several DPP4 substrates *in vitro*; however, based on the cytoplasmic location of these enzymes the significance of this is yet to be determined. Studies have identified a number of potential DPP8/9 substrates including calreticulin, chemokine CXCL10 and IL-1R antagonist (Table 1.6) that are implicated in IBD. Calreticulin and CXCL10 are associated with leukocyte trafficking and have been proposed as potential therapeutic targets for IBD (Ohkuro et al., 2018, Sandborn et al., 2017), while studies have found that polymorphisms within *IL-1RA* are associated with IBD (Vijgen et al., 2002, Carter et al., 2004a, Daryani et al., 2015).

Consequently, DPP8/9 regulation of these substrates may suggest DPP8/9 also have roles in IBD that require further investigation.

DPP8 and DPP9 are highly homologous, sharing a high degree of amino acid identity (Ajami et al., 2004) making it difficult for studies to discriminate between DPP8 and DPP9 activities. The homology between DPP8 and DPP9 and that of DPP4 (Abbott et al., 2000, Olsen and Wagtmann, 2002) is also significant with many DPP inhibitors also able to target DPP8/9 (Table 1.5). The 3D crystal structures of DPP8 and DPP9 were recently described by Ross *et al.* highlighting structural differences between these enzymes and DPP4 that will assist in the development of new selective inhibitors (Ross et al., 2018).

The use of potent selective DPP8/9 inhibitors, including 1G244 has proven a highly useful research tool for differentiating DPP activity relating to DPP8/9 from that of DPP4, and characterising DPP8/9 enzyme activity in tissues (Wu et al., 2009, Dubois et al., 2009, Matheeussen et al., 2013). DPP8 and DPP9 are found ubiquitously in normal tissues (Ajami et al., 2004, Olsen and Wagtmann, 2002, Harstad et al., 2013, Yu et al., 2009, Dubois et al., 2009) with expression particularly associated with mucosal and epithelial structures (Harstad et al., 2013, Yu et al., 2009) in animals. DPP8/9 activity has also been characterised in murine and human derived immune cells (Maes et al., 2007a, Matheeussen et al., 2013, Waumans et al., 2016). Matheeussen *et al.* found that DPP8/9 activity along with DPP9 protein was upregulated in *in vitro* differentiated and activated macrophages, suggesting roles for DPP9 in macrophage responses. The authors found that selective targeting of DPP9 using small interfering RNA, blocked the production of TNF-α and IL-6 in M1 macrophages (Matheeussen et al., 2013). Uncontrolled pro-inflammatory M1 responses are a feature of IBD and may suggest roles for DPP9 in regulating inflammatory responses in IBD. A separate study by Chowdhury *et al.* identified DPP8 and DPP9 mRNA within murine CD4+ lymphocytes, finding that DPP8 and

DPP9 were upregulated following activation in lymphocytes (Chowdhury et al., 2013). The authors found that the overexpression of active DPP9 was associated with apoptosis in these cells (Chowdhury et al., 2013) and may suggest roles for DPP9 in limiting lymphocyte responses through apoptosis.

The expression of DPP8 and DPP9 is also altered during disease. Separate studies from Sulda *et al.* and Wilson *et al.* have characterised DPP8 and DPP9 mRNA and protein expression in primary malignant B cells (Sulda et al., 2010) and panels of breast and ovarian carcinoma cell lines, finding that DPP8 and DPP9 are consistently expressed in these malignant cells. Roles for DPP8/9 have also been suggested in liver damage and disease, with lower levels of DPP8 and DPP9 mRNA found in livers of patients with end-stage primary biliary cirrhosis and during murine models (Chowdhury et al., 2013)**.** In a separate study, Schade *et al.* found DPP8 and DPP9 mRNA and protein levels were elevated in bronchial tissue of mice during asthma compared to control animals, with authors suggesting potential roles for DPP8/9 in the inflammatory response in this tissue (Schade et al., 2008). In addition to the work previously described by Yazbeck *et al.* these studies suggest roles for DPP8/9 in tissue inflammation and warrant further characterisation of DPP8/9 in human IBD tissues.

Table 1.6 Potential DPP8/9 substrates & physiological relevance to IBD.

Substrate	Relevance in IBD	Physiological roles	Effect of DPP8/DPP9 cleavage	Reference
Calreticulin	Small molecule inhibitor for calreticulin, identified as potential therapeutic target for IBD	Calcium binding protein in endoplasmic reticulum	Unclear	(Wilson et al., 2013, Ohkuro et al., 2018)
CXCL10 (IP-10)	Identified as a potential therapeutic target to modulate leukocyte infiltration in IBD	Chemokine - stimulates immune cell activation and migration	Unclear	(Zhang et al., 2015, Sandborn et al., 2017)
IL-1RA	Polymorphisms in IL-1RA associated with CD and UC	Modulates IL-1 related immune and inflammatory responses	Unclear	(Zhang et al., 2015, Daryani et al., 2015)
Renal ubiquitous 1 ($RU1_{34-42}$)	Unknown	Peptide antigen presented via MHC I molecules	DPP9 cleavage prevents MHC I presentation and CD8 stimulation	(Geiss-Friedlander et al., 2009)
Adenylate kinase	Unknown	Adenosine nucleotide regulation/metabolism and cellular energy	Increased activity and altered binding affinity. Activation of novel apoptosis pathway	(Wilson et al., 2013)

Collagen alpha-1 (VII) chain	Unknown	Cell adhesion	Unclear	(Zhang et al., 2015)
Nuleobindin-1	Unknown	Calcium binding and homeostasis	Unclear	(Zhang et al., 2015)
S100-A10 protein	Unknown	Calcium binding protein. Cell cycle progression	Unclear	(Zhang et al., 2015)

1.7.3 Dipeptidyl peptidase 2

Dipeptidyl peptidase 2 (DPP7 or quiescent cell proline dipeptidase), does not share structural homology to DPP4 and is not part of the S9B family; however, it does possess a DPP4-like enzyme activity cleaving post-proline residues (Maes et al., 2007b, Maes et al., 2005). DPP2 is a lysosomal enzyme, consistent with this its optimal activity is detected at acidic pH (Maes et al., 2005). DPP2 has the ability to cleave commonly used DPP4 colorimetric and fluorometric substrates such as -Ala-Pro-; however, its activity is diminished above pH 7, allowing for studies to discriminate between activity from DPP2 and other DPPs by raising the pH (Maes et al., 2005). DPP2 activity accounts for majority of DPP activity in primary immune cells and lymphocytic and monocytic lines (Maes et al., 2006), suggesting that DPP2 may have major roles in immune cells. A separate study using DPP2 knock down animals, found CD4+ T cells from DPP2$^{-/-}$ animals produced little IL-2 and IL-4 but high amounts of IL-17 and transcription factor *rorγt* that is characteristic of memory Th17 cells (Mele et al., 2011). Th17 cells are involved in driving inflammation in IBD (Bengsch et al., 2012) and may suggest roles for DPP2 in the regulation of T cell responses and the development of inflammatory conditions.

DPP2 has also been investigated within the plasma of patients with autoimmune and inflammatory conditions. Clinical studies in patients with rheumatoid arthritis and systemic sclerosis found significantly higher levels of plasma DPP2 activity compared to healthy controls (Hagihara et al., 1987). Work by Kamori et al. also found altered DPP2 activity within the synovial fluid of affected joints from patients with rheumatoid arthritis, and found positive correlations between the ratio of DPP2/DPP4 activity and clinical markers including erythrocyte sedimentation rate and CRP (Kamori et al., 1991) suggesting potential roles for both DPP2 in joint inflammation and its potential utility as a biomarker of inflammation. The expression of DPP2 within immune cells (Maes et al., 2007a, Mele et al., 2011) and data from

Yazbeck *et al.* suggest that DPP2 could have a role in intestinal inflammation; however, further characterisation is required in IBD clinical samples to understand the likely roles for DPP2 in IBD.

1.7.4 Prolyl endopeptidase

Prolyl endopeptidase (PREP) also known as prolyl oligopeptidase, is another serine protease of the S9 serine protease family (Polgar, 2002), that has similar DPP activity, cleaving endogenous post-proline bonds at the carboxyl end, with the exception of Pro-Pro bonds (Wilk, 1983, Gass and Khosla, 2007). PREP is structurally distinct from DPP4-related enzymes, existing as a monomer (Wilk, 1983) that is comprised of an α/β hydrolase fold and a propeller domain (Fülöp et al., 1998). Studies have identified a number of *in vivo* substrates including oxytocin, angiotensin II, neuropeptides (Wilk, 1983), gliadin (Matysiak-Budnik et al., 2005) and collagen (O'Reilly et al., 2009), with roles for PREP proposed in neurological development and disease, malabsorption, coeliac disease (Pyle et al., 2005), cancer (Suzuki et al., 2014) and inflammation (Abdul Roda et al., 2014). The broad physiological roles of PREP have stimulated interest regarding the potential of PREP as a biomarker, with studies investigating PREP activity in rheumatoid arthritis (Kamori et al., 1991), colorectal cancer (Larrinaga et al., 2014), psychiatric disorders (Maes et al., 1995) and Parkinson’s disease (Nagatsu, 2017); however, these studies have not progressed to clinical validation.

PREP has been identified as an important protease in the liberation of proline-glycine-proline from collagen (Abdul Roda et al., 2014, O'Reilly et al., 2009). This tripeptide has chemotactic effects via CXCR2, recruiting neutrophils to damaged sites (Abdul Roda et al., 2014). Abdul Roda *et al.* found that mice treated with PREP inhibitor, valporonic acid significantly reduced cigarette smoke induced neutrophilic infiltration measured within the bronchial alveolar fluid, suggesting that PREP may be an important target for modulating neutrophil infiltration.

However, work from Koelink *et al.* found that PREP activity was unchanged in tissue from IBD patients compared with healthy controls, despite increased levels of proline-glycine-proline (Koelink et al., 2014), suggesting that PREP activity is not associated with neutrophil infiltration via this mechanism. While not significant, the authors found PREP activity varied significantly in freshly isolated blood neutrophils from IBD patients, with a trend towards increased activity in patients with IBD compared to controls (Koelink et al., 2014), which could suggest some roles for PREP in IBD that warrant further investigation.

1.8 Summary & aims

DPPs are a unique family of serine proteases that have roles in gastrointestinal health and inflammation. The current literature has recognised that DPP4 has potential applications as a therapeutic target but also as a biomarker of disease, particularly those of inflammatory or autoimmune origin. While extensive work has been done characterising DPP4 in small intestinal disease, the expression and activity profiles of DPP4 during colonic inflammation and damage in patients is largely unclear. Further, studies have suggested roles for other DPP4-like enzymes in IBD; however, knowledge of the expression and activity profiles of these enzymes in healthy gastrointestinal tissues and during colonic IBD is lacking.

This study aimed to increase the current understanding of DPPs during gastrointestinal health and disease by comprehensively characterising the mRNA expression and enzyme activity profiles of DPPs in gastrointestinal health and disease. I specifically aimed to;

1. Characterise the mRNA expression and enzyme activity profiles of the dipeptidyl peptidase enzymes throughout healthy gastrointestinal tract and evaluate the clinical potential of DPP4 as a gastrointestinal marker (Chapter 2)
2. Explore the potential roles of dipeptidyl peptidase enzymes in gastrointestinal health and disease by examining the expression and activity profiles in gastrointestinal disease (Chapter 2, 3 & 4)
3. Characterise plasma DPP activity during inflammatory bowel disease, exploring disease specific factors that may be associated with plasma DPP activity (Chapter 3 & 4)

Chapter 2. Dipeptidyl peptidase expression and activity throughout the gastrointestinal tract

2.1 Introduction

The gastrointestinal mucosa is a complex architecture of cells that provide an interface between the external luminal environment and the underlying tissue. Damaged mucosa can result from iatrogenic (including chemotherapy and non-steroidal anti-inflammatory drugs), or idiopathic causes such as IBD, coeliac disease and Barrett's oesophagus. Current technologies for the assessment of gut health and function are largely restricted to invasive imaging methodologies such as endoscopy, colonoscopy, and radiography (Ludvigsson et al., 2014, Bird-Lieberman and Fitzgerald, 2009); however, these techniques are expensive, invasive, and are limited in the functional information they can provide. DPP4 is a serine protease that is differentially expressed in the mucosa throughout the gastrointestinal tract (Triadou et al., 1983, Sahara et al., 1983b) and may represent a novel biomarker of gastrointestinal health.

DPP4 is part of the S9B family of serine proteases that also includes DPP8, DPP9 and FAP (Gorrell, 2005). In the healthy gastrointestinal tract, there is an increasing gradient of DPP4 expression between the duodenum and the ileum, while low to absent DPP4 expression has been reported in the oesophagus and colon (Triadou et al., 1983, Goscinski et al., 2008c). Small intestinal DPP4 is localised to the small intestinal brush border enterocytes, where it has a critical role in the digestion and absorption of proline-rich dietary peptides (Tiruppathi et al., 1993). Dysregulated or altered DPP4 expression and/or enzyme activity has been previously described in oesophageal (Goscinski et al., 2008b, Goscinski et al., 2008c), small intestinal (Detel et al., 2007, Ziegler et al., 2003) and colonic diseases (Yazbeck et al., 2010b); however, the expression of other DPPs including DPP8, DPP9, FAP and PREP throughout the length of the gastrointestinal tract and during disease has been less described.

DPP4 expression and activity within the small intestine represents a candidate marker of intestinal function and integrity. Preclinical models of coeliac disease have reported lower DPP4 activity within the jejunum and the ileum of animals during gliadin induced enteropathy (Kozakova et al., 1998). While in patients with coeliac disease, small intestinal DPP4 activity inversely correlates with the degree of mucosal damage (Detel et al., 2007). PREP is a cytoplasmic serine protease with DPP4-like activity (Wilk, 1983, Gass and Khosla, 2007) that has also been investigated for its roles in the pathogenesis of coeliac disease (Garcia-Horsman et al., 2007, Matysiak-Budnik et al., 2005). Understanding the expression of DPP enzymes in healthy gastrointestinal tissues may reveal insights into their utility as biomarkers of gastrointestinal disease and function.

DPP4 expression is nearly absent from normal oesophageal (Goscinski et al., 2008b, Goscinski et al., 2008c), gastric and colonic mucosa (Sahara et al., 1983b), though is expressed in metaplastic and cancerous lesions of the oesophagus (Goscinski et al., 2008b, Augoff et al., 2014, Goscinski et al., 2008c), though its utility as a biomarker of oesophageal disease remains unknown. Barrett's oesophagus is a metaplastic condition where the normal squamous epithelium of the oesophagus is replaced by a simple columnar epithelium, and is associated with malignant progression to oesophageal adenocarcinoma (DeVault, 2000). Chaves *et al.* identified DPP4 expression on the surface of simple columnar cells present within lesions of Barrett's oesophagus (Chaves et al., 1999). Studies have also shown that DPP4 and FAP expression is also associated with regions of dysplasia (Goscinski et al., 2008a, Goscinski et al., 2008b), as well as tumour cells and tumour stroma in squamous cell carcinoma and adenocarcinoma of the oesophagus (Goscinski et al., 2008a, Augoff et al., 2014, Goscinski et al., 2008b, Goscinski et al., 2008c). The expression of DPP4 and FAP in dysplastic lesions as well as during malignancy may suggest utility for DPP4 and FAP as markers of neoplastic progression. DPP4 enzyme activity within these affected tissues has not been previously

quantified and may represent a unique measure of malignant progression that can be used clinically through breath testing applications (Jaenisch et al., 2016) (Appendix 6.1) and fluorescent imaging (Onoyama et al., 2016).

This study aimed to comprehensively characterise DPP mRNA expression and DPP enzyme activity in healthy gastrointestinal tissues as well as tissues from ileal CD and Barrett's oesophagus. This study also aimed to evaluate the clinical potential of DPP4 as a biomarker of gastrointestinal disease in an *in vitro* model of oesophageal disease.

2.2 Materials & Methods

2.2.1 Patient tissues

Oesophageal, stomach & duodenal tissues

Patients undergoing upper gastrointestinal endoscopy at Flinders Medical Centre, Adelaide, as part of the Barrett's Oesophagus Screening program (BOSS) or for other routine investigations were recruited to the study. Patients under the age of 18 and pregnant women were excluded from the study. Written and informed consent from patients for the collection of additional biopsies for research purposes was obtained prior to endoscopic procedures. This study was conducted in accordance with the guidelines set by the Southern Adelaide Clinical Human Research Ethics Committee (Ethics approval ID: 197.08).

Oesophageal, gastric and duodenal biopsies were collected from regions that appeared macroscopically normal during endoscopy. Oesophageal biopsies were also collected from affected oesophageal tissue in patients undergoing surveillance for Barrett's oesophagus. Biopsies were immediately snap frozen in liquid nitrogen for enzyme assays or immersed in RNA-later for qRT-PCR. All samples were stored at -80ºC until analysis.

Ileal and colonic tissues

Normal ileal and colonic biopsies were collected from non-IBD and IBD patients undergoing routine colonoscopy investigations at Flinders Medical Centre, Adelaide as part of our study detailed in Chapter 4 (Ethics approval ID: 135.15). All tissues were assessed endoscopically and determined to be normal by the treating gastroenterologist. Biopsies were immediately snap frozen in liquid nitrogen for enzyme assays or immersed in RNA-later for qRT-PCR. All samples were stored at -80ºC until analysis.

2.2.2 Cell culture conditions

Oesophageal cell lines FLO-1, OE33, OE21 and TE7 were kind donations from Dr Paul Drew, Basil Hetzel Institute, Adelaide, South Australia. Additional oesophageal cell lines OE19, JH-EsoAd1, GihTERT, GohTERT, ChTERT & QhTERT were kind donations from Dr Damian Hussey & Professor David Watson, Upper Gastrointestinal Research Laboratory, Flinders University, Adelaide. All cell lines described are adherent.

All cell culture chemicals and consumables were purchased from Sigma Aldrich (Castle Hill, Victoria, Australia). Flo-1, TE7, Caco-2 cells were cultured in Dulbecco's modified eagle media (DMEM) supplemented with 10% foetal bovine serum, 2mM L-glutamine, 1% non-essential amino acids and 1% sodium pyruvate. OE33, OE21 cells were maintained in RPMI 1640 supplemented with 10% foetal bovine serum and 2mM L-glutamine. JH-Eso-Ad1 cells were maintained in RPMI 1640 supplemented with 20% foetal bovine serum and 2mM L-glutamine. Barrett's cell lines (GihTERT, GohTERT, ChTERT & QhTERT) were cultured in LHC-9 basal medium supplemented with 5% foetal bovine serum. All growth medium was supplemented with penicillin/streptomycin and normocin. Cell lines were maintained in humid environment at 37ºC, 5% CO_2.

2.2.3 Sample preparation for enzyme activity assays

Gastrointestinal biopsies were homogenized in ice-cold 10mM Tris-HCl, pH 8.1 using an automated drill-bit pestle homogenizer. Tissue homogenates were separated into membrane and soluble fractions via ultracentrifugation 2 x [88,000 x g for 30 minutes, 4ºC] (Beckman Coulter, Optima Max-TL). Resultant fractions were separated and transferred into clean Eppendorf tubes and kept on ice, until analysis in the DPP enzyme assay. Membrane fractions were solubilized in 10mM Tris-HCl. This was tested in ileal, gastric and oesophageal biopsies

and deemed to be the most suitable method to quantify both protein and DPP activity in these small samples. Protein was quantified in membrane and soluble fractions by modified Bradford assay (Sigma Aldrich, Castle Hill, Australia).

Oesophageal cell lines were grown to confluence in T75 cell culture flasks under standard cell culture conditions. Cells were harvested and lysed by repeated freeze-thaw cycles. Soluble and membrane fractions were separated by centrifugation and stored at -20ºC for enzyme activity assays and protein quantification.

2.2.4 DPP enzyme activity assay

DPP enzyme activity in soluble and membrane fractions prepared from biopsies and cell lines was quantified by a kinetic colorimetric enzyme assay as previously described (Yazbeck et al., 2010b). The DPP substrate, H-Ala-Pro-pNA (extinction coefficient: $9450M^{-1}cm^{-1}$; Bachem, Switzerland) was used at a final concentration of 1mM in 10mM Tris-HCl, pH 8.1 for all enzyme assays. Dual absorbance readings were taken at 405 nm and 600 nm every 10 minutes for a total of 90 minutes using the ClarioStar (BMG Labtech, Germany). All samples were analysed in triplicate and each run included a positive control sample (Caco-2 lysate) to monitor assay validity and variability. Enzyme activity was expressed as µmoles of pNA released/min/mg of protein (U/mg of protein). Protein concentrations were determined as described below (Chapter 2.2.5).

To determine selective DPP4 activity, a final concentration of 1µM sitagliptin phosphate monohydrate (BioVision, United States) was included in the total reaction containing 10µL of sample, 10mM Tris-HCl pH 8.1 and 1mM H-Ala-Pro-pNA. Controls containing the sample without the inhibitor were run simultaneously for comparison to determine the proportion of activity due to DPP4 (Figure 2.1). It is important to note that at this concentration, sitagliptin

did not maximally inhibit recombinant DPP4 protein in our laboratory. Therefore, it is likely that residual DPP activity (Figure 2.1) is due to DPP4 along with non-DPP4 enzymes. Consequently, DPP4 activity presented here reflects pure DPP4 activity rather than total DPP4 activity.

The selective DPP8/9 inhibitor, 1G244 was a gift from our collaborator, Professor Mark Gorrell at the University of Sydney. 1G244 was originally diluted in DMSO though further dilutions were made using 10mM Tris-HCl pH 8, to achieve a final concentration of 10µM 1G244 (0.02% DMSO) (Wu et al., 2009) in the colorimetric assay. Appropriate DMSO controls (0.02%) were included in assays to determine activity attributable to DPP8/9 (Figure 2.1). As DPP4 is a transmembrane protein and DPP8/9 cytosolic proteins, (Gorrell, 2005) DPP4 and DPP8/9 specific activity was determined in membrane and cytosolic fractions respectively.

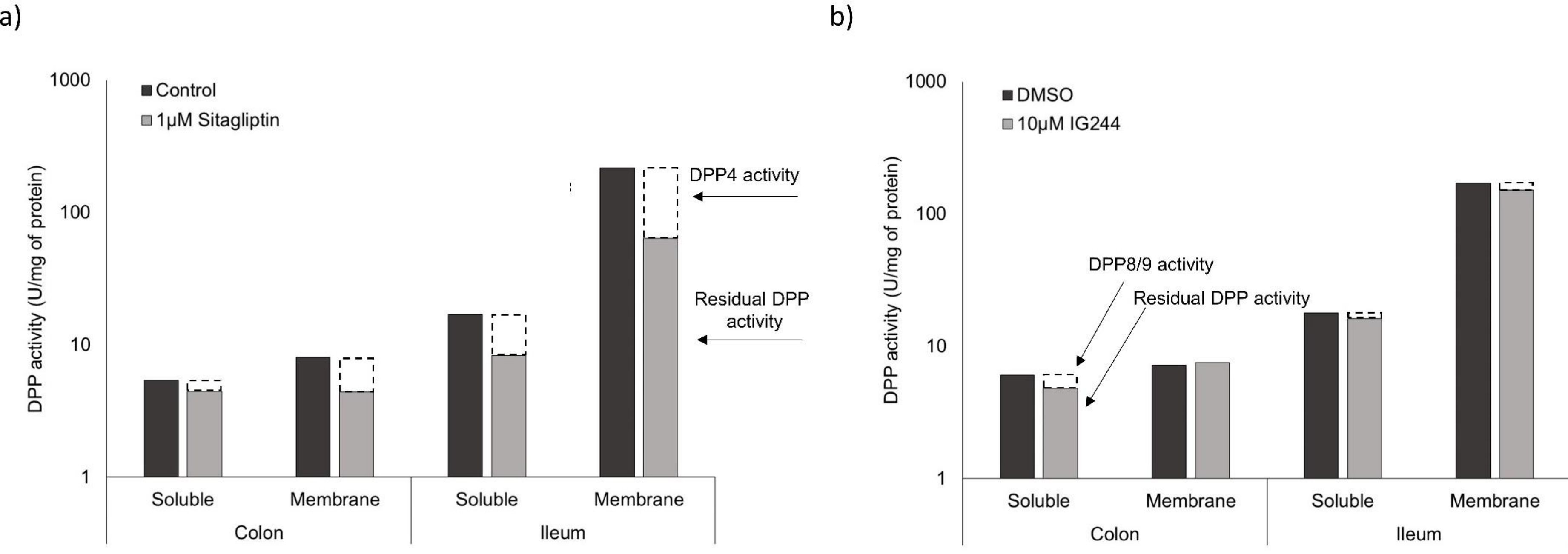


Figure 2.1 Schematic detailing how DPP4 and DPP8/9 activity was determined. Selective DPP4 and DPP8/9 inhibitors, sitagliptin and 1G244 were included in enzyme assays at final concentrations of 1μM and 10μM, respectively. To calculate specific activity related to DPP4 **a)** or DPP8/9 **b)**, activity in the inhibitor treated sample was subtracted from the respective control. Specific activity relating to DPP4 or DPP8/9 is indicated by dashed lines.

2.2.5 Protein assay

Protein was quantified in samples using a modified Bradford assay as per manufacturers' instructions for 96-well microplate assays (Bio-Rad Laboratories Pty Ltd, USA). Standard curves of BSA (0.05mg/ml to 0.5mg/ml) were used to determine protein concentration (mg/ml). Absorbance was measured at 595 nm on a ClarioStar plate reader (BMG Labtech, Germany).

2.2.6 qRT-PCR

DPP gene expression was measured in gastrointestinal biopsies by qPCR. Excess RNA later was removed, and biopsies were weighed and homogenized in 2mL round bottom snap-lock Eppendorf tubes using the TissueLyser bead-mill system (Qiagen, Germany). RNA was extracted using the RNA easy kit (Qiagen, Germany) according to the manufacturer's instructions. RNA was quantified using the spectrophotometer Nanodrop 2000 (Thermo Fisher Scientific, United States). RNA quality was assessed in approximately 25% of samples by 1% agarose gel electrophoresis.

Complementary DNA (cDNA) was synthesized from 1µg of RNA using Quantitect cDNA synthesis kit (Qiagen, Germany). The yield of cDNA at the end of the reaction was approximated by assuming that 1µg of RNA will yield 1µg of cDNA. Gene expression was quantified in approximately 10ng of cDNA using the real-time PCR cycler, Rotor Gene Q (Qiagen, Germany). 10µL reactions consisted of Kappa Universal SYBR Green Mastermix 1x (Sigma Aldrich, US), sterile RO water, and 100nM of primer, except for DPP2 that used 200nM (primer sequences in Table 2.1). PCR reaction cycling conditions were as follows: 95°C for 20 minutes, followed by 40 cycles of 95°C for 1 second, 65°C for 20 seconds and 95°C for 15 seconds. Each sample was analyzed in triplicate, and each run contained a positive control,

negative template control (NTC) and a non-reverse transcriptase control. Melt-curve analysis and 2% gel electrophoresis were performed on qPCR products to assess the identity of PCR products and ensure absence of contamination.

External gene standards consisting of purified PCR product of known concentration, were included in qPCR runs to determine the exact gene copy number in patient biopsies (Appendix 6.2). A minimum of four external gene standards ranging from $2x10^7$ copies to $2x10^1$ copies were included in each qPCR run to estimate gene copy number. Standards were made per previously described methods (Yin et al., 2001) (Appendix 6.2). Two reference genes, TATA and PPIA were quantified; however, PPIA was found to vary considerably between tissues. Consequently, all gene expression data is presented as copies of gene of interest normalized to the copies of reference gene, TATA (Sulda et al., 2010).

Table 2.1 Primer sequences for PCR and qPCR reactions

	Forward Primer (5'–3')	Reverse Primer (5'–3')	Product (bp)
DPP4	ACGCCGACGATGAAGACACCG	TTCAGCAGACCACGGGCACG	95
FAP	ATGGGGCTGGTCCTATGGAGGAT	GCTGGAGACTGGAGCCACTGC	97
DPP8	ACATGATGGCTAAGGCACCAATGA	CTGTTCTCACCAGACATGGCAAGG	106
DPP9	TTGCTCTGACCAGCGGTGAATG	GCCTCATAGCTGACCACGTAGA	142
PREP	CTGTGACCTACAGCAGGAATCC	AGTTGGGAGACTGGCGATTCGT	143
TATA	CGAAACGCCGAATATAATCCCAAGCG	GCCAGTCTGGATGTTCTTCACTCTT	137

2.2.7 *In vitro* detection of DPP enzyme activity using ^{13}C assay

Our laboratory has previously designed and validated a ^{13}C-labelled tri-peptide probe for the non-invasive quantification of DPP4 activity in metabolically active cells in real-time (Appendix 6.1). Using this novel assay method, DPP4 activity was quantified in sub-confluent cell monolayers by the addition of 2mM H-Gly-Pro-($^{13}C3$-Ala)-OH (Cambridge Isotopes, USA) dissolved in 3mL of cell culture media. 10mL gas samples were collected from the headspace of the cell culture flask as per previously described (Jaenisch et al., 2016). Gas samples were obtained every 20 minutes for 2 hours and subsequently analysed by isotope ratio mass spectrometer (IRMS) (ABCA, Europa Scientific, Sercon, Cheshire, UK), which determines a ratio of ^{12}C:^{13}C. Reference gas of 5% CO_2 was used to calibrate the IRMS prior to sample analysis. In addition, throughout the sample run, reference and quality control gas (High Purity Carbon Dioxide, BOC) was analysed every 10-20 samples to minimise drift. All samples were analysed within 24-48 hours of sample collection. Data is expressed as the mean $\delta^{13}CO_2 \pm$ standard deviation (SD). Data is representative of at least two separate experiments performed in triplicate.

2.2.8 Data analysis

Due to the low sample sizes and the observational nature of this study, non-statistical comparisons were made for expression and activity results and were based on tissue type or the presence of disease. Expression and activity changes of 3-fold were considered biologically significant (Harstad et al., 2013).

2.3 Results

2.3.1 Patients and tissues

Normal oesophageal, gastric and duodenal mucosal biopsies were collected from up to 14 different patients undergoing routine endoscopy procedures (Table 2.2). Eight oesophageal biopsies were also collected from patients with Barrett's oesophagus undergoing endoscopic surveillance as part of the BOSS program at Flinders Medical Centre. Evidence of intestinal metaplasia was confirmed in six of the Barrett's patients.

Table 2.2 Patient characteristics[a].

	Barrett's Oesophagus	Non-Barrett's
N	8	6
Age; range	51-77 years	45-65 years
Gender (F / M)	6 / 2	3 / 3

Colonic tissue was collected from three non-IBD patients who were macroscopically normal and had no evidence of diverticulitis or other inflammatory disease. Ileal tissue was collected from two separate IBD patients without evidence of small intestinal disease. Ileal involvement was evident in a single patient during colonoscopy and confirmed by pathology, for which matched biopsies were collected from inflamed and inactive ileal tissue.

[a] Normal stomach and duodenal biopsies were also collected from patients with Barrett's oesophagus and are included in the healthy analysis

2.3.2 Unique mRNA expression and enzyme activity profiles of DPPs in gastrointestinal tissues

To our knowledge, the mRNA expression of the DPP4 gene family has not been previously characterised in the upper gastrointestinal tract. Harstard *et al.* has extensively characterised DPP4 and DPP8/9 activity and mRNA expression in gastrointestinal tissues from mice and monkeys and while studies have quantified DPP activity in human gastrointestinal samples, such comprehensive characterisation of DPP mRNA expression and DPP activity has not yet been undertaken in human gastrointestinal tissues.

DPP4 mRNA expression was below the limits of detection in normal oesophageal mucosa and the majority (3/5) of gastric mucosal biopsies. DPP4 mRNA expression within the small intestine was more than 3-fold higher when compared to colonic and stomach biopsies (Table 2.3). Interestingly, the mRNA expression of DPP9 was only detected in ileal and colonic biopsies, with expression below the levels of detection in other gastrointestinal tissues (Table 2.3). DPP8 mRNA expression was identified throughout the gastrointestinal tract with a 3-fold higher expression detected within the duodenum compared with the ileum (Table 2.3). FAP and PREP mRNA was detected in all gastrointestinal tissues and was largely unchanged between different gastrointestinal tissues (Table 2.3).

Total membrane DPP activity and DPP4 activity was below the levels of detection in many normal oesophageal and gastric tissues (Table 2.4). Total membrane DPP activity was highest in the small intestine, whilst activity in the duodenum and ileum was relatively similar (Table 2.4). DPP4 activity was highest in the ileum and was increased up to 2.4-fold when compared to the duodenum and more than 32-fold when compared to the colon (Table 2.4). Total soluble

DPP activity and DPP8/9 activity was detected in majority of biopsy samples and activity levels were similar between the different gastrointestinal tissues (Table 2.4).

Table 2.3 DPP mRNA expression in the gastrointestinal tract. DPP gene copy number was determined in gastrointestinal mucosal biopsies using external standards included in each qPCR run. All data is normalized to the reference gene, TATA. Data is expressed as median [IQR, Q1-Q3][b].

	Oesophagus *n=3*	**Stomach** *n=2-5*	**Duodenum** *n=5*	**Ileum** *n=2*	**Colon** *n=5*
DPP4	UD[c]	1.68, 2.54	20.52 [14.76 – 26.65]	19.07, 32.80	1.33 [1.1 – 1.55]
FAP	0.15 [0.13 – 0.22]	0.12 [0.09 – 0.18]	0.14 [0.12 – 0.14]	0.09, 0.20	0.037 [0.031 – 0.038]
DPP8	4.48 [4.28 – 6.05]	3.81 [3.32 – 4.03]	6.70 [5.71 – 6.85]	1.47, 1.51	1.84 [1.70 – 1.91]
DPP9	UD	UD	UD	0.13, 0.12	0.12 [0.11 – 0.13]
PREP	1.89 [1.71 – 2.00]	1.31 [1.30 – 1.45]	2.72 [2.41 – 3.00]	0.56, 1.33	1.51 [1.30 – 1.62]

[b] For data where n = 2, individual data points are presented
[c]UD = undetected. mRNA expression was below the limits of detection for assay conditions.

Table 2.4. DPP enzyme activity in gastrointestinal tissues. DPP enzyme activity was determined in gastrointestinal mucosal biopsies collected from patients without macroscopic evidence of disease. Specific activity relating to DPP4 was determined in membrane fractions using 1µM sitagliptin, while specific activity relating to DPP8/9 was determined in soluble fractions using 10µM 1G244. Data is presented as median [IQR, Q1-Q3].[d]

	Oesophagus *n=6*	**Stomach** *n=8-13*	**Duodenum** *n=9-11*	**Ileum** *n=2*	**Colon** *n=4-5*[e]
Total soluble activity (U/mg of protein)	6.70 [5.46 – 9.56]	4.91 [4.20 – 5.41]	7.72 [7.30 – 8.47]	11.09, 16.90	5.78 [5.38 – 7.21]
Total membrane activity (U/mg of protein)	UD[f]	9.40 [5.90 – 11.16][g]	63.85 [43.53 – 70.75]	148.81, 218.20	10.21 [9.61 – 11.87]
DPP8/9 activity (U/mg of protein)	1.64 [1.01 – 2.85]	2.08 [1.19 – 2.39][h]	2.09 [1.28 – 2.74][i]	1.58, 5.60	2.34 [1.83 – 2.88]
DPP4 activity (U/mg of protein)	UD	2.18 [1.73 – 3.82][j]	43.62 [30.60 – 48.93]	110.84, 154.19	4.06 [2.85 – 5.20]

[d] For data where n=2, individual data points are presented
[e] Total membrane and DPP4 activity representative of n=4
[f] UD = Undetectable activity. Activity was below the detection limit of our enzyme assay and/or protein concentrations were too low for accurate estimation.
[g] Data representative of n=8
[h] Data representative of n=12
[i] Data is representative of n=9
[j] Data is representative of n=8

2.3.3 DPP enzyme activity is altered during gastrointestinal disease

2.3.3.1 Ileal Crohn's disease

DPP4 expression has been proposed as a candidate biomarker of small intestinal damage (Detel et al., 2007), with Moran *et al.* finding that DPP4 protein expression was lower in ileal biopsies from patients with active CD compared to controls (Moran et al., 2012). In biopsy material from a single patient with ileal CD, total DPP activity in soluble and membrane fractions was 33% and 56% lower in inflamed tissue compared to matched inactive tissue (Figure 2.2a, b).

Using DPP inhibitors to discriminate DPP activity, found similar levels of DPP8/9 activity in matched inactive (2.8 U/mg of protein) and active (3.1 U/mg of protein) ileal biopsies (Figure 2.2c), while DPP4 activity was found to be 62% lower in inflamed tissue (Figure 2.2d).

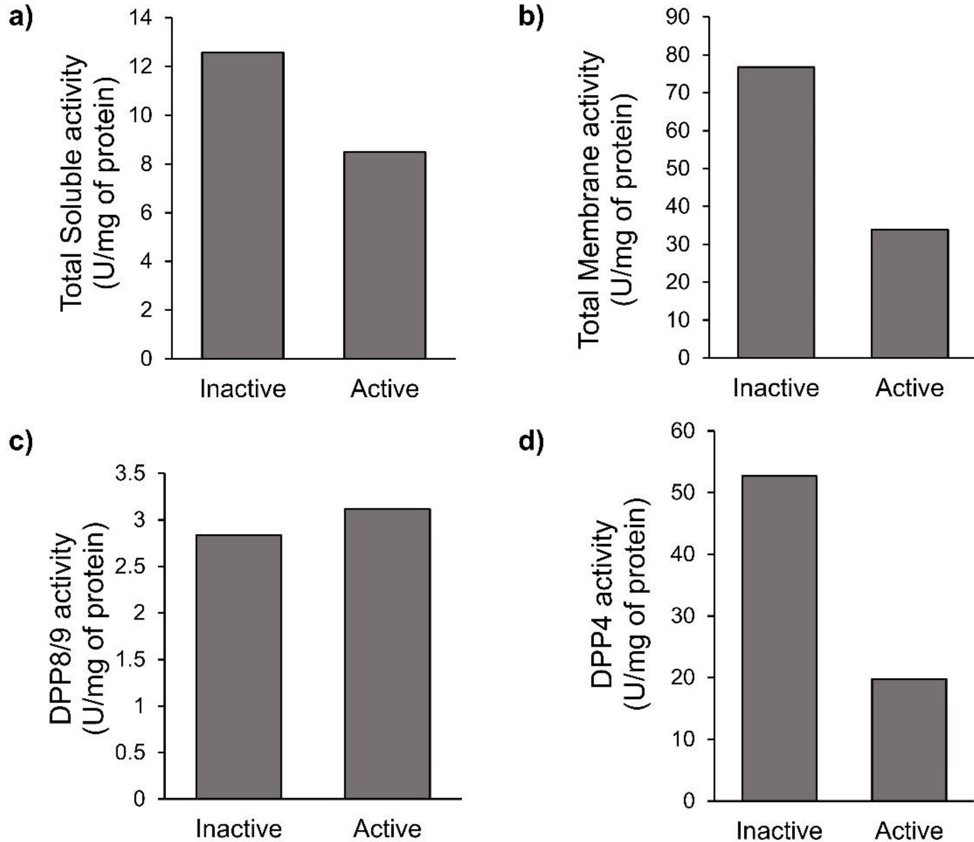


Figure 2.2 DPP enzyme activity in ileal tissue from a single CD patient. Biopsies from the terminal ileum were collected from areas of normal (inactive) and inflamed (active) tissue, (n=1). Total DPP activity within the soluble **a)** and membrane **b)** fractions was quantified. Selective DPP inhibitors, 10µM 1G244 and 1µM sitagliptin were used to quantify DPP8/9 **c)** and DPP4 **d)** activity in soluble and membrane fractions, respectively.

2.3.3.2 Barrett's oesophagus

DPP4 mRNA and protein expression has been previously identified in lesions of Barrett's oesophagus (Chaves et al., 1999); however, the specific enzyme activity of DPP4 and other DPPs has not yet been characterised in this premalignant condition. Soluble DPP activity and DPP8/9 activity was similar in oesophageal biopsies from control patients and Barrett's patients (Figure 2.3a). Total membrane DPP activity and DPP4 activity was detected in 50% of biopsies from Barrett's lesions, with no membrane DPP activity identified in healthy oesophageal tissue (Figure 2.3b).

DPP8 mRNA expression was identified in all biopsies from Barrett's lesions (2.42 ± 0.50 Relative DPP8 expression, data not shown), and was reduced by 1.2-fold compared to healthy oesophageal mucosa from control patients (5.39 ± 1.94 Relative DPP8 expression; Table 2.2). DPP4 mRNA was below the limits of detection in majority (4/5) of biopsies from Barrett's oesophagus lesions. Low DPP4 mRNA expression (35.4 gene copies / ng of cDNA) was detected in a single biopsy collected from Barrett's oesophagus (data not shown). Insufficient RNA concentrations prevented adequate quantification of other DPP genes including FAP and PREP.

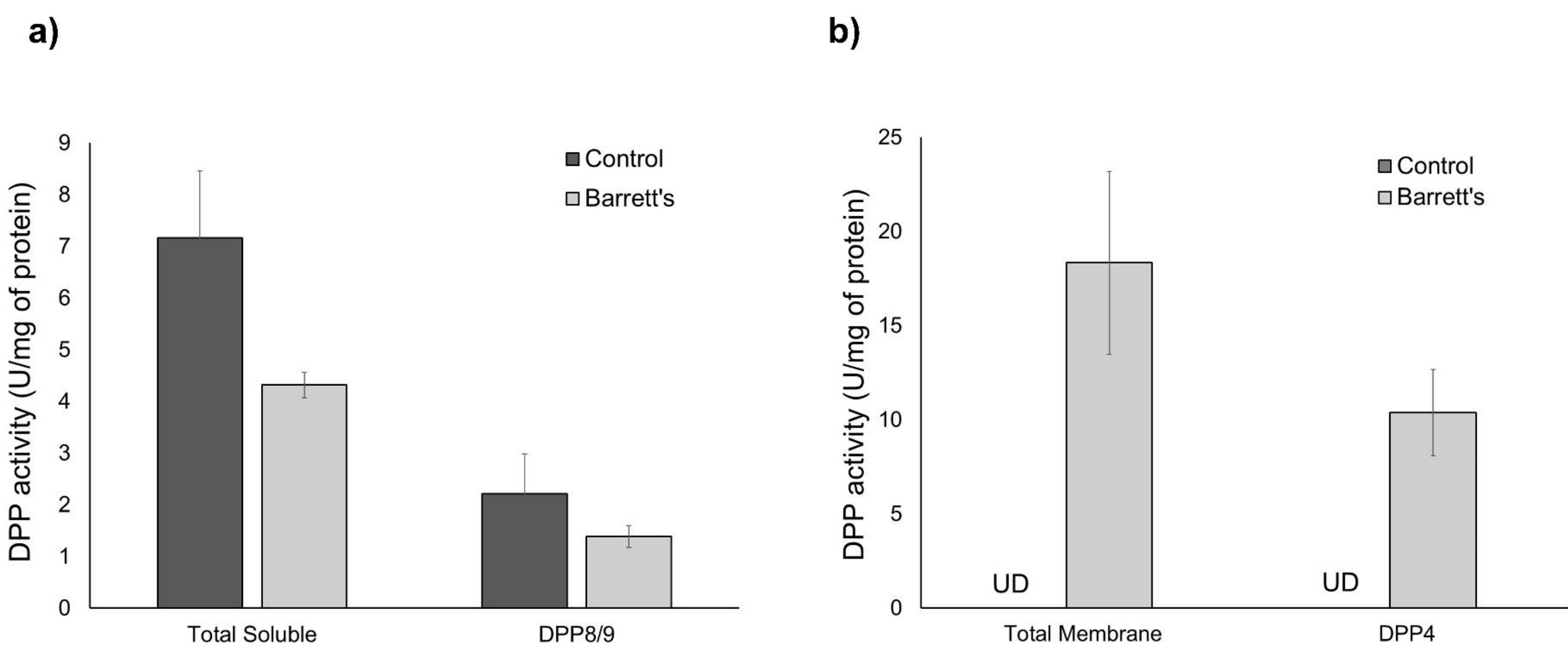


Figure 2.3 Tissue DPP enzyme activity in Barrett's oesophagus. DPP enzyme activity was determined in oesophageal tissue from control patients (n=6) and Barrett's oesophagus(n=4-8[k]). Total DPP enzyme activity and specific DPP enzyme activity (DPP8/9 or DPP4) was determined in soluble **a)** and membrane **b)** tissue fractions. The use of selective DPP inhibitors, 10µM 1G244 and 1µM sitagliptin, allowed for the determination of DPP8/9 and DPP4 activity in soluble and membrane fractions respectively. UD=undetected activity in control oesophageal tissues. Data is expressed as mean ± standard error of the mean.

[k] Total membrane DPP activity and DPP4 enzyme activity was only detected in 50% (n=4) Barrett's oesophagus tissue

2.3.4 DPP enzyme activity is increased in oesophageal adenocarcinoma derived cell lines

DPP4 has been identified as a candidate marker of Barrett's oesophagus (Chaves et al., 1999) and detection of DPP4 enzyme activity may represent a novel way for diagnosing and monitoring Barrett's. While tissue samples are preferable, it is not always feasible to collect matched tissues from patients, furthermore, quantity and size of biopsies can present technical difficulties for various experimental applications. Immortalized cell lines represent a useful alternative experimental model to biopsies, maintaining some characteristics of their originally derived tissue, they can be used to screen suitable biomarkers and develop assays that may not be appropriate in biopsy tissue.

DPP enzyme activity was quantified in a panel of Barrett's and oesophageal cancer derived cell lines. DPP activity was highest in the soluble fraction of Flo-1 cells (14.68 ± 4.96 U/mg protein; Figure 2.4a) and was comparatively low in the immortalized Barrett's cell lines, Gih-Tert, Goh-TERT, Ch-TERT and Qh-TERT, with activity ranging from 0.30 U/mg of protein to 0.77 U/mg of protein (Figure 2.4a). Membrane DPP activity was highest in OE33 cells (42.86 ± 1.60 U/mg of protein) while the lowest membrane DPP activity was detected in the Ch-TERT cell line (0.42 ± 0.15 U/mg of protein; Figure 2.4b).

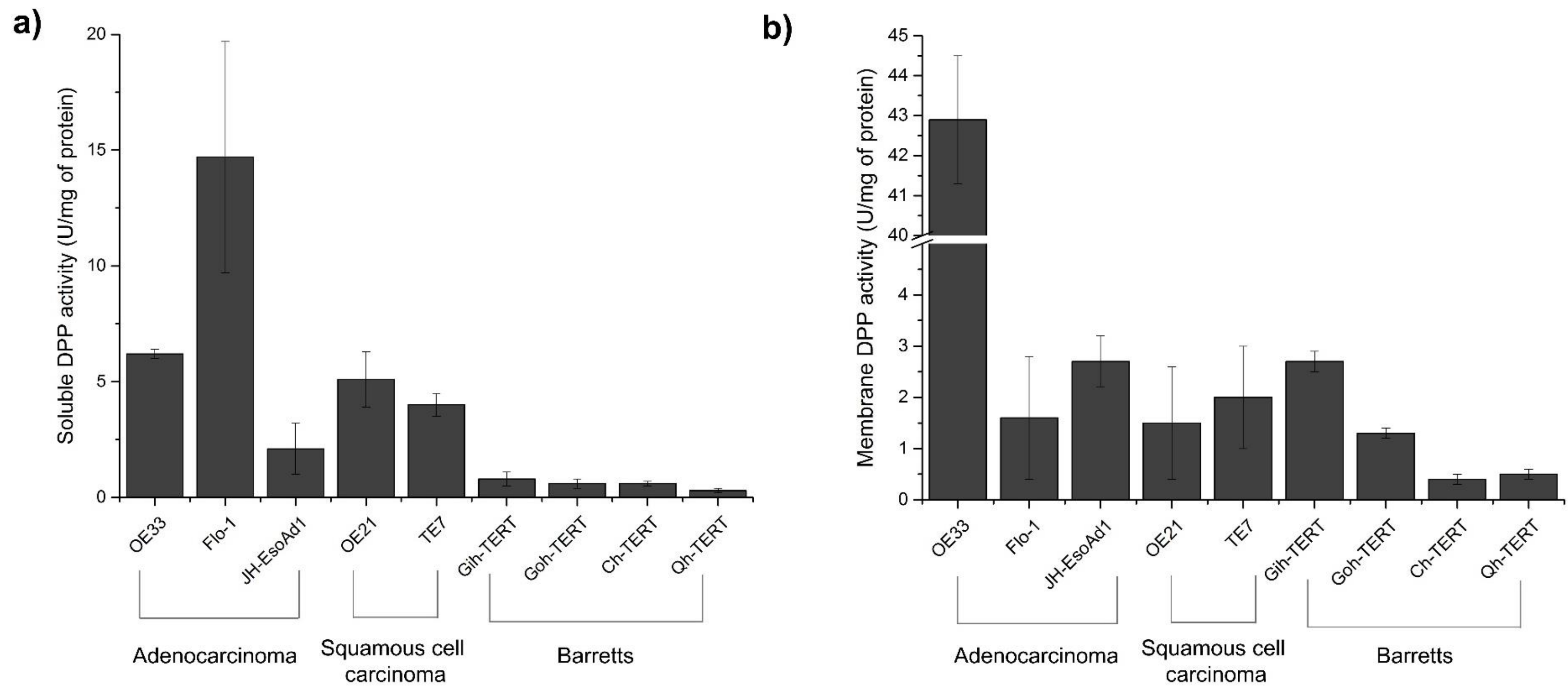


Figure 2.4 DPP enzyme activity in oesophageal cancer cell lines. DPP activity was determined in soluble **a)** and membrane **b)** fractions from oesophageal adenocarcinoma (OE33, Flo-1, JH-EsoAd1), squamous cell carcinoma (OE21, TE7), and Barrett's derived (Gih-TERT, Goh-TERT, Ch-TERT, Qh-TERT) cell lines. Results represents enzyme activity assessed in three separately growing T75 cultures. Sitagliptin and 1G244 were not utilized in this experiment. Data is presented as mean ± standard deviation.

2.3.5 Detection of differential DPP4 activity using novel ^{13}C-based technique

We have recently developed a non-invasive ^{13}C-stable isotope assay to non-invasively quantify DPP enzyme activity *in vitro* (Appendix 6.1). Following the addition of 2mM ^{13}C-labelled tripeptide to the cells, increasing $^{13}CO_2$ signals were detected over the 120-minute sampling period in OE33 cells and Flo-1 cells, while $^{13}CO_2$ signals in JH-EsoAd1 cells plateaued after 60 minutes (Figure 2.5). At the end of the 120-minute sampling period, the highest $^{13}CO_2$ signal was detected in OE33 cells (28.6 ± 8.0 $\delta^{13}CO_2$) compared to Flo-1 (14.02 ± 1.91 $\delta^{13}CO_2$) and JH-EsoAd1 (6.08 ± 1.26 $\delta^{13}CO_2$; Figure 2.5). The selective DPP4 inhibitor, sitagliptin was used to determine the selectivity of the ^{13}C-tripeptide assay. Unpublished work from our laboratory found that treatment with 10μM sitagliptin for 120 minutes reduced δ $^{13}CO_2$ signals in Caco-2 cells by up to 73% (Appendix 6.1). In this study, 10μM sitagliptin reduced δ $^{13}CO_2$ at 120 minutes in OE33, Flo-1 and JH-EsoAd1 cells by 80.1%, 64.2% and 36.4%, respectively (Figure 2.5).

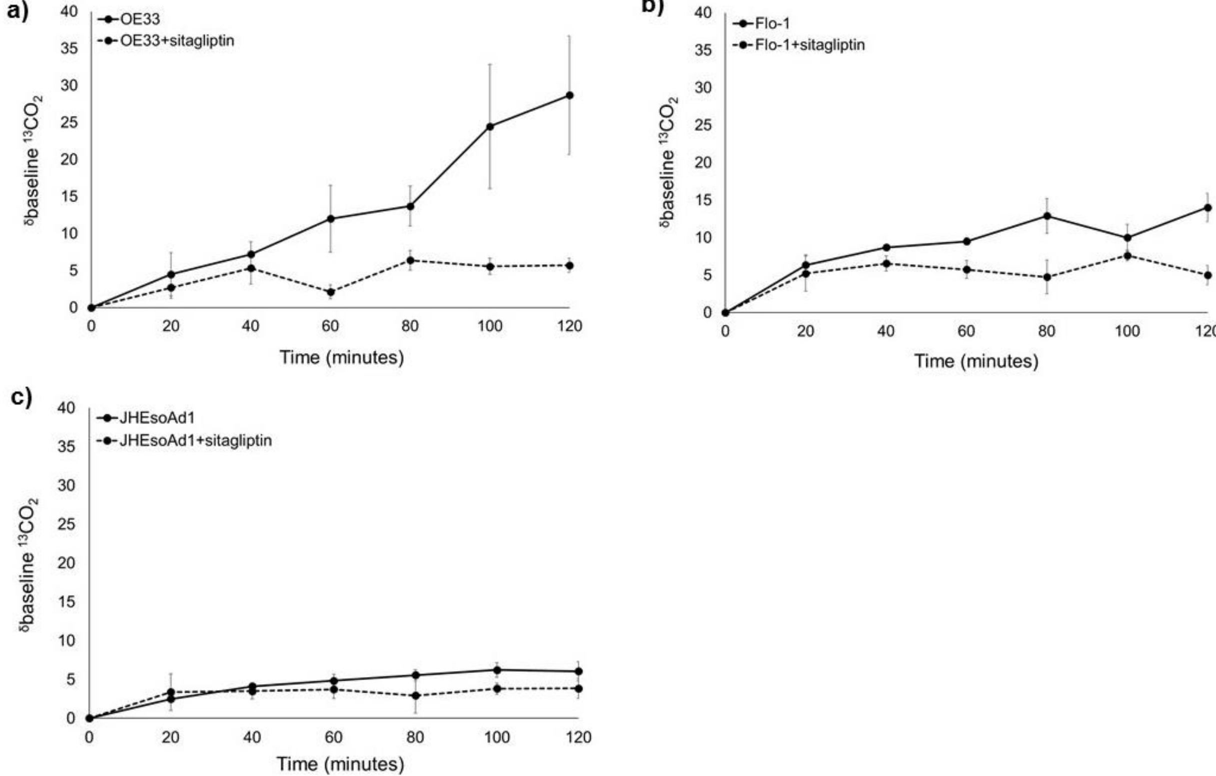


Figure 2.5 Real-time DPP4 activity using non-invasive ^{13}C-based technique *in vitro*. $^{13}CO_2$ signals were detected over 120 minutes in viable OE33 **a)**, FLO-1 **b)**, and JH-EsoAd1 **c)** cell line cultures as a result of ^{13}C-labelled tripeptide metabolism. Cells were treated with 10µM sitagliptin (dashed lines), to demonstrate selectivity of the assay for DPP4 activity. Data is representative of three separate experiments. Data points represent mean ± standard deviation.

2.4 Discussion

The current study has identified a distinct expression and activity profile of DPP4 throughout the gastrointestinal tract in both normal and diseased mucosa, highlighting the differential expression and activity of DPP4 based on tissue location and during disease. For the first time in this Chapter I have identified unique expression profiles of other DPPs including DPP8 and DPP9 throughout the gastrointestinal mucosa, suggesting potential site-dependent roles for these enzymes. Furthermore, using a novel, non-invasive ^{13}C-based *in vitro* assay, I have identified differential DPP4 activity in viable oesophageal cancer cell lines, a method that could have potential applications in diagnosis and monitoring of upper gastrointestinal diseases characterised by altered DPP4 activity.

In line with its roles in the assimilation and breakdown of dietary proteins (Tiruppathi et al., 1993), DPP4 is expressed at high levels in the small intestine (Triadou et al., 1983) and very low levels in normal oesophagus and stomach (Sahara et al., 1983a). Consistent with the study by Sahara *et al.* in rat tissues, we found low to undetectable levels of DPP4 mRNA and activity within oesophagus, stomach and colonic tissues of patients. The physiological function of DPP4 in the stomach and colon, where there is low levels of expression/activity, remains largely unclear. Previous studies investigating DPP4 during acute colonic inflammation have suggested that DPP4 may be involved in regulating localized acute inflammatory responses (Baticic et al., 2011, Detel et al., 2016) though this warrants further investigation. Understanding the normal physiological variation of DPP4 throughout the gastrointestinal tract (Figure 2.6) is important for biomarker investigations and highlights the importance of collecting appropriately matched tissue from patients with gastrointestinal disease.

DPP8 and DPP9 expression has been reported in several cell types including immune cells (Matheeussen et al., 2013, Chowdhury et al., 2013) and epithelial cells (Harstad et al., 2013), however, the physiological roles of these enzymes remain poorly understood. I found higher levels of DPP8 mRNA compared to DPP9 in normal oesophagus, stomach, duodenum, ileum and colonic tissues, which may suggest DPP8 accounts for majority of the DPP8/9 activity within healthy adult gastrointestinal tissues. Previous work from Harstad *et al.* shows that the mRNA expression of DPP8 within the colon and duodenum of rats did not correlate with detectable levels of DPP8 at the protein level (Harstad et al., 2013). Furthermore, the authors reported that enzyme activity due to DPP8/9 was below detectable limits within the duodenum, despite detecting both DPP8 and DPP9 mRNA (Harstad et al., 2013). While non-enzymatic roles for DPP8/9 have been proposed in adhesion, migration and apoptosis (Yu et al., 2006) deciphering the specific roles of DPP8/9 enzyme activity requires use of specific DPP8/9 inhibitors, such as 1G244 (Wu et al., 2009, Matheeussen et al., 2013) and identification of potential DPP8/9 substrates (Wilson et al., 2013, Zhang et al., 2015, Geiss-Friedlander et al., 2009).

DPP8 and DPP9 mRNA and protein expression has been reported within mucosal epithelium (Harstad et al., 2013) and in lymphocytes and monocytes/macrophages (Maes et al., 2007a, Matheeussen et al., 2013, Chowdhury et al., 2013), with studies suggesting roles for DPP8 and DPP9 in immunity (Yazbeck et al., 2010b, Schade et al., 2008) and metabolism (Chen et al., 2016). DPP8 mRNA was expressed throughout the mucosa of the gastrointestinal tract, with the highest expression in the duodenum and the oesophagus. The mucosa of the gastrointestinal tract exhibits high cellular turnover to assist in maintaining barrier integrity (Wong and Wright, 1999). Recent work from Zhang *et al.* identified S100-A10, a calcium binding protein with roles in cell cycle progression as a potential substrate of DPP8 (Zhang et al., 2015), which may suggest roles for DPP8 in cellular proliferation. The low levels of DPP9 mRNA found

throughout normal adult gastrointestinal mucosa might suggest limited roles for DPP9 in these tissues. Previous work from Chen *et al.* found that DPP9 is vital for the regulation of metabolic processes within the gastrointestinal tract of neonates (Chen et al., 2016), which may also suggest more important roles for DPP9 at various stages of development. Understanding the expression and activity profiles of DPP8 and DPP9 during normal physiological conditions and in diseased tissue is important for deciphering the roles of these enzymes and may also give insights into DPP4 inhibitors the likelihood of non-specific inhibition in various tissues.

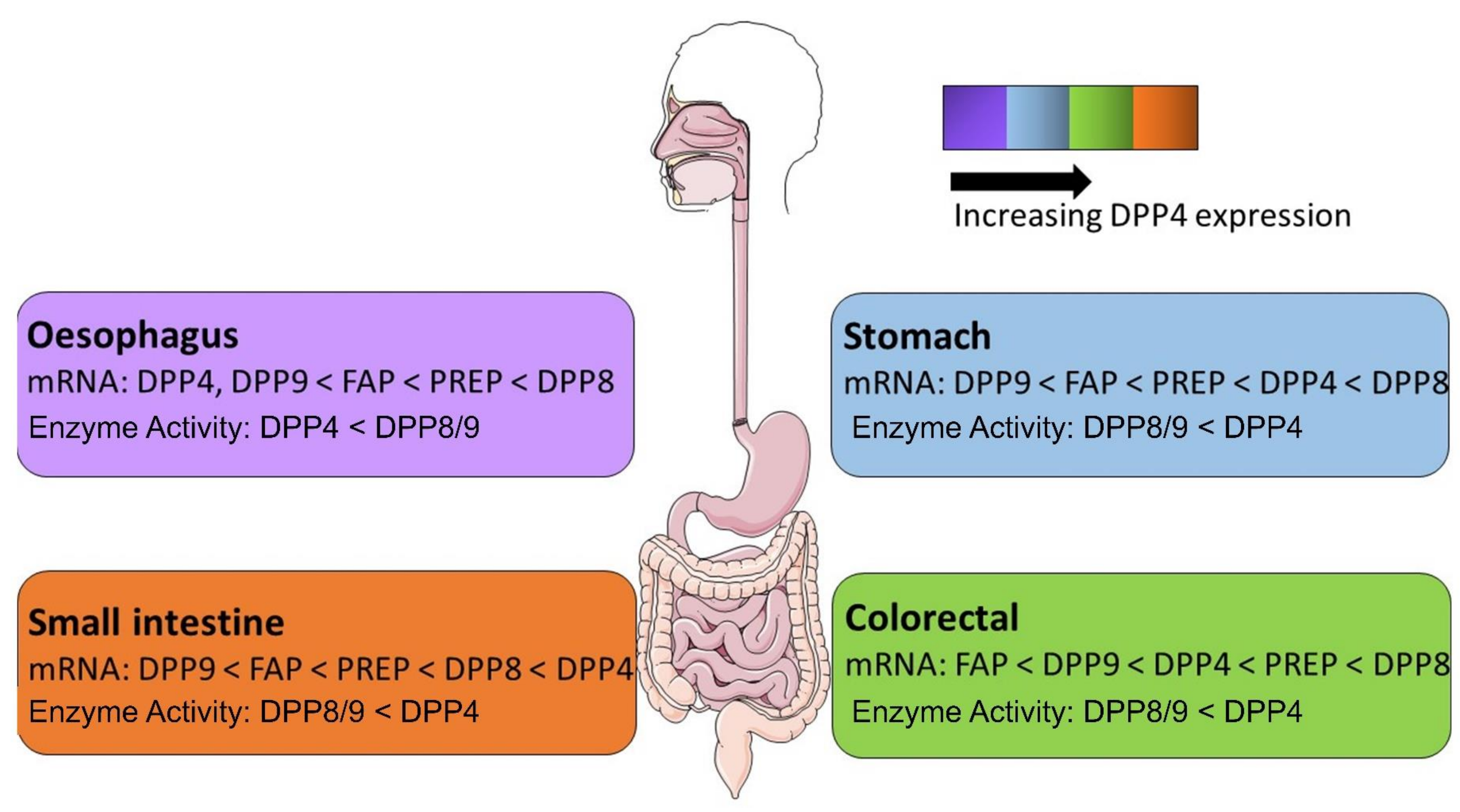


Figure 2.6 Schematic showing DPPs throughout the gastrointestinal tract. DPP4 expression and DPP4 enzyme activity is highest in the small intestine, with much lower expression and enzyme activity levels found in other gastrointestinal tissues. In contrast, other DPPs exhibit similar mRNA expression profiles in normal gastrointestinal tissues. Images are derived and modified from Les Laboratories Servier.

DPP4 protein expression is reduced during active ileal CD (Moran et al., 2012). Consistent with this, I found total membrane activity, total soluble activity and DPP4 activity was reduced in inflamed ileal tissue compared with matched unaffected ileal tissue. Studies of small intestinal damage associated with coeliac disease (Detel et al., 2007) and drug induced villous-atrophy (Ziegler et al., 2003) have also reported significant reductions in DPP4 protein expression, and was inversely associated with the degree of mucosal damage. DPP4 may represent a biomarker of small intestinal damage/integrity and function. The emergence of new non-invasive tools to measure DPP4 activity may support the inclusion of DPP4 as a routine biomarker of small intestinal damage, assisting in improvements to the clinical management of small intestinal pathologies.

DPP4 protein and mRNA expression has been previously identified in Barrett's oesophagus (Chaves et al., 1999, Nancarrow et al., 2011). Chaves *et al.* previously found DPP4 immunoreactivity in both the cell cytoplasm and along the apical membrane of columnar cells present in Barrett's lesions with and without evidence of small intestinal metaplasia (Chaves et al., 1999). Consistent with this, DPP4 mRNA was absent from normal oesophageal mucosa with variable DPP4 mRNA expression detected in Barrett's lesions. Further studies characterising DPP4 expression/activity and how DPP4 is related to oesophageal malignant progression will help to determine the potential clinical utility of DPP4.

^{13}C-based assays are safe, novel, non-invasive tests that are cost effective and highly suitable for clinical applications. Our laboratory has previously optimised and validated a ^{13}C-based assay for the detection of DPP4 activity *in vitro* (Appendix 6.1), revealing good correlations between DPP4 activity detected using the colorimetric assay and the ^{13}C-tripeptide assay. Using the ^{13}C-DPP4 assay, differential DPP4 activity was detected in oesophageal cancer cell lines that was consistent with membrane DPP activity detected via the colorimetric assay.

Unexpectedly, the $^{13}CO_2$ signal plateaued in the JH-EsoAd1 cell line after just 60 minutes. Similar $^{13}CO_2$ profiles are produced from HeLa cells (Appendix 6.1) that have low levels of DPP4 expression, suggesting that the JH-EsoAd1 cells may also have low levels of active membrane DPP4. However, further studies characterising DPP activity in live cells using both colorimetric assays and this new technique are required to better understand DPP activity in live cells.

The results also suggest that the high levels of soluble DPP activity present in Flo-1 cells, was not detected by the ^{13}C-DPP4 assay, highlighting the specificity of the assay for membrane DPPs. Additionally, the use of sitagliptin, a highly selective inhibitor for DPP4, reduced the $^{13}CO_2$ signal in all cell lines, highlighting the selective nature of the ^{13}C-based *in vitro* assay for DPP4. A non-invasive ^{13}C-DPP4 assay would represent a novel diagnostic tool that could have applications throughout the gastrointestinal tract to evaluate, diagnose and monitor conditions such as Barrett's oesophagus and IBD. As DPP4 is expressed ubiquitously in the human body, validation studies are necessary to ensure correct delivery of the substrate to the target organ and that the expired $^{13}CO_2$ signals reflect metabolism in that organ.

This study highlights the distinct expression and activity profile of DPP4 with respect to other DPP enzymes throughout the gastrointestinal tract, suggesting the importance of DPP4 in gastrointestinal health and disease. The results show that DPP4 expression and activity is differentially altered in diseased gastrointestinal tissues and that DPP4 expression may represent unique marker of damage, inflammation or pre-malignant progression in different tissues. A non-invasive ^{13}C-based assay for the detection of DPP4 activity could have significant applications in gastrointestinal disease and disorders; however, extensive validation is necessary to optimize substrate delivery and ensure a thorough understanding of $^{13}CO_2$ signals.

Chapter 3. Differential dipeptidyl peptidase expression and activity in Crohn’s Disease and Ulcerative Colitis

3.1 Introduction

IBD is a chronic and debilitating condition characterized by mild to severe, relapsing gastrointestinal symptoms that include abdominal pain, bloating, bleeding and fatigue (Strober et al., 2007, Wallace et al., 2014). There are two main disease sub-types, CD and UC, which have distinct clinical presentation, aetiology, pathogenesis and disease management. IBD is diagnosed and monitored using a multifaceted approach that includes a detailed patient history, physical and endoscopic examinations, histology and assessment of minimally invasive laboratory parameters such as CRP and faecal calprotectin (Laass et al., 2014, Conrad et al., 2014). Biomarkers such as faecal calprotectin and CRP, are useful in guiding clinical decisions; however, they lack disease specificity, limiting their application in IBD (Vermeire et al., 2006). Treatment options for IBD can include immunosuppressant's and immunomodulatory drugs or the more recent 'biological therapies' such as the anti-TNF-α agents, Infliximab and Adalimumab (Burger and Travis, 2011, Scott and Osterman, 2013). The efficacy of existing treatments for IBD has been previously reported (Dassopoulos et al., 2013, Pithadia and Jain, 2011, Blomqvist et al., 2001); however, these therapies are associated with various side-effects (Stein and Hanauer, 2000, Buisson et al., 2013) that further increase the burden of disease and the likelihood of poor compliance, disease relapse and the need for surgical resections (Solina et al., 2016).

DPP4 is a ubiquitously expressed serine protease with specific enzyme activity, cleaving dipeptides from the N-terminus of peptides with a proline or alanine in the penultimate position (Yazbeck et al., 2009a). DPP4 substrates (Chapter 1, Table 1.3) include chemokines, growth factors, incretin hormones and neuropeptides (Mentlein, 1999). The roles of GLP-2 (Buchman et al., 2010, Sigalet et al., 2013, Wu et al., 2015a), NPY (Chandrasekharan et al., 2008) and vasoactive intestinal peptide (Abad et al., 2003, Wu et al., 2015b) in gastrointestinal health and

inflammation have been examined previously, with studies suggesting roles for these peptides as therapeutic agents to manage intestinal inflammation and IBD. DPP4 is part of the larger DPP4 gene family of structurally homologous enzymes that includes FAP, DPP8 and DPP9 (Yazbeck et al., 2009a, Gorrell, 2005), that despite sharing similar enzymatic activity, have unique substrate profiles based on their cellular location and expression within the body. FAP has been extensively characterized and is highly expressed at sites of wound healing and tissue remodeling (Gorrell, 2005, Levy et al., 1999). Recent studies have suggested DPP8 and DPP9 could be involved in immune regulation and inflammation (Matheeussen et al., 2013).

Yazbeck *et al.* reported differential DPP enzyme activity and gene expression in a DSS mouse model of colitis (Yazbeck et al., 2010b). Reduced mRNA expression of DPP4, FAP and DPP9 was found in colonic tissue of mice with colitis compared to controls, while DPP8 and DPP2 expression was increased. Colonic MPO activity was also significantly reduced in mice receiving a non-selective DPP inhibitor, suggesting potential roles for the DPP enzymes in the regulation of colonic inflammation. Furthermore, work from this group found that DPP inhibitors reduced the severity of DSS colitis, suggesting a role for this enzyme family in IBD pathogenesis (Yazbeck et al., 2010b, Yazbeck et al., 2011).

Other studies have since supported our original findings, using selective inhibitors including anagliptin and specific peptide inhibitors of DPP4 respectively, to ameliorate disease activity in DSS colitis (Mimura et al., 2013) and TNBS colitis (Salaga et al., 2017). DPP4 inhibition has also prevented intestinal ulcer formation induced by the NSAID, indomethacin, in both mice (Fujiwara et al., 2015) and rats (Inoue et al., 2014). Treatment with the DPP inhibitors has been reported to improve intestinal architecture, increase reparative processes (Yazbeck et al., 2010b, Yazbeck et al., 2011, Salaga et al., 2017, Mimura et al., 2013, Ban et al., 2011), and modify immune parameters such as neutrophil infiltration (Yazbeck et al., 2010b, Yazbeck et

al., 2011, Salaga et al., 2017) and cytokine expression (Sakanaka et al., 2015) in models of damage and inflammation, which suggests potential clinical applications for DPP4 inhibitors in human IBD.

DPP4 enzyme activity and expression has also been investigated as a potential disease biomarker for a range of diseases, including IBD (Yazbeck et al., 2017). Xiao *et al.* have reported decreased plasma DPP activity in IBD patients compared to healthy patients (Xiao et al., 2000). A more comprehensive study by Hildebrandt *et al.* found that plasma DPP activity inversely correlated with disease activity scores and inflammatory markers in CD and UC patients (Hildebrandt et al., 2001). DPP4 protein expression and enzyme activity was also decreased in the plasma and biopsy tissue from active intestinal lesions of CD patients compared to healthy controls (Moran et al., 2012). However, none of these studies defined the expression of all DPP4 gene family members during IBD. Furthermore, substrates, H-Ala-Pro- and H-Gly-Pro- that are often used to quantify DPP enzyme activity are not selective for DPP4 and may also be cleaved by other DPPs including DPP8, DPP9 and FAP (Bjelke et al., 2006) .

Selective DPP4 inhibitors have recently been used to estimate specific DPP4 and residual enzyme activity within plasma samples. Quantification of specific DPP4 and residual DPP activities represents a novel technique that may have applications in DPP4 inhibitor development and biomarker discovery (Matheeussen et al., 2012). Using the selective DPP4 inhibitor, vildagliptin, Matheeussen *et al.* were able to differentiate plasma DPP enzyme activity of healthy mice into selective DPP4 (inhibited fraction) and residual DPP activity (remaining activity after inhibition) (Matheeussen et al., 2012). Specific DPP4 activity and residual activity have not been previously reported in human plasma in healthy or disease paradigms.

The aim of the current study was to characterize the gene expression of DPP4 gene family members in colorectal tissue derived from IBD patients. Furthermore, I aimed to further characterize plasma DPP activity in patients with IBD, assessing the relationship between plasma DPP activity and other clinical and physiological factors in these patients.

3.2 Materials & Methods

3.2.1 Patient samples

Colorectal tissue for qPCR

DPP mRNA expression was determined in bio-banked colorectal biopsies from patients with CD, UC and those without IBD (non-IBD). Biopsies were donated by the Gastroenterology Department at the Queen Elizabeth Hospital, Adelaide, South Australia, and were collected during standard colonoscopy procedures. Use of these samples had been approved for use by the Central South Australian Clinical Human Research Ethics Committee.

Plasma Samples for DPP Enzyme Assays

Bio-banked plasma samples were obtained from the Gastroenterology and Hepatology Department at the Flinders Medical Centre, Adelaide, South Australia. Plasma samples were collected from patients with CD and UC during routine clinic visits between 2008-2015 and stored at -80°C. Information on disease activity (active or inactive) and bowel resections was available for a sub-set of patients (Table 3.2). Clinician's notes from the time of blood collection were assessed by a separate physician to determine patient disease activity status. Clinical notes included: physical examination, patient general wellbeing and blood analysis. These samples were approved for research use by the Flinders Clinical Research Ethics Committee (Study ID: 13/08).

Venous blood was collected from non-IBD adult donors presenting for blood donation (for routine diagnostic services) at the Women's and Children's Hospital under the guidelines and approval of the Women's and Children's Health Network Research Ethics Committee. Written informed consent was obtained from all participants. No additional information was collected from this group of donors. Following collection, blood samples were centrifuged (800xg for 30 minutes) to separate plasma from cellular components. Plasma was subsequently harvested and stored at -80°C.

3.2.2 qPCR

DPP gene expression was assessed in non-IBD, CD and UC colorectal patient samples by qPCR. The qPCR experiments described here were the first qPCR experiments performed in this thesis and are slightly different to the method described in Chapter 2 and Chapter 4. Points of difference include the qPCR machine, qPCR reaction components, use of a Beta actin as a reference gene and use of the comparative Ct method instead of absolute quantitation method used Chapter 2 and Chapter 4. DPP primer sequences described here are identical to other Chapters, though were re-synthesized for experiments described in Chapter 2 and Chapter 4 following the loss of a freezer.

Patient biopsies were stored in RNA later at -20°C until use. RNA was isolated from colorectal biopsies using the RNA easy kit (Qiagen, Germany) as per manufacturer's instructions. Complementary DNA (cDNA) was immediately synthesized from 1µg of RNA using Quantitect cDNA synthesis kit (Qiagen, Germany). Relative mRNA expression of DPPs was determined in 25ng of cDNA by qPCR using SYBR green technologies. 10µL reactions consisted of SYBR Green 2x Mastermix (Life

Technologies, United States) ultrapure water and 150nM of primer (Table 3.1). PCR reaction cycling conditions were as follows: 95°C for 20 minutes, followed by 40 cycles of 95°C for 1 second, 65°C for 20 seconds and 95°C for 15 seconds. All qPCR reactions were run using the Fast protocol on the Viia7 (Life Technologies Gene expression data was analyzed using the comparative Ct method ($2^{-\Delta CT}$). Reference genes beta actin, cyclophilin A (PPIA) and ribosomal large protein (RPLPO) were quantified in all samples. RPLPO varied between disease states and was unsuitable for normalization. Beta actin was chosen for normalization as the expression profile was more consistent with the genes of interest. Data is presented as Relative Expression compared to reference gene beta actin, referred to intext as arbitrary units (AU) (Schmittgen and Livak, 2008).

Table 3.1 PCR primer sequences used to quantify DPP mRNA expression.

	Forward Primer (5'–3')	**Reverse Primer (5'–3')**	**Product (bp)**
DPP4	ACGCCGACGATGAAGACACCG	TTCAGCAGAACCCGGGCACG	95
FAP	ATGGGGCTGGTCCTATGGAGGAT	GCTGGAGACTGGAGCCACTGC	97
DPP8	ACA TGATGGCTAAGGCACCACATGA	CTGTTCTCACCAGACATGGCAAGG	90
DPP9	GGATCATTTCCAGGCCACGCC	ATGCCGAAGACCCCCAGGCG	90
DPP2	CGCTTCCTGGTGTCGGACAGG	GCCGAGTTGTTGGCGAAGGCC	101
Beta Actin	GAAGAGCTACGAGCTGCCTGAC	TGCCACAGGACTCCATGCCCA	110

3.2.3 DPP enzyme activity assays

DPP enzyme activity in human plasma samples was quantified by a kinetic colorimetric enzyme assay as previously described (Yazbeck et al., 2010b). The DPP substrate, H-Ala-Pro-pNA (extinction coefficient: $9450M^{-1}cm^{-1}$; Bachem, Switzerland) was used at a final concentration of 1mM in 0.1M sodium phosphate buffer for all enzyme assays. Dual absorbance readings were taken at 405 nm and 600 nm every 10 minutes for a total of 90 minutes using the ClarioStar (BMG Labtech, Germany). All samples were analyzed in triplicate. Enzyme activity was expressed as µmoles of pNA released/min/Litre of plasma (U/L).

H-Ala-Pro-pNa has been commonly used for the detection and quantification of DPP4 enzyme activity(Yazbeck et al., 2010b). However, it may also be hydrolyzed by other DPP enzymes including DPP8, DPP9, and FAP (Bjelke et al., 2006, Aertgeerts et al., 2005). Studies in DPP4 knock out animals have reported unchanged levels of plasma DPP activity compared to wild-types (Geier et al., 2005), suggesting the presence of multiple DPP enzymes within the plasma. Consequently, activity determined by the hydrolysis of H-Ala-Pro-pNA will be referred to as total DPP activity (U/L).

To better define plasma DPP activity and discriminate between DPP4 activity and that of other DPPs, the selective DPP4 inhibitor, sitagliptin phosphate monohydrate (BioVision, United States) was incorporated into enzyme assays. A final concentration of 1µM Sitagliptin was chosen to maintain selectivity over other DPP enzymes that may be present (Kim et al., 2005). The amount of activity inhibited by sitagliptin was termed ‘DPP4 activity’ and remaining activity referred to as ‘residual activity’ (Figure 2.1).

Sitagliptin concentration

The Caco-2 cell line has high DPP4 expression (Darmoul et al., 1992), and was used to assess the inhibition profile of Sitagliptin under conditions described above in 3.2.3. Caco-2 cells were grown in T75 flasks (n=6) under standard cell culture conditions to five-days post-confluence. Whole cell extract was collected by lysing cells in RIPA buffer (Sigma Aldrich, Castle Hill, NSW) and samples were pooled before being clarified by centrifugation and stored in aliquots at -20ºC.

DPP enzyme activity was determined in 5µL of Caco-2 lysate as described above. Concentrations of 1µM and 10µM sitagliptin were tested to determine the percentage of DPP4 activity inhibited at each concentration. Data is as DPP enzyme activity (U/mg of protein) and is the mean of n=8 independent replicates.

Inhibition of DPP4 activity by sitagliptin was also determined in recombinant DPP4 protein. Enzyme assays were performed on 20ng, 10ng and 5ng of human recombinant DPP4 protein (Abnova, Taiwan) as described above, with and without 1µM or 10µM of sitagliptin.

3.2.4 Statistics

Data normality was determined by analysis of frequency histograms and quantile-quantile plots. Non-parametric comparisons between multiple groups for qPCR and enzyme activity data were made by Kruskal-Wallis test with a Mann-Whitney post-hoc and Bonferroni correction. Comparisons between active and inactive disease groups were made by Mann-Whitney test. For all analyses, $p<0.05$ was considered significant. All data is expressed as median [IQR, Q1- Q3]. Statistical comparisons were made using IBM SPSS Statistics V23 (Armonk, New York).

3.3 Results

3.3.1 DPP4 and FAP are differentially expressed in IBD vs Non-IBD colonic tissue

DPP4 protein is reduced in affected ileal tissue during CD (Moran et al., 2012); however, the expression and activity of DPPs during colorectal IBD has not been previously characterised. The relative mRNA expression of DPP4 was significantly lower in tissue from UC patients (0.45 [0.41 - 0.63] AU) compared to non-IBD patients (2.17 [1.11 - 2.48] AU, $p<0.05$); however, no change in DPP4 mRNA expression was observed between the CD (0.79 [0.39 – 1.37] AU) and non-IBD patients (Figure 3.1a). FAP mRNA expression was 3.7-fold higher in UC patients (0.48 [0.30 – 0.62] AU, $p<0.05$) compared with non-IBD patients (0.04 [0.03 – 0.098] AU) whereas no changes in FAP expression were observed between the CD (0.06 [0.05 – 0.24] AU) and non-IBD patient groups (Figure 3.1d). Expression of DPP8 (Figure 3.1b), DPP9 (Figure 3.1c) and DPP2 (Figure 3.1e) was unchanged between non-IBD and IBD patient groups.

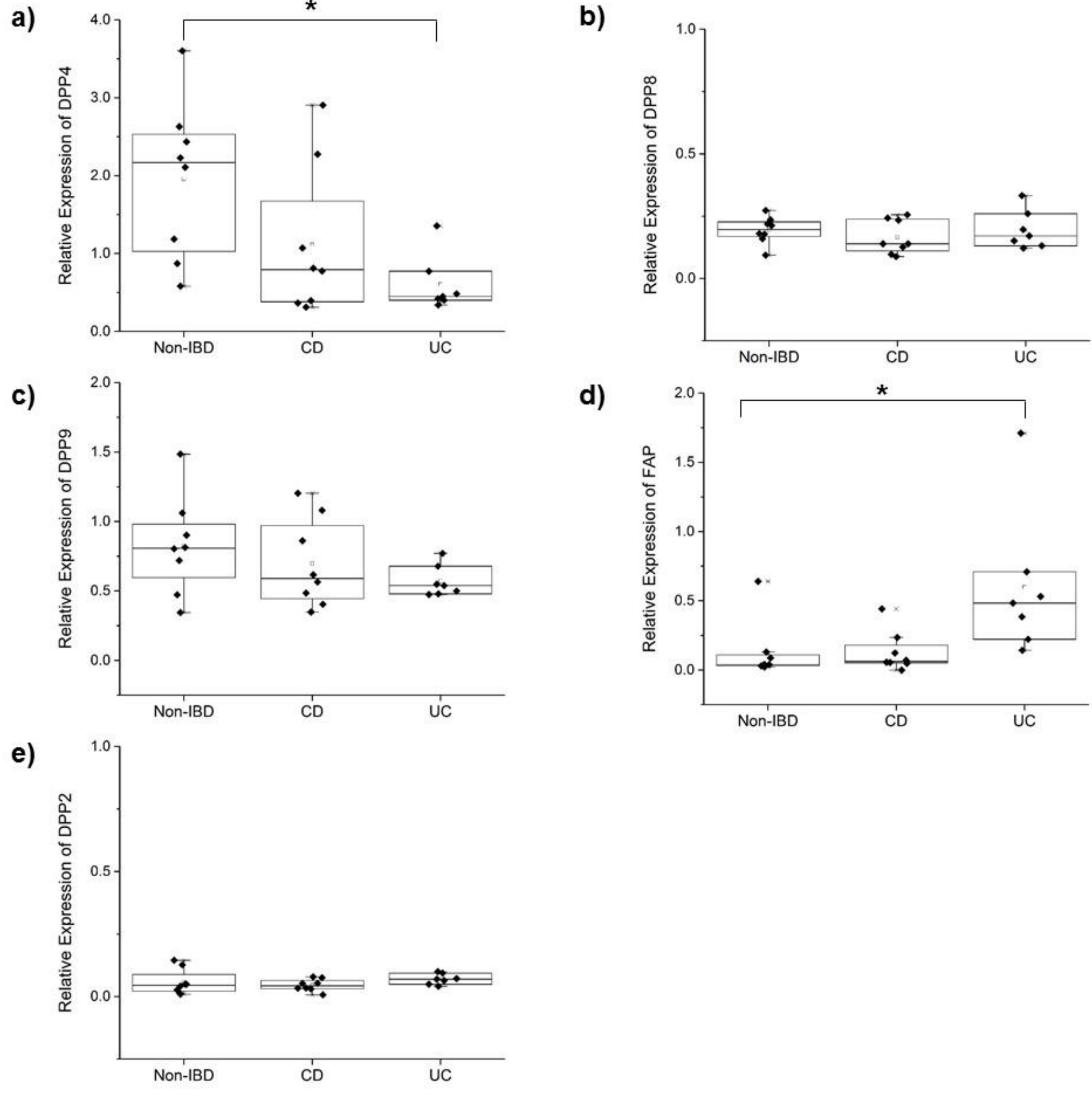


Figure 3.1 mRNA expression of DPPs in IBD and non-IBD colorectal biopsies. The mRNA expression of genes DPP4 **a)**, DPP8 **b)**, DPP9 **c)**, FAP **d)** and DPP2 **e)** relative to reference gene, beta actin, was determined using qPCR and calculated using the comparative Ct method, $2^{-\Delta CT}$ (n=8/non-IBD, n=8/CD, n=7/UC). Each data point represents DPP mRNA expression in a single patient. Box-plot overlay represents the median and IQR, whiskers represent the range excluding outliers. Relative expression referred to intext as AU. **p<0.05 for DPP4, Non-IBD vs UC and FAP, Non-IBD vs UC.*

3.3.2 Defining total, DPP4 and residual enzyme activity

The IC50 reported for sitagliptin ranges between 18-29nM for DPP4 under various laboratory conditions (Kim et al., 2005, Kirby et al., 2010). DPP activity was reproducibly detected in Caco-2 cells, and was inhibited by a mean of 66.1% and 90% by 1µM and 10µM sitagliptin respectively (Figure 3.2a).

Recombinant DPP4 activity increased in a concentration dependent manner, and 1µM sitagliptin inhibited 57%, 58% and 62% in 5ng, 10ng and 20ng recombinant DPP4 respectively (Figure 3.2b). 10µM sitagliptin inhibited DPP4 activity by 82%, 88% and 91% in 5ng, 10ng and 20ng recombinant DPP4 respectively (data not shown). Despite that lack of complete inhibition of DPP4, subsequent experiments in human plasma continued to use 1µM sitagliptin to minimize any non-specific inhibition of other DPPs.

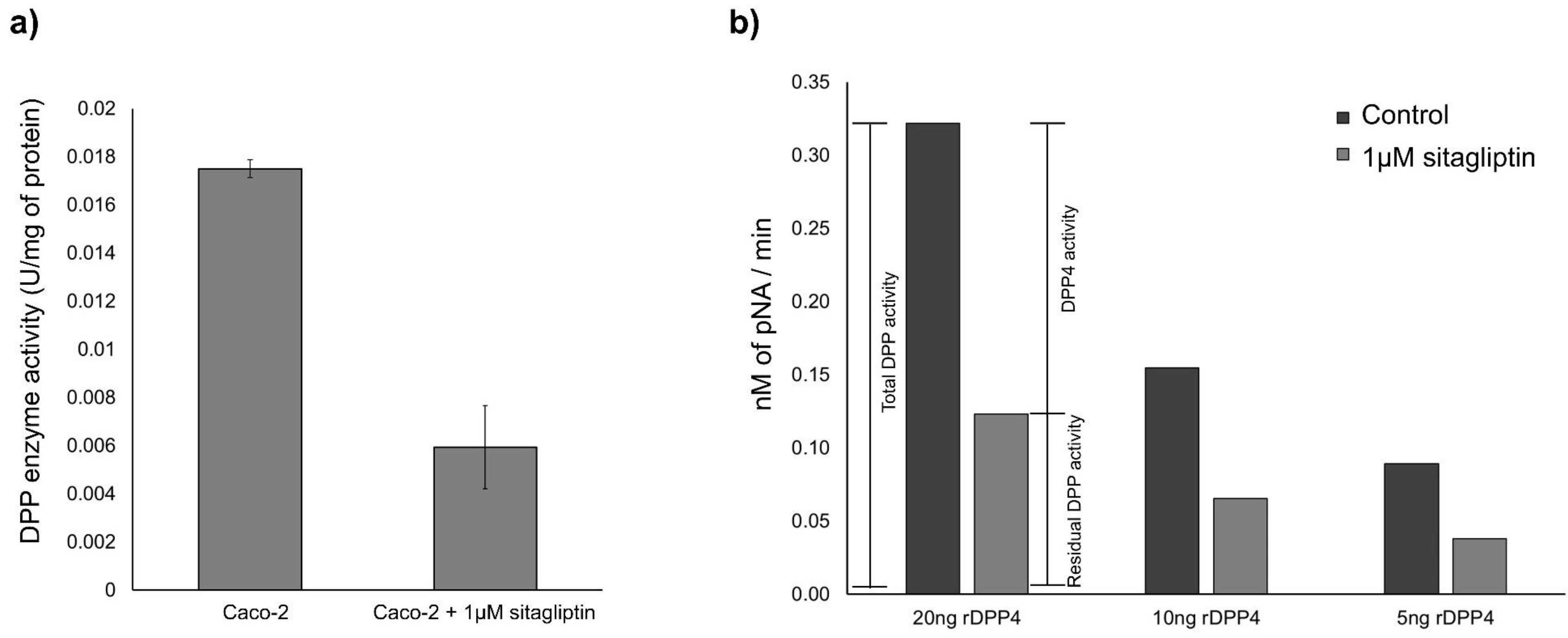


Figure 3.2 Determining DPP4 enzyme activity using 1μM sitagliptin. **a)** DPP enzyme activity in Caco-2 cell extracts. The relative contribution of DPP4 was determined by 1μM sitagliptin. **b)** DPP4 enzyme activity was further quantified in 20ng, 10ng and 5ng of human recombinant DPP4 with and without 1μM sitagliptin. Total DPP, DPP4 activity and residual activity are indicated by black bars. All samples were assayed in triplicate.

3.3.3 Patient characteristics

Plasma samples from 130 patients with CD, and 90 patients with UC, were collected during routine clinic visits to the Gastroenterology Department at Flinders Medical Centre between 2008 and 2015. A total of 37 CD and 19 UC patients were characterized according to disease activity (active or inactive) at the time of blood collection (Table 3.2), information for other patients was unavailable due to inconsistencies associated with data collection at the time of blood collection.

Table 3.2 Information on the Retrospective IBD cohort

	Crohn's Disease	**Ulcerative Colitis**
Total Number of Patients (n)	130	90
Age median; range	37; 18 - 73	48; 18 - 75
Disease Activity (Active vs Inactive)	18 vs 19	9 vs 10
Resection History (Resection vs no resection)	10 vs 16	14 vs 37

3.3.4 Plasma DPP activity is reduced in CD and UC patients

Total DPP activity was lower in the plasma of CD (14.1 [10.9 – 17.1] U/L) and UC patients (14.9 [12.4 – 18.3] U/L) compared to non-IBD patients (17.7 [14.5 – 20.2] U/L; $p<0.05$; Figure 3.3a). DPP4 activity was determined by the addition of the DPP4 selective inhibitor, sitagliptin, to the enzyme activity assays. Plasma DPP4 activity was significantly lower in CD (7.5 [5.7 – 10.0] U/L) compared with non-IBD patients (9.5 [7.9 – 12.0] U/L, $p<0.05$; Figure 3.3b). Residual DPP activity was also significantly lower in the plasma of CD (6.2 [4.7 – 7.5] U/L) and UC (6.0 [4.9 - 7.3] U/L) compared to non-IBD patients (7.6 [5.9 - 8.3] U/L, $p<0.05$; Figure 3.3c).

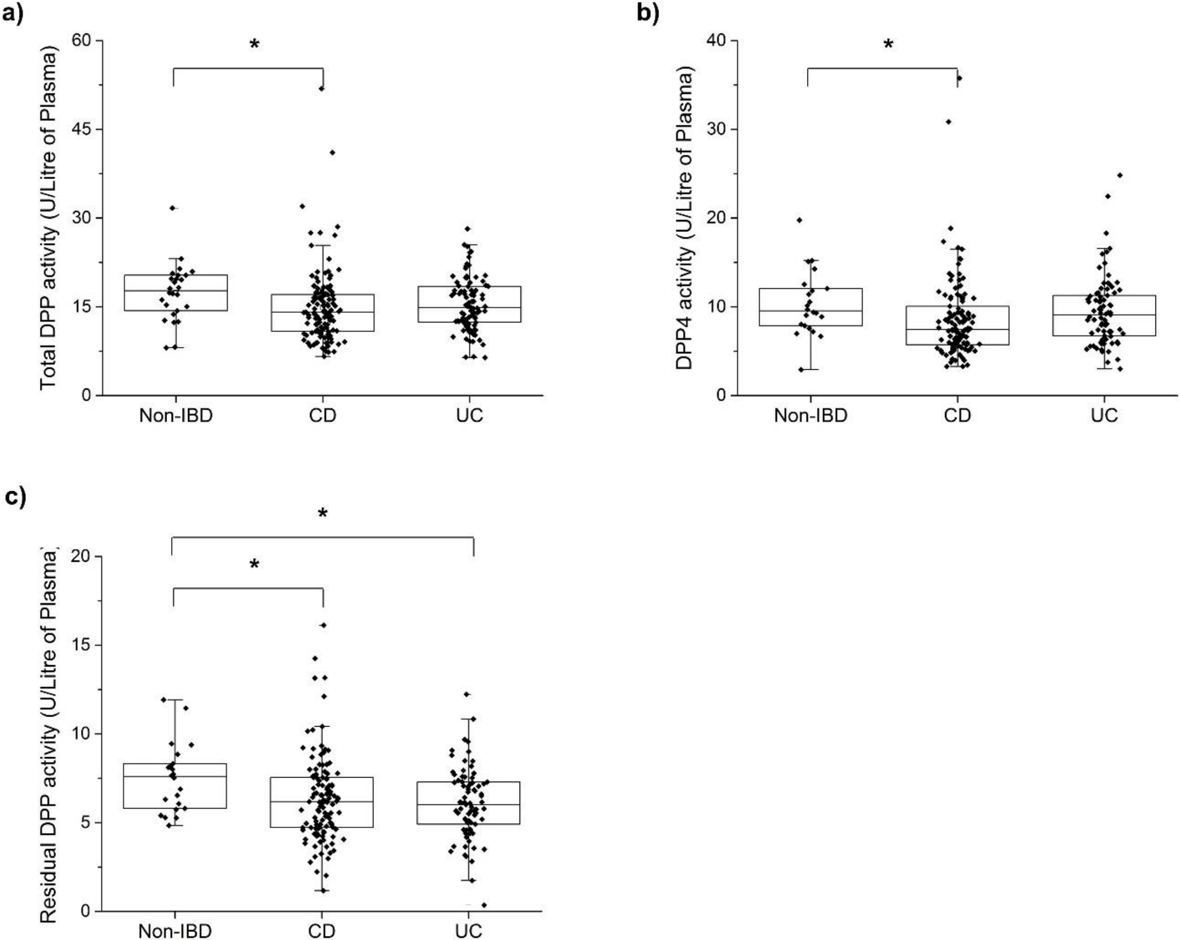


Figure 3.3 Plasma DPP enzyme activity in non-IBD, CD and UC patients. Plasma DPP activity was quantified as total (n=26/non-IBD; n=130/CD; n=90/UC) **a)**, DPP4 **b)** and residual DPP activity (n=22/non-IBD; n=121/CD; n=81/UC) **c)**, respectively, using 1µM sitagliptin. Each data point represents the result of a single patient. Box-plot overlay represents the median and IQR, whiskers represent the range excluding outliers. **p<0.05 compared to non-IBD.*

3.3.5 Active CD and UC have differential plasma DPP activity profiles

IBD patient cohorts were further classified into those with active and inactive disease at the time of blood collection. Total DPP activity was 30% lower in CD patients with active disease compared to patients with inactive disease, 11.7 [9.6 – 13.8] U/L vs 14.4 [12.5 – 20.1] U/L respectively (Figure 3.4a, $p<0.05$). However, DPP4 activity and residual DPP activity was unchanged between CD patients with active and inactive disease (Figure 3.4b and 3.4c).

There was no difference in total DPP activity (Figure 3.5a) and DPP4 activity (Figure 3.5b) between UC patients with active and inactive disease. However, residual DPP activity was significantly lower in UC patients with active disease (4.9 [3.7 – 5.7] U/L) compared to those with inactive disease (7.3 [6.2 – 7.7] U/L, $p<0.05$; Figure 3.5c).

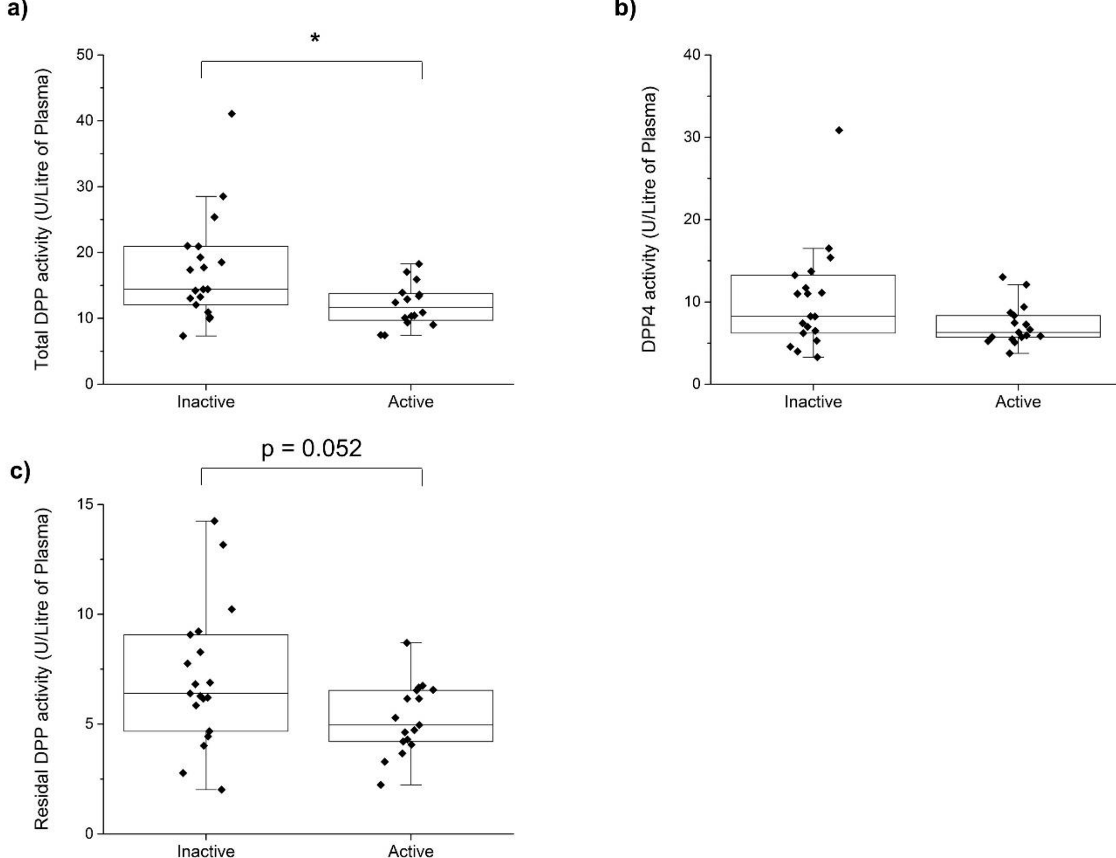


Figure 3.4 Plasma DPP enzyme activity in patients with inactive and active CD. Plasma DPP activity was quantified as total **a)**, DPP4 **b)** and residual DPP activity **c)** respectively, using 1µM sitagliptin, (n=16/active; n=19/inactive). Each data point represents the result of a single patient. Box-plot overlay represents the median and IQR, whiskers represent the range excluding outliers. **p<0.05 active vs inactive.*

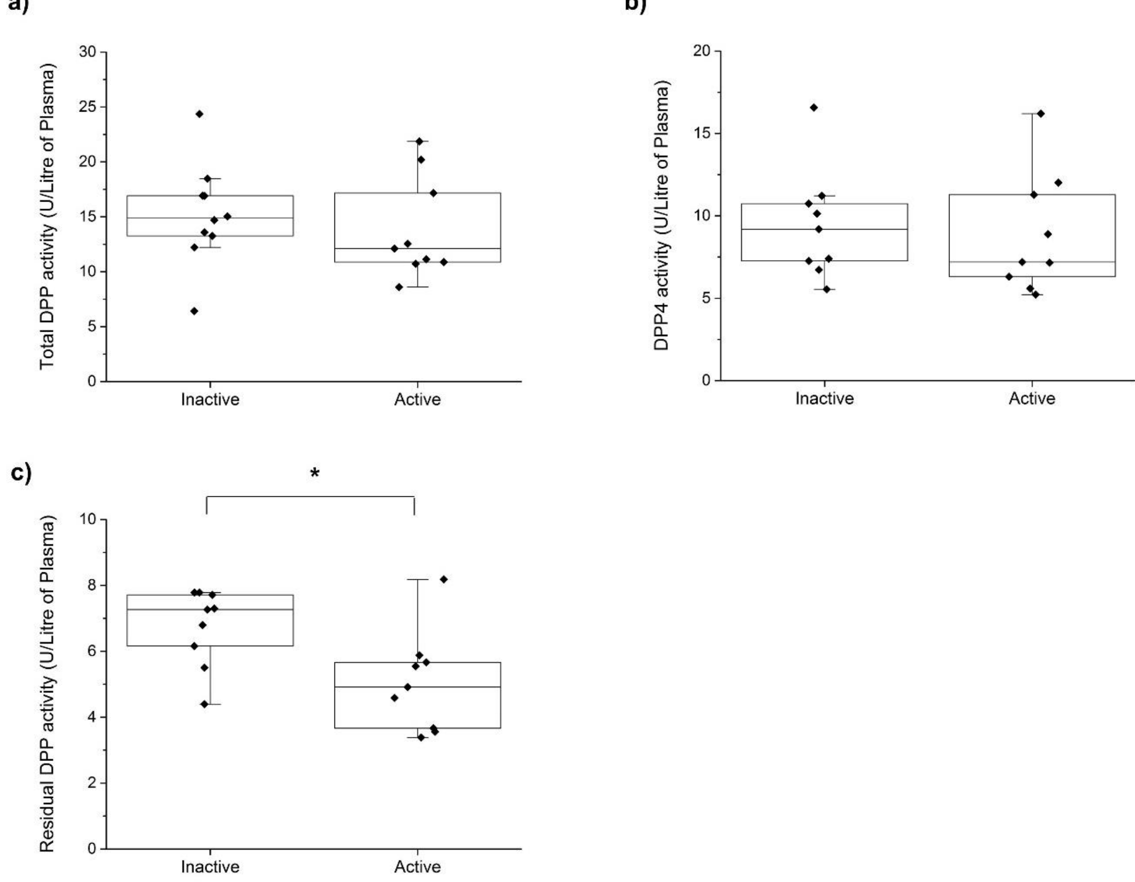


Figure 3.5 Plasma DPP activity in patients with inactive and active UC. Plasma DPP enzyme activity was quantified as total **a)**, DPP4 **b)** and residual DPP activity **c)** respectively using 1μM sitagliptin, (n=9/active; n=9/inactive). Each data point represents the result of a single patient. Box-plot overlay represents the median and IQR, whiskers represent the range excluding outliers. **p<0.05 active vs inactive.*

3.3.6 Bowel resections and patient age have no effect on plasma DPP activity in IBD

It has been previously reported that small intestinal DPP4 mRNA and protein expression is reduced following bowel resection and/or tissue damage, correlating with intestinal adaptation (Ziegler et al., 2003, Dunphy et al., 1999); however, the effect of bowel resection on plasma DPP activity has not been previously reported. CD and UC patients were separated into those with and without previous bowel resection. Time since the resection ranged from 1-14 years with the average time between bowel resections and plasma collection 5.9 years. We found no difference in plasma total DPP (17.0 [12.9 – 20.0] U/L vs 14.4 [11.9 – 18.4] U/Litre of plasma), DPP4 (9.3 [6.7 – 12.5] U/L vs 8.9 [7.0 – 11.3] U/L) or residual DPP activity (6.5 [4.6 – 7.2] U/L vs 5.7 [4.6 – 7.3] U/L) between IBD patients with and without previous bowel resection respectively (data not shown). Furthermore, there was no relationship between the time that had elapsed since surgery and plasma enzyme activity.

Human DPP enzyme activity has also been reported to decline with age (Durinx et al., 2001). To assess to whether age contributed to plasma DPP activity of IBD patients in this study, we excluded all patients with known active disease, and analyzed DPP enzyme activity according to defined age groups (Table 3.3). No differences were found between IBD patients of any age group (Table 3.3). Furthermore, plasma DPP activity did not correlate with patient age.

Table 3.3 The effect of patient age on plasma DPP activity.

Age (yrs)	*n*	Total DPP activity	DPP4 activity	Residual DPP activity
<20	9	15.1 [13.2 – 17.4]	10.5 [7.9 – 12.2]	5.7 [3.9 – 9.7]
20-29	39	14.9 [12.1 – 17.5]	8.3 [5.7 – 10.1]	6.5 [5.1 – 7.5]
30-39	38	13.3 [10.1 – 16.7]	6.6 [5.7 – 10.3]	6.2 [4.2 – 7.1]
40 - 49	42	13.4 [11.1 – 16.9]	7.2 [5.6 – 9.8]	5.6 [4.6 – 7.1]
50-59	35	16.3 [12.8 – 19.6]	9.3 [7.5 – 11.9]	6.1 [4.9 – 7.5]
60<	28	14.3 [12.2 – 17.0]	8.8 [7.1 – 10.6]	6.2 [4.9 – 7.8]

Data expressed as median [IQR, Q1-Q3]

3.4 Discussion

This is the first study to report colorectal tissue mRNA expression of the DPP4 gene family in human IBD, finding reduced DPP4 and increased FAP gene expression in UC patients. Furthermore, this study is the first to discriminate human plasma DPP activity into DPP4 and residual activity, reporting differential plasma DPP activity patterns in CD and UC patients during active disease and remission.

DPP4 expression is dysregulated in a number of inflammatory and autoimmune diseases (Yazbeck et al., 2017) including rheumatoid arthritis (Kamori et al., 1991) and multiple sclerosis (Tejera-Alhambra et al., 2014). Furthermore, Yazbeck *et al.* has previously reported differential DPP gene expression in the colon of mice with experimental colitis (Yazbeck et al., 2010b), implicating this enzyme family in gastrointestinal inflammation. Moran *et al.* have previously characterized DPP4 protein expression using western blotting in ileal tissues from patients with CD, finding DPP4 protein was 2.7-fold lower in ileal tissue from CD patients with active disease compared to non-IBD controls (Moran et al., 2012). In the current study, we found detectable levels of DPP4 mRNA in colorectal tissue from IBD and non-IBD patients, with expression down-regulated in tissue from UC patients. In the non-IBD group, two defined patient clusters of high (> 2) and low relative expression (< 2) were identified. Owing to the retrospective nature of the tissue samples, the exact anatomical location of each biopsy segment was not available. In Chapter 2, it was reported that DPP4 mRNA expression is more than 10-fold higher in the ileum compared to the colon, highlighting the importance of biopsy location when interpreting DPP4 expression. It is possible that some of biopsy material from non-IBD and CD patients may have come from more proximal regions such as the ileum, this would explain the variation in DPP4 expression observed in these two patient groups. As per Chapter 2, the expression of other DPP genes particularly, FAP, DPP8 and DPP9 is more consistent

between ileal and colonic tissues and would have been unaffected by biopsies from different regions. More comprehensive studies are indicated to prospectively determine DPP gene expression in tissue collected from defined gastrointestinal regions of IBD patients and during active disease.

The intestinal mucosa is a complex milieu of cells that includes epithelial cells, fibroblasts and immune cells that are dysregulated in IBD. The immune profiles of the two main IBD sub-types, CD and UC are distinct and are characterized by the uncontrolled activation of the mucosal immune system, in particular, mucosal immune cells — such as macrophages, T cells and innate lymphoid cells (ILCs). M1 macrophages and CD4+ Th1/Th17 cells have been implicated in mucosal inflammation associated with IBD (Schenk et al., 2007, Kamada et al., 2008, Jiang et al., 2014). Zhong *et al.* reported that DPP4 expression is upregulated on the surface of human derived peripheral blood monocytes, macrophages and dendritic cells following 24-hour activation *in vitro* with lipopolysaccharide. In this context, DPP4 may act as a co-stimulatory molecule of adenosine deaminase present on the surface of T-cells, inducing T-cell proliferation. Furthermore, DPP4 is highly expressed on activated CD4+ T-lymphocytes such as Th17 cells, further implicating DPP4 in regulating intestinal immunity (Zhong et al., 2013, Bengsch et al., 2012, Salgado et al., 2000). DPP4 expression is also dysregulated on peripheral blood lymphocytes from IBD patients (Rybaczyk et al., 2009), suggesting DPP4 may have a role in driving the inflammation associated with IBD.

FAP shares 67% structural homology to DPP4 and has similar substrate specificity; however, FAP expression is limited to cells of mesenchymal origin, including fibroblasts and myofibroblasts that are present at sites of tissue remodeling and malignancy (Gorrell, 2005, O'Brien and O'Connor, 2008). Intestinal fibrosis is defined as the excessive deposition of extracellular matrix as a result of chronic inflammation such as that associated with IBD, and

is associated with complications such as stiffening of the colon, strictures, fistulas and stenosis (Speca et al., 2012, Latella et al., 2014). Rovedatti *et al.* have previously reported increased FAP protein expression in the submucosa and muscularis layers of strictured tissue from CD patients, and low to undetectable expression in colonic tissue of inflamed UC patients and non-IBD controls (Rovedatti et al., 2011). Higher levels of FAP mRNA was found in colonic biopsies from UC patients compared to CD and non-IBD controls, suggesting there may be disease specific factors involved in the regulation of FAP expression. Although information regarding fibrosis and stricture formation was unavailable, elevated FAP expression may be indicative of tissue repair processes (O'Brien and O'Connor, 2008). Further studies are needed to comprehensively localize and quantify FAP mRNA and protein expression, as well as enzyme activity (Keane et al., 2013), to better understand the role of FAP in IBD pathogenesis. Chapter 4 expands on this and further characterizes FAP mRNA expression in IBD tissues as well as FAP plasma activity in patients with IBD.

Plasma DPP enzyme activity has been investigated as a serology marker in several different paradigms, including chronic inflammatory diseases, cancer and psychosocial disorders (Yazbeck et al., 2017). DPP enzyme activity has previously been measured by fluorometric and colorimetric assays, using non-selective substrates that do not discriminate between members of the DPP gene family (De Meester et al., 1992). Using the selective DPP4 inhibitor, sitagliptin, plasma DPP activity was discriminated into total, DPP4 and residual DPP enzyme activity. It is important to note that the concentration of sitagliptin used here did not completely inhibit that of purified recombinant DPP4,consequently, DPP4 activity reported here represents pure DPP4 activity rather than total DPP4 activity, with residual activity likely containing activity from DPP4 as well as other DPPs. Sitagliptin was used at 1µM in enzyme assays to selectivity inhibit DPP4 over other DPP enzymes (Kim et al., 2005).

Hildebrandt *et al.* and Rose *et al.* have previously reported lowered plasma DPP enzyme activity in CD and UC patients. Consistently, lower levels of total DPP enzyme activity were found in plasma from CD patients compared to non-IBD controls; however, only residual activity was significantly lowered in UC plasma, suggesting other DPP enzymes were contributing to this change. When CD and UC groups were further divided into active and inactive disease groups, total DPP activity was significantly lower in the active CD group, but only residual activity was lower in active UC group. Lower levels of residual activity could be the result of higher proportions of DPP4 compared to other DPP enzymes within the plasma of these patients; however, further characterization of the DPP enzymes that constitute plasma DPP activity is required.

This study highlights the complex roles of DPPs in gastrointestinal inflammation. This Chapter highlights the differential expression of DPP4 and FAP in colorectal tissue from CD and UC patients that warrants further comprehensive studies to understand the role of these enzymes within the gastrointestinal tract. Consistent with previous studies, plasma DPP activity was lower in the IBD cohort compared with non-IBD controls. Discriminating between plasma total DPP, DPP4 and residual activity, has identified further differences between CD and UC patients that require further analysis to completely elucidate the other enzymes that constitute plasma DPP activity. Better understanding the expression profile and function of the DPP enzyme family during human IBD may identify new opportunities for therapeutic intervention and novel biomarkers to differentiate pathophysiological aspects of the disease.

Chapter 4. Enhanced DPP expression and activity during human colorectal IBD; roles for DPPs in colorectal inflammation

4.1 Introduction

IBD is a chronic and debilitating condition characterized clinically by relapsing symptoms of diarrhea, rectal bleeding, abdominal pain and fatigue (Strober et al., 2007, Vatn, 2009). The clinical management of IBD involves consideration of clinical factors including disease phenotype, location, severity, complications, response rate, patient tolerance and access to medical facilities for diagnosis and treatment with the overall goal to achieve symptomatic and clinical remission (Bernstein et al., 2010). A recent study by Colombel *et al.* found that frequent monitoring of serological and faecal biomarkers, CRP and faecal calprotectin alongside the Crohn's disease activity index (CDAI), compared to CDAI alone, was associated with higher rates of mucosal healing as a result of earlier and more intensive clinical management (Colombel et al., 2018). This suggests that frequent and proactive monitoring of specific biomarkers could improve clinical management and outcomes in IBD patients.

Biomarkers are quantifiable units that reflect specific biological processes or physiological states and are sensitive to the presence of abnormalities or disease, making them clinically useful for screening, diagnosis and disease monitoring (Rifai et al., 2006). Ideal biomarkers are highly specific and selective, minimally invasive, cheap, quick and easily quantified without the need for specialist equipment or specific training, providing clear, insightful and clinically relevant information that assists in forming clinical decisions and predicting disease behavior (Boyapati et al., 2016).

A number of serological and faecal markers have been investigated for IBD including CRP, erythrocyte sedimentation rate, serum albumin, faecal calprotectin, lactoferrin and S100A10 (Boyapati et al., 2016, Galgut et al., 2017, Li et al., 2008). To date, markers of generalized inflammation, CRP and erythrocyte sedimentation rate remain the most commonly used

biomarkers to inform clinical decision making in IBD (Carter et al., 2004b, Iskandar and Ciorba, 2012), highlighting the need for new, specific and comprehensively validated biomarkers of IBD that could assist in the diagnosis, prognosis, monitoring and treatment of IBD (Boyapati et al., 2016, Rieder et al., 2013).

DPP4 is a multifunctional serine protease that has specific roles in metabolism, immunity and gastrointestinal health. DPP4 is ubiquitously expressed on the surface of epithelial, endothelial and immune cells (Gorrell, 2005). DPP4 is highly expressed along the small intestinal brush-border, with an increasing gradient of expression and activity reported from the duodenum to the terminal ileum (Darmoul et al., 1994). In contrast, low to undetectable levels of DPP4 are found on the surface of normal colonic epithelium (Sahara et al., 1983a). Chapter 2 also confirmed these findings, reporting considerable higher levels of DPP4 mRNA expression as well as DPP4 enzyme activity within the small intestine compared to other tissues. To date, studies have focused on small intestinal DPP4 expression and activity during damage and inflammation, with studies finding lower levels of DPP4 protein (Moran et al., 2012, Ziegler et al., 2003) and DPP4 enzyme activity (Detel et al., 2007) Chapter 2) in affected small intestinal mucosa. In contrast, the roles of DPP4 during colonic disease remain unclear, with studies in animal models of colitis reporting inconsistent results (Yazbeck et al., 2010b, Sakanaka et al., 2015). Chapter 3 suggested variation to DPP4 expression during IBD; however, the retrospective nature of the samples prevented adequate conclusions and suggests the need for more comprehensive studies.

DPP4 regulates a number of immunologically relevant and gastrointestinal regulatory peptides including GLP-2, VIP, NPY and GIP (Yazbeck et al., 2009a) (Table 1.3), and has been previously investigated for its roles in gastrointestinal dysfunction and disease (Baticic et al., 2011, Bank et al., 2008, Dunphy et al., 1999, Moran et al., 2012, Detel et al., 2007). Studies in

DPP4$^{-/-}$ mice with experimental colitis have reported altered levels of cytokines, immune cells and gastrointestinal peptides within the circulation and tissue of animals (Baticic et al., 2011, Detel et al., 2016) despite no reported changes to disease severity. In contrast, the use of non-selective and selective DPP4 inhibitors in animals has been associated with improvements to disease severity and intestinal functions during models of colitis (Yazbeck et al., 2010b, Mimura et al., 2013), short bowel syndrome (Okawada et al., 2011) and mucositis (Yamazaki et al., 2004). In addition to improvements to clinical disease severity, our laboratory has previously reported lower levels of colonic MPO activity, a commonly used marker of neutrophils, and higher proportions of peripheral blood T regs in mice treated with non-selective DPP inhibitors (Yazbeck et al., 2010b). A separate study by Bank *et al.* found that co-treatment with a non-selective DPP inhibitor and an aminopeptidase inhibitor during DSS colitis significantly reduced colonic inflammatory infiltrates and enhanced the expression of T reg related genes, whilst reducing disease progression (Bank et al., 2006). Anagliptin has also been associated with improved healing and colonic epithelial proliferation during the recovery phase of murine DSS colitis (Mimura et al., 2013). Together, these studies suggest a potential role for DPP4 and other DPP enzymes in mediating intestinal inflammation, and highlighting DPP inhibitors as potential therapeutic agents for intestinal disease.

A soluble form of DPP4 has been described in serum (Iwaki-Egawa et al., 1998) which has been proposed as a potential biomarker in a number of disease states (Yazbeck et al., 2017). In IBD, plasma DPP activity and DPP4 protein concentration inversely correlated with clinical disease activity indices and markers of inflammation such as CRP and orosomucoid (Moran et al., 2012, Xiao et al., 2000, Hildebrandt et al., 2001). In Chapter 3, lower levels of plasma DPP activity were found in CD and UC patients compared with non-IBD patients. Furthermore, plasma DPP activity was notably reduced in IBD patients with active disease, with unique

plasma DPP activity profiles identified in CD and UC patients, and may suggest potential roles for plasma DPP enzymes in diagnostic applications and disease monitoring of IBD.

The primary aim of this prospective, observational study was to comprehensively characterize plasma DPP activity and tissue DPP mRNA expression as well as DPP enzyme activity during IBD.

4.2 Materials & Methods

4.2.1 Patient recruitment

CD, UC and non-IBD patients were recruited from the endoscopy clinic at Flinders Medical Centre, Adelaide, South Australia between December 2015 and April 2017. Power calculations were performed by a statistician at the Basil Hetzel Institute, Adelaide, South Australia using plasma DPP activity as the primary endpoint. Calculations determined that n = 24/group would give 90% statistical power, this was inflated to n=30/group to allow for possible patient withdrawals. Suitable patients were identified from endoscopy lists based on clinical reasons for endoscopy and previous medical diagnosis. Exclusion criteria included patients under the age of 18, undergoing treatment with DPP4 inhibitors and pregnant women. Study participants were recruited to the study by a research nurse, and consent obtained on the day of endoscopy procedure. All participants were fasted and had undergone bowel preparation prior to their colonoscopy. Participants also completed a brief questionnaire assessing lifestyle factors including smoking (Appendix 6.3). All patient samples were de-identified in compliance with the ethical guidelines set by the Southern Adelaide Clinical Human Research Ethics Committee (SAC HREC 135.15).

Disease activity was determined endoscopically by the treating gastroenterologist and in the majority of patients, disease activity was confirmed by routine pathology. Clinical disease scoring indices, Harvey Bradshaw and the Mayo index were conducted by clinical research nurses and the treating gastroenterologist as a secondary means of assessing disease activity in IBD patients. Active disease was defined as a Harvey Bradshaw score of >4 (Walsh et al., 2016) or a Mayo score of >2 (Lewis et al., 2008).

Sample collection

Up to 40 mL blood was collected into K_2EDTA blood collection tubes prior to endoscopy. Blood samples were centrifuged (800 x g, 20 minutes, room temperature) to separate plasma, white and red blood cells. Plasma was then aliquoted and stored at -80°C until analysis. Up to ten biopsies were collected from each IBD and non-IBD patient. Disease activity was assessed visually by the treating physician during the colonoscopy procedure. Inflammation was identified by the presence of odema, friability, erythema, granularity and increased vascularity. In consultation with the treating gastroenterologist, up to five biopsies were collected from inflamed tissue regions (ileum, colon or rectum) with up to five matched biopsies collected from adjacent inactive tissue regions and random inactive tissue sites. Random biopsies from endoscopically normal tissue were collected throughout the ileum, colon and rectum from patients without inflammation. Biopsies were stored in cryovials containing RNA later, snap frozen in liquid nitrogen or placed in plastic tissue molds containing OCT and snap frozen for later analysis (Figure 4.1).

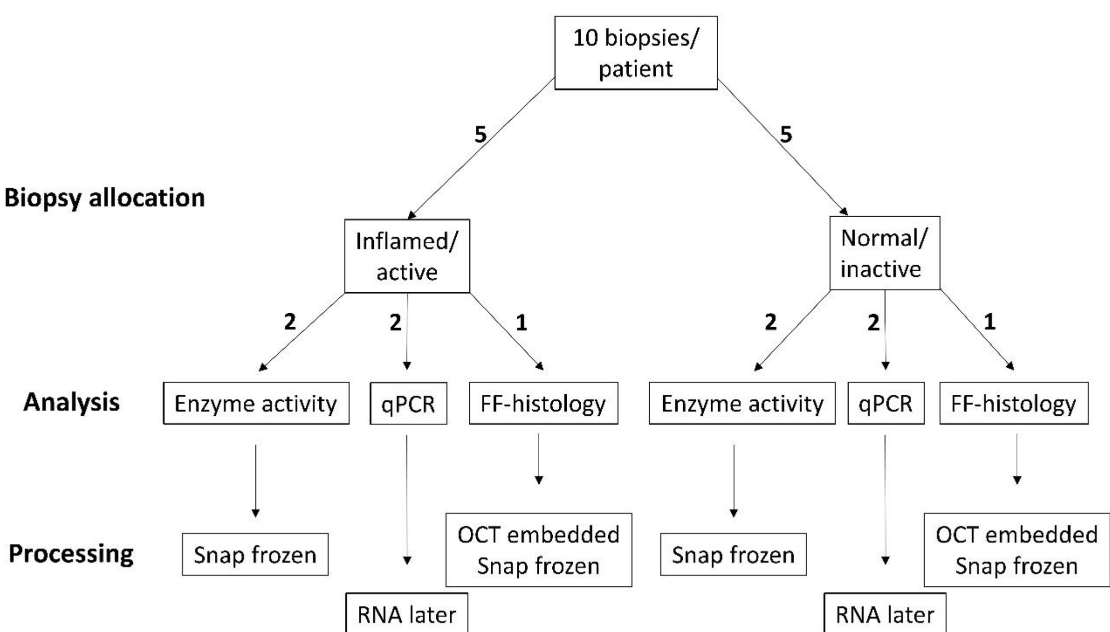


Figure 4.1 Schematic detailing 'ideal' biopsy collection and processing. When the collection of 10 biopsies was not suitable as per opinion of the treating gastroenterologist, biopsies were preferentially collected for enzyme activity and qPCR over those for analysis by histology techniques.

4.2.2 Enzyme activity

Tissue homogenization and preparation of fractions

Tissue samples were processed as per Chapter 2.2.3. Biopsies were homogenized in 200µL of ice-cold 10mM Tris-HCl buffer pH 8. Membrane and soluble cell fractions were separated by ultracentrifugation, 2 x (88,000 x g for 30 minutes, 4°C) (Beckman Coulter, Optima Max-TL). Membrane fractions were solubilized in 100-200µL of ice-cold 10mM Tris-HCl. Tissue homogenate fractions were transferred into clean Eppendorf tubes and kept on ice for immediate analysis by enzyme assay.

Enzyme assay procedure

DPP enzyme activity in human plasma samples was performed as previously described in Chapter 3.2.3; however, 10mM Tris-HCl pH 8 was utilized as assay buffer. 1µM sitagliptin was incorporated into assays to determine total DPP activity, DPP4 activity and residual DPP activity within plasma samples.

DPP enzyme activity was quantified in soluble and membrane tissue fractions as described in Chapter 2.2.4. 1µM sitagliptin and 10µM 1G244 were used to determine DPP4 and DPP8/9 specific activity respectively. Protein concentrations were determined using modified Bradford assays as per Chapter 2.2.5.

The colorimetric substrate H-Ala-Pro-pNA (Bachem, Switzerland) was used in all enzyme assays at a final concentration of 1mM. Caco-2 cell lysates were used as positive control to monitor assay validity. Enzyme activity within plasma samples is expressed as µM of pNA

released/min/Litre of plasma (U/L). Enzyme activity within tissue fractions is expressed as µM of pNA released/min/mg of protein, (U/mg of protein).

4.2.3 FAP enzyme activity

FAP enzyme activity assays were performed by Professor Mark Gorrell at the Centenary Institute, University of Sydney. FAP activity was measured in plasma samples using the previously validated fluorogenic substrate 3144-aminomethylcoumarin (AMC) (Keane et al., 2013). 100µL reactions consisted of diluted plasma, phosphate buffered saline and 150M AMC substrate. The microplate was read in a Polarstar plate reader (BMG Labtech, Germany) at excitation of 355 nm and emission of 450 nm every 5 minutes for 1 hour at 37ºC. Data was expressed as U/L of plasma.

4.2.4 C reactive protein

CRP is an acute phase reaction protein that is elevated in a number of inflammatory conditions including sepsis and IBD (Pepys and Hirschfield, 2003). Frozen plasma samples were thawed at room temperature and clarified by centrifugation (7,500 x g for 10 minutes) to remove any debris. CRP was quantified using a particle enhanced immune-turbidimetric assay, and samples were measured using the Cobas 8000 modular analyzer series (Roche, USA). CRP concentration is expressed as mg/L.

4.2.5 qPCR

DPP gene expression was measured in colorectal tissue samples stored in RNA-later by qPCR. Methods and conditions for all experiments were identical to that described in Chapter 2.2.6, with the additional quantification of DPP2 mRNA and a secondary reference gene, PPIA (Table 4.1, additional primer sequences). PPIA primers were used at a final concentration of 100nM while DPP2 primers were used at 200nM. The number of gene copies was determined by the use of external gene standard (Chapter 2.2.6, Appendix 6.2). All gene expression data is presented as copies of gene of interest normalized to the copies of reference genes, TATA and PPIA (Vandesompele et al., 2002), written as AU in text. Due to variation of mRNA levels of TATA and PPIA within colorectal tissues, the geometric mean was used in normalization calculations.

Table 4.1. Primer sequences.

	Forward Primer (5'–3')	Reverse Primer (5'–3')	Product (bp)
DPP2	CGCTTCCTGGTGTCGGACAGG	GCCGAGTTGTTGGCGAAGGCC	101
PPIA	GGCAAATGCTGGACCCAACACA	TGCTGGTCTTGCCATTCCTGGA	161

4.2.6 Statistics

Data normality was determined by analysis of frequency histograms and quantile-quantile plots. Comparisons were not made for $n \leq 5$. Non-parametric comparisons between multiple groups for qPCR and enzyme activity data were made by Kruskal-Wallis test with a Mann-Whitney post-hoc and Bonferroni correction. Comparisons between active and inactive disease (non-matched) groups were made by Mann-Whitney test. Non-parametric comparisons were also made between matched active vs inactive disease using Wilcoxon Signed Ranks Test. For all analyses, $p<0.05$ was considered significant. All data is expressed as median and interquartile range [IQR, Q1 – Q3] unless otherwise indicated. Statistical comparisons were made using IBM SPSS Statistics V23 (Armonk, New York).

4.3 Results

A total of 46 patients, 20 non-IBD, 13 CD and 13 UC were recruited into this study (Table 4.2). Active inflammation was identified in 50% (5/10) of CD patients and 53.8% (7/13) of UC patients (Table 4.2). Co-morbidities were most commonly associated with non-IBD patients, with type-2 diabetes and arthritis the most common conditions (Table 4.2). Analysis of other non-IBD related factors found current smokers have lower levels of total plasma DPP activity compared with patients identified as never smokers. (Appendix 6.4, Table 6.2.3). Other factors including patient age, gender and previous cancer diagnosis were not associated with altered plasma DPP activity (Appendix 6.4).

Table 4.2 Baseline patient characteristics

	non-IBD	**CD**	**UC**
Sample size *(n)*	20	13[l]	13
Age; mean (range)	59 (30-77)	43 (25-79)	47 (27- 69)
Gender (Female/ Male)	6 / 14	7 / 6	5 / 8
Time since diagnosis (yrs) mean, (range)	-	15, (1-30)	11, (1-24)
Inflammation during scope (%)	0%	50%	53.8%
Disease activity indices			
Harvey Bradshaw > 4	-	3/13	-
Mayo Score > 2	-	-	5/11
IBD Medications			
Immuno-suppressants[m]	1	8	3
Anti-inflammatory[n]	-	4	5
Biologicals[o]	-	2	3
Steroids	1	2	-
Combination therapy	-	6	2
Comorbidities & smoking status			
Diverticulitis	11	-	3
Arthritis	3	-	1
Type 2 diabetes	3	-	-
Previous Cancer	3	2	-
Smoking status (current, ex-smoker, never)	3 , 8 , 9	4 , 2 , 7	1 , 3 , 9

[l] A total of 13 CD patients were recruited into the study and suitable for tissue enzyme activity and qPCR analysis. Due to difficulties associated with blood collection, plasma was only available for 10/13 CD patients.
[m] Immunosuppressants included azathioprine, methotrexate and mercaptopurine
[n] Anti-inflammatory medications included mesalazine and sulfasalazine
[o] Biologicals included humira/infliximab and adalimumab

4.3.1 Plasma DPP activity is not altered in IBD or during endoscopic colonic inflammation

Previous studies have investigated the utility of circulating and tissue DPP4 as a biomarker of gastrointestinal diseases (De Chiara et al., 2010). In Chapter 3, it was found that plasma DPP activity was lower in patients with CD and UC compared to non-IBD patients. In this study, total plasma DPP activity, DPP4 activity and residual DPP activity was unchanged between non-IBD, CD and UC patients (Table 4.3). Interestingly, there was a similar level of DPP4 activity and residual DPP activity between all patient groups (Table 4.3). Consistent with Chapter 3, disease duration in IBD had no effect on plasma DPP, DPP4 or residual activity (data not shown). Plasma DPP activity was also unchanged in IBD during active disease as defined during colonoscopy (Figure 4.2a-c) or by clinical disease activity indices (Appendix 6.5).

Table 4.3. Plasma DPP enzyme activity in non-IBD and IBD patients.

	n	**Total DPP activity (U/L)**	**DPP4 activity (U/L)**	**Residual DPP activity (U/L)**
non-IBD	20	14.0 [12.7 – 17.2]	7.6 [6.3 – 10.3]	7.0 [5.7 – 7.8]
CD	10	13.4 [11.0 – 16.6]	6.7 [4.6 – 8.5]	6.8 [6.1 – 8.1]
UC	13	14.4 [12.8 – 16.0]	6.5 [5.8 – 8.3]	7.2 [6.4 – 8.6]

Median [IQR, Q1-Q3]

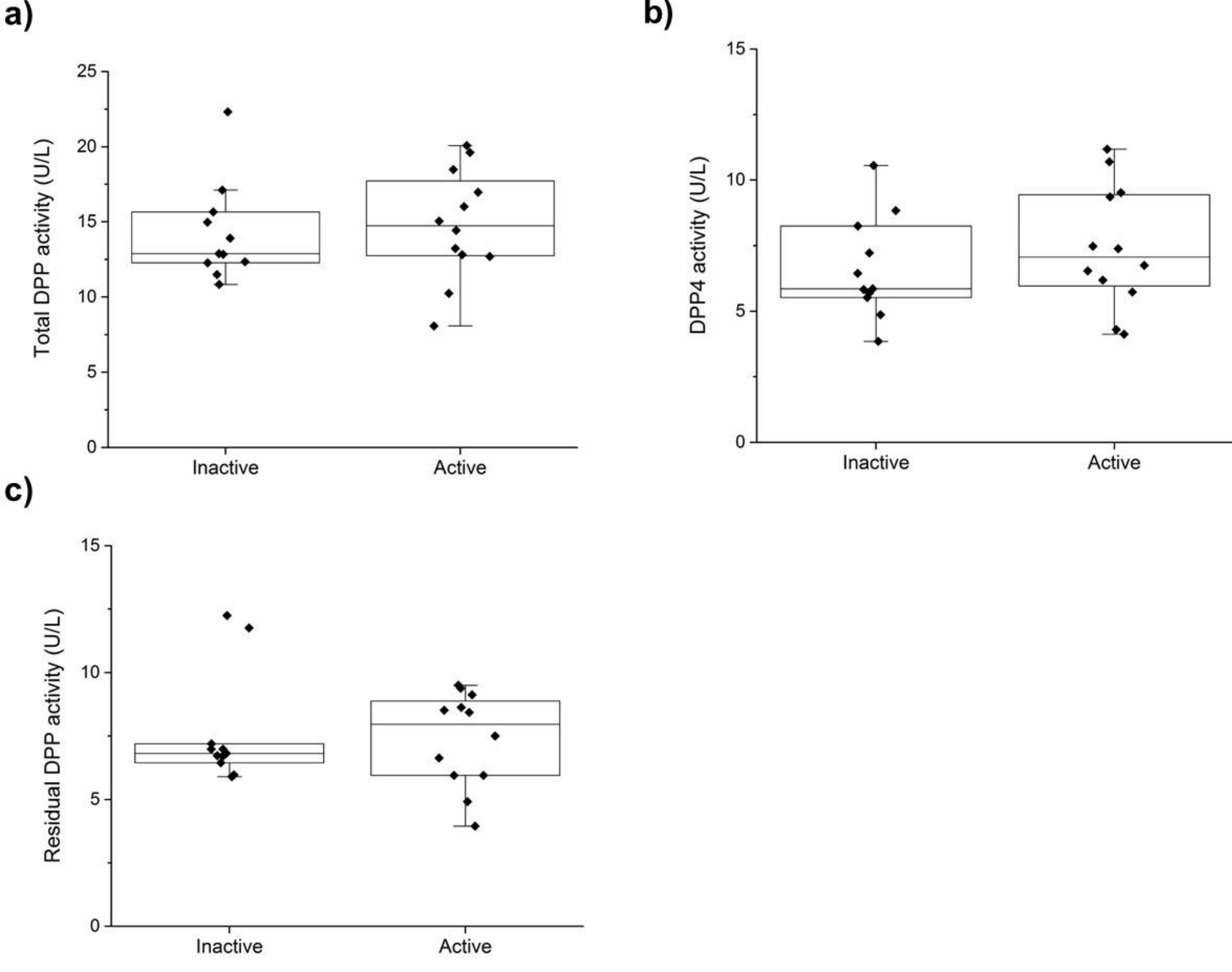


Figure 4.2 Plasma DPP enzyme activity during colorectal inflammation in IBD. Plasma DPP activity was quantified as total **a)**, DPP4 **b)** and residual DPP activity **c)** respectively using 1µM sitagliptin. Patients were classified as inactive (n=11; n=6/UC, n=5/CD) or active (n=12; n=7/UC, n=5/CD) depending on the presence of endoscopic inflammation as determined by an experienced gastroenterologist. Box-plot overlays represent median and IQR, whiskers represent range excluding outliers.

4.3.2 Plasma DPP activity is reduced in patients with elevated CRP

CRP is an acute phase reaction protein and was measured to provide an objective measure of inflammation. Elevated plasma CRP (>10mg/L) was found in 5/42 patients, that included four patients with IBD and a single non-IBD patient (Table 4.4). Total DPP activity was 28% lower in patients with elevated CRP (>10mg/L) compared to patients with normal CRP values (Figure 4.3a, $p<0.05$). Plasma DPP4 activity was also reduced in patients with elevated CRP values compared to patients with normal values by up to 34% (Figure 4.3b, $p<0.05$). Residual DPP activity was unchanged by CRP levels (Figure 4.3c).

Table 4.4 CRP levels in non-IBD and IBD patients.

	non-IBD	**CD**	**UC**
n	19	10	13
CRP (mg/L)	1.84 (0.16 – 25.57)	3.71 (0.06 – 69.89)	0.73 (0.08 – 11.55)
Elevated CRP (CRP > 10mg/L)	1/19	3/10	1/13

Median (range)

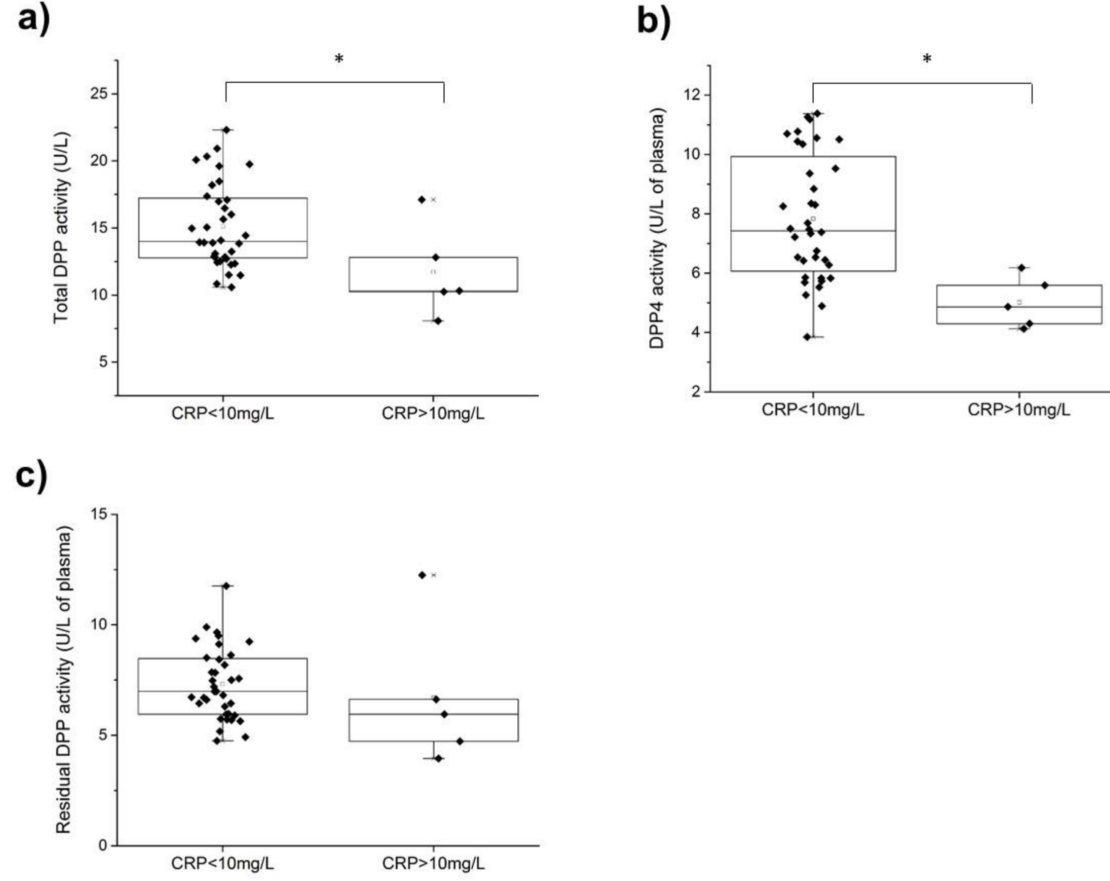


Figure 4.3 Plasma DPP enzyme activity and CRP in IBD and non-IBD patients. Plasma DPP activity was quantified in plasma samples as total **a)**, DPP4 **b)**, and residual DPP activity **c)** using 1µM sitagliptin. CRP was analyzed in non-IBD and IBD patient plasma samples. CRP values were classified as CRP < 10 mg/L, normal, (n=37; n=18/non-IBD, n=12/UC, n=7/CD) and CRP>10mg/L, elevated, (n=5; n=1/non-IBD, n=1/UC, n=3/CD). Box-plot overlays represent median and IQR, whiskers represent range excluding outliers. **p<0.05, CRP < 10mg/L vs CRP > 10mg/L*

4.3.3 Plasma FAP activity in IBD patients

Plasma FAP activity has also been identified as a potential biomarker of inflammatory and fibrotic conditions (Sinnathurai et al., 2016, Uitte de Willige et al., 2017). Here we found FAP activity was unchanged in plasma from non-IBD, CD and UC patients (Appendix 6.6). There was no difference in FAP activity between IBD patients with active and inactive disease as determined by endoscopy (Figure 4.4a). However, FAP activity was approximately 47% lower in patients with elevated CRP values (>10mg/L) compared to patients with normal CRP values (Figure 4.4b, $p<0.05$).

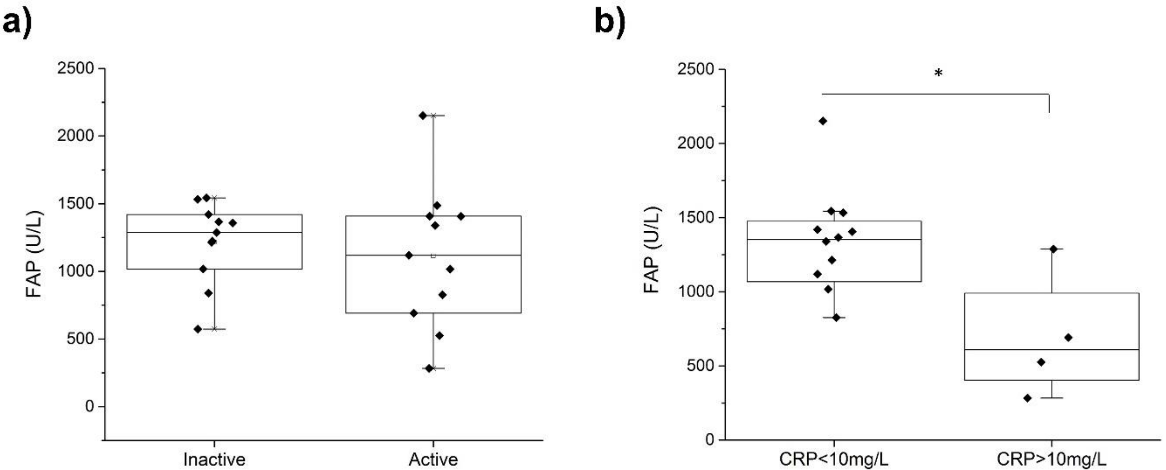


Figure 4.4 Plasma FAP activity in active IBD. Disease activity, inactive (n=11) or active (n=11), was determined during endoscopy by treating gastroenterologists **a)**. Measurement of CRP, a non-specific marker of inflammation was also used to gauge the presence of inflammation. CRP values (>10 mg/L) were considered elevated (n=4), while values of <10mg/L were considered normal (n=18) **b)**. Box plots represent median and IQR, whiskers represent range excluding outliers. **p<0.05, CRP <10mg/L vs CRP >10mg/L*

4.3.4 Colonic expression of DPP4, FAP and DPP8 mRNA is altered in IBD

DPP4 gene expression was 150% higher in normal colorectal tissue from IBD patients compared to non-IBD patients (Figure 4.5a, $p<0.05$). Higher levels of FAP (4.3 [2.9 - 5.2] AU vs 2.4 [2.0 – 2.8] AU, $p<0.05$; Figure 4.5b) and DPP8 (154.0 [134.0 – 201.1] AU vs 125.4 [106.6 – 140.1] AU, $p<0.05$; Figure 4.5c) mRNA expression were also found in colorectal tissue from IBD patients compared with non-IBD patients respectively. The mRNA expression levels of DPP9, DPP2 and PREP was unchanged in colorectal tissue isolated from IBD patients compared to non-IBD patients (Figure 4.5d-f).

DPP mRNA expression was also compared in matched inactive and active colorectal tissues from three separate IBD patients (Appendix 6.7). Due to the low sample size, statistical analysis was not performed. However, there was a trend towards increased expression of all DPPs in active colorectal tissue compared to the matched inactive tissue (Appendix 6.7).

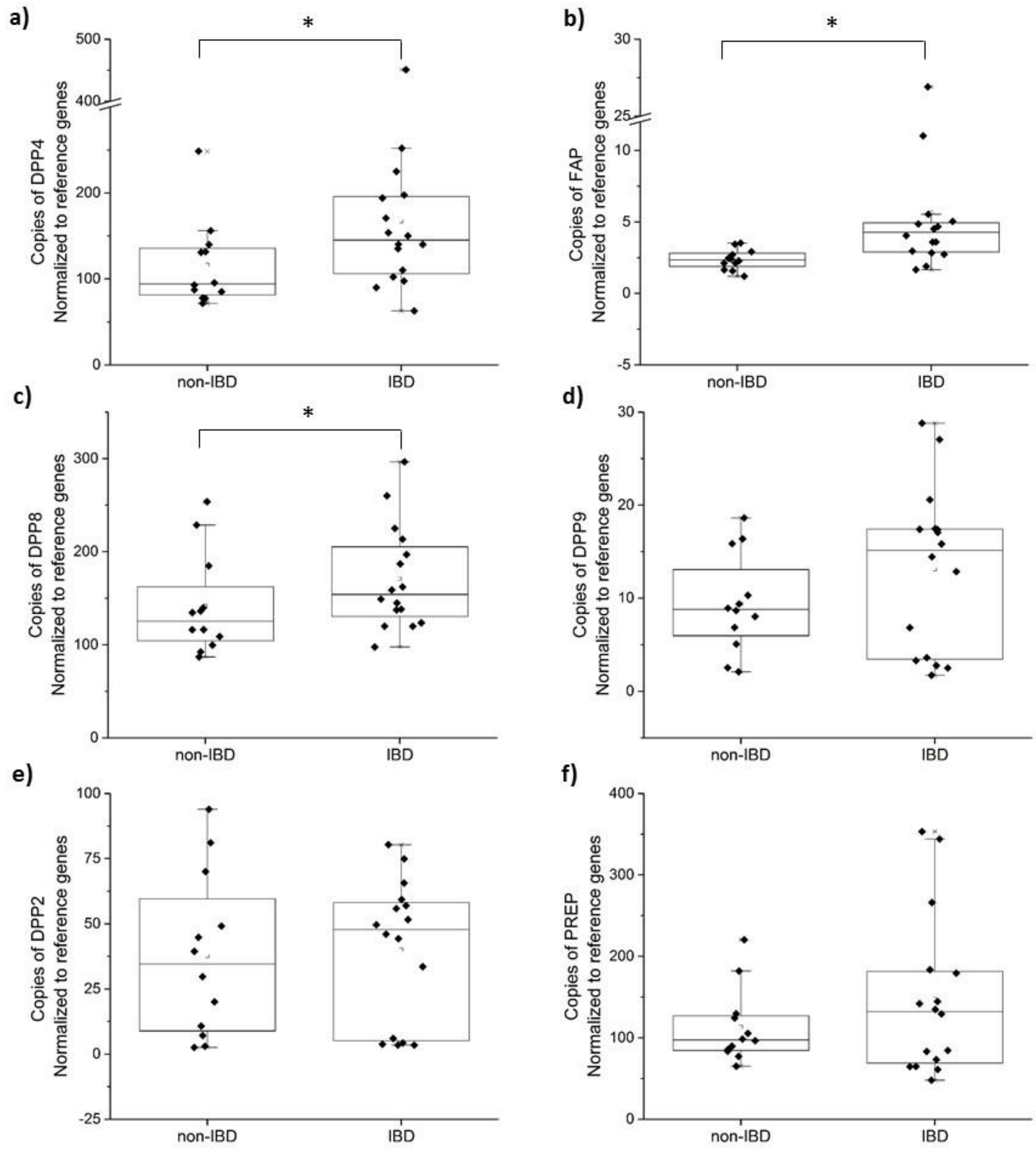


Figure 4.5 DPP mRNA expression in normal colorectal tissue from non-IBD and IBD patients. Gene copy number for genes DPP4 **a)**, FAP **b)** DPP8 **c)**, DPP9 **d)**, DPP2 **e)** and PREP **f)** was determined by qPCR with the use of external PCR standards. Gene copy number is expressed normalized to reference genes, PPIA and TATA. Colorectal biopsy samples were collected from IBD (n=12) and non-IBD (n=16; n=9/CD, n=7/UC) patients from endoscopically normal regions. Box plots represent median and IQR, whiskers represent range excluding outliers. **p<0.05, non-IBD vs IBD*

4.3.5 Colorectal tissue DPP activity in IBD

Yazbeck *et al.* previously characterized colonic DPP activity in a murine model of experimental colitis, finding lower levels of membrane activity and higher levels of soluble activity in diseased animals (Yazbeck et al., 2010b). In normal colorectal tissue from IBD patients, specific DPP8/9 activity was increased 2.2-fold compared to non-IBD patients (3.5 [2.3 – 4.3] U/mg of protein vs 1.1 [1.6 – 3.2] U/mg of protein, $p<0.05$; Figure 4.6). Total soluble activity, membrane activity and specific DPP4 activity in normal colorectal tissue was unchanged between non-IBD and IBD patients (Figure 4.6).

However, when matched IBD patient samples of active and inactive tissue were compared, total soluble DPP activity was significantly higher in biopsies from active regions compared to inactive tissue (7.9 [7.5 – 8.2] U/mg of protein vs 6.4 [6.0 – 6.8] U/mg of protein, $p<0.05$; Figure 4.7a). Total membrane activity was also increased in active colorectal tissue compared with inactive tissue from the same patient, though this failed to reach statistical significance (20.9 [10.1 – 28.5] U/mg of protein vs 9.9 [7.2 – 14.9] U/mg of protein, $p=0.091$; Figure 4.7b). There was no difference in DPP8/9 or DPP4 activity between matched inactive and active colorectal tissue from IBD patients (Figure 4.7c, d).

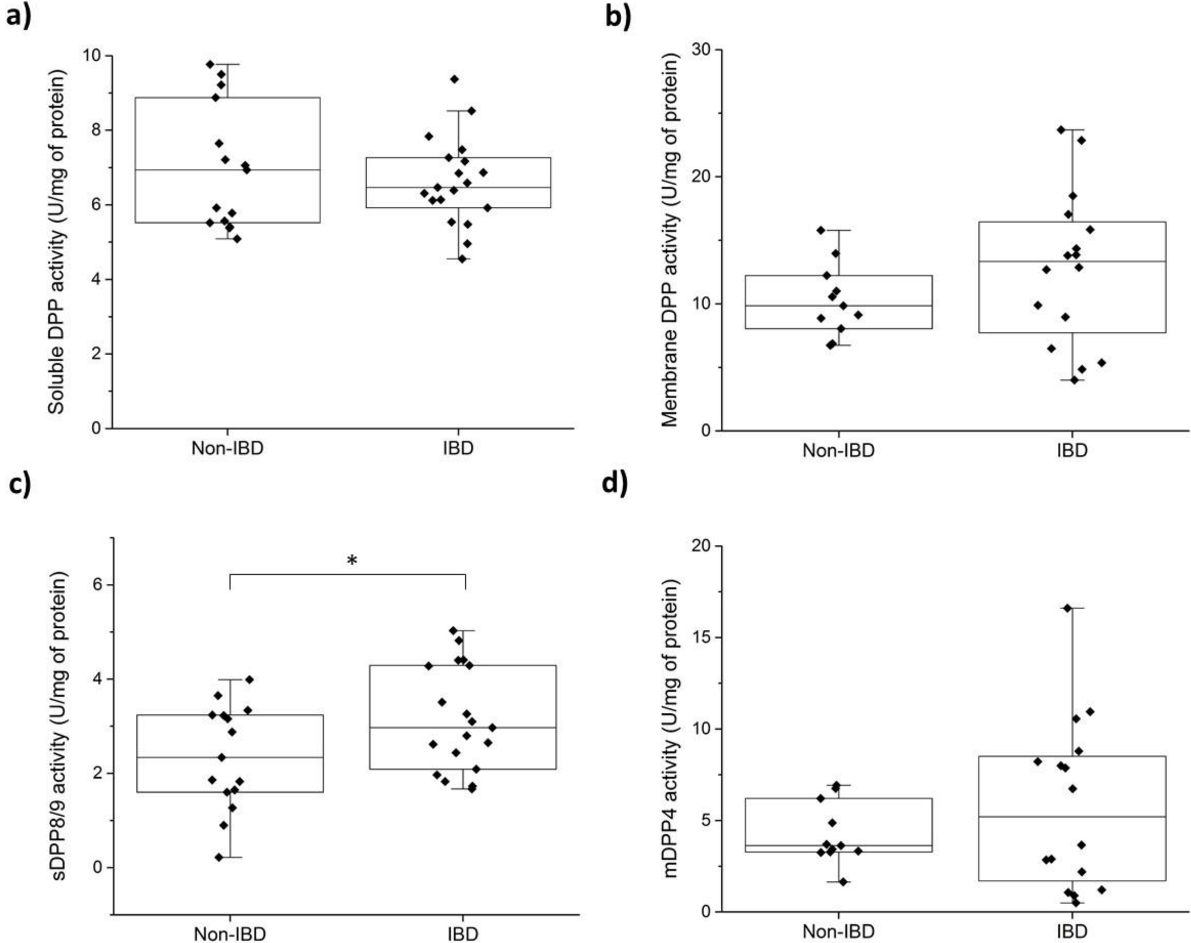


Figure 4.6 DPP enzyme activity in colorectal tissue from non-IBD and IBD patients. Total DPP enzyme activity was determined in soluble **a)** and membrane **b)** fractions from colorectal biopsies. The use of selective inhibitors, 10µM 1G244 and 1µM sitagliptin, allowed for the selective quantification of DPP8/9 **c)** and DPP4 **d)** activity respectively (n=14/non-IBD, n=19/IBD). Box-plot overlays represent median and IQR, whiskers represent range excluding outliers. **p<0.05, non-IBD vs IBD*

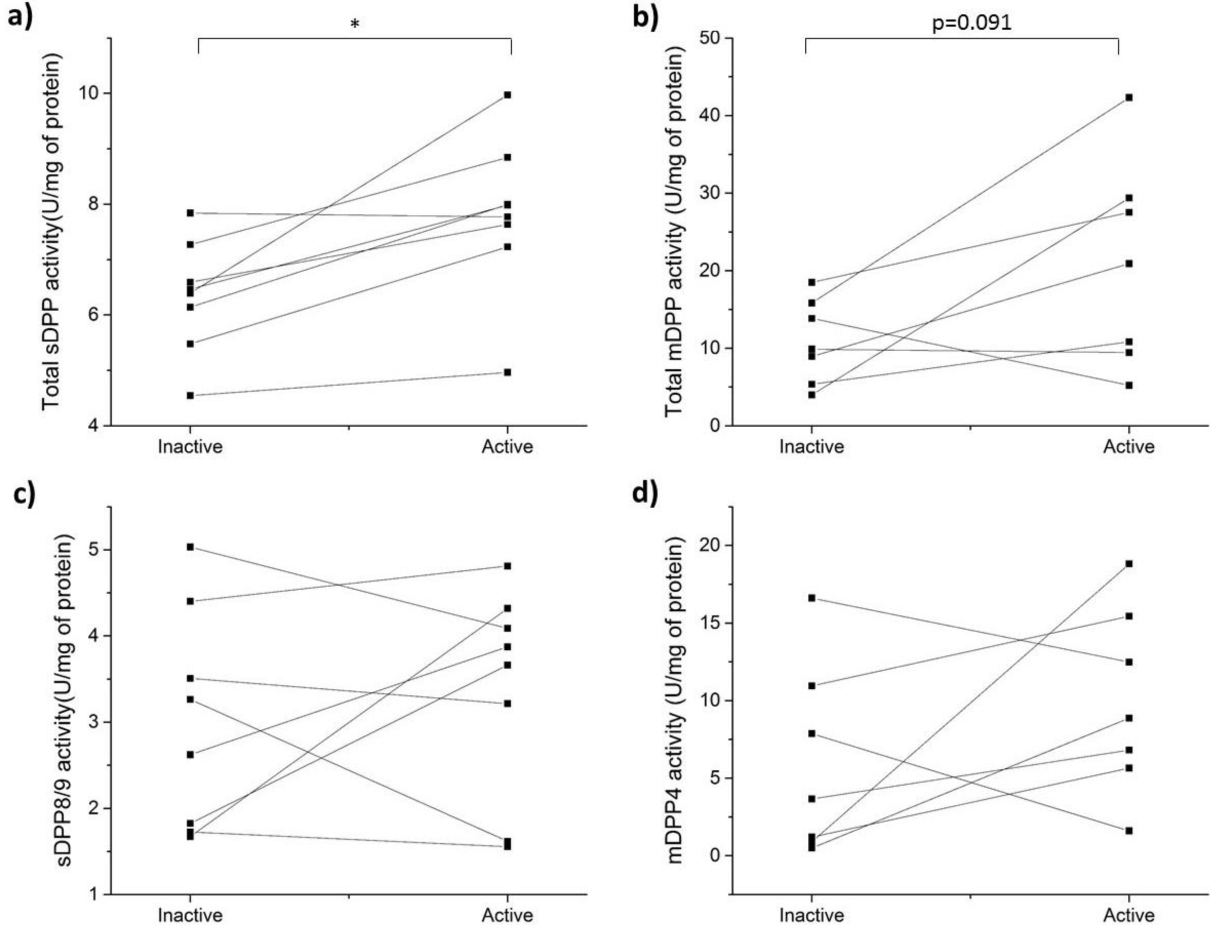


Figure 4.7 DPP enzyme activity in inactive and active colorectal biopsies during IBD. DPP enzyme activity was assessed in endoscopically inactive and active lesions from matched IBD patients (indicated by lines). Total DPP enzyme activity was determined in soluble **a)** and membrane **b)** fractions from colorectal biopsies. The use of selective inhibitors, 10µM 1G244 and 1µM sitagliptin, allowed for the selective quantification of DPP8/9 **c)** and DPP4 **d)** activity respectively (n=3/CD, n=5/UC). Box-plot overlays represent median and IQR, whiskers represent range excluding outliers. **p<0.05, inactive vs active*

4.4 Discussion

This is the first study to comprehensively quantify DPP mRNA and DPP enzyme activity in colorectal tissues during IBD, identifying distinct changes to DPP mRNA and DPP enzyme activity within the tissues of IBD patients compared to non-IBD patients. The use of matched tissues from patients with IBD found further differences in DPP enzyme activity between active and inactive colorectal tissue. In contrast to the results found in Chapter 3, here there was no significant difference in plasma DPP activity including total DPP activity, DPP4 activity and residual DPP activity between IBD and non-IBD patients.

Plasma DPP activity is modified in a number of disease states and has been previously shown to correlate with disease activity and severity in IBD (Hildebrandt et al., 2001). In Chapter 3, significantly lower levels of plasma DPP activity were found in IBD patients compared to non-IBD patients, with specific differences also observed between patients with active and inactive disease. However, in the current study, no differences were found in plasma DPP activity or plasma FAP activity between IBD and non-IBD patients or between active and inactive disease. Most of the IBD cases presented as 'mild' in severity, consistent with CRP values for the majority of IBD patients falling within normal limits (CRP < 10mg/L) (Reeves, 2007). The small sample size combined with the mild cases of IBD likely contributed to the lack of difference in plasma DPP activity reported here. Larger, longitudinal studies are required to better ascertain the relationship between plasma DPP activity and disease flare, and determine the potential clinical utility of plasma DPP activity in IBD.

Previous studies in IBD (Moran et al., 2012, Hildebrandt et al., 2001) and rheumatoid arthritis (Sromova et al., 2015, Sinnathurai et al., 2016) reported inverse correlations between plasma DPP activity, plasma FAP activity and inflammation indicated by CRP concentrations. Despite

the limited numbers of study participants with raised CRP, lower levels of plasma DPP activity and FAP activity were found in patients with elevated CRP (CRP > 10mg/ml) compared to patients with normal CRP levels. CRP is an acute phase reaction protein that is released primarily from hepatocytes in response to increased concentrations of IL-6 and is elevated during tissue damage, infection and inflammation (Henriksen et al., 2008, Pepys and Hirschfield, 2003). While CRP can provide useful clinical information, studies have found poor associations between CRP and endoscopic inflammation in IBD (Miranda-Garcia et al., 2016, Jones et al., 2008), which may limit its application as a singular biomarker in IBD. This study and others (Hildebrandt et al., 2001) have highlighted relationships between plasma DPP activity and disease activity in IBD, CRP, orosomucoid as well as clinical indices such as CDAI in IBD. This might suggest that plasma DPP activity could be an important component of a biomarker panel alongside these markers like CRP. Further studies should investigate the potential clinical utility of monitoring plasma DPP activity alongside CRP for diagnostic and prognostic outcomes in IBD.

DPP activity can be detected in various biological samples using a relatively simple and robust biochemical assay (Yazbeck et al., 2010b, Hildebrandt et al., 2001). Despite good correlations between plasma DPP activity and DPP4 protein, it is widely accepted that other DPPs found within serum/plasma may contribute to this soluble DPP activity (De Chiara et al., 2009). The importance of distinguishing the specific enzyme activities has only been previously investigated by Matheeussen and colleagues, who specified potential applications for this sub-analysis in the realm of DPP4 inhibitor development (Matheeussen et al., 2012). In Chapter 3, the discrimination of plasma DPP activity into total, DPP4 and residual DPP activity, highlighted specific differences between CD and UC that could reflect altered levels of plasma DPPs between these patients. Despite the small sample size reported here, changes to plasma DPP4 activity though not residual DPP activity, were found in patients with elevated CRP

levels, which may suggest that DPP4 but not other circulating DPPs are related to this inflammatory marker. Interestingly, plasma FAP activity was also lower in patients with raised CRP levels. It is possible that FAP activity was detected in the plasma of patients here (Aertgeerts et al., 2005); however, it would then be expected that residual DPP activity would also be lower. Further classification and identification of plasma DPP activity and the enzymes that constitute DPP enzyme activity is necessary to understand the importance of differentiating plasma DPP activity and whether this may have clinical applications.

DPP4 tissue expression and activity has been investigated in small intestinal diseases (Detel et al., 2007, Ziegler et al., 2003, Moran et al., 2012); however, the expression and activity profiles of the DPP enzymes during colorectal IBD has not been previously characterized. In the current study, higher levels of DPP4, DPP8 and FAP mRNA, and DPP8/9 enzyme activity were found in the colorectal mucosa from IBD patients, which could suggest that these DPPs are constitutively altered in IBD. The use of matched colorectal tissue found increased DPP mRNA and DPP enzyme activity in endoscopically active tissue compared with inactive tissue from IBD patients. Without matched histological analysis it is difficult to interpret whether these changes are related to inflammation, tissue damage or other pathological changes. Consequently, future studies are planned to visualize the cells and structures associated with DPP activity in frozen tissue sections from IBD patients using enzyme histochemistry (Appendix 6.8).

In small intestinal diseases, reductions to tissue DPP4 expression and DPP activity are associated with the degree of mucosal damage (Detel et al., 2007, Moran et al., 2012, Ziegler et al., 2003), and suggest that DPP4 is a useful marker of intestinal integrity and damage within the small intestine. In murine experimental colitis, studies have reported increased mRNA expression of DPP8, DPP2 (Yazbeck et al., 2010b) as well as DPP4 and FAP mRNA (Sakanaka

et al., 2015) within affected colonic tissues. Yazbeck *et al.* found the changes to DPP8 and DPP2 mRNA expression coincided with histological changes including crypt damage and increased inflammatory infiltrates, and may suggest that changes to DPP8 and DPP2 could be related to some of these pathological changes. However, further studies localizing the expression of DPPs using immunostaining techniques is necessary to identify if there is a relationship between DPP expression and pathological changes in colorectal tissue.

The current study is subject to a number of limitations that must be considered when interpreting the data. Primarily the small sample sizes reported here have contributed to the lack of statistical power, and limited sub-group analysis to assess the impact of drug therapy or disease phenotype on plasma DPP activity. This study included patients with more mild-moderate disease activity and did not include patients with complicated or severe disease phenotypes such as fistulizing and stenosing disease, that may have further contributed to the lack of significance reported here. Large longitudinal studies are required to determine how plasma DPP activity is altered with disease activity, severity and phenotype and to understand the clinical relevance of plasma DPP activity in IBD. In this study most IBD cases were limited to the colon and rectum, though small intestinal involvement was present in a small number of CD patients, the poor sample size of biopsies collected from this region prevented statistical comparisons. Biopsies collected for analysis in this study were collected randomly throughout the colon in accordance with the opinion of the gastroenterologist. Due to the lack of matched samples collected for histological analysis, it is possible that some non-inflamed IBD biopsies may contain microscopic levels of inflammation. It is currently unclear how the degree of inflammation effects DPP activity and expression, further studies should assess DPP expression in histological sections to better understand the relationship between DPP expression and activity and inflammation and mucosal damage within the colon.

Colorectal DPP expression and activity is increased during inflammation associated with IBD. Further studies involving more matched tissue samples are necessary to determine whether DPP expression during colorectal IBD is associated with the degree of inflammation and to identify the cell types responsible for DPP expression in IBD. Plasma DPP activity was not altered during IBD in this study; however, further longitudinal studies are necessary to understand the relationship between plasma DPP activity and the relapsing-remitting nature of inflammation in IBD. A better understanding of the role of DPPs in localized tissue inflammation and how DPPs are regulated in response to inflammation will help to identify the clinical relevance of DPP expression and activity and whether DPPs represent a unique biomarker for tissue inflammation.

Chapter 5. Conclusions & Future directions

5.1 Summary

Current techniques used to measure gastrointestinal function, damage and disease are limited to invasive endoscopic technologies that are expensive and time consuming, representing a cost to both patients and the economy. Less invasive biomarkers have the capacity to provide real-time, functional information of physiological states, and could assist in guiding medical decision making related to diagnosis, prognosis and formulating treatment plans (Appendix 6.1). This thesis aimed to understand the role of DPPs in gastrointestinal health by characterising expression and activity of DPPs in both normal mucosa and diseased mucosa from Barrett's oesophagus and IBD, and the potential for DPPs as biomarkers of gastrointestinal health. The thesis has reported novel insights into the roles of DPPs during inflammation and immune responses, and suggests that DPPs could be used as biomarkers of gastrointestinal inflammation and damage.

Chapter 2 sought to understand the normal expression and activity profiles of DPP enzymes throughout the gastrointestinal tract, and how these are altered in oesophageal and ileal diseases. We found that DPP4 was expressed along an increasing gradient of expression and activity from the proximal small intestine to the distal ileum, with little to no expression in the upper gastrointestinal tract or colon. For the first time, this research has reported high gene expression of DPP8 relative to other DPPs throughout the gastrointestinal mucosa, which may suggest a potential role for DPP8 in gastrointestinal health. Distinct changes to DPP mRNA expression and enzyme activity were detected in both Barrett's oesophagus and inflamed ileal tissues, suggesting potential utility for these markers in diseases throughout the gastrointestinal tract.

The detection of DPP4 activity in the mucosa of the gastrointestinal tract is currently limited to bench-top biochemical assays performed in biopsy homogenates. Our laboratory has designed, developed and validated a non-invasive ^{13}C-based assay that can selectively quantify membrane DPP4 activity *in vitro* (Appendix 6.1). The results from the ^{13}C-DPP4 assay correlate with colorimetric enzyme activity data (Appendix 6.1) and suggest that the ^{13}C-DPP4 assay can be used to non-invasively quantify DPP4 activity *in vitro* and *in vivo*. The ^{13}C-DPP4 assay was used to quantify DPP4 activity in a panel of oesophageal cancer cell lines, identifying differential levels of activity in individual cell lines (Chapter 2). The reported findings in Chapter 2 present compelling evidence for the application of a ^{13}C-DPP4 breath test to non-invasively quantify modified gastrointestinal pathologies associated with Barrett's oesophagus, oesophageal cancer, and small intestinal damage.

Chapter 3 characterised the activity and expression of DPPs in IBD using bio-banked plasma and colorectal tissue samples respectively, from two separate patient cohorts. DPP4 and FAP mRNA expression were found to be altered in UC patients compared to controls, and may represent a unique molecular characteristic of UC. Using the selective DPP4 inhibitor, Sitagliptin, this study was the first to specifically quantify DPP4 and residual activity in the plasma of patients with IBD, finding lower levels of DPP4 activity in patients with CD, but not UC, compared to controls. It is estimated that residual DPP activity described here contains some DPP4 activity as well as non-DPP4 activity that could be the result of activity from DPP8/9 and FAP. Consequently, changes to the proportion of DPP4 and other DPPs in the plasma would explain some of the variation in residual activity between patients, although further studies are needed to confirm this, and characterise the DPP proteins that constitute plasma DPP activity.

Stratification of patients according to disease activity, found lower levels of residual activity in both CD and UC patients with active disease, compared to those with inactive disease. These results highlight the potential importance of discriminating between DPP enzyme activities using selective DPP4 inhibitors, and could suggest further utility for this method in studies investigating biomarkers or DPP4 inhibitors.

Chapter 4 comprehensively characterised the expression and activity profiles of DPP enzymes in the plasma and tissue of IBD patients. In contrast to Chapter 3, no specific differences in plasma DPP activity were identified in this IBD cohort, even after stratification based on disease activity information; however, it is likely that the small sample size reduced the studies power. Interestingly, when results were correlated with CRP concentrations, it was found that patients with elevated CRP (>10mg/ml) had lower levels of plasma DPP activity, suggesting that plasma DPP activity was reduced during inflammation. Additionally, this was the first study to report higher levels of DPP4, FAP and DPP8 mRNA expression in colorectal tissue from IBD compared to control patients. Increased levels of mucosal DPP activity and DPP mRNA expression (Appendix 6.7) were also found within active lesions from IBD patients compared to matched control tissue, suggesting DPPs may have a direct role in colorectal inflammation that warrants further investigation.

This thesis has identified unique expression and activity profiles during gastrointestinal health and disease, suggesting potential for this unique class of enzymes as biomarkers for the assessment of gastrointestinal health and disease states. Further validation studies and research into the sources and regulation of plasma and tissue DPPs are required to better understand the clinical utility of DPPs as biomarkers of gastrointestinal health.

5.2 DPPs as clinical biomarkers

Biomarkers are classified as any objective measure that accurately reflects a physiological process, function or disease (Strimbu and Tavel, 2010). Consequently, biomarkers have applications in diagnosis, prognosis and the monitoring of disease progression and/or treatment efficacy (Strimbu and Tavel, 2010). The incorporation of biomarkers into clinical practice requires vigorous testing and analysis to ensure sensitivity, specificity and clinical usefulness (Figure 5.1) (Pepe et al., 2001, Ensor, 2014, Rifai et al., 2006). To date, investigations involving plasma DPP4 and plasma DPP activity have yet to progress to clinical validation stages (Figure 5.1)(Yazbeck et al., 2017) with larger population studies required.

Consistent with the findings reported in this thesis, previous studies have reported low plasma DPP activity in patients with IBD (Hildebrandt et al., 2001, Moran et al., 2012, Xiao et al., 2000), suggesting that plasma DPP activity may have utility as a biomarker in IBD and inflammation. Plasma DPP activity from control patients was broadly distributed, as reported in Chapters 3 and 4, despite the absence of any evidence of active inflammation, suggesting that outlying values could be associated with other unknown pathologies, lifestyle factors or physiological phenomena. If plasma DPP activity is to be considered as a biomarker, larger population studies are needed to comprehensively understand the normal distribution of DPP activity, and the factors that may influence it. DPP4 gene polymorphisms have been associated with altered protein expression of plasma DPP4 (Thanapirom et al., 2015, Aghili et al., 2012), and could explain the disparate levels of plasma DPP activity in some patients. Genetic profiling of the DPP4 gene within patients may help to decipher the extremes of plasma DPP activity that exist, and why plasma DPP activity in these patients may not correlate well with inflammatory measures in all patients.

Inverse relationships between inflammatory parameters and plasma DPP activity have been previously reported in patients with IBD (Hildebrandt et al., 2001, Rose et al., 2002), RA (Hagihara et al., 1987, Busso et al., 2005) and MS (Tejera-Alhambra et al., 2014), suggesting the utility of plasma DPP activity as a biomarker of inflammatory conditions (Yazbeck et al., 2017). Consistent with this, inverse associations between plasma DPP activity and inflammation in patients with IBD were reported in Chapter 3 and 4; however, how accurately and the degree to which plasma DPP activity reflects inflammatory changes is unclear and requires further characterisation. Longitudinal monitoring of plasma DPP activity and its association with inflammatory parameters would assist in defining the utility of plasma DPP activity as a biomarker of inflammation. The acute LPS induced sepsis model causes rapid immunological changes and endotoxemia (Fink, 2014, Kroller-Schon et al., 2012) and could be useful to further evaluate the relationships between plasma DPP activity and inflammation. Alternatively, the TNBS model of colitis can be evaluated over longer periods of time (Cury et al., 2013) could assist in determining the time-course relationship between plasma DPP activity and inflammation.

While a relationship between plasma DPP activity and inflammation has been suggested in Chapters 3 and 4, the sources of plasma DPP4, the mechanisms regulating plasma DPP activity and the roles of plasma DPP activity remain poorly understood. Studies have suggested a number of cellular sources for soluble DPP4 and have found that changes to glucose concentrations can affect DPP4 surface shedding (Wang et al., 2014, Nargis et al., 2017, Chowdhury et al., 2016, Rohrborn et al., 2014). However, the effects of inflammation on DPP4 surface shedding are currently unclear and could provide insights into the regulation of plasma DPP4 during inflammatory disease. *In vitro* experiments using primary and secondary cells could examine the effect of various cytokines on the cellular expression of DPP4 and whether it is shed into the culture media as a result of changes to cytokine environments. Understanding

whether cytokines involved in propagating inflammation during IBD play a role in regulating DPP4 shedding would provide useful information about the potential roles of plasma DPP4 during IBD as well as its utility as a biomarker of IBD.

As reviewed in Chapter 1, DPP4 can cleave a wide array of biological substrates with a many of these relevant to IBD (Table 1.3) (Mulvihill and Drucker, 2014). It has been proposed by Xiao *et al*. that lower levels of plasma DPP activity found in IBD patients may form part of a regulatory mechanism to increase the concentrations of biologically active GLP-2 and assist in healing and repair in IBD (Yazbeck et al., 2010a, Xiao et al., 2000). Lower levels of plasma DPP4 activity have also been associated with elevated levels of NPY and vasoactive intestinal peptide (VIP) in patients with RA (Buljevic et al., 2013). Given the broad substrate repertoire of DPP4 (Table 1.3) (Mulvihill and Drucker, 2014), it is likely that the lower levels of plasma DPP4 activity found in patients with active IBD may also correlate with altered levels of other DPP4 substrates such as chemokines that are involved in IBD pathogenesis. Identifying substrates that correlate with plasma DPP4 activity may identify roles for DPP4 in the pathogenesis of IBD, and new potential therapeutic targets for the management of IBD. The prospective study (Chapter 4) collected and stored patient plasma with sitagliptin for later quantification of various DPP4 substrates including GLP-2, NPY and SDF-1. Due to time restraints and limited availability of resources at the time, these experiments are yet to be performed. However, identification of substrates and pro-inflammatory molecules that correlate with plasma DPP activity should be a focus for future studies.

This is the first study to comprehensively quantify the DPP mRNA expression and DPP enzyme activity in colorectal tissues from patients with IBD. The use of matched tissue in this study suggests that the mRNA expression of all DPP enzymes (Appendix 6.7) and that DPP enzyme activity (Chapter 4) is increased in inflamed colorectal mucosa from patients with IBD.

Elevated DPP mRNA expression and DPP activity could be the result of increased numbers of infiltrating immune cells expressing DPPs or alternatively upregulation of DPP expression and DPP enzyme activity in resident mucosal cells, or a combination of these. FAP expression is reportedly confined to mucosal myofibroblasts in IBD, with authors identifying FAP as a potential therapeutic target for the management of complicated fibrotic disease (Truffi et al., 2018). Consequently, identifying the specific cells that express DPP enzymes may identify new therapeutic targets for the management of IBD. To determine the cellular expression of DPPs where suitable antibodies are available studies could utilize flow cytometry and immunofluorescent co-staining to define the cellular expression of DPP4 in these tissues. Alternatively, laser dissection microscopy is a useful tool to visualise and isolate cells of interest from tissue sections. Following the isolation of specific cells or structures using laser dissection microscopy, mRNA can then be quantified using qPCR technologies (A. et al., 2002). Consequently, laser dissection microscopy may represent a useful tool to define the cellular expression of DPP enzymes, particularly DPP8 and DPP9 where suitable antibodies are currently lacking.

Identification

Observational studies
In vitro, in vivo derived samples.

Identify candidate markers. Often uses cell, tissue homogenates

Samples less readily available in patients

Qualification & verification

Retrospective observational studies

Confirm differential expression profiles in patient cohorts

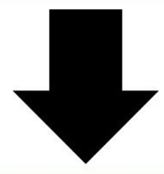
Selection bias, sample type & quality, multiplicities

Validation

Large scale population based prospective studies.

Establish assay validity & clinical utility & value of marker.

Time consuming, costly, multiplicities



Regulatory body approval

Figure 5.1 Development and validation of clinical biomarkers. Flow chart detailing major steps and hurdles associated with development and validation of clinical biomarkers (Pepe et al., 2001, Rifai et al., 2006, Ensor, 2014).

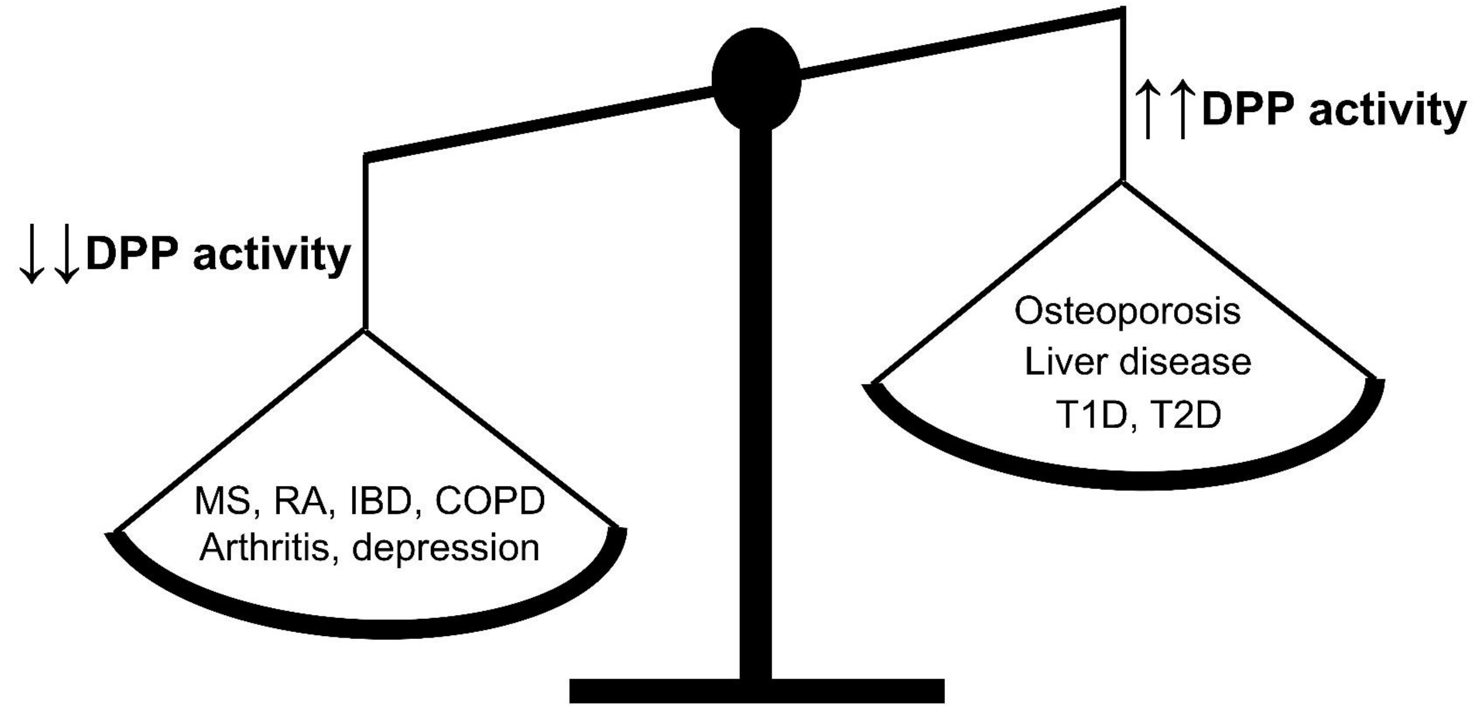


Figure 5.2 The delicate balance of plasma DPP activity. Altered plasma DPP activity has been suggested as a biomarker for a number of disease states (Chapter 1). Increased levels of plasma DPP activity are found in osteoporosis (Zheng et al., 2015), obesity (Kirino et al., 2012), and hyperglycaemia (Mannucci et al., 2005, Ryskjaer et al., 2006). In contrast, plasma DPP activity is lower in inflammatory conditions including IBD (Hildebrandt et al., 2001, Moran et al., 2012), MS (Tejera-Alhambra et al., 2014), RA (Hagihara et al., 1987, Busso et al., 2005) and depression (Maes et al., 1995, Maes et al., 1991) and is inversely associated with inflammatory parameters (CRP and disease activity measures). Understanding how plasma DPP activity is regulated and its sources may help to determine the clinical utility of plasma DPP activity as a biomarker.

Fibrotic complications in CD are primarily diagnosed through radiological assessment (Laass et al., 2014, Rieder et al., 2011), while evaluation of clinical factors remains the most accurate method for identifying patients at risk of developing complicated disease phenotypes (Rieder et al., 2011). FAP expression has been previously identified in tissues from CD patients, though not in UC, with expression limited to myofibroblasts (Rovedatti et al., 2011, Truffi et al., 2018). More recently, Truffi *et al*. found that antibodies targeting FAP enhanced the wound healing and cellular migration of primary myofibroblasts isolated from CD patients with stenosing disease, suggesting that targeting FAP may be useful for the management of fibrotic complications in CD (Truffi et al., 2018). The findings reported here suggest that altered FAP mRNA expression is common to both CD and UC and could have utility in both conditions (Chapter 4). Given the association between FAP and malignancy (Kilvaer et al., 2015, Kim et al., 2014, Hua et al., 2011) and fibrosis and its potential utility as both a therapeutic target and a biomarker, it is vital to understand the relationship between both plasma and tissue FAP and clinical parameters in IBD (i.e. disease severity, flare duration, number of flares) to decipher if FAP can provide any insight into patients that are at risk of severe complicated forms of IBD. Further preclinical studies are necessary to investigate the expression of FAP throughout inflamed mucosa and how neutralizing FAP antibodies could affect healing and tissue repair. The mouse strain, SAMP1/YitFc, produces spontaneous ileitis characterised by discontinuous transmural inflammation, with fibrotic complications including perianal fistula and intestinal stricture present in 5-10% and 50% of the animals respectively (Cominelli et al., 2017). This model of ileitis closely mimics human CD and is the only murine model where fibrotic complications are currently observed (Cominelli et al., 2017) and therefore, represents a unique and highly useful preclinical model for examining the roles of FAP during IBD.

5.3 A ^{13}C-DPP4 breath test and its clinical application

Stable isotope breath tests are a non-invasive modality to rapidly and reliably quantify disease biomarkers abundance and functionality. We have developed and validated, *in vitro,* a ^{13}C-assay for DPP4, using Sitagliptin and p32/98 to demonstrate its selectivity for membrane DPP4. (Figure 5.3). The specific activity and organ specific expression of DPP4 makes it an ideal target for a ^{13}C-based breath test, however, applying this test may require specific considerations to assess DPP4 activity in each tissue site.

Delivering appropriate concentrations of substrate to target tissues and ensuring maximum exposure is an important consideration when developing a ^{13}C-based approach to the detection of gastrointestinal mucosal DPP4 activity. Mucoadhesive compounds and formulations represent a possible option for the delivery of drugs to gastrointestinal tissues, maximising their exposure to target tissues and are a suitable option for administering the ^{13}C- substrate. Compound structure and the properties of mucosal surfaces (i.e. pH, presence of mucous layers, disease states) can also contribute to the retention of compounds to mucosal surfaces (Boddupalli et al., 2010, Richardson et al., 2005, Dunnhaupt et al., 2011). As a result, different mucoadhesive formulations may be necessary for applications in oesophageal, gastric, small intestinal and colonic tissues.

Upper gastrointestinal

Results presented in Chapter 2 identified DPP4 activity in biopsies from Barrett's oesophagus; however, DPP4 activity was absent from normal oesophageal mucosa, suggesting the potential for DPP4 as a biomarker of Barrett's oesophagus. Previous studies conducted by Chaves *et al.* and Nancarrow *et al.* have also identified DPP4 protein and DPP4 mRNA respectively, in Barrett's lesions, though were unable to detect DPP4 within healthy oesophageal tissue, further

corroborating the unique expression profile of DPP4 and its potential as a biomarker. Differential levels of DPP activity in oesophageal cancer cell lines reported in Chapter 2, could also suggest that monitoring DPP4 activity could have applications in the diagnosis or monitoring of oesophageal malignancies.

A non-invasive ^{13}C-based breath test that has the ability to detect small levels of DPP4 activity within the oesophagus, such as occurs in Barrett's oesophagus and malignant changes, could have applications in diagnostics and monitoring of these conditions. To maximise exposure of the ^{13}C-DPP4 substrate to the oesophageal epithelium, mucoadhesive formulations should be explored. Sodium alginate has been previously identified as a mucoadhesive compound and has shown good retention to the oesophageal epithelium (Richardson et al., 2005) that could aid the delivery and maximise the exposure of the ^{13}C-DPP4 substrate to the area.

Prior to combining our substrate with adhesive formulations, proof-of-concept studies are required to demonstrate that this technique will be able to detect differential $^{13}CO_2$ signals in healthy patients and those with Barrett's oesophagus, dysplasia or malignancy. Consequently, future proof-of-concept studies conducted in patients could administer the ^{13}C-DPP4 substrate to the oesophageal mucosa as a spray during endoscopic procedures. Alternatively, *in vitro* models for evaluating mucoadhesive compounds to oesophageal mucosa (Smart et al., 2013) could be modified to evaluate the ^{13}C-DPP4 assay also in a similar closed system to the *in vitro* ^{13}C-assay (Appendix 6.1). This *in vitro* method could also be applied in oesophageal-gastric surgical resection specimens from patients with cancer (Lagergren et al., 2017). Resection specimens are considerably larger than biopsy samples and it is possible, depending on the degree of pathology and the proportion of healthy tissue, that application of the ^{13}C-DPP4 assay would generate detectable a $^{13}CO_2$ signal. The use of resection specimens would also allow for

subsequent analysis of histology and DPP4 activity levels that would provide useful information for the validation of the ^{13}C-DPP4 assay.

Lower gastrointestinal

Chapter 2 reported reduced small intestinal DPP4 activity during small intestinal disease which could be due to either a damaged small intestinal mucosa, or a down-regulation of DPP4 as part of an overall intestinal adaptive response (Yazbeck et al., 2009b, Dunphy et al., 1999). The data presented in Chapter 2 is from a single patient with active small intestinal disease and suggests that mucosal DPP activity is reduced during active CD. Previous studies have also found that mucosal DPP4 activity and/or protein expression is reduced in small intestinal damage induced by coeliac disease (Kozakova et al., 1998, Detel et al., 2007), CD (Moran et al., 2012) and villous atrophy (Ziegler et al., 2003) and may suggest that DPP4 represents a useful marker of mucosal integrity or damage within the small intestine. In contrast, Chapter 4 reported that DPP4 mRNA expression and DPP activity is higher in colorectal tissues from IBD patients compared to control patients, and that colorectal DPP4 could be useful as a marker of colorectal inflammation or IBD. Studies looking to further develop mucosal DPP4 as a marker of gastrointestinal health, damage and/or inflammation need to consider appropriately matched tissue controls and should apply immunohistochemical and enzyme histochemical methods to demonstrate the relationship between DPP4 expression/activity and pathology findings.

A ^{13}C-DPP4 breath test could have potential applications in both small intestinal and colorectal disease conditions; however, the substrate delivery and dose optimisation need to be considered before such applications. As DPP4 is expressed throughout the gastrointestinal mucosa, targeted delivery of the substrate using microencapsulation techniques will ensure that regionally specific DPP4 activity is being measured by the breath test. Specific microsphere

polymer formulations have been developed that are sensitive to changes in pH (Deore et al., 2013) or the presence of specific microorganisms that are localized within the colon (Jin et al., 2016), limiting the specific release of the microsphere contents to a particular region. pH dependent microspheres are useful for specific targeting of the gastrointestinal tract due to the specific variation in pH between regions of the stomach, small intestine and colon (Evans et al., 1988). A breath test looking to specifically target ileal DPP4 could utilize polymers sensitive to a pH of approximately 7.5 (Evans et al., 1988) leading to the targeted release of the ^{13}C-DPP4 substrate in the ileum.

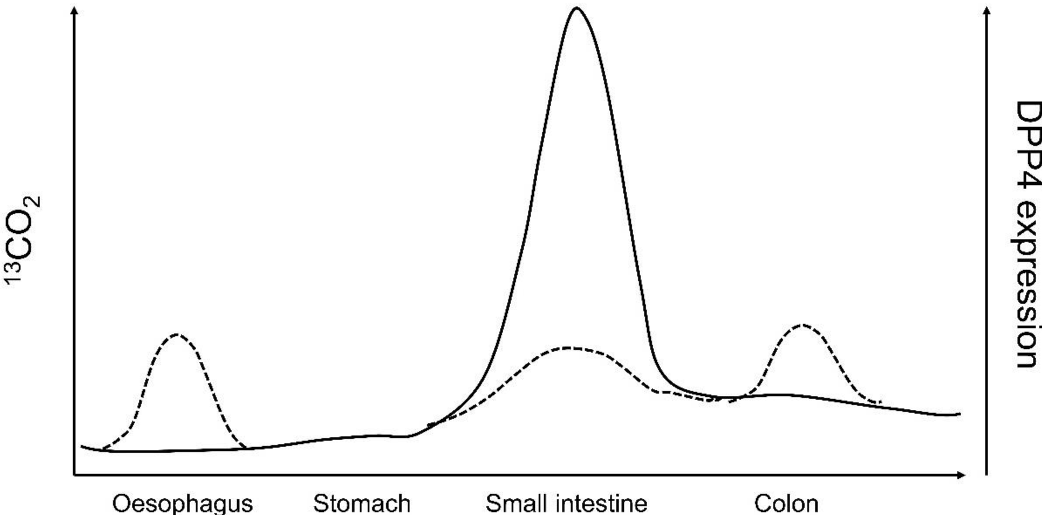


Figure 5.3 Schematic representation of ^{13}C-DPP4 breath tests in clinic. Full black line shows expected $^{13}CO_2$ signals from healthy gastrointestinal tissues and how they correlate with DPP4 expression. Dashed black lines indicate the presence of disease in the oesophagus, small intestine and colon characterised by altered DPP4 expression. During gastrointestinal disease $^{13}CO_2$ signals from the ^{13}C-DPP4 test would also change according to DPP4 expression.

5.4 DPP4, immunity and inflammation

As reviewed in Chapter 1, the DPP enzyme family is strongly associated with adaptive and innate immune responses. Neutrophils are strongly involved in mediating tissue damage and inflammation in IBD, and previous data from our laboratory suggested that DPP inhibition reduces colonic myeloperoxidase activity during colitis (Yazbeck et al., 2010b). A number of studies have since suggested that DPP4 inhibitors modulate neutrophil chemotaxis, adhesion and ROS production (Schurmann et al., 2012, Kroller-Schon et al., 2012, Jungraithmayr et al., 2010). Preliminary investigations conducted as part of this candidature suggested that DPP4/CD26 is upregulated on the surface of freshly isolated neutrophils following *in vitro* activation (Appendix 6.9); however, the roles of CD26 in neutrophil functions remains poorly understood.

Preliminary investigations conducted as part of this candidature (Appendix 6.9) examined the direct role of DPP4 inhibitors on neutrophil responses; however, it is possible that DPP4 inhibitors can alter neutrophil responses indirectly through effects on T cells, endothelial cells or epithelial cells that also express DPP4. *In vitro* co-culture techniques represent a useful method for evaluating the effects of secreted compounds released from one cell on another (Watanabe et al., 2004, Mochizuki et al., 2004). This system could be used to determine if DPP4 inhibitors cause changes in endothelial, T cells or macrophages that impact the responses of surrounding cells such as neutrophils. Due to the short half-life of freshly isolated neutrophils, the use of immortalized cell lines Jurkat (T cell), THP-1 (monocyte), HL-60 (neutrophils) may be more appropriate for co-culture experiments. Evaluating the responses to infectious challenge with organisms responsible common infections such as *Streptococcus pneumoniae, Haemophilus influenza and E. coli* in murine models during DPP4 inhibitor

treatment may also provide insight into the clinically relevant effects of DPP4 inhibitors on the innate immune system.

A recent study by Abrahami *et al.* found that the use of DPP4 inhibitors was associated with an increased risk of developing IBD (Abrahami et al., 2018). In contrast, *in vitro*, preclinical and clinical studies suggest that DPP4 inhibitors have anti-inflammatory effects and may prevent or treat inflammatory and autoimmune conditions (Chapter 1). Preliminary *in vitro* data in freshly isolated neutrophils (Appendix 6.9) suggests that CD26 expression and DPP activity in resting and activated neutrophils varies in healthy donors. One potential explanation for this result is the presence of individual differences. The risk of IBD in patients using DPP4 inhibitors was low (Abrahami et al., 2018), suggesting that only certain patients are predisposed to adverse reactions. Changes to the plasma and cellular expression and activity of DPP4 could alter the effectiveness of DPP4 inhibitors as anti-hyperglycaemic agents and predispose patients to adverse immunological or gastrointestinal effects. Further work is necessary to identify patients that may be predisposed to developing IBD as a result of DPP4 inhibitor therapy.

Promising work from Matheeussen *et al.* and Waumans *et al.* has identified roles for DPP9 in the M1 differentiation of macrophages, with DPP9 inhibition reducing the M1 response of macrophages *in vitro* (Matheeussen et al., 2013, Waumans et al., 2016), highlighting potential for selective targeting of DPP9 to treat conditions characterised by aberrant M1 responses such as IBD (Campos et al., 2011, Caprioli et al., 2013, Elliott et al., 2015, Gren and Grip, 2016). There are currently no selective inhibitors available that can selectively target DPP9, though the recent characterization of the crystal structures of DPP8 and DPP9 will likely lead to the development of new selective inhibitors (Ross et al., 2018). Furthermore, the results presented throughout this thesis suggest that while DPP9 is only expressed at low levels in the lower

gastrointestinal tract, which might suggest that DPP9 could be a useful therapeutic target, with inhibition unlikely to cause additional toxic effects in the gastrointestinal tract. Further characterisation of DPP9 expression and activity in human tissues would be necessary to elucidate if DPP9 inhibition would likely cause widespread biological effects.

5.5 Concluding remarks

This thesis provides important insights into the roles of DPPs in gastrointestinal health and the potential for DPPs as clinical biomarkers of gastrointestinal health and damage. The results presented here show that mucosal DPP4 is differentially altered during inflammation and tissue damage in oesophageal, ileal and colorectal tissues, and may represent a useful biomarker of gastrointestinal disease. This thesis further suggests that DPP4 activity could be detected using a ^{13}C-non-invasive assay, and with further development and validation, a ^{13}C-DPP4 could have clinical utility in the monitoring of gastrointestinal integrity and disease states. Characterisation of DPP enzyme activity using selective DPP inhibitors, in addition to DPP mRNA expression has identified new roles for DPP8 in colorectal IBD and could suggest utility of DPP8 as a biomarkers or therapeutic target in IBD.

This thesis also suggests potential utility for plasma DPP activity as a biomarker for active disease and inflammation in IBD, and that differentiating plasma DPP activity into DPP4 and residual may be useful in defining IBD subtypes. Inconsistencies between studies may suggest that plasma DPP4 activity is not a suitable sole biomarker for disease activity in IBD; however, further studies are warranted to define whether incorporating plasma DPP activity alongside current clinical and laboratory parameters has clinical utility in IBD.

This thesis has contributed to the knowledge of DPPs in gastrointestinal health and disease, and is the first study to comprehensively characterise all DPP enzymes in the gastrointestinal tract and during IBD. While further studies are necessary, this thesis suggests compelling evidence for DPPs as serological and tissue biomarkers of gastrointestinal disease and has further identified a novel non-invasive method for detection that could greatly enhance the clinical applications of DPPs.

Chapter 6. Appendix

6.1 Development of a ^{13}C stable Isotope Assay for Dipeptidyl Peptidase-4 Enzyme Activity

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Presented here as draft manuscript and references are presented in accordance with the styling of Diabetes. Author contributions are listed below.

The work in this manuscript preceded that presented in Chapter 2 and Chapter 4 of this Thesis. The methods described throughout this thesis (enzyme assays, qPCR and the *in vitro* ^{13}C-DPP4 assay) were optimized and validated are part of this manuscript prior to use in the clinical samples presented throughout this thesis. The work presented here also provided the necessary validation work to begin investigating the potential of the ^{13}C-DPP4 assay (Chapter 2).

Throughout my candidature I was involved in the conceptualization and planning of future experiments and also spent considerable time undertaking experimental work relating to this manuscript: assisting in cell culture & *in vitro* ^{13}C assays, performing enzyme activity analysis and qPCR analysis of mRNA expression, as well as corresponding data analysis. I have also been involved in the editing of manuscripts.

Author Contributions

R.Y. conceptualized the project and overall study design, performed data analysis and interpretation, drafted and completed the final manuscript

S.J. completed PCR experiments and enzyme activity assays, assisted with *in vitro* headspace analysis studies, assisted with data analysis and was involved in editing of the manuscript

M.S. completed *in vitro* headspace analysis studies, assisted with the enzyme activity assays and contributed to the experimental design

C.A.A. contributed to experimental design, data analysis and interpretation, and read draft versions of the manuscript

D.A.B. contributed to experimental design, data analysis and interpretation, and read draft versions of the manuscript

E.P.L. contributed to experimental design, data analysis and interpretation, and read draft versions of the manuscript

R.N.B. conceptualised the project and initial concepts, contributed to the data interpretation and provided input to the final manuscript

Abstract

Dipeptidyl peptidase-4 inhibitors (DPP4i) are a class of orally available, small molecule inhibitors for the treatment and management of Type-II diabetes A rapid, real-time, functional breath test for DPP4 enzyme activity could help to define DPP4i efficacy in patients that are refractory to treatment. We aimed to develop a selective, non-invasive, stable-isotope ^{13}C breath test for DPP4.

In vitro experiments were performed in triplicate (n=3/group/cell line) using high DPP4 expressing Caco-2 and low DPP4 expressing HeLa cells. DPP gene expression in cell lines was determined by quantitative real-time PCR. A ^{13}C-tripeptide was designed with selectivity for DPP4. 3mM ^{13}C-tripeptide was added to cells in the presence and absence of the selective DPP4 inhibitor, Sitagliptin, to determine assay selectivity. 10ml gas samples were collected from the headspace every 20min for a total of 120min. $^{13}CO_2$ content was quantified by isotope ratio mass spectrometry. At the conclusion of the gas-sampling period, cell extracts were collected and DPP4 enzyme activity was quantified by colorimetric enzyme assay. The ^{13}C-DPP4 breath test was also tested in a human case study for proof-of-concept in the presence and absence of sitagliptin.

DPP4 was highly expressed in Caco-2 cells and very low expression was detected in HeLa cells. Following addition of the ^{13}C-tripeptide, a high $^{13}CO_2$ signal was detected from Caco-2 cells, whilst no signal was detected in HeLa cells. Inhibiting DPP4 activity with sitagliptin reduced the $^{13}CO_2$ signal from Caco2 cells, comparable to that observed from HeLa cells. DPP4 activity measured by ^{13}C-assay correlated positively with enzyme activity measured by colorimetric assay. We have developed a selective, non-invasive, stable isotope ^{13}C-assay for

DPP4 that could have broad translational applications, with further *in vivo* pre-clinical and clinical validation studies indicated.

Introduction

Dipeptidyl peptidase-4 inhibitors (DPP4i) are a class of orally available, small molecule inhibitors for the treatment and management of Type-II diabetes that have been on the market for over 10 years. In 2006, Merck received approval from the FDA for their first in class DPP4i, Januvia® (Sitagliptin), with several other candidates, referred to broadly as the 'gliptins' that include vildagliptin, saxagliptin and linagliptin, since approved [1, 2]. Several meta-analyses have summarised the clinical efficacy of DPP4i, with almost all studies reporting overall improvements in the primary clinical endpoints of blood glucose, indicated by haemoglobin A_{1C}, and bodyweight[3-5]. A sub-set of diabetic patients have been classified as either poor or non-responders to DPPi[6-8], possibly suggesting differential DPP4 inhibition in non-responders. A rapid, real-time, functional test of DPP4 activity could help to define DPP4i efficacy in patients.

The current, gold standard for quantification of DPP4 enzyme activity is by fluorometric or colorimetric enzyme assays[9, 10]. However, these methodologies are cumbersome and are restricted to a single measurement from biological (blood and tissue) samples collected at a single point in time. Breath analysis represents a novel paradigm for the non-invasive detection and monitoring of health and disease[11-14]. Breath analysis has been widely used to non-invasively detect functional pathophysiological changes[15-19], and represents a novel, non-invasive method to quantify DPP4 enzyme activity.

Stable isotope breath tests are primarily dependent on the ingestion of a specific isotopically labelled substrate, whose subsequent metabolism and incorporation into CO_2 can be quantified in the exhaled breath (Figure 6.1.1)[20]. ^{13}C stable isotope breath tests have had the broadest clinical application to date, and have been used to detect changes in liver function, exocrine

pancreatic function, gastric emptying, and *Helicobacter pylori* infection[15-19]. There is an emerging recognition of the value of ^{13}C-breath tests to rapidly and non-invasively predict and monitor response to pharmacological drugs.

Substrate design and delivery is critical for the specificity of any breath test. Ideally, a candidate non-invasive biomarker should possess unique functional characteristics that are altered when homeostasis is disturbed, such as altered metabolic pathways, differential enzyme expression, or a modified physiological state that ultimately leads to production of a CO_2 bi-product, which is detectable on the breath [20]. DPP4 is a membrane bound serine protease with unique substrate specificity, cleaving dipeptides from the N-terminal of proteins with a penultimate proline or alanine residue[21, 22], and represents an excellent candidate for detection by ^{13}C stable isotope breath testing.

A stable isotope breath test for DPP4 enzyme activity could have broad research and clinical applications. As new DPP4i are developed, a non-invasive, functional breath-test could provide real-time information on inhibitor selectivity and pharmacokinetics and individual response to DPPi therapy. The current study aimed to develop, *in vitro*, a ^{13}C stable isotope enzyme assay that could detect DPP4 enzyme activity. Furthermore, we aimed to determine assay selectivity using the selective DPPi, Sitagliptin, and how the assay correlated to the existing, gold-standard, colorimetric enzyme assay for DPP4.

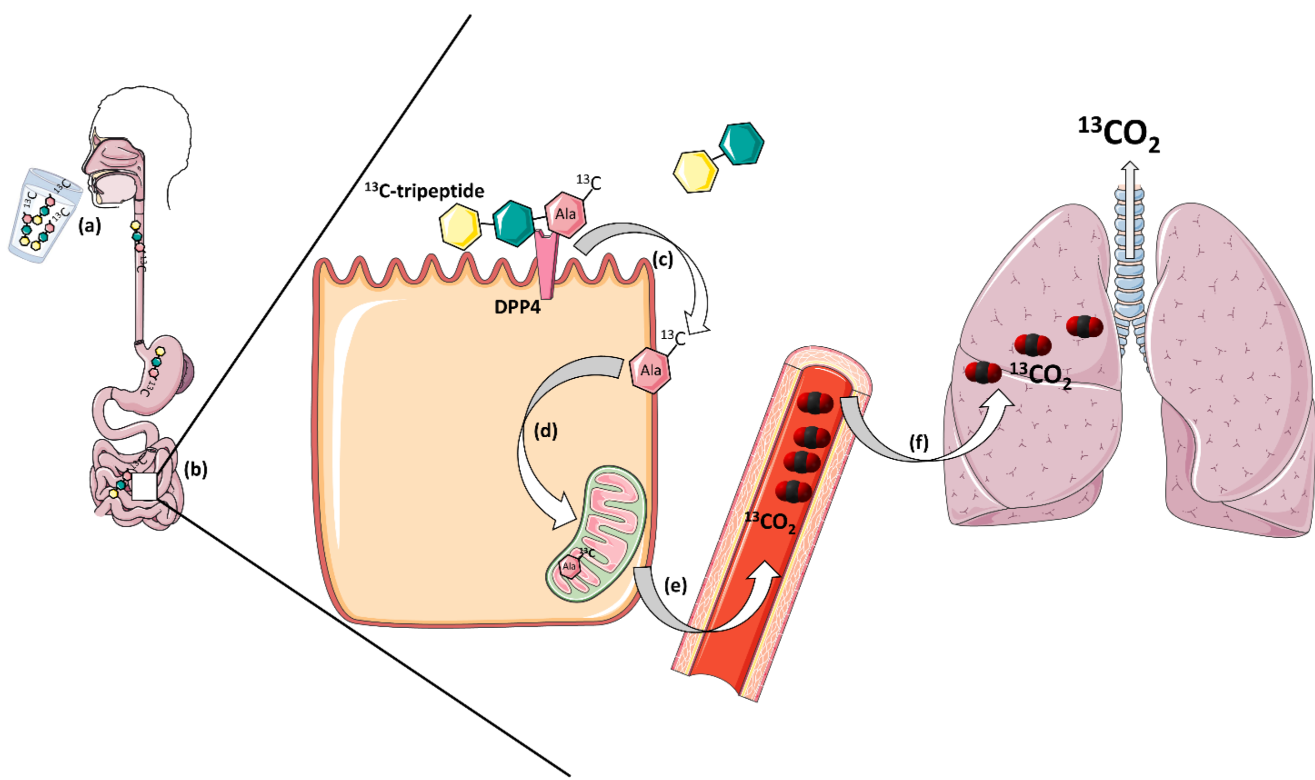

Figure 6.1.1 Schematic outlining the principle of a DPP4 breath test. **(a)** Following ingestion of the ^{13}C-tripeptide in solution (water), **(b)** the ^{13}C-tripeptide empties from the stomach into the duodenum where **(c)** it undergoes hydrolysis by DPP4 expressed on the epithelial cells of the intestinal brush border. **(d)** the liberated ^{13}C-alanine is then absorbed by the cell where it is metabolised, leading to the formation of a $^{13}CO_2$ bi-product, which **(e)** is transported via the blood **(f)** to the lungs where it is exhaled via the breath for collection and analysis by IRMS.

Materials & Methods

Cell culture

All cell lines were purchased from CellBank Australia (New South Wales, Australia). Immortalised cell lines with high and low DPP4 expression and activity were selected for development of a DPP4 stable isotope assay. Caco-2 cells are derived from a colorectal adenocarcinoma and have high membrane DPP4 expression[23]. In contrast, HeLa cells are derived from cervical squamous cell carcinoma, and have very low to no membrane DPP4 expression or activity[24].

Cells were grown in T75 tissue culture flasks under standard cell culture conditions in a 5% CO_2, 37°C incubator. Caco-2 cells were maintained in Dulbecco's Modified Eagle's Minimum Essential Media (DMEM) supplemented with 10% Foetal Bovine Serum (FBS), 1% sodium pyruvate, 1% non-essential amino acids and 20mM L-glutamine. HeLa cells were grown in Eagle's Minimum Essential Medium, supplemented with 10% foetal bovine serum and 20mM L-glutamine.

Absolute mRNA expression by qRT-PCR using external standards

RNA was extracted from Caco-2 and HeLa cells using the RNeasy Kit (Qiagen, Germany) combined with the Qia-shredder (Qiagen, Germany) in accordance with manufacturer's instructions. Complementary DNA (cDNA) was synthesised from 1µg of RNA using the Quantitect Reverse Transcription Kit (Qiagen, Germany). 2.5ng of cDNA was analysed using qPCR in real-time on the Rotor Gene Q (Qiagen, Germany). 10uL qPCR reactions consisted of 100nM primer, 1x Kapa SYBR Fast qPCR Universal Mastermix (Kapa Biosystems, USA). Primer sequences are detailed in Table 6.1.1. Cycling conditions were as follows: 1 cycle at 95°C for 3 minutes for enzyme activation, followed by 45 cycles of denaturation at 95°C for 3

seconds and Annealing/Extension/Data Acquisition performed at 68°C for 30 seconds. External PCR standards for each gene were included in each run. These consist of purified PCR products of known gene copy number prepared using previously published methods[25]. All samples and standards were analysed in triplicate. Data is expressed as a ratio of the number of copies of the gene of interest to the number of copies of the tata box protein (TATA). Error bars represent standard deviation of samples performed in triplicate.

Table 6.1.1 Primer sequences for quantitative real-time PCR reactions

Gene	*Primer Sequences (5'-3')*	*Product length (bp)*
DPP4	F: ACG CCG ACG ATG AAG ACA CCG R: TTC AGC AGA ACC ACG GGC ACG	95
DPP8	F: ACA TGA TGG CTA AGG CAC CAC ATG A R: CTG TTC TCA CCA GAC ATG GCA AGG	106
DPP9	F: TTGCTCTGACCAGCGGTGAATG R: GCCTCATAGCTGACCACGTAGA	142
FAP	F: ATG GGG CTG GTC CTA TGG AGG AT R: GCT GGA GAC TGG AGC CAC TGC	97
TATA	F: CGA AAC GCC GAA TAT AAT CCC AAG CG R: GCC AGT CTG GAC TGT TCT TCA CTC TT	137

Design and optimisation of gas sampling method

Gas sampling lids were custom designed for all gas collection experiments. Briefly, the lid of a solid (non-vented) T75 tissue culture flask was pierced with an 18-gauge needle, and silicone gel was then used to seal the area around the needle and secure its position. A two-way tap was attached to the needle to facilitate gas collection by 10ml syringe (Figure 7.1.2).

As all mammalian cells actively metabolise glucose, D-1-^{13}C-glucose (Cambridge Isotopes, USA) was used to optimise and validate the headspace collection method. Briefly, flasks (n=3/cell line) containing confluent Caco-2 or HeLa cells were incubated for 20 minutes at 37°C in a 5% CO_2 incubator, and a baseline (t=0) 10ml gas sample was collected into 12ml

evacuated vacutainers (Exetainer, USA). 10ml of room air was then injected into the cellular headspace to replace the sample volume of gas (Figure 6.1.2). The original cell media was then discarded and replaced with 2mM D-1-^{13}C-glucose dissolved in 3ml of fresh cell media.

Gas sampling lids were then re-attached and flasks returned to the 5% CO_2 incubator. Gas samples were collected every 20 minutes for a total of 120 minutes. $^{13}CO_2$ was quantified in gas samples by ABCA isotope ratio mass spectrometry (IRMS) (Europa Scientific, Sercon, Cheshire, UK). Concurrent with every experiment, headspace samples were collected from cells incubated with media only.

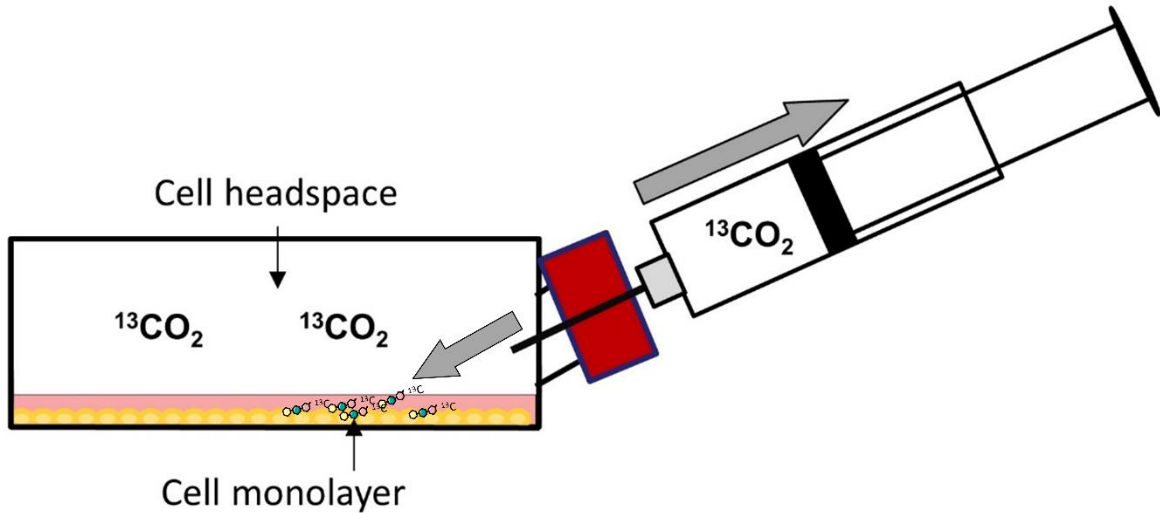

Figure 6.1.2 Headspace sampling from T75 tissue culture flasks. Following injection of the ^{13}C-substrate into the flask, gas samples are collected by withdrawing 10ml of gas via the syringe connected to a two-way tap. An equivalent volume of room air is then used to replace the gas volume lost from the flask.

Design and optimisation of DPP4 selective ^{13}C-tripeptide

Alanine is a non-essential amino acid whose metabolism via the glucose-alanine cycle leads to the production of a CO_2 bi-product. ^{13}C-alanine (Isotec, USA) was selected as the reporter moiety of the ^{13}C-tripeptide. To confirm that there was no differences between Caco-2 and HeLa cells in alanine catabolism, Caco-2 and HeLa cells were incubated with 1mM, 2mM or 5mM ^{13}C-alanine, as previously described, and headspace samples were collected every 20 minutes for a total of 120 minutes.

The ^{13}C-tripeptide was subsequently designed, with a proline in the penultimate position to confer selectivity for DPP4. The full tripeptide sequence was H-Gly-Pro-($^{13}C3$-Ala)-OH and was synthesised by Cambridge Isotopes (USA). 3mM H-Gly-Pro-($^{13}C3$-Ala)-OH dissolved in 3ml of media was then added to confluent flasks of Caco-2 and HeLa cells and headspace samples collected every 20 minutes, for 120 minutes as previously described.

H-Gly-Pro-($^{13}C_3$-Ala)-OH assay selectivity

Assay selectivity was determined using the selective inhibitors of DPP4 and DPP enzyme activity sitagliptin phosphate monohydrate (Biovision, USA, #1757) [26] and p32/98 (Enzo Life Sciences, USA, #BML-PI142-0050)[26]. Caco-2 and HeLa cells were treated with 1nM, 10nM, 1μM and 10μM sitagliptin or p32/98 for 5 hours prior to headspace analysis. After collection of the baseline headspace sample (t=0), the media was discarded and replaced with 3ml of fresh media containing 3mM H-Gly-Pro-($^{13}C3$-Ala)-OH and the corresponding concentration of sitagliptin or p32/98.

Gas sample analysis

$^{13}CO_2$ was quantified in headspace samples by ABCA isotope ratio mass spectrometry (IRMS) (Europa Scientific, Sercon, Cheshire, UK), which measures the ratio of ^{12}C:^{13}C. 5% CO_2 reference gas was used to calibrate the IRMS prior to sample analysis. In addition, throughout the sample run, reference and quality control gas (High Purity Carbon Dioxide; BOC, Australia) was analysed every 10–20 samples to minimise drift. All samples were analysed within 24–48 hours of sample collection. All data was expressed as the mean δbaseline $^{13}CO_2$ of n=3 replicates per cell line per experiment ± standard error of the mean, and is representative of at least two repeated experiments.

DPP enzyme activity assays

Flasks of confluent cells were rinsed with 7ml cold phosphate buffered saline (PBS) then scraped into 10ml PBS and centrifuged at 15,000G for 10 minutes at 4°C. Cell pellets were resuspended in 1-1.5ml phosphate buffered saline with protease inhibitors, and extracts separated using the 'freeze-thaw' method. Samples were again centrifuged for ten minutes at 15,000G at 4°C. The supernatant (representing the cytoplasmic fraction) was then removed and stored at -80ºC. The remaining pellet was resuspended in 200µl PBS with 1% TritonX-100 and then centrifuged. The supernatant (representing the membrane fraction) was aspirated and stored at -80ºC until further analysis.

DPP enzyme activity was measured in cell cytoplasmic and membrane fractions using a colorimetric enzyme assay, modified from the original method described by Hopsu Havu et al[27]. The colorimetric DPP substrate, H-Gly-Pro-p-nitroalinilide (pNA; extinction coefficient: $9450M^{-1}cm^{-1}$) (Bachem, Switzerland, #L-1100) was used at a final concentration of 1mM in all assays. Dual absorbance readings were taken at 405 nm and 600 nm every 10 minutes at

37°C for a total of 90 minutes using a ClarioStar microplate reader (BMG Labtech, Germany). DPP8/9 activity was determined by addition of the selective DPP4i, Sitagliptin and the non-selective inhibitor, p32/98 at concentrations of 1nM, 10nM, 1μM and 10μM.

Protein content was determined in 10μL of sample using a BioRad Detergent Compatible (DC) Assay kit with Bovine Serum Albumin (BSA) standards. Final enzyme activity was expressed as μmol/min/mg of protein.

Results

DPP4 is highly expressed in Caco-2 cells

To confirm high and low DPP4 expression in Caco-2 and HeLa cells, DPP gene expression was determined in both cell lines respectively. DPP4 was highly expressed in Caco-2 cells, whilst very low expression was identified in HeLa cells (Table 6.1.2). Relatively high expression of DPP8 was also observed in both Caco-2 and HeLa cells (Table 6.1.2). DPP8 and DPP9 expression was lower in HeLa cells compared to Caco-2 cells. Relatively low expression of FAP was detected in HeLa cells, compared to Caco-2 cells, where FAP expression was almost undetectable.

Table 6.1.2 DPP mRNA expression in Caco-2 and HeLa cells

	DPP4	**DPP8**	**DPP9**	**FAP**	**PREP**	**TATA**
Caco-2	13742.16 ± 444.37	4186.82 ± 143.22	707.51 ± 24.34	0.18 ± 0.17	5267.54 ± 254.25	6020.55 ± 178.49
HeLa	38.07 ± 1.58	1444.92 ± 127.32	106.71 ± 10.62	391.52 ± 42.90	1173.73 ± 72.79	3075.45 ± 153.69

Data is expressed as mean (n=3 replicates) gene copy number/1μg RNA ± SEM

^{13}C-alanine is metabolised in vitro by Caco-2 and HeLa cells

The headspace sampling method was first optimised and validated in Caco-2 and HeLa cells using D-1-^{13}C-glucose. Both Caco-2 and HeLa cells rapidly metabolised glucose, as indicated by an increasing δbaseline $^{13}CO_2$ signal over the two-hour sampling protocol (Figure 6.1.3a). The rate of increase in δbaseline $^{13}CO_2$ was comparable between the two cell lines, and was higher in HeLa cells compared to Caco-2 cells by the conclusion of the sampling period (Figure 6.1.3a). A background of 4-6 δbaseline $^{13}CO_2$ signal was observed in all headspace samples taken from cells not receiving a ^{13}C-substrate (Figure 6.1.3b).

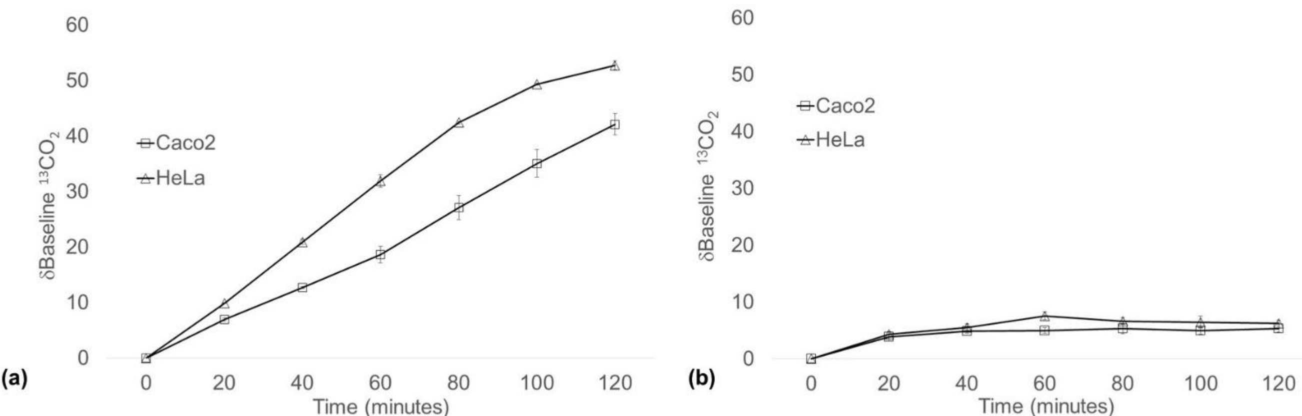

Figure 6.1.3 Metabolism of ^{13}C-glucose *in vitro*. Headspace δbaseline $^{13}CO_2$ of Caco-2 and HeLa cells after 2h incubation with **(a)** ^{13}C-glucose or **(b)** with media only. After baseline gas collection, cells received 2mM ^{13}C-glucose in fresh media. 10ml gas samples were then collected from the cellular headspace over 2h. Data is representative of a minimum of three separate experiments. Data expressed as mean ± SEM, n=3 biological replicates

^{13}C-alanine metabolism was then determined in Caco-2 and HeLa cells. An increasing δbaseline $^{13}CO_2$ was observed in both Caco-2 and HeLa cells over the 120 minute collection period for all ^{13}C-alanine concentrations tested (Figure 6.1.4). The δbaseline $^{13}CO_2$ signal was highest in Caco-2 cells (108±3.53; Figure 6.1.4b) at the highest concentration of 5Mm ^{13}C-alanine compared to HeLa cells (55.66±1.72; Figure 6.1.4a). However, the δbaseline $^{13}CO_2$ was more comparable between cell lines at 1mM and 2mM ^{13}C-alanine, with a $\delta^{13}CO_2$ of 36.69±2.83 and 26.55±095 in Caco-2 and HeLa cells respectively at 2mM ^{13}C-alanine. This informed the concentration of H-Gly-Pro-($^{13}C_3$-Ala)-OH used in subsequent experiments.

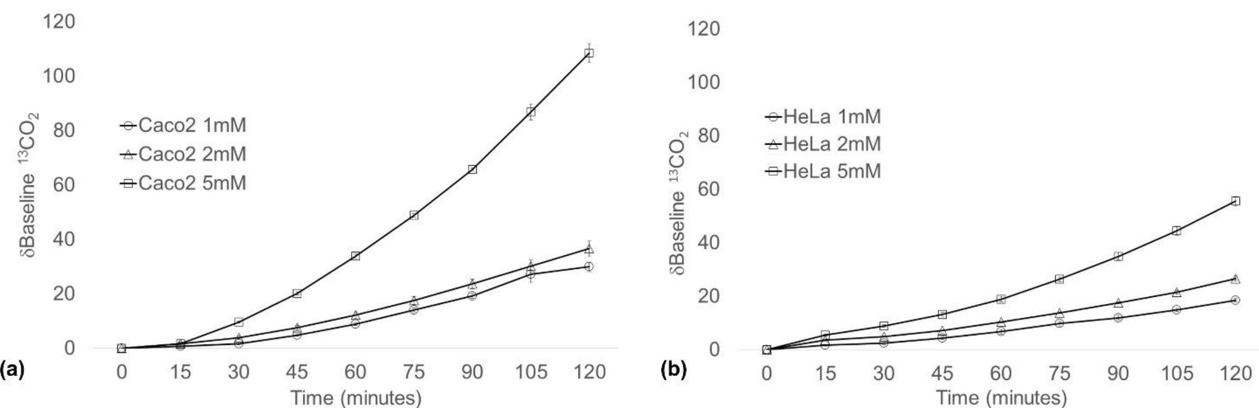

Figure 6.1.4 Metabolism of ^{13}C-alanine *in vitro*. Headspace δbaseline $^{13}CO_2$ of **(a)** Caco-2 and **(b)** HeLa cells after 2h incubation with ^{13}C-alanine. After baseline gas collection, cells received 1mM, 2mM or 5mM ^{13}C-alanine in fresh media. 10ml gas samples were then collected from the cellular headspace over 2h. Data is representative of a minimum of three separate experiments. Data expressed at mean ± SEM, in n=3 flasks (biological replicates)

DPP4 activity can be detected in vitro by ^{13}C-assay

The H-Gly-Pro-($^{13}C_3$-Ala)-OH substrate was subsequently tested in Caco-2 and HeLa cells. An increasing δbaseline $^{13}CO_2$ was observed in Caco-2 cells over the 120 minute collection period (Figure 6.1.5a and 6.1.5c). In contrast, DPP activity, as indicated by the δbaseline $^{13}CO_2$, was unchanged in HeLa cells, and was almost identical to the signal observed in non-H-Gly-Pro-($^{13}C_3$-Ala)-OH controls (Figure 6.1.5b and 6.1.5d).

The selectivity of the ^{13}C-assay for DPP4 was determined using the highly selective DPP4i, sitagliptin. δbaseline $^{13}CO_2$ increased to a maximum of 39.58±0.97 and 10.83±1.18 in Caco-2 and HeLa cells respectively, and addition of 1nM or 10nM concentrations of sitagliptin made no change to the δbaseline $^{13}CO_2$ signal (Figure 6.1.5a and 6.1.5b). However, δbaseline $^{13}CO_2$ was significantly reduced in Caco-2 cells treated with either 1μM and 10μM sitagliptin by up to 73% compared to non-sitagliptin treated cells at 120 minutes (Figure 6.1.5c). δbaseline

$^{13}CO_2$ was largely unchanged in HeLa cells over the collection period, and remained unchanged with addition 1μM or 10μM sitagliptin (Figure 6.1.5d).

DPP4 enzyme activity was then measured by the colorimetric assay in the corresponding Caco-2 and HeLa membrane extracts. Similar to the pattern of activity measured by ^{13}C-assay, high DPP activity was observed in the Caco-2 membrane fraction (44.57ng/min/mg protein) compared to low activity in HeLa cells (0.62ng/min/mg protein). Addition of 1μM or 10μM sitagliptin to Caco-2 cell membrane extracts inhibited activity by 71% and 96% respectively, while there was no observable change in HeLa cells.

DPP enzyme activity was lower in Caco-2 cytoplasm compared to HeLa cells (Figure 6.1.5e,f). Addition of 1μM or 10μM sitagliptin to the cytoplasmic fraction reduced activity by 49% and 68% respectively (Figure 6.1.5f). In contrast, DPP enzyme was higher in the HeLa cytoplasmic fraction (4.99ng/min/mg protein) compared to the HeLa membrane fraction, and addition of 1μM or10μM sitagliptin did not modify DPP activity.

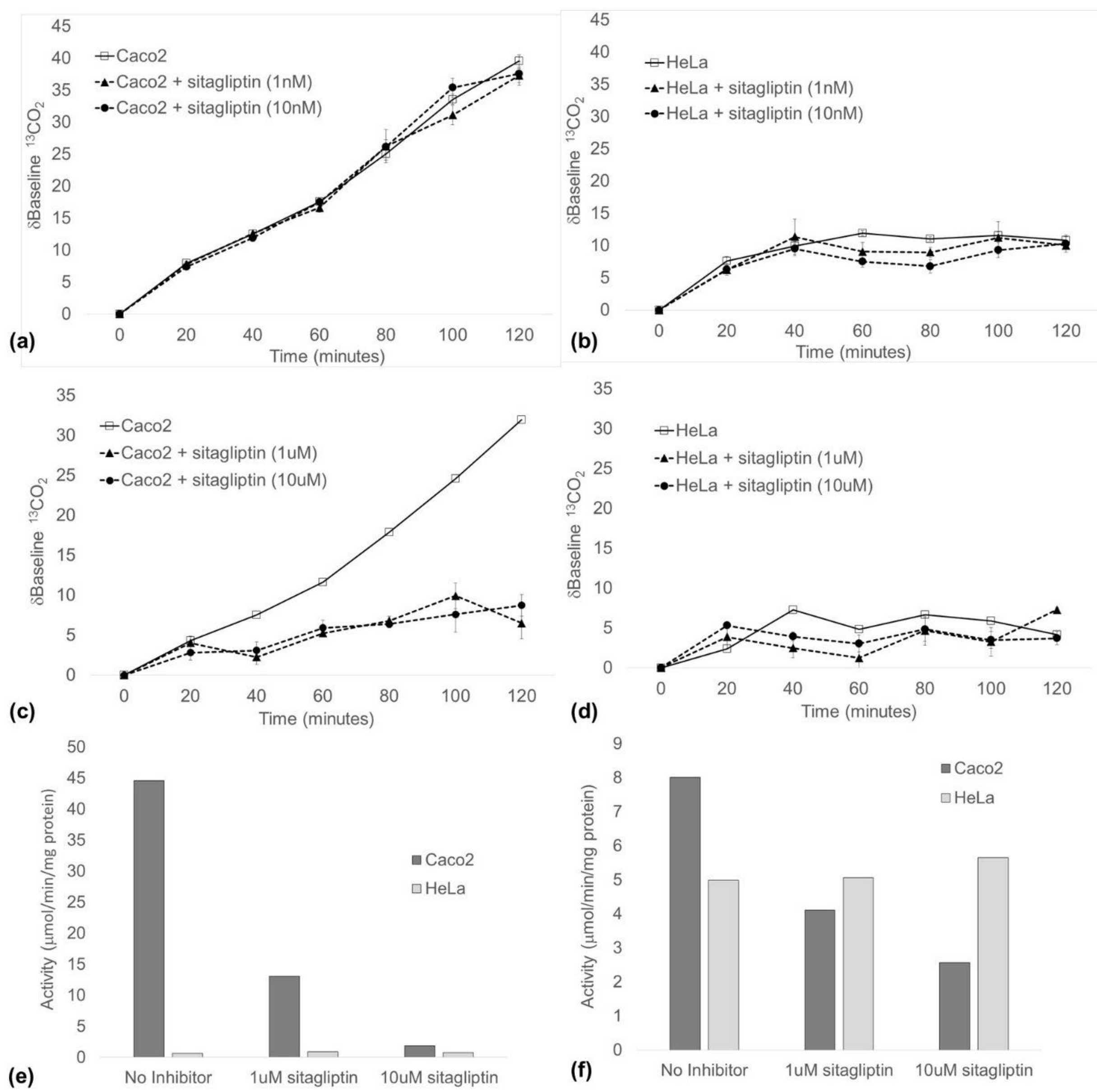

Figure 6.1.5 Defining selectivity of H-Gly-Pro-($^{13}C_3$-Ala)-OH using sitagliptin. Headspace δbaseline $^{13}CO_2$ in n=3 flasks of **(a)** & **(c)** Caco-2 and **(b)** & **(d)** HeLa cells after 2h incubation with H-Gly-Pro-($^{13}C_3$-Ala)-OH. ^{13}C-assay selectivity was determined by addition of the selective DPP4i, Sitagliptin. After headspace collection was completed, cell extracts were collected, and DPP4 enzyme activity quantified in **(e)** membrane and **(f)** cytoplasmic fractions by the 'gold-standard' H-Gly-Pro-pNA colorimetric assay, with and without Sitagliptin. Data is representative of a minimum of three separate experiments. Data expressed at mean ± SEM

The selectivity of the H-Gly-Pro-($^{13}C_3$-Ala)-OH substrate for DPP4 over other DPP enzymes was further defined using the less selective DPP inhibitor, p32/98 (Figure 6.1.6). Nanomolar concentrations of p32/98 had no observable effect on enzyme activity measured by the ^{13}C-assay (Figure 6.1.6a and 6.1.6c). The δbaseline $^{13}CO_2$ at 120 minutes was reduced by 44% only at the highest concentration (10μM) of p32/98 in Caco-2 cells (Figure 6.1.6c).

When measured by colorimetric assay, Caco-2 membrane DPP activity was similarly reduced at the highest concentration of p32/98 by 33%. Cytoplasmic DPP activity was again lower in Caco-2 cells compared to membrane activity; however, DPP activity was higher in the cytoplasmic fraction of HeLa cells. Addition of p32/98 did not significantly modify cytoplasmic DPP activity in the cytoplasmic fraction of both cell lines.

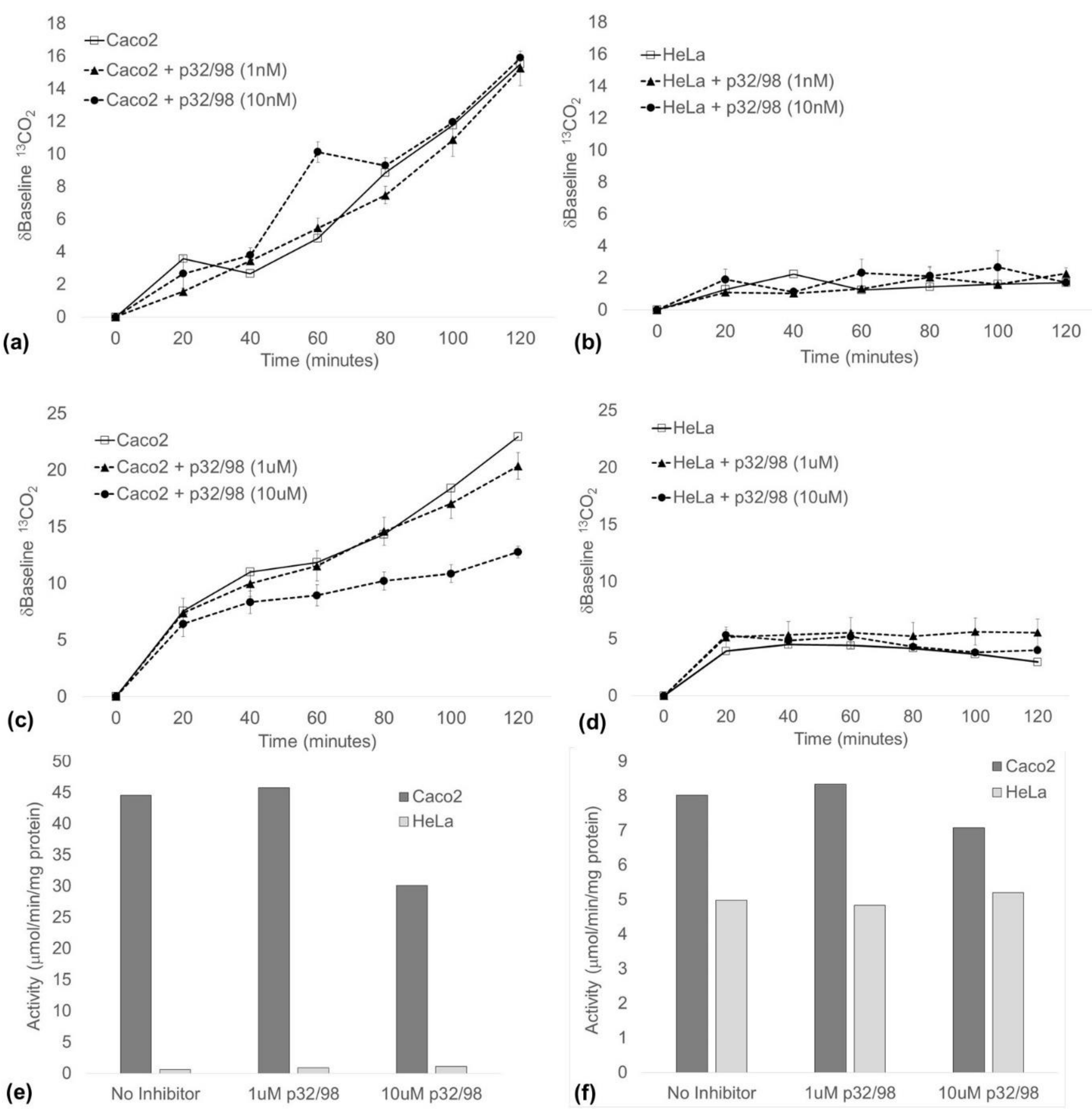

Figure 6.1.6 Defining selectivity of H-Gly-Pro-($^{13}C_3$-Ala)-OH using P32/98. Headspace δbaseline $^{13}CO_2$ in n=3 flasks of **(a)** & **(c)** Caco-2 and **(b)** & **(d)** HeLa cells after 2h incubation with H-Gly-Pro-($^{13}C_3$-Ala)-OH. ^{13}C-assay selectivity was determined by addition of the selective DPP4i, p32/98. After headspace collection was completed, cell extracts were collected, and DPP4 enzyme activity quantified in **(e)** membrane and **(f)** cytoplasmic fractions by the 'gold-standard' H-Gly-Pro-pNA colorimetric assay, with and without p32/98. Data is representative of a minimum of three separate experiments. Data expressed at mean ± SEM.

The ^{13}C-assay positively correlates with the gold-standard assay

To determine how well the ^{13}C-assay correlated with the DPP4 gold standard, colorimetric assay, a Pearson correlation was performed between the two methods (Figure 6.1.7). There was a strong correlation between activity measured by H-Gly-Pro-($^{13}C_3$-Ala)-OH assay and colorimetric assay for both sets of experiments ($r=0.91$ and $r=0.79$ respectively, $p<0.0001$)

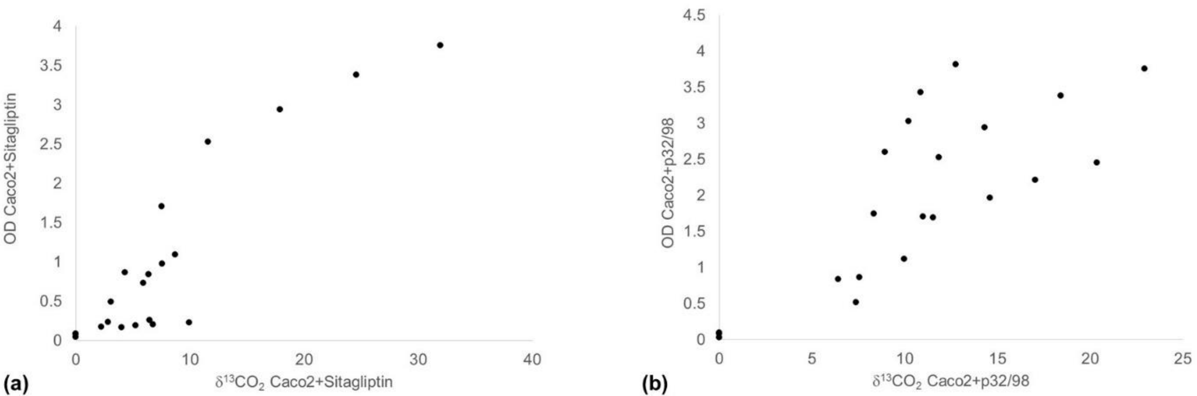

Figure 6.1.7 Relationship between ^{13}C-DPP4 assay and colorimetric assay. Correlation between mean DPP4 activity measured by H-Gly-Pro-($^{13}C_3$-Ala)-OH and by H-Gly-Pro-pNA colorimetric assay with the addition of either **(a)** sitagliptin ($r=0.91$) or **(b)** p32/98 ($r=0.79$), $p<0.0001$.

Discussion

To our knowledge, this is the first study to describe a non-invasive assay for the quantification of DPP4 enzyme activity in live cells using a ^{13}C-labelled substrate. Using selective inhibitors of DPP4, we have demonstrated the specificity of the ^{13}C based assay for DPP4 in live cell lines. Furthermore, we also demonstrated the *in vivo* specificity of the assay in a human case study. The current study highlights the ^{13}C assay for DPP4 as a useful tool for the non-invasive detection and monitoring of DPP4 activity in the presence and absence of DPP4i, with further *in vivo* pre-clinical and clinical studies indicated.

The ^{13}C-tripeptide was designed with a substrate specificity for DPP4, incorporating a proline in the penultimate position, with hydrolysis of the prolyl bond by DPP4 liberating the ^{13}C-alanine, leading to the subsequent production of a $^{13}CO_2$ bi-product. Alanine is a non-essential amino acid that is converted to pyruvate before entry into the citric acid cycle, where it is completely oxidized to CO_2[28]. We first determined the capacity of our chosen cell models to metabolise alanine, as well as ensure that both Caco-2 and HeLa cells metabolised alanine at a similar rate. At the highest concentration of ^{13}C-alanine, the $^{13}CO_2$ signal was approximately 2-fold higher in Caco-2 compared to HeLa cells. Differing alanine transport mechanisms have been reported in Caco-2 and HeLa cells[29, 30], and may, in part, explain these observations. At lower concentrations of ^{13}C-alanine, the $^{13}CO_2$ signal was more comparable between the two cell lines, and this was used to guide the concentration of ^{13}C-tripeptide used in subsequent experiments.

Addition of the ^{13}C-tripeptide to Caco-2 cells led to an increasing $^{13}CO_2$ over the sample collection period. In contrast, the $^{13}CO_2$ signal was unchanged in HeLa cells, correlating to membrane DPP enzyme activity, as measured by the gold-standard colorimetric assay. ^{13}C-

DPP4 assay selectivity was determined using the DPP4 inhibitor, sitagliptin, a small molecule, competitive binding inhibitor of DPP4, with high selectivity for DPP4 over other DPP family members[31]. When Caco-2 cells were treated with either 1μM or 10μM sitagliptin, the $^{13}CO_2$ signal was similar to that observed from HeLa cells and correlated positively to enzyme activity measured by the colorimetric assay, suggesting that the ^{13}C-DPP4 assay was specific to DPP4. p32/98 is a less selective inhibitor for DPP4, and has been shown to also inhibit other members of the DPP enzyme family, with IC50 values of 420nM for DPP4, 2180nM for DPP8 and 1600nM for DPP9 previously reported[26]. The ^{13}C-DPP4 assay detected a lowered $^{13}CO_2$ signal from Caco-2 cells treated with the highest dose of p32/98, again correlating to activity measured by the colorimetric assay. The consistent background $^{13}CO_2$ produced by Caco-2 and HeLa cells in cell media is likely due to the naturally enriched ^{13}C-glucose present in the cell media. However, the DPP4 ^{13}C-assay achieved a high signal to noise ratio, highlighting the specificity of the ^{13}C-assay for DPP4 and its potential for direct translational applications.

Cytoplasmic DPP activity was almost 5-fold lower in Caco-2 cells, compared to the corresponding membrane fraction measured by the colorimetric assay. Addition of sitagliptin reduced DPP activity by more than 70%, suggesting there is a significant intracellular store of DPP4. In contrast, cytoplasmic DPP activity was almost 10-fold higher in HeLa cells when compared to the corresponding membrane fraction. Neither sitagliptin nor p32/98 reduced cytoplasmic DPP activity in HeLa cells, suggesting that all of the activity was likely from other DPP enzyme family members and not DPP4. This observation was further supported by qRT-PCR data demonstrating relatively high DPP8 expression in HeLa cells. Interestingly, no $^{13}CO_2$ signal was observed in HeLa cells, despite DPP activity detected in the cytoplasm, and DPP8 expression detected by PCR, suggesting that the ^{13}C-DPP4 assay was selective for membrane bound DPP4.

DPP4i have emerged as an effective, orally available treatment modality for type-II diabetes[2, 32]. However, a sub-set of patients are refractory to DPP4i, and individual variability between patients has been reported[6, 8, 33]. Previous studies have investigated predictive clinical parameters for the therapeutic benefits of DPP4i, including age and body mass[5]; however, to our knowledge, the level of DPP4 inhibition in individual diabetic patients treated with a DPP4i has not been reported. It is conceivable that differential systemic inhibition of DPP4 could result in differing glucoregulatory profiles. A ^{13}C-DPP4 breath test represents a non-invasive, functional test of DPP4 activity that could be used to longitudinally quantify response to DPP4 inhibitors in type-II diabetics, potentially informing response to therapy and dose adjustment. Furthermore, as new DPP4i continue to be developed, a DPP4 breath test represents a novel, non-invasive tool to investigate pre-clinical and clinical efficacy of new drug candidates. Pre-clinical studies are required to further validate the DPP4 breath test against a panel of clinically relevant DPPi, comparing systemic and oral administration of the H-Gly-Pro-($^{13}C_3$-Ala)-OH.

The intestinal lumen contains one of the largest pools of DPP4, playing a critical role in digestion and protein assimilation[34, 35]. It has been the long held consensus that DPPi modulate blood glucose concentrations by prolonging the action of incretin hormones glucagon-like peptide-1 (GLP-1) and gastric-inhibitory polypeptide (GIP)[29]. Waget *et al* used low dose sitagliptin in mice to selectively inhibit small intestinal DPP4 activity, achieving significant glucoregulation[36]. Their findings suggest that gastrointestinal DPP4 may be important for mediating, in part, the glucoregulatory action of DPPi[36]. A breath test for DPP4 may have application in human subjects to better dissect the glucoregulatory mechanism of DPPi.

^{13}C breath tests have had broad translational application, correlating to more invasive methodologies such as endoscopy and serology testing for *Helicobacter pylori* or gastric emptying by scintigraphy. Our *in vivo* proof-of-concept experiment has demonstrated that not

only can a $^{13}CO_2$ signal be detected after oral ingestion of Gly-Pro-Ala-^{13}C, but that this signal is specific to DPP4. Past studies have characterised DPP4 enzyme activity along the length of the gastrointestinal tract, reporting little to no activity in either the oesophagus or stomach. In our case study, Gly-Pro-Ala-^{13}C was orally ingested while in the left recumbent position and breath samples were collected for the first 30 minute while remaining in left recumbent in order to delay gastric emptying and differentiate upper gastrointestinal DPP4 activity from small intestinal DPP4 activity[37]. The δbaseline $^{13}CO_2$ remained relatively low for the first 14 minute of the test, before steadily increasing thereafter. These results suggest that a DPP4 breath test may also have specific application for the non-invasive assessment of gut function and protein assimilation in individuals with suspected functional gastrointestinal disorders.

The translational application and interpretation of a DPP4 breath test will largely depend on the mode of substrate delivery. For example, assessment of DPPi response and efficacy in pre-clinical or clinical studies would likely seek to administer the Gly-Pro-Ala-^{13}C via a sub-cutaneous route to measure systemic DPP4 activity. In contrast, the gradient of DPP4 activity along the gastrointestinal tract could be targeted using pH sensitive encapsulation techniques that specifically release the substrate in defined regions within the gastrointestinal lumen. The 50mg dose of Gly-Pro-Ala-^{13}C elicited a relatively high $^{13}CO_2$ signal, suggesting that dose adjustments could be made to reduce cost and refine delivery. Future iterations of the ^{13}C-tripeptide may incorporate novel chemical design elements that specifically target intestinal vs systemic DPP4.

In conclusion, we have developed a ^{13}C stable isotope assay that could be used to selectively quantify DPP4 activity *in vitro* and *in vivo*, with broad research and translational application in the development and assessment of new and existing DPPi. Furthermore, the significant pool of DPP4 in the small bowel suggests a DPP4 breath test could also have potential application

as a non-invasive method to measure intestinal function. Further pre-clinical and clinical studies are indicated to further validate the application of a ^{13}C-DPP4 breath test.

Acknowledgements

Dr Yazbek is supported by a Catherine Marie Enright Kelly research fellowship from the Flinders University, Department of Surgery. We would like to thank Ms Michelle Parsons (Flinders University) for running and maintaining the IRMS.

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6.2 Determining gene copy number using external gene standards

Synthesis of PCR gene standards for qPCR

The synthesis of external gene standards was performed per published methods from (Yin et al., 2001)[16]. Three 50µL PCR reactions consisting of Phusion DNA polymerase (New England Biolabs), 500nM primers, 200µM dNTPs, 1xPhusion HF buffer, nuclease free water and template cDNA derived from human cell lines, Caco-2 or HeLa cells were run on the Thermohybaid Px2 thermocycler (ThermoFisher Scientific, Scoresby, VIC) for each gene of interest and reference gene. Two step PCR cycling conditions were as follows with initial denaturation was conducted at 98ºC for 30 seconds, prior to 35 cycles at 98ºC for 10 seconds (denaturation), 72ºC for 45 seconds (annealing & extension), with a final extension at 72ºC for 10 seconds. Three step PCR was used for FAP and DPP4 primer sets with annealing conducted at 68ºC and all other temperatures kept consistent with 2-step PCR.

All PCR products were confirmed by 2% agarose gel electrophoresis, 100V for 40 minutes. PCR products with a single visible band by electrophoresis were purified directly from the tube using SV Gel and PCR Clean-up system (Promega, Perth, WA) as per manufacturer's instructions. Purified PCR products were quantified using Qubit Fluorometer (Life Technologies/ThermoFisher Scientific, Scoresby, VIC). Samples were sent to the Australian Genome Research Facility for sanger sequencing to confirm the identity of expected gene products.

[16] YIN, J. L., SHACKEL, N. A., ZEKRY, A., MCGUINNESS, P. H., RICHARDS, C., PUTTEN, K. V., MCCAUGHAN, G. W., ERIS, J. M. & BISHOP, G. A. 2001. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I. *Immunol Cell Biol,* 79**,** 213-21.

Gene copy number was calculated for the remaining purified samples per the following equation:

$$\text{Gene copy number}/\mu\text{L} = \frac{\text{Concentration of PCR product (ng}/\mu\text{L) x Avogadro's number}}{\text{Molecular weight of the PCR product x } 10^9}$$

where Avogadro's number is 6.023×10^{23} molecules/mole and molecular weight of the PCR product was estimated by multiplying the length of the PCR product (base pairs) by 660 Da (average weight of a single DNA base pair).

PCR products were diluted in sterile 10mM Tris 0.1mM EDTA buffer, with serial dilutions made ranging from 10^7 -10^1 gene copy number/µL. PCR gene standards were transferred into single use aliquots and stored at -20ºC for use in qPCR reactions.

Initial qPCR reactions were run to gauge the relative expression of the patient samples and ensure that each patient sample fits within the standard curve. Starting gene copy number was determined in patient cDNA samples using generated standard curves (Figure 6.2.1) produced in the Rotorgene-Q (Qiagen) software package (V2.0).

Figure 6.2.1 PCR external gene standards used to estimate starting gene copy number. Figure represents gene standards for all genes of interest (DPP4, FAP, DPP8, DPP9, DPP2 and PREP) and reference genes (TATA and PPIA). Rotorgene Q software package V2.0 (Qiagen, Chadstone, VIC) was used to derive equations for gene copy number. Standard dilutions were modified as necessary to ensure all patient samples fell within the working range of the standard curve. A minimum of 4 standards were used to estimate gene copy number in patient samples. Data points represent average Ct value of sample run in triplicate in single qPCR run. Error bars = standard deviation

6.3 IBD prospective observational study questionnaire

Below is a copy of the questionnaire relating to the recruitment of patients in Chapter 4. IBD research nurses involved in the study ensured the adequate completion of the questionnaire.

IBD prospective observational study patient questionnaire

Study: Investigating innate immune responses in Inflammatory Bowel Disease

Study ID: ____________ Date:_______________

Patient information:

1. Age at diagnosis:____
2. DOB:____
3. Gender: ____
4. Ethnicity:____
5. Current drug therapies:____

Medications

This section refers to the use of over-the-counter medications,

1. Have you taken any non-steroidal anti-inflammatory (pain killers) medication in the last 48 hours? Examples: paracetamol, aspirin, ibuprofen. **If yes, please indicate below and how long ago.**

   ____

2. Do you have type II diabetes

   YES **NO**

   If you answered yes to this question, please proceed to question 3. If you answered NO please proceed to question 4.

3. Are you currently taking medication for your condition, such as sitagliptin/januvia?

   YES **NO**

Co-morbidities

This section would like to know if you have been diagnosed with any other gut related diseases or immune related diseases.

4. Have you previously received a diagnosis of any of the following gastrointestinal conditions (circle below if relevant)

Irritable bowel syndrome Coeliac's disease

5. Have you previously received a diagnosis of cancer? (if yes, please indicate which type)

____

6. Do you suffer from allergies, asthma or hay fever? (please circle the relevant option if appropriate)

7. Have you received a diagnosis for an autoimmune or inflammatory disorder. *Please circle the relevant option below if appropriate*

Multiple sclerosis, psoriasis, rheumatoid arthritis, osteoarthritis, Graves disease, systemic lupus erythematosus, type I diabetes, heart failure, atherosclerosis, periodontitis, chronic obstructive pulmonary disease

8. Have you experienced a bacterial infection in the last month that required antibiotic treatment?

YES NO

Lifestyle factors

3. Are you a smoker? (Please circle below)
   Non-smoker Smoker

4. Only for those that answered smoker in question 8. Please indicate how long you have been a smoker

   ____

5. For those that answered smoker in question 8. Please indicate the frequency you smoke (Circle)
   Less than a pack a day Pack a day More than a pack a day

6.4 Effect of age, gender, smoking and previous cancer diagnosis on plasma DPP activity

Age and gender have no effect on plasma DPP activity

Plasma DPP activity is reportedly altered with age and gender (Durinx et al., 2001) [17]. Total plasma DPP activity was slightly reduced in patients 75< years of age <35, though this failed to reach statistical significance (Table 6.2.1). No other differences were observed between age and plasma DPP activity. Comparison of male and females from non-IBD and IBD groups of this study found no differences between males and females for total, DPP4 or residual DPP plasma activity (Table 6.2.2).

[17] DURINX, C., NEELS, H., VAN DER AUWERA, J. C., NAELAERTS, K., SCHARPE, S. & DE MEESTER, I. 2001. Reference values for plasma dipeptidyl-peptidase IV activity and their association with other laboratory parameters. *Clin Chem Lab Med,* 39**,** 155-9.

Table 6.2.1 Effect of patient age on plasma DPP enzyme activity.

Age (yrs)	***n*** **(non-IBD, IBD)**	**Total DPP activity (U/L)**	**DPP4 activity (U/L)**	**Residual DPP activity (U/L)**
<35	9 (1, 8)	13.9 [11.5 – 15.0]	6.5 [5.5 – 7.2]	6.7 [5.9 – 7.0]
36-45	5 (1, 4)	12.8 [12.2 - 16.0]	6.5 [5.8 – 7.4]	6.4 [6.3 – 8.6]
46-55	12 (7, 5)	15.5 [13.6 – 17.1]	7.5 [6.4 – 9.8]	7.0 [5.6 – 8.6]
56-65	5 (3, 2)	18.2 [14.1 – 18.5]	9.4 [8.3-10.3]	7.9 [7.2 – 9.1]
66-75	9 (6, 3)	15.4 [12.8 – 17.8]	7.3 [5.8 – 10.4]	7.5 [6.6 – 8.1]
76<	3 (2, 1)	12.8 [12.6 – 13.0]	6.2 [5.7 – 6.9]	6.6 [5.7 – 7.2]

1μM sitagliptin was used to determine DPP4 and residual DPP activity. Data presented as median [IQR, Q1 – Q3]

Table 6.2.2 Effect of gender on plasma DPP enzyme activity.

	n **(non-IBD, IBD)**	**Total DPP activity (U/L)**	**DPP4 activity (U/L)**	**Residual DPP activity (U/L)**
Male	27 (14, 13)	13.9 [12.5 – 16.2]	7.3 [5.8 – 9.2]	7.0 [5.8 – 7.8]
Female	16 (6, 10)	15.2 [12.8 – 17.5]	7.2 [6.2 – 9.6]	7.1 [6.2 – 9.3]

1μM sitagliptin was used to determine DPP4 and residual DPP activity. Data is expressed as median [IQR, Q1-Q3]

DPP4 mRNA expression is upregulated in the lungs of smokers compared with non-smokers (Seys et al., 2018)[18]. Smoking is thought to have roles in the pathogenesis of IBD; however, to our knowledge the effect of cigarette smoking on plasma DPP activity has not previously been assessed. Smoking history was available for all patients recruited as part of the prospective IBD study (Chapter 4, Table 4.2). The non-IBD patient group contained the highest proportion of current and ex-smokers (55%) compared to 45% and 30% for CD and UC respectively (Chapter 4, Table 4.2). To determine the relationships between smoking and plasma DPP activity, IBD and non-IBD patients were pooled for subsequent analysis. Total plasma DPP activity was 18.1% lower in current smokers compared with patients that had never smoked (12.5 ± 2.4 U/L vs 15.4 ± 3.2 U/L) (Table 6.2.3). Plasma DPP4 and residual DPP activity were unchanged by patient smoking status (Table 6.2.3).

[18] SEYS, L. J. M., WIDAGDO, W., VERHAMME, F. M., KLEINJAN, A., JANSSENS, W., JOOS, G. F., BRACKE, K. R., HAAGMANS, B. L. & BRUSSELLE, G. G. 2018. DPP4, the Middle East Respiratory Syndrome Coronavirus Receptor, is Upregulated in Lungs of Smokers and Chronic Obstructive Pulmonary Disease Patients. *Clinical Infectious Diseases,* 66**,** 45-53.

Table 6.2.3. Effect of cigarette smoking on plasma DPP enzyme activity (U/L).

	n (non-IBD, IBD)	**Total DPP activity (U/L)**	**DPP4 activity (U/L)**	**Residual DPP activity (U/L)**
Current smoker	8 (3, 5)	11.9 [10.8 – 13.5]*	6.0 [5.0 – 7.2]	6.0 [6.0 – 6.3]
Ex-smoker	13 (8, 5)	13.1 [12.5-14.4]	7.3 [5.7 – 9.5]	7.0 [5.2 – 7.6]
Never smoked	25 (9, 16)	15.2 [12.8 – 17.5]	7.3 [6.4 – 9.6]	7.5 [6.6 – 8.8]

*1µM sitagliptin was used to determine DPP4 and residual DPP activity. Data is expressed as median [IQR, Q1-Q3], *p<0.05 - vs never smoker*

Previous cancer diagnosis does not alter plasma DPP activity

Studies have suggested that plasma DPP activity may represent a novel marker of malignancy (Yazbeck et al., 2017)[19]. Five of the patients involved in the study were previously diagnosed with cancer. Comparisons between patients with previous cancer diagnosis and the remaining non-IBD patients found no differences in total, DPP4 or residual DPP plasma activity (Table 6.2.4).

Table 6.2.4 Effect of previous cancer diagnosis on plasma DPP activity.

	n	**Total activity (U/L)**	**DPP4 activity (U/L)**	**Residual DPP activity (U/L)**
Previous diagnosis	5	17.0 [13.1 – 18.2]	7.7 [7.5 – 10.3]	7.9 [7.8 – 9.5]
No prior diagnosis	16	14.1 [12.8 – 17.4]	7.5 [6.4 – 10.4]	6.6 [5.7 – 8.1]

1µM sitagliptin was used to determine DPP4 and residual DPP activity. Data presented as median [IQR, Q1-Q3]

[19] YAZBECK, R., JAENISCH, S. E. & ABBOTT, C. A. 2017. Potential disease biomarkers: dipeptidyl peptidase 4 and fibroblast activation protein. *Protoplasma*.

6.5 Plasma DPP activity does not correlate with clinical disease severity scores

Clinical disease scoring systems for IBD are useful for the evaluation of disease severity, taking into consideration patient symptoms and clinical parameters (Walsh et al., 2016)[20] (Figure 6.3.1). Using Harvey Bradshaw and Mayo indices, for CD and UC respectively, two CD and five UC patients were classified as active. Plasma total, DPP4, and residual DPP activity were unchanged between patients classified as inactive and active disease defined by the clinical scoring systems (Figure 6.3.1).

[20] WALSH, A. J., BRYANT, R. V. & TRAVIS, S. P. 2016. Current best practice for disease activity assessment in IBD. *Nat Rev Gastroenterol Hepatol,* 13**,** 567-79.

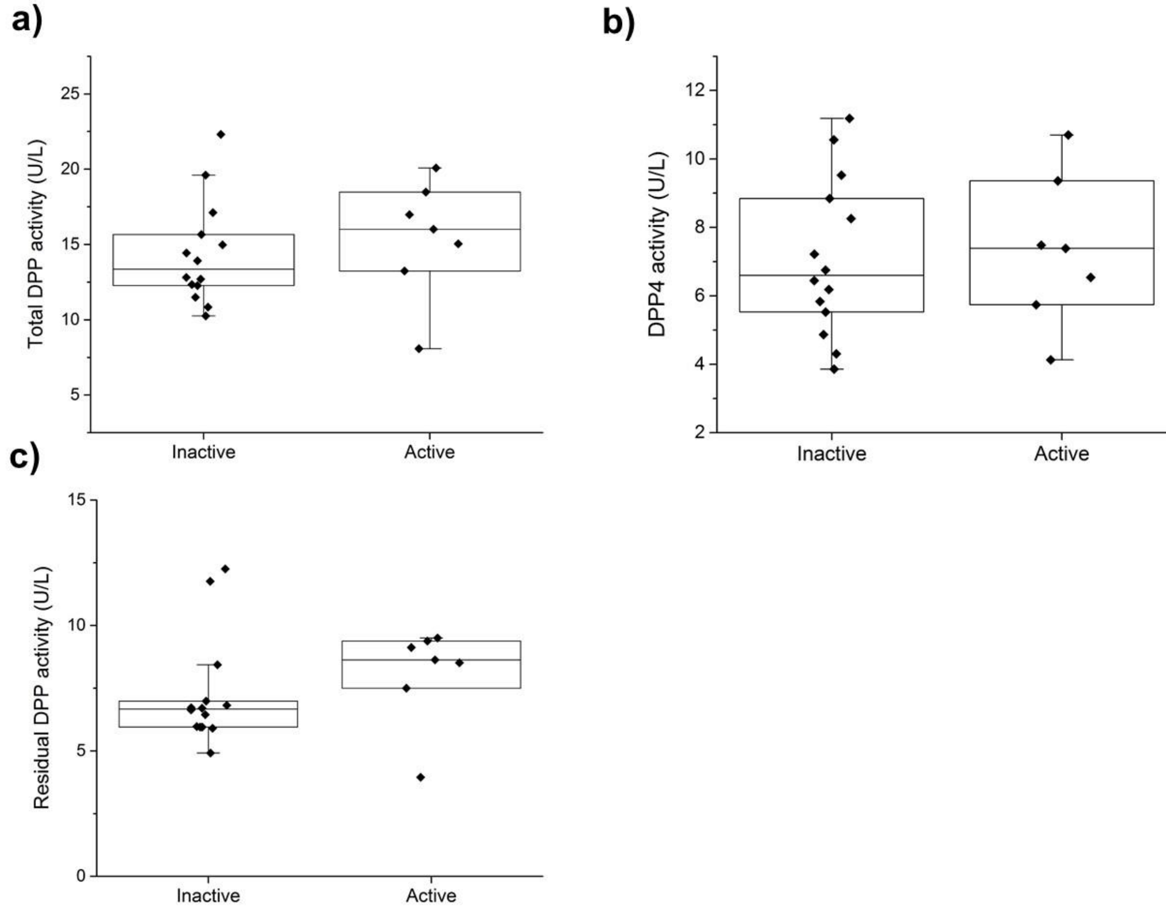

Figure 63.1 Plasma DPP enzyme activity and disease activity using clinical scoring indices. Plasma DPP activity was quantified in IBD patient plasma as total **a)**, DPP4 **b),** and residual DPP activity **c)** respectively using 1µM sitagliptin. Disease severity was assessed using clinical scoring systems, Harvey Bradshaw for CD and Mayo for UC. Scores of <4 for Harvey Bradshaw and <2 for Mayo, were classified as inactive (n=14; n=6/UC, n=8/CD). Scores >4 and >2 were classified as active (n=7, n=5/UC, n=2/CD). Box-plot overlays represent median and IQR, whiskers represent range excluding outliers.

6.6 Plasma FAP activity and patient baseline parameters

FAP plasma activity was not significantly altered by baseline characteristics including age, gender, or previous cancer history (Table 6.6.1). Plasma FAP activity was found to be reduced in current smokers compared to patients who had never smoked and ex-smokers (Table 6.3).

Table 6.3 Plasma FAP activity (U/L) and baseline patient characteristics

Patient Characteristics	FAP activity (U/L)	p<0.05
Age (years)		
<35	11.5 [8.3 – 13.4]	Ns
36-45	13.3 [10.6 – 16.0]	Ns
46-55	13.1 [10.2 - 14.3]	Ns
56-65	16.2 [15.4 - 16.3]	Ns
66-75	12.9 [11.9 – 15.6]	Ns
76<yrs of age	9.5 [8.2 – 9.6]	Ns
Gender		
Male	12.2 [9.7 – 14.9]	Ns
Female	13.3 [10.7 – 14.5]	Ns
Smoking status		
Current	9.8 [6.6 – 10.2]*#	p<0.05
Ex-smoker	13.6 [10.2 – 16.2]	Ns
Never	13.2 [11.5 – 14.2]	Ns
Cancer history		
Previous diagnosis	14.1 [9.7 – 15.3]	Ns
Never	13.1 [11.1 – 16.3]	Ns

*Data presented as median [IQR, Q1-Q3], *p<0.05 - vs never smoker, #p<0.05 – vs ex-smoker, Ns =p >0.05*

6.7 DPP mRNA expression during colorectal inflammation of IBD patients

Matched colorectal tissue from both inflamed and un-involved lesions was available from three IBD patients. Due to the low sample size, statistical analysis was not performed. In general, the mRNA expression of DPP genes including DPP4, FAP, DPP8, DPP9, DPP2 and PREP was higher in tissues collected from sites of active inflammation compared with matched inactive tissue (Figure 6.4.1).

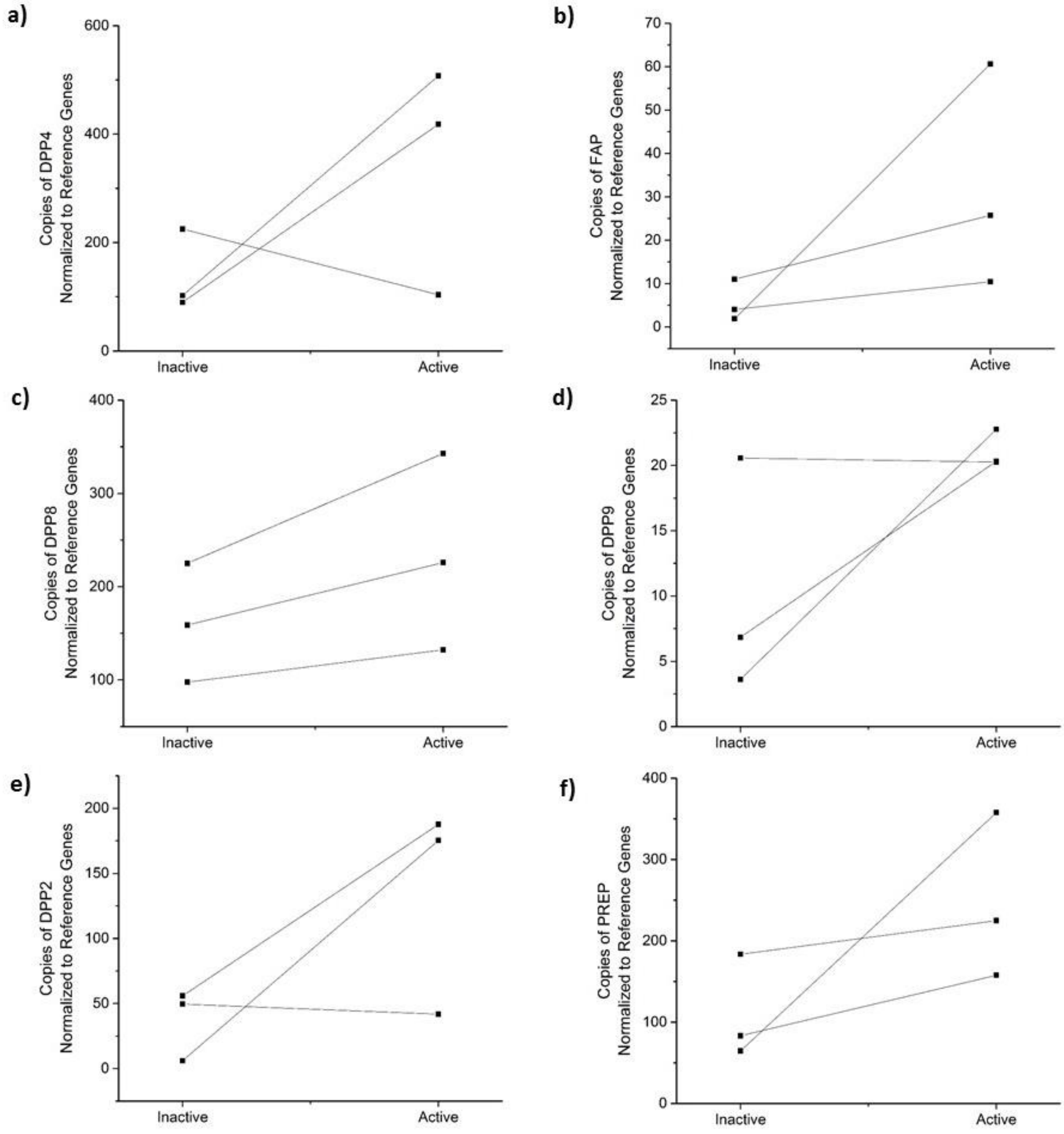

Figure 6.4.1 DPP mRNA in matched inactive and active colorectal tissue during IBD. Gene copy number for genes DPP4 **a)**, FAP **b)**, DPP8 **c)**, DPP9 **d)**, DPP2 **e)** and PREP **f)** was determined by qPCR and the use of external PCR standards. Gene copy number is expressed normalized to reference genes, PPIA and TATA. The presence of endoscopic inflammation was determined by an experienced gastroenterologist. Matched inactive and active tissue samples (n=3,n=2/CD, n=1/UC) are connected by lines.

6.8 Initial optimisation of DPP enzyme histochemistry

DPP enzyme histochemistry methods

Enzyme histochemical staining for DPP4 was performed as per previously published methods with minor modifications (Matsumoto et al., 1992)[21].10µM tissue sections were cut using the Lecia CM1850 cryostat and stored in an airtight container at -20ºC for less than a month before processing. Sections were fixed using acetone chloroform mixture (1:1; v:v) for 10 minutes at -20ºC. 3mg of H-Gly-Pro-4-MβNA (Bachem, Switzerland) was dissolved in *N,N*-dimethylformamide. 0.01g of Fast Blue RR salt was added to the substrate mixture and diluted in 5mL of 0.1M sodium phosphate buffer, pH 7.4. Sections were pretreated for 10 minutes at room temperature with 0.1M sodium phosphate buffer or 10µM sitagliptin to determine staining due to DPP4. Tissue sections were subsequently incubated for 30 minutes at room temperature with the staining solution. Slides were immediately rinsed in tap water and counterstained with Harris hematoxylin for 30 seconds (Fronine, Thermofisher Scientific). Slides were mounted in buffered glycerol and analyzed immediately using the BX53 Brightfield microscope. Images were obtained using the Olympus DP27 digital camera system and Olympus cellSens software package. Negative controls consisted of the staining solution without H-Gly-Pro-4-MβNA. Rat small intestine was included as a positive day control to monitor assay validity. Positive enzyme staining appears pink – red. Colour saturation of all images was increased to 200% to assist in the identification of positive enzyme staining regions.

[21] MATSUMOTO, Y., BISHOP, G. A. & MCCAUGHAN, G. W. 1992. Altered zonal expression of the CD26 antigen (dipeptidyl peptidase IV) in human cirrhotic liver. *Hepatology,* 15**,** 1048-53.

DPP enzyme histochemistry in rat small intestine

OCT embedded, snap frozen rat small intestine was used as a positive control for DPP enzyme histochemistry staining. Positive, red-pink staining (arrows) attributable to DPP4 was observed in the brush border of the intestinal villi (Figure 6.5.1). Some regions of the muscularis (arrows) also stained positive for DPP enzyme activity, these regions tended to be on the edge of the tissue section (Figure 6.5.1). 10μM sitagliptin reduced the amount of staining related to DPP4 activity, with less staining evident within the intestinal villi and reduced colour intensity associated with the muscularis (Figure 6.5.1).

Initial DPP enzyme histochemistry analysis of human colorectal sections show limited positive staining, with evidence of non-specific staining and brown-precipitate in majority of human colorectal samples (data not shown). Further experiments optimizing this technique for human colorectal samples are required. It is possible that this technique is may not be sufficiently sensitive to detect the low levels of DPP enzyme present in colorectal tissue. Further studies should also explore the potential for fluorescent probes to detect the presence of DPP activity in human colorectal tissues.

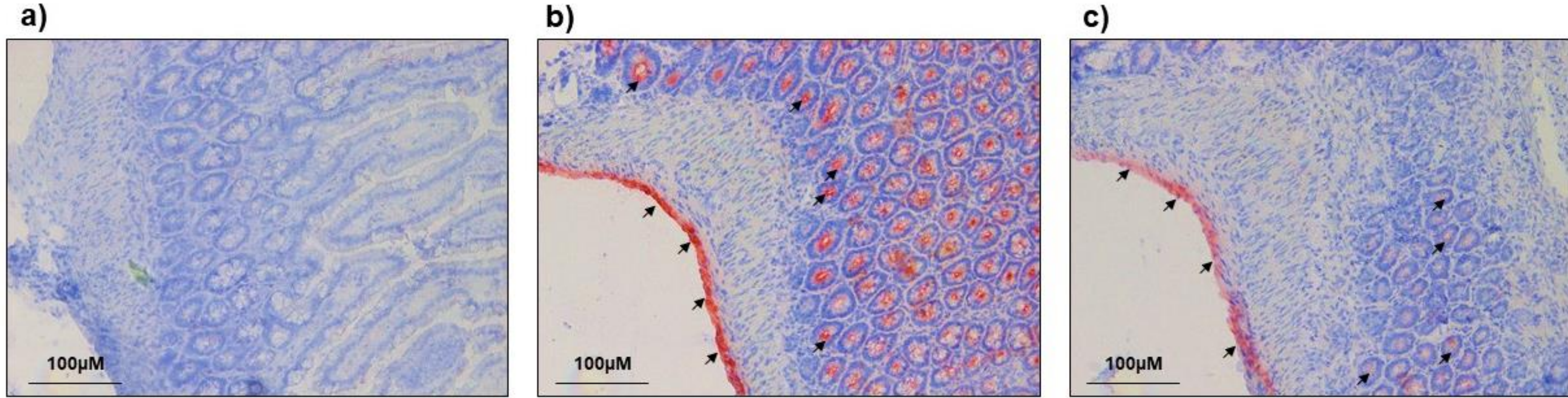

Figure 6.5.1 DPP4 enzyme histochemistry within rat small intestine. 10µM serial sections of rat small intestine were stained for DPP4 enzyme activity using the substrate H-Gly-Pro-MβNA. Three serial sections were stained representing the DPP negative control **a)**, DPP activity **b)** and 10µM sitagliptin treated **c)** sections. Images are representative of n=3 experiments on small intestine of healthy rats.

6.9 Preliminary study investigating DPP4 in neutrophils

The DPP family has broad physiological roles and has been implicated in adaptive, and more recently, innate immune responses. Our group has previously reported that the non-selective DPP inhibitors, Ile-Pyrr-(2-CN)*TFA and Ile-Thia were associated with lowered colonic myeloperoxidase activity in mice with DSS induced colitis (Yazbeck et al., 2010), suggesting that DPPs may modulate neutrophil recruitment or activity. Jungraithmayr *et al.* have also reported that DPP4 inhibition using AB192, reduced neutrophil infiltration in a model of lung graft failure (Jungraithmayr et al., 2010).

It has also been reported that DPP4 inhibitors may also affect specific neutrophil functions. Kroller-Schon *et al.* found that linagliptin dose-dependently reduced ROS production in LPS-stimulated neutrophils; however, DPP4 inhibitors, sitagliptin, vildagliptin, alogliptin and saxagliptin, had little effect on *in vitro* ROS production (Kroller-Schon et al., 2012). Linagliptin also reduced the adhesion of LPS-activated human neutrophils to an endothelial monolayer *in vitro* (Kroller-Schon et al., 2012), suggesting that DPP4 inhibitors may directly modulate neutrophil functions.

Neutrophils are a major component of the immune system and are the first responders against invading pathogens and microbes (Mocsai, 2013, Kumar and Sharma, 2010). Neutrophils are also implicated in chronic inflammatory responses (Smith, 1994), and have been associated with IBD (Verspaget et al., 1988, Nielsen et al., 1994) and asthma (Monteseirin, 2009). Understanding the roles of DPP4 in neutrophil responses and further characterizing the effects of DPP4 inhibitors on neutrophils may identify DPP4 inhibitors as a unique treatment modality for inflammatory diseases. With the increasing use of DPP4 inhibitors in type-2 diabetes, it is important to fully understand any potential off-target effects that these drugs may have.

These series of experiments conducted early within my PhD candidature aimed to investigate the role of DPPs during *in vitro* neutrophil responses of healthy donors. It was hypothesized that DPPs would have a specific role in neutrophil responses.

Materials & Methods:

Collection of patients and samples:

Venous blood was collected from anonymous adult donors presenting for blood donation (for routine diagnostic services) at the Women's and Children's Hospital under the guidelines and approval of the Women's and Children's Health Network Research Ethics Committee. Written informed consent was obtained from all participants. No identifying information or clinical information was collected for this group of donors.

Isolation of neutrophils from whole blood:

Using methods described previously by (Paton and Ferrante, 1983), whole blood was layered onto a Ficoll-Hypaque density gradient of specific gravity 1.114. Centrifugation of samples at 600 x g for 30-35 minutes resolved white blood cells into two distinct layers containing polymorphonuclear cells (PMNs) and mononuclear cells (MNCs). PMNs and MNCs were carefully harvested from appropriate layers and washed three times in RPMI 1640 prior to resuspension for functional assays.

Expression of CD26

The surface expression of CD26/DPP4 was quantified in freshly isolated neutrophils from healthy donors using flow cytometry techniques. Freshly isolated PMNs were resuspended in phosphate buffered saline containing 0.5% albumin. To assess CD26 expression in activated neutrophils, phorbol myristate acetate (PMA) ($1x10^{-5}$M) was used to activate PMNs for 20 minutes prior to staining with CD26-PE (#555437, BD Biosciences). T cells express present within the MNC layer express CD26 and were used as a positive CD26 antibody control. Resting MNCs were resuspended in phosphate buffered saline containing 0.5% albumin and

stained with CD45-PercP (#347464, BD Biosciences), CD3-FITC (#349201, BD Biosciences), CD8-PE-Cy7 (#557746, BD Biosciences) and CD26-PE. T cells were gated according to CD45, CD3 and CD8 expression. Fluorescence was measured using the BD FACs Canto (BD Biosciences, US) and analysed using BD FACs Diva Software (BD Biosciences, US). Data is expressed as the Median Fluorescence Intensity (MFI).

DPP Enzyme Activity in PMN and MNC lysates

Enzyme activity was quantified in freshly isolated PMN and MNC whole cell lysates. $1x10^7$ PMNs were activated with PMA, for 20 minutes. PMNs and MNCs were immediately lysed in ice-cold RIPA buffer (Sigma Aldrich) and purified by centrifugation to remove the nucleic acid precipitates.

DPP enzyme assay were performed as per previously described methods in Chapter 2. Enzyme activity is expressed as µM of pNA released / minute / mg of protein (U/mg of protein). Protein concentrations were determined using Pierce BCA assay to (Thermofischer Scientific) as per manufacturers' instructions.

Viability assay

Immune cell viability during DPP4 inhibitor treatment was assessed by 7-aminoactinomycin D (7-AAD) (BD Pharmigen) staining. Following treatment with various concentrations of DPP4 inhibitors, 7-AAD was added to the cell suspension and incubated in the dark before analysis on the BD FACS Canto. Increased fluorescence indicates the presence of dead cells.

Oxidative Burst

Oxidative burst was assessed using the dihydrorhodamine-123 (DHR-123) assay. $5x10^5$ PMNs were incubated with 1µM, 10µM or 100µM DPP inhibitor and 2µM DHR-123 for 15 minutes in the dark at 37°C. PMNs were then stimulated with $1x10^{-5}$M PMA for a further 15 minutes. Cells were then washed, resuspended and analyzed by flow cytometry using the BD FACS Canto. Neutrophil populations were gated according to forward scatter and side scatter. The median fluorescence intensity (MFI) of the FITC channel is proportional to the production of oxidative species. Data is presented as ROS production relative to controls.

Adhesion assay

CD11b (Mac-1) is an integrin expressed by neutrophils with major roles in *in vitro* adhesion (Detmers et al., 1990) and its expression was used as an indicator of neutrophil adhesion. Freshly isolated PMNs were treated for 15 minutes with DPP inhibitors at final concentrations of 1µM, 10µM or 100µM. The PMNs were then stimulated with PMA for 15 minutes, before subsequent incubation for 20 minutes with anti-human CD11b-PE. Cells washed & centrifuged before analysis on the BD FACS Canto.

Results

CD26 expression in neutrophils

Prabhash *et al.* previously reported DPP4/CD26 surface expression in circulating immune cells including neutrophils from cancer patients and healthy donors (Prabhash et al., 2010). The roles of CD26 in neutrophil responses; however, remain unclear. CD26 expression was high on the surface of resting MNCs (2,682.8 ± 262.7, median fluorescent intensity (MFI) ± standard deviation, n=1), while much lower levels of CD26 where found on the surface of resting PMNs from all donors (Table 6.4.1). Stimulation of PMNs with PMA increased CD26 expression in a single donor, while in two separate donors CD26 expression remained unchanged (Table 6.4.1).

Table 6.4.1 CD26 expression on the surface of PMNS. CD26 expression was determined on the surface of resting and PMA stimulated PMNs isolated from three separate donors, using flow cytometry

	Donor 1	Donor 2	Donor 3
Resting	83 ± 125	134 ± 262	197 ± 75
PMA stimulated	270 ± 133	126 ± 130	172 ± 128

Data presented as Median Fluorescence Intensity (MFI) ± coefficient of variation from a single experiment.

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DPP enzyme activity in neutrophils

DPP enzyme activity was quantified in PMN and MNC lysates. DPP activity was detected in resting MNC lysates, and approximately 70% of DPP activity was inhibited by sitagliptin (Figure 6.6.1a). Resting PMNs were found to have less than half the activity of resting MNCs, with only 7% of DPP activity inhibited by sitagliptin (Figure 6.6.1b). However, DPP activity more than doubled in PMNs stimulated with PMA compared to resting cells, with up to 44% of activity inhibited by sitagliptin (Figure 6.6.1b).

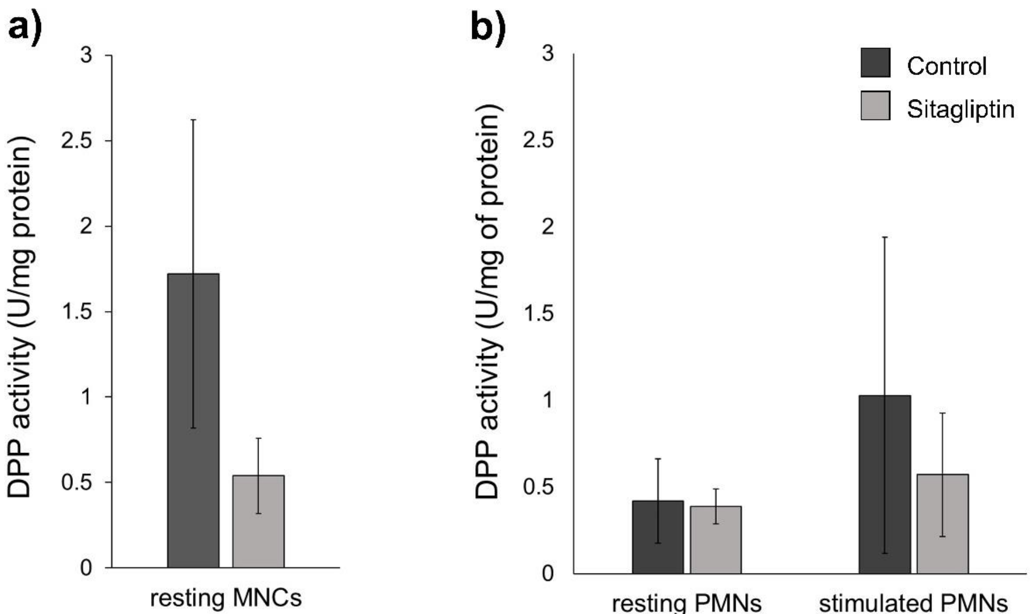

Figure 6.6.1 DPP enzyme activity in MNCs and PMNs. DPP enzyme activity was quantified in freshly isolated MNCs (n=4) **a)** and PMNs (n=3) **b)** from healthy donors. DPP activity was measured in resting (n=3) and PMA stimulated (n=2) PMNs using 1mM H-Ala-Pro-pNA, 10mM Tris-NaCl, pH 8.1. Selective DPP4 inhibitor, sitagliptin was incorporated into assays to determine selective DPP4 activity. Data presented is mean ± stdev.

Functional consequences of DPP4 inhibition

All donor PMNs produced ROS in response to PMA (Figure 6.6.2). The addition of DPP4 or DPP8/9 inhibitors produced varied responses in patients, with some trends towards elevated ROS production seen amongst the treated donor neutrophils (Figure 6.6.2). Conversely, treatment with non-selective DPP inhibitor P32/98 at 100µM appeared to reduce ROS production by approximately 20% (Figure 6.6.2).

Using CD11b as a marker of *in vitro* neutrophil adhesion, we found that stimulation with PMA and LPS upregulated the expression of CD11b on the surface of donor PMNs (Table 6.4.2). Pre-treatment with linagliptin did not alter the surface expression of CD11b in resting or stimulated PMNs *in vitro* (Table 6.4.2).

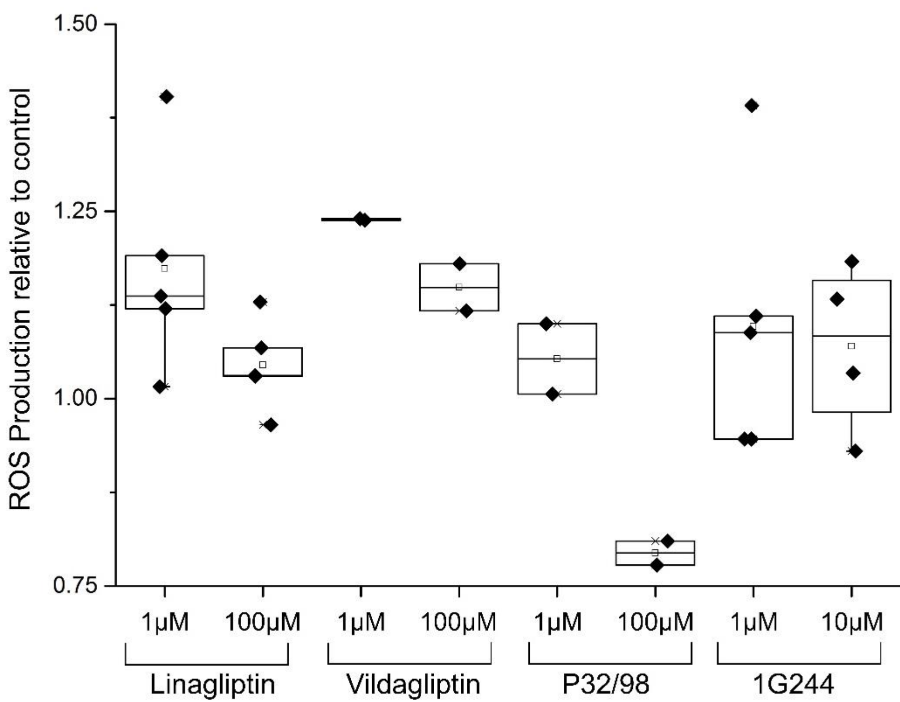

Figure 6.6.2 Effect of DPP inhibition on *in vitro* ROS production. PMNs isolated from healthy donors (n=2-6) were treated with control buffer, DPP4 inhibitors (linagliptin, vildagliptin), a DPP8/9 inhibitor (1G244) or a non-selective DPP inhibitor (P32/98) prior to stimulation with PMA. ROS production was measured using DHR-123 dye, resultant fluorescence in FITC channel is proportional to ROS production. ROS production is presented relative to control. Box plot overlays represent median and IQR, whiskers represent range excluding outliers. Cell viability > 90% for all treatments.

Table 6.4.2 Effect of linagliptin on CD11b expression in PMNs

	Resting	PMA stimulated	LPS stimulated
Control	4,919±72.9	17,594±43.8	16,355±50.6
1µM linagliptin	5,963±79.7	17,712±47.3	17,301±48.4
100µM linagliptin	4,894±70.2	18,121±38.4	16,391±60.5

Data shows the MFI ± % coefficient of variation. Data is from a single representative donor. Experiment was repeated in n=3 separate donors.

Discussion

This preliminary study suggests that DPP4 inhibitors may modify neutrophil functions *in vitro*; however, further studies are required to decipher the specific mechanisms by which DPPs regulate neutrophil responses.

The original aims of this study were to determine the role of DPPs in neutrophil functions in healthy individuals and patients with IBD. Neutrophil functions such as chemotaxis have been shown to decay over time *in vitro* (Ferrante et al., 1980), suggesting the need for neutrophil functional assays to be conducted immediately following blood collection. Unfortunately, throughout patient recruitment as part of the study detailed in Chapter 4, there was substantial delay in processing blood samples that limited the assessment of neutrophil functions from these patients. Instead, PMN and MNC fractions were harvested from patients and stored with the potential for later profiling of surface markers and DPP enzyme activity in these patients. This work is yet to be commenced.

Throughout this preliminary study, we found CD26 expression, DPP activity and the effect of DPP inhibitors on ROS production varied amongst our donor population. The variation in our results could have been related to errors in methodology or may suggest the presence of patient differences. The anonymous nature of the healthy donors prevented any stratification of the data and identification of any obvious physiological differences that may have influenced neutrophil functions. It is also important to note that in these preliminary experiments assessing CD26 expression, an isotype control was not included. As a result, CD26 expression could be considerably lower than anticipated from this data. It is vital that the reproducibility of this data be assessed in larger donor cohorts with appropriate controls. Furthermore, due to the limited amount of blood donated for research purposes of which this study was included, it was not

possible to conduct all neutrophil functional assays in each donor. Furthermore, assessing multiple functions in each donor may help to explain some of the variations between patients.

Neutrophil adhesion is a critical step in neutrophil extravasation and infiltration, and patients with neutrophil adhesion defects experience severe and recurrent infections (Etzioni et al., 1992). CD11b (Mac-1) is a major integrin that is upregulated on the surface of activated neutrophils (Detmers et al., 1990) and that roles in binding fibrinogen and ICAM-1 (Diamond and Springer, 1993). In the study by Kroller-Schon *et al. in vitro* neutrophil adhesion to endothelial cells was assessed using LPS as a stimulant, finding much lower levels of adhesion in linagliptin treated cells (Kroller-Schon et al., 2012). However, our study found no change to the expression of CD11b in resting, LPS stimulated or PMA stimulated neutrophils treated with linagliptin. The lack of variation to CD11b expression following treatment with linagliptin may suggest that linagliptin alters neutrophil adhesion via other mechanisms. Consequently, future studies should look to examine the expression of other major neutrophil integrins LFA-1 or p150,95 and endothelial adhesion molecules ICAM-1, as well as E-selectin and P-selectin (Diamond and Springer, 1993, Futosi et al., 2013) to better decipher the mechanisms by which DPP4 inhibitors alter neutrophil adhesion.

Neutrophil respiratory burst plays a vital role in intracellular killing, and disruption to this pathway is associated with defective microbial killing and clearance (McCaffrey and Allen, 2006). DPP4 inhibition was previously found to reduce the respiratory burst of neutrophils induced by lipopolysaccharide and zymosan A [3]. During an *in vivo* model of sepsis, the authors also reported changes to vascular ROS production and myeloperoxidase content within the vasculature (Kroller-Schon et al., 2012), which could suggest broad roles for DPPs in mediating ROS production in multiple cell types. Our data suggests some trends towards increased ROS production following DPP4 inhibitor treatment, however, the variation between

individual donor responses, combined with the small sample size highlight the need for further studies in a larger number of donors. Interestingly, P32/98 when used at 100µM reduced ROS production in two separate donors. P32/98, also known as Ile-Thia (Yazbeck et al., 2010), is a non-selective DPP inhibitor (Lankas et al., 2005) and can inhibit activity relating to DPP4, DPP8 and DPP9, which may have contributed to the lower levels of ROS in treated donor neutrophils. However, this is a preliminary result that needs to be replicated in larger numbers of donors.

The expression and activity of DPP8 and DPP9 has been previously identified in lymphocytes and monocyte/macrophages (Maes et al., 2007), with studies suggesting major roles for DPP9 in macrophage responses (Matheeussen et al., 2013). Our study identified DPP activity present in resting and stimulated neutrophils that was not inhibited by sitagliptin, and could be related to other DPP enzymes including DPP8/9. Characterizing the expression and activity of other DPP enzymes using western blotting and applying the selective DPP8/9 inhibitor, 1G244 during enzyme activity analysis will help to determine the main DPPs present expressed by neutrophils.

While *in vitro* studies provide a convenient platform for investigating the role of DPP enzymes in neutrophil functions, it is important to acknowledge that many of these studies are unlikely to accurately reflect the roles of DPP enzymes or DPP4 inhibitors on *in vivo* neutrophil responses. In our studies, functional assays were conducted on purified neutrophil preparations, reflecting the sole functions of neutrophils and does not consider the complex interactions that exist *in vivo* between neutrophils, other cells and soluble factors. Furthermore, we also observed considerable variability in donor responses, this may have been related to the variability in age of our donors, highlighting the need for further studies in healthy, age-matched donors. While understanding the roles of DPPs in specific neutrophil functions is

important, identifying any clinically relevant effects of DPP4 inhibitors on the immune system and the implications for type-2 diabetic patients is imperative and future studies in DPP4 inhibitor treated animals could examine the overall ability of animals to cope and clear bacterial, viral and parasitic infections. Given the increasing role of DPP4 inhibitors as anti-hyperglycaemic agents, studies could also look to characterize and monitor the functions of immune cells isolated from type-2 diabetic patients undergoing DPP4 inhibitor therapy, providing a unique insight into the impact of chronic DPP4 inhibition on immune responses.

The data presented here is in its preliminary state though suggests a role for DPP enzymes in major neutrophil functions such as the production of ROS. One of the major limitations of this study was the small sample sizes, the limited functions examined. Further investigations should build upon this study, characterizing DPP expression and activity in neutrophilic cell lines (HL 60) as well as larger numbers of donors. Further exploration of DPP4 inhibition and its effects on neutrophil functions will help to define the roles for DPP4 and identify whether DPP4 represents a suitable therapeutic target for inflammatory diseases.

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