



**Towards local delivery of therapeutics for the
treatment of eosinophilic oesophagitis**

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PhD Thesis**

Declaration

‘I, Farhan Kayyum Taherali, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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~ Hussein, I hope this contribution signifies the importance of knowledge and that one should strive to make a difference in whatever they do.

Most of all, do what you love and love what you do ~

Abstract

Eosinophilic oesophagitis (EoE) is a chronic inflammatory disease of the oesophagus leading to lifelong morbidity. EoE has an increasing prevalence with the current mainstay of therapy being oral corticosteroid, budesonide, intended for topical action. This contribution examines the potential for local application of tacrolimus and monoclonal antibody (mAb), infliximab to the oesophagus. Tacrolimus, is used topically for atopic dermatitis and has shown to ameliorate, IL-5 and IL-13 in a mouse model of EoE whereas mAbs are administered parenterally for treating EoE. Local application of tacrolimus and mAbs to the oesophagus, would serve as options for non-responder EoE patients or those suffering from side effects of steroids. Using permeation system was used to develop an ex-vivo injury model to mimic the histopathology of EoE; dilated intercellular spaces. Porcine oesophageal tissue (surrogate for human) was exposed to biorelevant gastroduodenorefluxate elements, until the drop in transepithelial electrical resistance (TEER) was irreversible, indicating opening of tight junctions. Tissue and basolateral accumulation after 30 minutes, was quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS) for budesonide and tacrolimus and ELISA for infliximab. A significant increase ($p < 0.05$) in tissue accumulation of tacrolimus (approx. 2-fold) and infliximab (approx. 7-fold) was observed in the injured tissue versus control but no significant difference ($p > 0.05$) was observed for budesonide. This led to the hypothesis that the efficacy of budesonide formulations may be owing to a systemic rather than local effect. However, the poor perfusion of the oesophagus challenged this hypothesis. Tail vein budesonide injection into the Sprague Dawley rat (similar oesophageal perfusion to human) showed that budesonide accumulated in the oesophagus. It is likely that currently available formulations of budesonide are absorbed and acting systemically. Molecules like tacrolimus and mAbs owing to their greater tissue accumulation in an injured epithelium, are better suited towards site specific local delivery to the oesophagus while avoiding systemic side effects.

Impact statement

Eosinophilic oesophagitis (EoE) is a chronic inflammatory disease of the oesophagus affecting people of all ages. The prevalence of EoE across Europe and North America is 450,000 and steadily increasing since the term was coined in 1998. The disease affects quality of life owing to painful swallowing and food impaction. Oral corticosteroids have been the mainstay of therapy for local treatment of EoE; budesonide viscous suspension (BVS) is used off label and recently a budesonide orally dissolving tablet (BET) has gained regulatory approval under 'orphan designation' in the EU.

Budesonide has been the drug of choice for topical use, owing to its high mucosal to systemic affinity ratio. Side effects such as adrenal suppression have been reported from prolonged use of BVS and plasma $C_{\max}$ of 0.44 ± 0.31 ng/mL have been reported from a single 1mg dose of BET in healthy subjects. Monoclonal antibodies (mAbs) of varying target specificities have undergone clinical trials with anti IL-4 and anti IL-13 (Dupilumab) showing best in class efficacy for treatment of EoE. However, mAbs are administered parenterally and have drawbacks of severe systemic side effects namely immunosuppression and cancer. Furthermore, only a small fraction of the dose may distribute into the oesophagus. EoE is classed as an atopic disease. Tacrolimus is used in moderate to severe atopic dermatitis and has shown to ameliorate IL-5 and IL-13 in a mouse model of EoE.

The work in this thesis uses highly sensitive analytical techniques (LC-MS/MS and ELISA) to quantify the accumulation of budesonide, tacrolimus and mAb (infliximab) directly in porcine oesophageal tissue (a surrogate to human oesophageal mucosa). An ex-vivo model using porcine oesophageal tissue; mimicking the hallmark feature of EoE, the dilated intercellular spaces (injured tissue) was developed using Ussing diffusion chambers. When permeation was compared between the intact and injured tissue, tacrolimus and infliximab showed a ~2 and ~7 fold greater tissue accumulation respectively, while no difference was observed for budesonide. To our knowledge, this work has for the first time shown the ability of a 150 kDa mAb, budesonide and tacrolimus to permeate the oesophageal epithelium. BVS has a short transit through the oesophagus with majority of the dose deposited in the stomach. Conversely, as

suggested by its pharmacokinetics, Jorveza[®] dissolves and probably undergoes buccal absorption and avoidance of the first pass hepatic metabolism. Their efficacy may arise majorly owing to a systemic effect. However, the concern that the oesophagus is a poorly perfused organ challenged the hypothesis of a systemic effect. Using the rat, it was shown that budesonide can accumulate in the oesophagus. The implications of these results support the hypothesis that the currently available formulations of budesonide are not offering exclusively a topical action. Molecules like tacrolimus and mAbs owing to their greater tissue accumulation in an injured epithelium, are better suited towards site specific local delivery to the oesophagus while avoiding systemic side effects.

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List of abbreviations:

ADCC – antibody dependent cellular cytotoxicity
ADCP- antibody dependent cellular phagocytosis
ANOVA – Analysis of variance
APC – antigen presenting cells
BET – budesonide effervescent tablet
BVS – budesonide viscous suspension
CDC – complement dependent cytotoxicity
CYP3A4 – cytochrome P450 3A4
cGVHD – chronic graft versus host disease
DLS – Dynamic light scattering
ELISA – Enzyme linked immunosorbent assay
EoE – eosinophilic oesophagitis
Eos - eosinophil
eos/hpf – eosinophils per high power field
GERD – gastro oesophageal reflux disease
HPLC - SEC – High performance liquid – size exclusion chromatography
HPLC – High performance liquid chromatography
IFX – infliximab
IL - interleukin
KBR – Kreb’s bicarbonate ringer’s solution
LC-MS/MS – liquid chromatography tandem mass spectrometry
mAb – monoclonal antibody
mHanks – modified Hank’s buffer
MRI - magnetic resonance imaging
MRM – multiple reaction monitoring
P-gp – P- glycoprotein
PET-CT – positron emission computed tomography
SGF – Simulated Gastric Fluid
SMG – sub mucosal glands
TEER – transepithelial electrical resistance
Th2 – T helper 2 cells
TNF - α - Tumor necrosis factor alpha
TSLP – Thymic stromal lymphopoietin

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CHAPTER 1 : INTRODUCTION

1.1 Overview

Drug delivery research encompasses the development of new technologies to improve therapeutic outcomes. Gastro-retentive systems as well as delayed release systems such as colon specific drug delivery systems are available for local treatment of diseases affecting the gastrointestinal tract (Durán-Lobato et al., 2020, Kirtane et al., 2018). However, the oesophagus (food pipe) is a forgotten organ despite being affected by diseases spanning from inflammation to cancer; this is mainly owing to the challenge of local delivery owing to short transit time (Batchelor, 2005). One such disease that affects people of all ages is eosinophilic oesophagitis (EoE), a chronic inflammatory disease of the oesophagus which has been coined since the year 1998 and consensus recommendations published in 2007 (Shaheen et al., 2018). A brief description of the history of EoE and coining of the term is illustrated in Figure 1-1. The abbreviation used for eosinophilic oesophagitis in the UK and rest of the world is EoE. However, some authors from the USA write it as EE. This probably arises as a result of the way **oesophagus** is spelled in the UK as opposed to USA where it is **esophagus**.

EoE presents with symptoms such as difficulty and pain during swallowing (dysphagia), vomiting and food impaction due to strictures (Furuta and Katzka, 2015). The prevalence of EoE is about 0.5 million people across Europe and North America with a rising incidence across Japan and Australia (Dellon, 2014, Dellon et al., 2014). Overall, incidence rates for EoE are coming steadily closer to that recently reported for Crohn's disease in North America, Europe, Switzerland and Spain (Molina-Infante et al., 2017). The increasing incidence and therefore prevalence is as a result of tools and expertise available to diagnose EoE. Off-label budesonide viscous suspension is currently the mainstay of treatment. However, recently, an orally dissolving tablet (ODT) of budesonide, Jorveza® has gained regulatory approval in the EU including orphan designation (Lucendo, 2020).

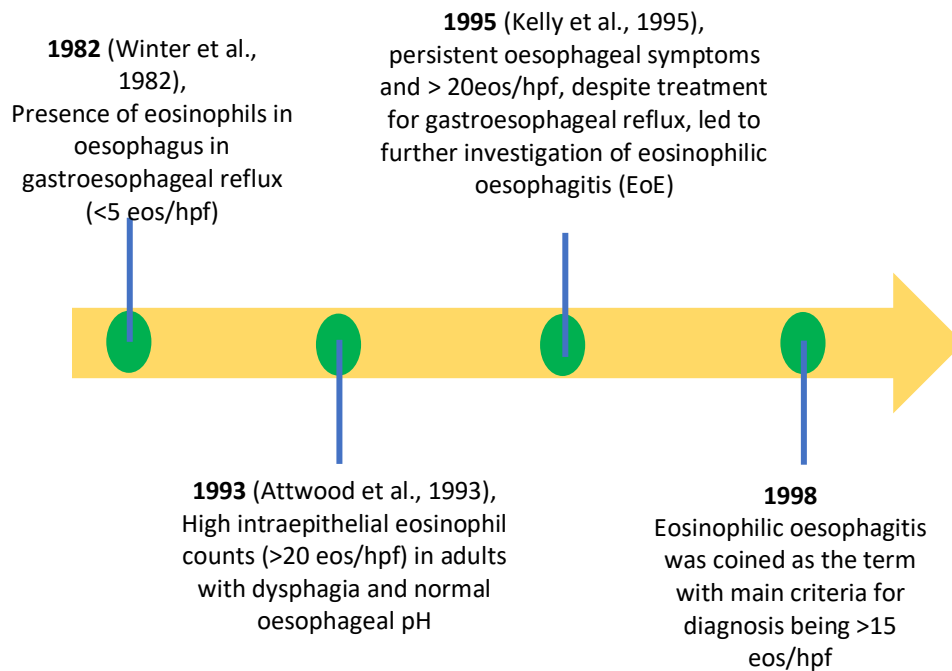


Figure 1-1: History of timeline for eosinophilic oesophagitis (EoE)

The oesophagus has a very short transit time in the order of a few seconds compared to other regions of the gastrointestinal tract which is in the order of a few hours; making the desired local application of drugs extremely challenging (Batchelor, 2005). Significant efforts have been made towards understanding how to increase the residence time of formulations for drugs to exert their local effect on the oesophagus. Various bioadhesive polymers have been studied both on ex-vivo porcine tissue using a flow model and in vivo in humans by using imaging techniques such as endoscopy and gamma scintigraphy (Richardson et al., 2005, Batchelor et al., 2002, Collaud et al., 2007, Dellon et al., 2012, Laquintana et al., 2019). However, while all these endeavours have led to treatment modalities such as oral gels, viscous liquids, buccal tablets, oral films, orally dissolving tablet, chewable tablets and chewing gums; none of these are actually proven to exert their effects entirely locally. This observation comes from the fact that systemic side effects and poor efficacy rates are observed when these drug delivery approaches are used (Zhang et al., 2008).

Budesonide was selected to be used off-label on the basis of its efficacy in other atopic conditions like asthma, due to the high mucosal to systemic affinity ratio (Brattsand R

Fau - Thalén et al., 1982). However, side effects such as adrenal suppression have been reported owing to its prolonged use as a viscous suspension in EoE (Golekoh et al., 2016, Harel et al., 2015). On this note, it is questioned whether the efficacy of oral forms of budesonide is really due to local action as a virtue of its properties. Therefore, the aim is to understand the permeation of drugs in an ex vivo model mimicking histopathological features of EoE and compare against the ‘gold standard’ budesonide since it is used in clinic. Drugs such as tacrolimus which is a BCS class II drug with a poor solubility is 0.00402 mg/mL but high logP of 3.3 and infliximab (a large 150kDa monoclonal antibody), have poor or no gastrointestinal absorption and therefore should mainly act locally. Furthermore, tacrolimus is a potent drug used at very low dosages of 0.1% w/w in atopic dermatitis. 1 gram ointment contains 1mg tacrolimus normally applied over a surface the equivalent of two adult’s hand (approximately 260 cm²) (Agarwal and Sahu, 2010). Therefore, 1mg can be the dose applied to the oesophagus which has a surface area of 200cm² (Helander and Fändriks, 2014). Tacrolimus ointment applied to adults with moderate to severe atopic dermatitis achieved tissue levels of about 100 ng/cm³ after 24 hours but none achieved systemic tacrolimus exposure above 1ng/ml (Undre et al., 2008). The blood trough concentrations of tacrolimus is 5ng/ml. Therefore, the 1mg dose can be extrapolated to treat EoE which is also an atopic inflammatory disease. Tacrolimus has recently shown to ameliorate EoE via suppression of IL-5 and IL-13 in an *Aspergillus fumigatus* mouse model of EoE (Kandikattu and Mishra, 2020). As such, it is expected that a low dose in the order of 1mg per day would exclusively act locally and not achieve therapeutic concentrations. This is because it would largely undergo first pass tissue metabolism and therefore not elicit a systemic side effect (Lemahieu et al., 2005). Monoclonal antibodies are large 150kDa hydrophilic therapeutic proteins which have high target affinity. However, these molecules are generally given at high concentrations via the parenteral route to cater for the poor tissue distribution. Major drawbacks of mAbs given parenterally include adverse events like immunosuppression, cancer, severe conjunctivitis and cardiac events. Furthermore, there are also reports of loss of response owing to anti-drug antibodies (ADAs) (EMC, 2019a). However, monoclonal antibodies have the potential to be applied directly, locally to the oesophagus at lower concentration, of course dependent on permeation rate and time. TNF- α is upregulated in EoE and highly expressed by epithelial cells

of the oesophagus in patients with active EoE (Straumann et al., 2008b). Furthermore, infliximab is an IgG1 monoclonal antibody and it can be used to investigate the permeation of monoclonal antibodies (mostly of IgG class) thereby serving as a surrogate. On reaching the stomach mAbs would get degraded owing to the low pH and proteolytic enzyme, pepsin. This therefore poses an advantage thereby preventing their systemic uptake and preventing adverse events.

Table 1-1: Physicochemical properties of budesonide, tacrolimus and infliximab ((Goyon et al., 2017, DrugBank, 2021b, DrugBank, 2021a)

Drug	Log P	Solubility (mg/ml)	Isoelectric point	Molecular weight (g/mol)
Budesonide	2.42	0.0457	-	430.5
Tacrolimus	3.3	0.00402	-	804.0
Infliximab	-0.441	Freely soluble	7.7	144190.3 Da

1.2 Anatomy and physiology of the oesophagus

The oesophagus is a tubular structure connected to the pharynx and is approximately 2.5 cm in diameter and 25 cm in length, providing a pathway via which food and drink are carried to the stomach. The surface area is estimated to be about 200 cm² (Helander and Fändriks, 2014). It is lined by non-keratinized squamous epithelium with an average thickness of 500-800µm, lying over a lamina propria and the muscularis mucosa. The squamous epithelium is a tough lining able to resist distress caused by bolus abrasion (Diaz del Consuelo et al., 2005). The top third of the oesophagus is made of striated and smooth muscle therefore undergoing voluntary contractions whereas the bottom two-thirds is smooth muscle so involuntary contraction (Braden Kuo, 2006).

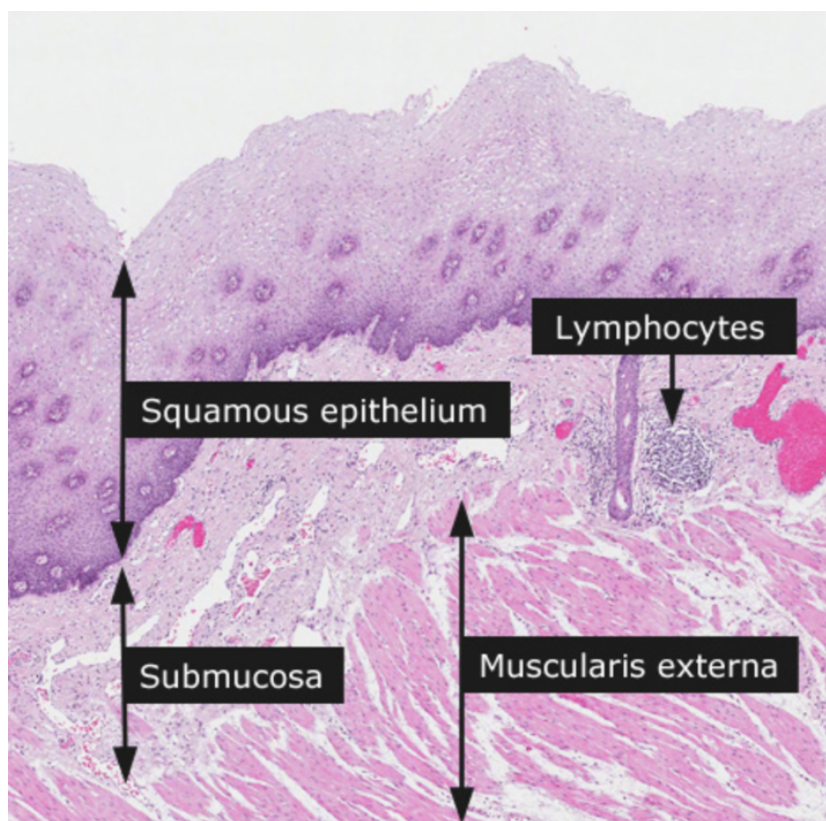


Figure 1-2: Histology of the human oesophagus (Atlas, 2020)

Salivary glands secrete mucins MUC5B which lubricate the masticatory bolus which can easily propel through the oesophagus. The oesophagus has submucosal glands which secrete a bicarbonate rich fluid but is not mucin like in nature. The pH within

the oesophagus is similar to that of saliva at about 6-7 (Neena Washington, 2001). The oesophageal sub-mucosal glands (SMGs) reside, within the submucosa of the mammalian oesophagus. Each SMG culminates in a single duct, which collects the acinar secretions and delivers them to the oesophageal lumen. It was shown that the SMGs secrete water, electrolytes (including bicarbonate ions), mucin like substance, epidermal growth factor and prostaglandins. It also secretes mucin like substances as evidenced by staining of the cells of the SMGs by Periodic acid Schiff and Alcian blue (Abdulnour-Nakhoul et al., 2007, Orlando, 2006). The secretion of mucin like substance from the SMGs show that it is released at a rate of $0.23 \pm 0.03 \text{ mg cm}^{-2} \text{ min}^{-1}$ (Namiot et al., 1994). However, it is unlikely that this mucin has a role in protecting the normal oesophagus against acid reflux. In a study by Dixon et al., it was observed that there was a complete absence of adherent mucus gel layers on normal human, pig, and rat oesophagi. They concluded that a secretory mucin, with an analysis distinct from that of gastric or salivary mucin, is present in very small quantities on the oesophageal mucosa and in amounts insufficient to form an adherent gel layer (Long and Orlando, 1999; Dixon *et al.*, 2001).

1.3 Transit time and contractility

The oesophagus acts as a conduit to the passage of solid bolus and liquids. Swallowing is a highly complex process with a normal adult swallowing 100-600 times per day. One-third of these events accompany eating and drinking and the remaining events occur when breathing out. Less than 10% of swallows occur during sleep (Wilson et al., 2011). During a swallow the upper oesophageal sphincter relaxes and the peristaltic wave is generated at 2-6 cm/s (Neena Washington, 2001). The lower oesophageal sphincter relaxes to allow for the passage of food into the stomach. When swallows are repeated in quick succession, then the contraction of the oesophagus is inhibited temporarily. The oesophageal transit time as seen in Figure 1-3 is in the order of 10-15 seconds with varying pressures generated by the sphincters and mid region. The maximal residence is along the distal part of the oesophagus as seen in Figure 1-4.

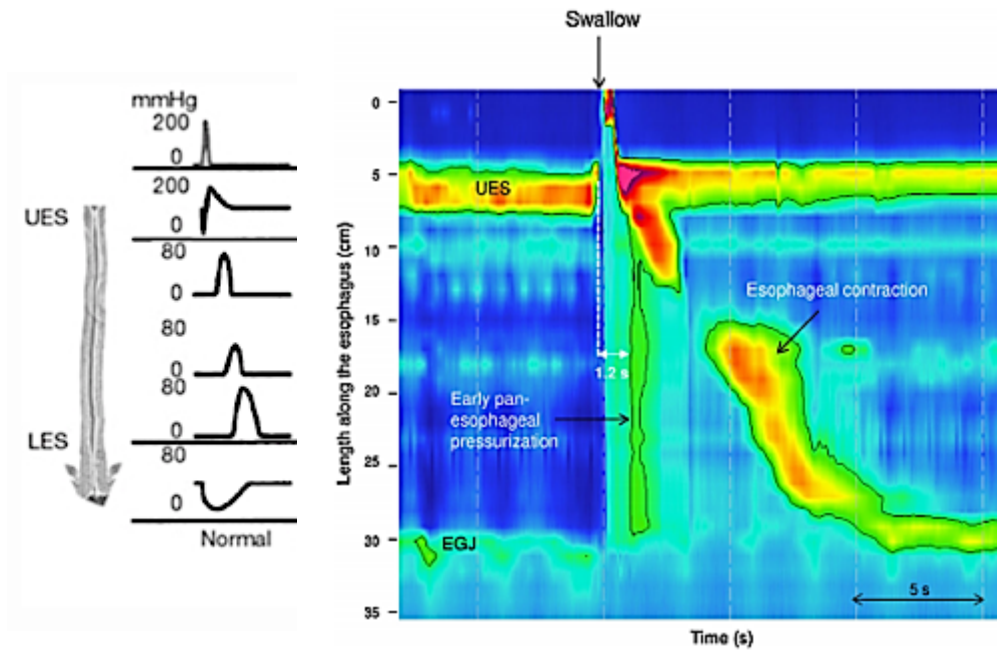


Figure 1-3: Oesophageal manometry across the length of the oesophagus after a normal swallow (Kasper and Harrison, 2005, W.G. Paterson, 2006)

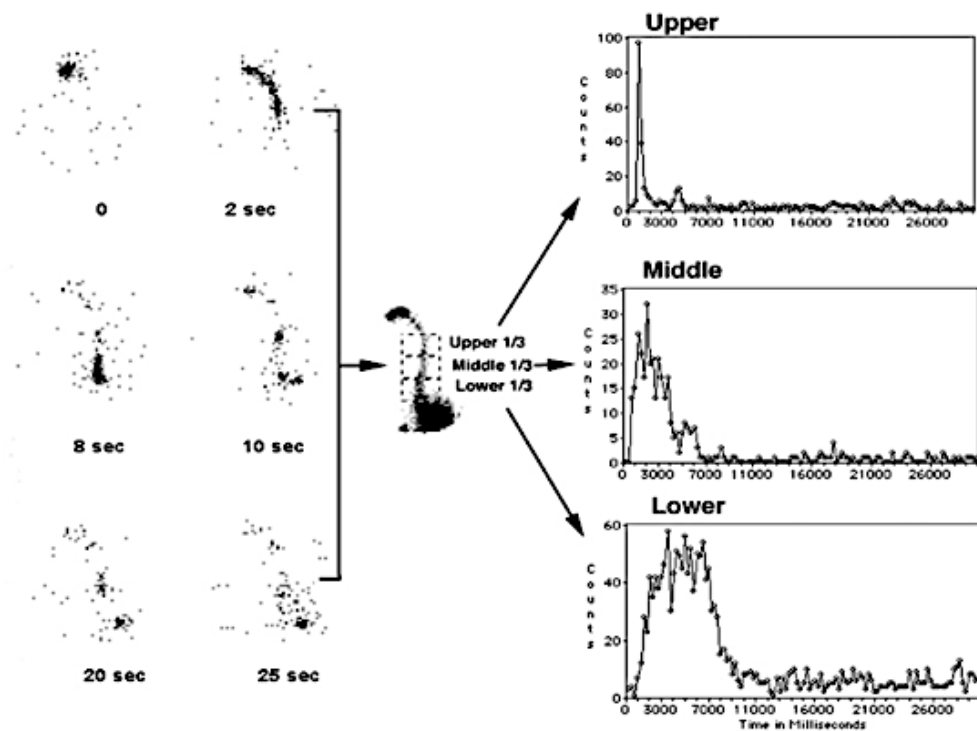


Figure 1-4: Sequential dynamic images by gamma scintigraphy demonstrate normal bolus transit through the oesophagus. In the right, time activity curve shows counts in each region indicating residence of the bolus in that region (Maurer, 2015)

Various studies as seen in Table 1-2 using magnetic marker monitoring and video fluoroscopy (Osmanoglou et al 2004) have been performed to assess the transit of food bolus and dosage forms (size 2 gelatine capsule) through the oesophagus. Residence time is generally in the order of a few seconds although the supine position does have a marginal prolonging effect on transit time. Furthermore, a greater retention rate of capsules was observed when taken with low volumes of water and in supine position.

Table 1-2: Effect of body position and liquid bolus volume on oesophageal transport of solid dosage forms (size 2 gelatine capsules) (Osmanoglou et al., 2004)

S – supine, U - upright

Quantity	5ml		25ml		50ml	
	S	U	S	U	S	U
Velocity (cm/s)	2.3	7.5	10.2	12.4	23	40
Transit time (s)	8	7	9	7	6	3
Retention rate	60%	45%	35%	7%	5%	0%

1.4 Diseases affecting the oesophagus

The oesophagus although a conduit for the passage of food and medicines (tablets, capsules and oral solutions/suspensions etc.), is a crucial organ to the overall health of an individual. Inability to administer food due to diseases affecting this organ, affects nutrient intake via food thereby leading to malnutrition. Like other lower gastrointestinal regions; the oesophagus is also affected by infectious diseases such as oesophageal candidiasis, motility disorders such as achalasia, diffuse oesophageal spasm and nutcracker oesophagus, malignancies (oesophageal cancer being the 14th most common cancer affecting the UK) and inflammatory conditions such as gastroesophageal reflux disease and eosinophilic esophagitis Figure 1-5 (Cancer ResearchUK, 2020, Zhang et al., 2008).

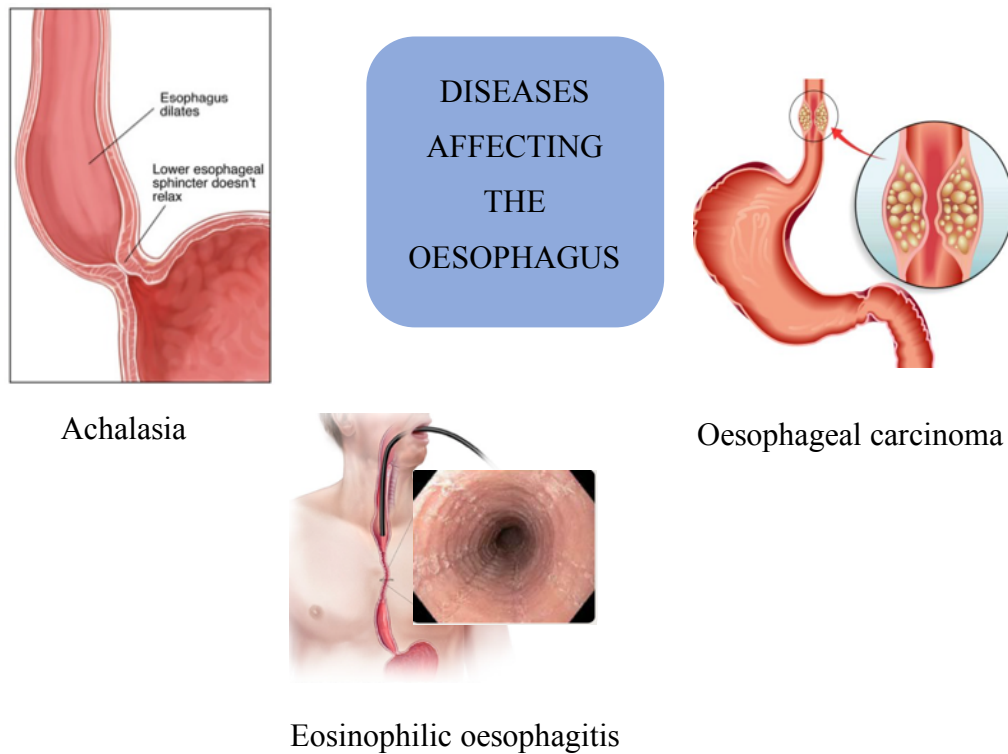


Figure 1-5: Representation of some diseases affecting the human oesophagus (Bariatrics, 2020, Furuta and Katzka, 2015)

From the diseases mentioned, eosinophilic esophagitis (EoE) is the disease that has been the sole focus of the work described in this thesis. EoE is a chronic disease affecting people of all ages, i.e. children to adults, and there is a clear unmet medical need (de Rooij et al., 2019). The mainstay of therapy is currently budesonide only. Budesonide has shown to have local and systemic side-effects and corticosteroid resistance occurs. Currently, no FDA approved product for topical use is available on the market as therapeutic option for EoE. However, Jorveza[®], an orally dissolving tablet of budesonide, has gained regulatory approval in the EU under orphan designation. Therefore, the aim is to investigate the potential of the local delivery of other therapeutic agents, including tacrolimus and monoclonal antibodies, such as infliximab.

1.5 Pathophysiology of EoE

An increased number of eosinophils in the oesophageal epithelium, which is a mucosa that is typically devoid of eosinophils, is the histologic hallmark of eosinophilic esophagitis. A cut-off value of at least 15 eosinophils per high-power field (x400 magnification, 0.55 mm²) establishes the histologic diagnosis of eosinophilic esophagitis (Collins et al., 2016).

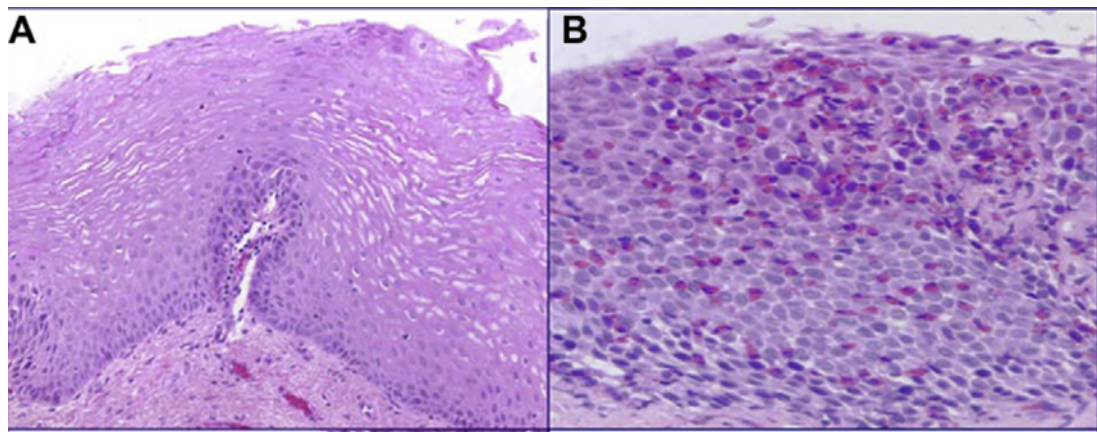


Figure 1-6: Histology of the oesophagus (mucosal biopsy specimens). A, Normal oesophagus (H&E magnification x100). B, Eosinophilic oesophagitis (H&E magnification x400). (Liacouras et al., 2011)

The disease predominantly affects males as compared to females at a ratio of 3:1 (Cianferoni and Spergel, 2016). The male predominance could be owing to a single nucleotide polymorphism that codes for the TSLP gene (Soumelis et al., 2002). TSLP (Thymic stromal lymphopoietin) is a cytokine primarily secreted by epithelial cells at barrier surfaces such as skin, gut, and lung in response to danger signals, infectious agents, atopic cytokines (IL-4, IL-13, TNF α), and environmental allergens. It is similar to IL 7, and it binds to TSLP receptor. TSLP is important for the maturation of antigen-presenting cells (APCs) and it is a master regulator of T-helper 2 (Th2) type allergic inflammation. Single nucleotide polymorphisms (SNPs) on TSLP gene, which are associated with its dysregulated expression, have been linked to atopic diseases such as eczema, asthma, allergic rhinitis, and EoE. In particular, TSLP is increased in the oesophageal biopsies of EoE subjects compared to non-EoE subjects (Soumelis and Liu, 2004)

As seen in

Figure 1-7, inflammation triggers, including food and possibly aeroallergens, activate T-helper 2 (Th2) cells in the lamina propria of the oesophagus, thereby stimulating the release of IL-13 and IL-5. In addition to Th2 cytokines,, TNF- α is upregulated in EoE and highly expressed by epithelial cells of the oesophagus in patients with active EoE (Straumann et al., 2008b). IL-5 is involved in the maturation, homing and release of eosinophils into the circulation. IL-13 induces oesophageal epithelial cells to express eotaxin-3, an eosinophil chemoattractant. IL-13 also affects the oesophageal epithelial barrier by upregulating periostin and downregulating filaggrin thereby increasing epithelial permeability. Downregulation of desmoglein-1 (DSG1) by IL-13 results in impaired barrier function (IBF) and increased antigen exposure as well as the expression of pro-allergic mediators including periostin (POSTN), forming a pathogenic cycle to further exacerbate allergic inflammation (Sherrill et al., 2014). Activated eosinophils release cytotoxic granules, including major basic protein (MBP). MBP, along with B-cell-associated IgE, activates mast cells, which release prostaglandin D2 (PGD2). PGD2 binds to the receptors CRTH2 (chemoattractant receptors on Th2 lymphocytes and eosinophils), promoting both chemotaxis and activation. Transforming growth factor beta (TGF- β) released by activated eosinophils induces fibrosis which can lead to stricture (Furuta and Katzka, 2015, O'Shea et al., 2018, Chen and Kao, 2017).

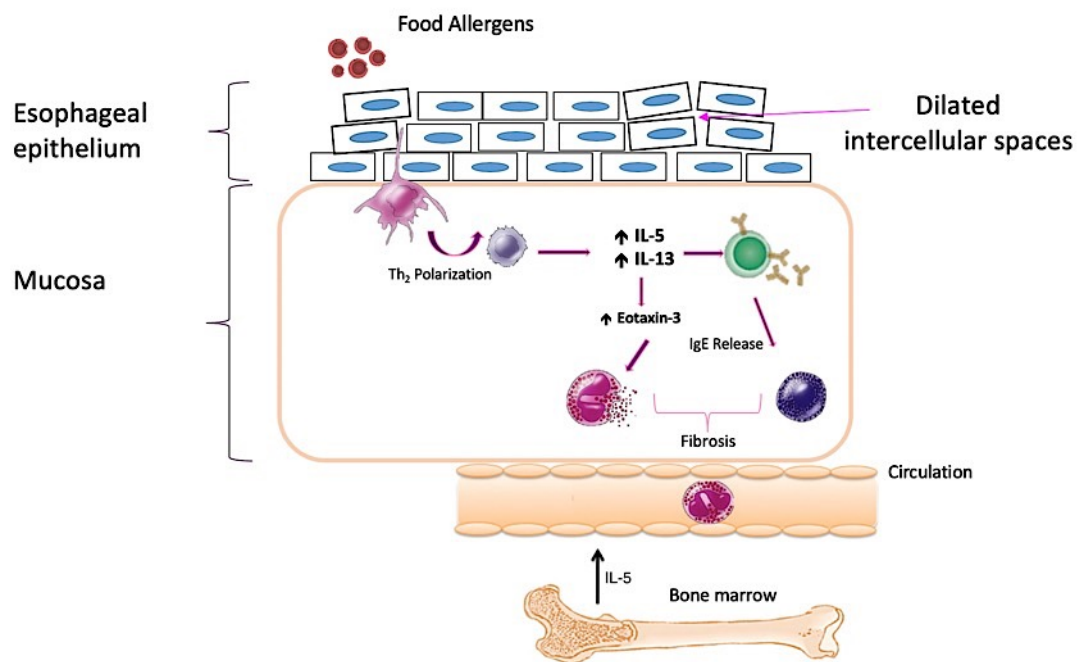


Figure 1-7: Pathophysiology of eosinophilic oesophagitis adapted from (Cianferoni et al., 2015, Chen and Kao, 2017, Kern and Hirano, 2013)

Table 1-3: Differences between eosinophilic esophagitis and healthy oesophagus

Comparison element	Healthy oesophagus	Oesophagus in EoE	Oesophagus in GERD
<i>Epithelial layer thickness</i>	500-800 μm ^a	$\uparrow$ ^d	$\uparrow$ ^g
<i>Oesophageal barrier integrity</i>	No DIS (intercellular space 0.45-0.56 μm) ^e	Impaired barrier function. DIS ($2.24 \pm 0.28 \mu\text{m}$) ^{b,e}	Impaired barrier function. DIS ($1.67 \pm 0.81 \mu\text{m}$) ^{c,e}
<i>DIS causing factors</i>	N/A	IL-13 ^d DSG-1 $\downarrow$ ^a Calpain-14 ^d	Gastric reflux (acid, pepsin, bile acids, trypsin) ^{c, g}
<i>Immunological response</i>	N/A	Th-2 mediated ^c	Epithelial cells mediated ^f
<i>Eosinophilic count</i>	No eosinophil ^a	High (≥ 15 eos/hpf) ^e	Low (0-7 eos/hpf) ^e
<i>Inflammatory cytokines</i>	None	Eotaxin-3, IL-5, and IL-13 ^{a, d}	IL-1, IL-4, IL-6, IL-8 ^f

^a (Sherrill et al., 2014), ^b (Ravelli et al., 2014), ^c (Tobey et al., 1996), ^d (Davis et al., 2016), ^e (Mueller et al., 2008),

^f (Jovov et al., 2011), ^g (Björkman et al., 2013)

EoE – eosinophilic oesophagitis, GERD – gastro oesophageal reflux disease, DIS – dilated intercellular spaces, IL – interleukin, DSG-1 – desmoglein 1, eos – eosinophil, hpf – high power field, CYP3A4 – cytochrome P450 3A4

1.6 Off-label use and drugs currently used in clinical trials

Proton pump inhibitors, corticosteroids and elimination diets are currently the mainstay of treatment for EoE. About 40% patients also respond to proton pump inhibitors known as proton pump inhibitor responsive oesophageal eosinophilia (PPI-REE) (van Rhijn et al., 2014). Elimination diets are used but lead to serious nutritional deficiencies in the long term hence corticosteroids are the main treatment option and are used off-label; these include budesonide viscous slurry and swallowed budesonide/fluticasone by metered dose inhaler (MDI). Jorveza® is the only EU approved dosage form not used off-label to treat EoE (Lucendo, 2020).

Currently, there are numerous clinical trials ongoing for various drugs to treat EoE. However, the ones that have completed Phase II and III are for an orally disintegrating budesonide effervescent tablet (Jorveza®) and budesonide viscous slurry respectively. Jorveza® is available in the market in several EU countries and Switzerland. The efficacy rates between the two formulations of budesonide have varied with the budesonide effervescent tablet (BET) showing higher efficacy compared to the viscous suspension. The Table 1-4 is a summary of the trials conducted for budesonide formulations. The Table 1-5 below summarizes all the other small molecules consisting mainly of corticosteroid fluticasone that have undergone clinical trials. A brief summary of the outcomes is presented.

It should be noted that the best histological remission, 95%, was obtained with the budesonide effervescent tablet (BET). Budesonide viscous slurry and fluticasone MDI have given similar histologic response rates (50 vs 64% respectively) (Dellon et al., 2019, Lucendo et al., 2019).

1.6.1 Small molecules

Table 1-4: Clinical studies and trials of budesonide viscous suspension (OVB/BVS) and orally dissolving tablets (BET)

Reference	N	Study design	Drug Dose	Duration	Study outcome
(Dohil et al., 2010)	24 C	Double blind RCT	OVB 1 mg or 2 mg daily v placebo	12 weeks	Improvement in clinical response. Histological response in 86.7%
(Straumann et al., 2010a)	36	RCT placebo-controlled trial	BVS 1 mg BID v placebo	15 days	Improvement in dysphagia scores, Histological response improved in BVS group
(Dellon et al., 2012)	22	Prospective open label RCT	NEB v OV B 1 mg BID	8 weeks	Improvement in dysphagia in both groups. Histological improvement in OV B but not NEB group
(Gupta et al., 2015)	71 C	Double blind RCT	BVS v placebo	12 weeks	Significant histological improvement seen in medium-high dose BVS (~50%)

Reference	N	Study design	Drug Dose	Duration	Study outcome
(Miehlke et al., 2016)	76	Double blind RCT (Phase II)	BET 2×2mg /day, BVS 2×5 mL (0.4mg/ mL)/ day	14 days	Symptomatic relief (dysphagia), Histological remission 94.7% in BET and BVS group compared with 0% in placebo group
(Dellon et al., 2017)	93	Double blind randomized controlled trial (Phase II)	BVS 2mg BID	12 weeks	Symptomatic improvement, Histological response of 39%
(Lucendo et al., 2019)	88	Double blind randomized controlled trial, (Phase III)	BET 1mg BID	12 weeks	Remission 85%, Histological response 95%
(Dellon et al., 2019)	64	Double blind, Double dummy Trial (Phase II)	BVS 1mg/4ml BID	8 weeks	Histological response 50%

C- children, Where there is no C it means the trial was in adults, RCT – Randomized controlled trial, OVB/BVS – oral viscous budesonide, BET – budesonide effervescent tablet

Table 1-5: Other small molecules and corticosteroids that have undergone clinical trials for eosinophilic esophagitis

Reference	Design	N	Drug dose	Class	Duration	Study outcome
(Konikoff et al., 2006)	Double blind RCT	36 C	Fluticasone 880 µg BID v placebo	Corticosteroid		clinical improvement, <2 eos/hpf 50% vs 9% (P=0.047)
(Peterson et al., 2010)	Prospective RCT	30 A	Fluticasone 440 µg BID v esomeprazole 40 mg QD	Corticosteroid/PPI	8 weeks	No difference between PPI or fluticasone groups
(Alexander et al., 2012)	Double blind RCT	42 A	Fluticasone 880 µg BID v placebo	Corticosteroid	6 weeks	62% histological response in fluticasone group and 0% in placebo
(Moawad et al., 2013)	Single blind RCT	42 A	Fluticasone 440 µg BID v esomeprazole 40 mg QD	Corticosteroid/PPI	8 weeks	No difference between steroid and PPI groups
(Butz et al., 2014)	Double blind RCT	42 A & C	Fluticasone 880 µg BID v placebo	Corticosteroid	3 months	65% histological response in fluticasone group, 0% in placebo
(Schroeder et al., 2012)	Clinical study	4 A	Ciclesonide (Swallowed from metered dose inhaler) 80 µg/BID or 160 µg/BID	Corticosteroid	2 months	Symptoms resolved, Eosinophil numbers decreased significantly from 71 ± 25.5 eos/hpf before treatment to 1.75 ± 2 eos/hpf; P =.007 after treatment

Reference	Design	N	Drug dose	Class	Duration	Study outcome
(Straumann et al., 2013)	Double blind RCT	26 A	OC000459 (Tablets) 100mg/BID	CRTH2 antagonist	8 weeks	No difference between drug and placebo, 36.2% reduction in eosinophil count compared with placebo

A- Adults, C – children, BID – twice daily, QD – once daily, RCT – Randomized controlled trial, CRTH2 – chemoattractant receptor on Th2 cells/Prostaglandin D2 receptor

1.6.2 Monoclonal antibodies (mAbs)

Based on the pathophysiology of EoE, monoclonal antibodies have been evaluated as an approach to inhibit the specific cytokines (IL-4, IL-5, and IL-13) and/or their receptors involved in the disease pathogenesis (Eskian et al., 2018). Based on this premise, targeting these key effectors by monoclonal antibodies might break the inflammatory cascade of EoE and eventually reverse the tissue remodeling process which is responsible for disease symptoms. The use of monoclonal antibodies has been studied in similar disease conditions including asthma with various degrees of success. Monoclonal antibodies are a highly efficacious class of biotherapeutics and account for 7 of the 10 top selling marketed drugs; with their market projected to reach about USD130 billion by 2023 (Ecker et al., 2015).

Various monoclonal antibodies have been assessed in clinical trials for the treatment of eosinophilic esophagitis. IL-5 is a Th2 cell pro-inflammatory cytokine and has a critical role in eosinophil activation, migration, and tissue survival. Anti IL-5 mAbs have shown reduction of eosinophils and mast cells with mild adverse events. In patients with EoE, anti IL-5 therapies, such as Reslizumab a fully humanized IgG4 mAb has shown decreases in eosinophil counts compared to patients on placebo, albeit not below the <15 eos/hpf requirement. Moreover it was not found to be effective for induction of clinical response. On the other hand, Mepolizumab a fully human IgG1 anti IL-5 mAb, showed clinical response (dysphagia) at week 4 but peak eosinophil counts remained above 20 eos/hpf (de Rooij et al., 2019).

IL-13 secreted by Th2 cells and activated eosinophils plays a vital role in the pathogenesis of EoE by increasing eotaxin-3 and periostin, factors which increase eosinophil recruitment (chemotaxis) to the oesophagus. IL-13 affects the barrier integrity by downregulating the basement membrane protein desmoglein-1, filaggrin and involucrin (Sherrill et al., 2014). IL-13 has been shown to induce EoE in mouse models with similar pathology to human EoE (Mishra and Rothenberg, 2003). Furthermore, IL-13 was found to be markedly overexpressed in the oesophagus of patients with EoE (Blanchard et al., 2007). Similarly, IL-4, a cytokine that causes

naïve T-cells to differentiate into Th2 cells and activated B-cells; is found in increased concentrations in patients with EoE. QAX 576 and RPC 4046 are anti IL-13 mAbs with the latter preventing the binding of IL-13 to IL-13 receptor alpha 1 and 2 and the former directly inhibiting IL-13. In a Phase II study QAX 576 showed improvement in histological response but did not meet the criteria of >75% reduction in peak eosinophil count and also did not show improvement in clinical symptoms i.e dysphagia. Similar results were observed for RPC 4046 in a Phase II study (Rothenberg et al., 2015). Dupilumab, a mAb blocking IL-4R α and thus modulating signalling of both the IL-4 and IL-13 pathways, showed in a Phase II study improvement in clinical response after 10 weeks and eosinophil counts were significantly reduced at week 12 compared to placebo (92 vs 15%, $p<0.0001$). Dupilumab's (Dupixent®) promising efficacy results have allowed it to progress into a Phase III study (Hirano et al., 2020).

Some have suggested that similar to asthma, the activation of mast cells in EoE is an IgE dependent process. The observation that food and aero-allergen IgE mediated hypersensitivity is more frequent in patients with EoE than in the general population, supports this concept. Omalizumab is a humanized monoclonal anti-immunoglobulin E (IgE) antibody. It binds to IgE and hence prevents activation of mast cells. However, this mAb showed no statistically significant reduction in clinical symptoms or tissue eosinophil counts when compared against a placebo (Loizou et al., 2015, Clayton et al., 2014, Foroughi et al., 2007).

TNF- α is a pro-inflammatory mediator that has been found to be upregulated in biopsies of EoE patients. It is thought to be a Th1 cytokine which generates production of other downstream cytokines. Anti TNF- α mAbs such as infliximab and adalimumab have shown to be highly effective in moderate to severe forms of inflammatory bowel disease (IBD); Crohn's disease and Ulcerative Colitis (Yadav et al., 2016b). Straumman and co-workers investigated the therapeutic efficacy of infliximab in three corticosteroid dependent EoE patients. Infliximab did not manage to successfully reduce eosinophil numbers nor induce a clinical response. Reduction in TNF- α in the oesophageal tissue was not observed (Straumann et al., 2008a). That said, the experience of TNF- α should be interpreted with caution because of the small

number of patients evaluated or because of the poor distribution of infliximab in the oesophagus when given by injection.

Table 1-6: Clinical trials of monoclonal antibodies with primary focus on histologic outcomes

Reference	Age group	N	Study type	Dose	Duration	Clinical response	Histologic/serologic response	Adverse events
Anti IL-5								
(Stein et al., 2006)	Adults	4	open label safety and efficacy (Phase II)	Mepolizumab IV 750mg	8, 12, 16 weeks	improve QOL	↓ peripher al & oesophageal eos	mild
(Straumann et al., 2010b)	Adults	11	Randomized double blind placebo controlled	Mepolizumab IV 750mg	weekly x 2 doses	limited improvement	↓peripheral eos, ↓peak/mean oesophageal eos (not meeting endpoint of >5 eos/HPF)	mild
(Assa'ad et al., 2011)	Children	59	Randomized double blind parallel group	Mepolizumab 0.55, 2.5 or 10mg/kg	every 4 weeks x 3 doses	No clinical improvement	8.8% reached remission	87% reported vomiting, diarrhoea and abdominal pain
(Spergel et al., 2012)	Children	226	Randomized double blind placebo controlled	Reslizumab 1,2 or 3mg/kg	every 4 weeks x 3 doses	No clinical improvement	↓ peak oesophageal eos	Mild

(Otani et al., 2013)	Pediatric	43	Randomized double blind	0.55, 2.5, or 10 mg/kg mepolizumab	every 4 weeks x 3 doses	No improvement	40% ↓ eos, 77% ↓ mast cells	Mild
Anti IL-13								
(Rothenberg et al., 2015)	Adults	23	Randomized double blind placebo controlled	QAX 576 IV 6mg/kg	every 4 weeks x 3 doses	Improvement in dysphagia	33% achieved >75% reduction in oesophageal eos	mild
(Dellon et al., 2016)	Adults	90	Randomized double blind placebo controlled	RPC 4046 180 or 360 mg	IV then SC weekly	Improvement during duration of study	Significantly ↓ mean oesophageal eos for both doses	headache, mild
(Hirano et al., 2019)	Adults	99	Randomized double blind placebo controlled	RPC 4046 180 or 360 mg	5mg/kg IV + 180mg SC (LD) then 180mg SC weekly x 15 doses	Improvements in dysphagia and endoscopic scores	Significantly ↓ mean oesophageal eos for both doses (not <15eos/hpf)	mild
Dupilumab (anti IL-4 and IL-13)								
(Hirano et al., 2020)	Adults	47	Phase 2 placebo controlled RCT	300mg weekly SC	12 weeks	Reduction in dysphagia score	Eosinophil reduction by 107%	Nasopharyngitis, erythema at injection site
Anti IgE								

(Clayton et al., 2014)	Adults	30	Randomized double blind placebo controlled	Omalizumab SC 0.016mg/kg (n=16)	every 2-4 weeks for 16 weeks	No improvement	No improvement	not available
(Loizou et al., 2015)	Children and adults	15	Open label single arm	Omalizumab 1mg/kg SC	every 4 weeks x 3 doses	50% improvement	33% achieved < 15 eos/hpf + ↓ eso IgE + ↓ mast cells	No serious adverse events
(Foroughi et al., 2007)	Adults	7	Open label single arm	Omalizumab dose different for each subject	every 2 weeks for 16 weeks	No clinical improvement	eso eos remain unchanged	No serious adverse events
Anti TNF-α								
(Straumann et al., 2008b)	Adults	3	Prospective study	Infliximab IV 5 mg/kg	week 0 and 2	No improvement	eos remain unchanged	No serious adverse event

RCT – Randomized controlled trial, SC- subcutaneous, IV- intravenous, LD- loading dose, eso – oesophageal, eos/hpf – eosinophil per high power field, QOL – Quality of life

1.7 Side effects arising from the systemic use of corticosteroids, tacrolimus and monoclonal antibodies

Corticosteroids are used over long periods for bringing the patient in remission. Reports suggest that budesonide viscous suspension has caused adrenal insufficiency in children and adults alike (Harel et al., 2015, Golekoh et al., 2016). This therefore indicates that current formulations are not entirely acting locally as also evidenced by their short transit times through the oesophagus (Dellon et al., 2012). Furthermore, systemic side effects arising from corticosteroid use lead to hypertension and osteoporosis, diseases which have to be managed in addition to the one being treated i.e EoE.

Tacrolimus is used systemically to prevent graft versus host tissue rejection in transplant recipients. However, severe side effects have been reported from the systemic use of tacrolimus. Its immunomodulatory properties causes significant immunosuppression which can lead to secondary infections and cancers. Furthermore, it is also nephrotoxic and can cause renal damage leading to kidney failure (Mihatsch et al., 1998).

Monoclonal antibodies that have been used this far have shown mild adverse events over short term use. However, over longer term use they may exhibit severe side effects such as lymphomas, heart failure, opportunistic infections as a result of immunosuppression (Yadav et al., 2016b). This has been observed in patients being treated with anti TNF- α inhibitors. Furthermore, the systemic administration of mAbs may achieve lower accumulation of the drug in the oesophagus while the rest distributes to other parts of the body thereby causing adverse effects. The low tissue to serum anti TNF- α concentration of infliximab has been observed in the inflamed colonic tissues of ulcerative colitis patients (Yarur et al., 2015).

1.8 Rationale for local delivery of tacrolimus and monoclonal antibodies for the treatment of EoE: a pharmacological perspective

Corticosteroids have been the mainstay of therapy in treatment of EoE. However, of these, budesonide is the one approved for off-label use by US FDA and approved in the EU in the form of an orally dissolving tablet, Jorveza[®]. Corticosteroids inhibit mRNA responsible for interleukin-1 formation and arachidonic acid thereby producing anti-inflammatory and immunosuppressive effects (Kragballe, 1989) as seen in Figure 1-8. As discussed earlier, corticosteroids do not seem to act entirely locally and therefore cause systemic side effects such as adrenal suppression. Their local action leads to mucosal thinning and oesophageal candidiasis thereby having to stop them and consider other options which are either less efficacious or need to be given parenterally e.g monoclonal antibodies.

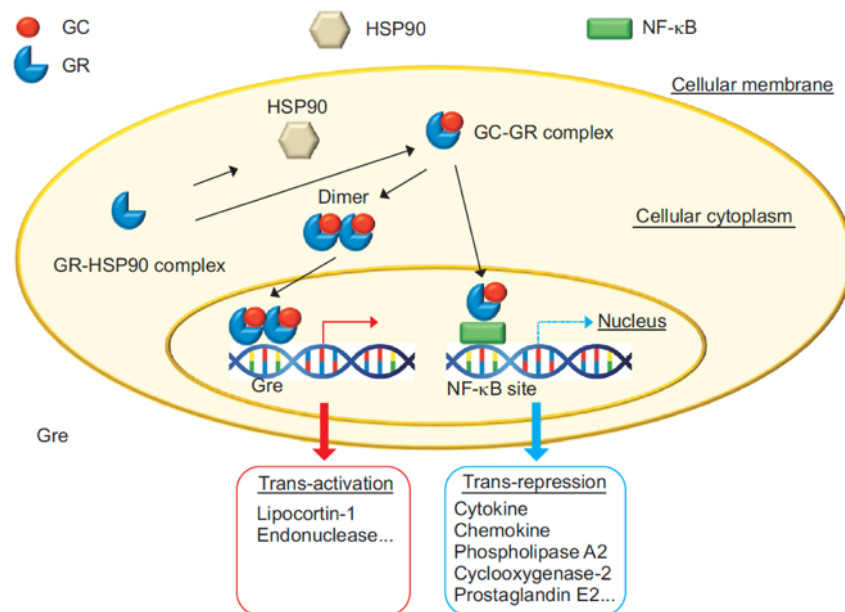


Figure 1-8: Mechanism of action of corticosteroids (Nakase, 2019)

GC, glucocorticoid; HSP90, heat shock protein 90; NF-κB, nuclear factor-kB; GR, glucocorticoid receptor; Gre, glucocorticoid response element.

Tacrolimus is a drug that acts intracellularly in the T- lymphocyte. It is a small molecule of 804 Da and inhibits T-lymphocyte activation by first binding to an

intracellular protein, FKBP-12. A complex of tacrolimus-FKBP-12, calcium, calmodulin, and calcineurin is then formed and the phosphatase activity of calcineurin is inhibited. This prevents the dephosphorylation and translocation of nuclear factor of activated T-cells (NF-AT), a nuclear component thought to initiate gene transcription for the formation of lymphokines Figure 1-9. Tacrolimus also inhibits the transcription of genes encoding IL-2, IL-3, IL-4, IL-5, IL-13 and $\text{TNF-}\alpha$, all of which are involved in the early stages of Th 2-cell activation. (Liu *et al.*, 1991)

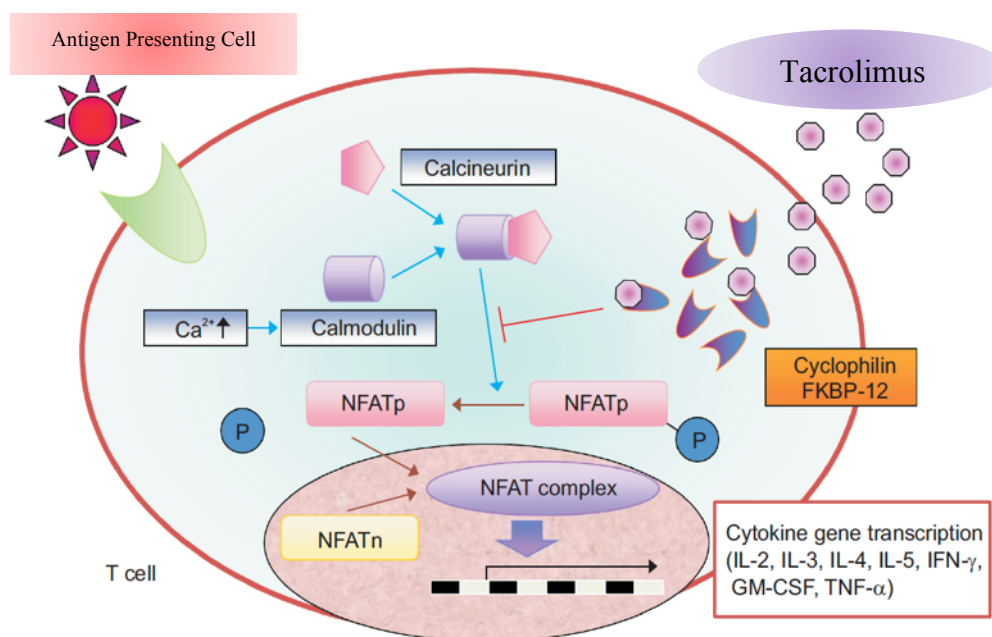


Figure 1-9: Mechanism by which tacrolimus inhibits the formation of inflammatory cytokines (Nakase, 2019)

Therefore, tacrolimus is a pharmacologically sound alternative as EoE has predominantly Th2 cytokines involved in its pathogenesis. Furthermore, tacrolimus is lipophilic making it appropriate for the local treatment of EoE. Furthermore, low doses can be applied topically owing to its potency such that any tacrolimus that is absorbed into the blood side would not elicit any adverse side effects due to being below the trough therapeutic concentrations.

In addition to tacrolimus, monoclonal antibodies such as the anti IL-13 and anti IL-13 and IL-4 (Dupilumab) given parenterally have shown good efficacy in both clinical and histological response. However, mAbs are given parenterally and low tissue distribution has been reported (Li et al., 2016). Thus, topical use of monoclonal antibodies where potentially a smaller dose could be administered might be an interesting option. Even if reaching the stomach, mAbs will get completely degraded in the hostile gastric environment (Yadav et al., 2016a). Monoclonal antibodies exert effector functions by binding with their Fc region to Fc γ Rs on immune cells, thereby eliciting antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent complement cytotoxicity (CDC), both mechanisms leading to the apoptosis of the target cells (Kellner et al., 2017, Kolbeck et al., 2010) Herein, the potential to locally deliver infliximab to the oesophagus is investigated. TNF- α is upregulated in EoE and highly expressed by epithelial cells of the oesophagus in patients with active EoE (Straumann et al., 2008b). Furthermore, infliximab is an IgG1 monoclonal antibody and it can be used to investigate the permeation of monoclonal antibodies thereby serving as a surrogate.

1.9 Aims and objectives

I) To investigate the ex vivo tissue accumulation and/or permeability of three drugs budesonide, tacrolimus and infliximab in intact (healthy) porcine oesophageal tissue with a focus on mucosal contact time and sex (males and females). Ex vivo tissue accumulation/permeability of budesonide and tacrolimus is also investigated in porcine oesophageal tissue injured by acid and pepsin.

II) To investigate ex vivo tissue accumulation and/or permeability of a large 150 KDa monoclonal antibody, infliximab, into the porcine oesophageal tissue with a focus on understanding any underlying mechanisms promoting its uptake.

III) To develop an ex-vivo injury model using porcine oesophageal tissue with the main aim being to mimic the dilated intercellular spaces as observed in human biopsy samples of EoE. Subsequently, to investigate the tissue accumulation and/or permeability of budesonide, tacrolimus and infliximab in the injured porcine oesophageal tissue and comparing it to intact (healthy) tissue. The aim of this is to understand if the injured tissue is allowing significantly greater tissue accumulation/permeation compared to control (intact tissues).

IV) In vivo studies to understand whether the efficacy arising from the topical formulations of budesonide is as a result of a systemic rather than local effect using the Sprague Dawley rat as an animal model

CHAPTER 2 : TISSUE ACCUMULATION AND/OR PERMEABILITY OF SMALL MOLECULES, BUDESONIDE AND TACROLIMUS

2.1 Introduction

Small molecules such as corticosteroids, primarily budesonide and in other instances, fluticasone have been used as mainstay of treating EoE. However, these molecules as discussed in chapter 1 have shown varying efficacy rates and based on the outcomes of the studies, it can be deduced that the differences in efficacy, especially for budesonide, may be arising as a result of differences in formulation.

In this chapter, the aim is to understand the permeability of budesonide and tacrolimus in the porcine oesophageal mucosa with the aim to understand their potential for local delivery to the oesophagus.

2.2 Transit of budesonide viscous suspensions (BVS) and marketed orally dissolving tablets (ODTs) by gamma scintigraphy

Budesonide viscous suspension (BVS) is a combination of budesonide respules (0.5 mg/2 ml) and sucralose as sweetener. Two respules (4ml consisting of 1mg budesonide) are added to 10 grams of sucralose (Splenda) to make a slurry of 8ml (Dohil et al., 2010). Gamma scintigraphy was conducted and compared against swallowed nebulized budesonide. The oesophageal transit time of the BVS was 90 seconds and most of the administered volume accumulated in the stomach. In contrast, more than 50% of the administered swallowed nebulized budesonide entered the lungs (Dellon et al., 2012).

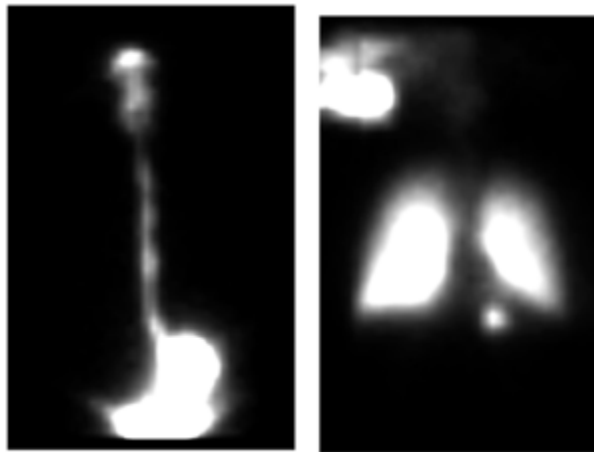


Figure 2-1: Gamma scintigraphy showing the distribution of budesonide in the oesophagus via a) administration of budesonide viscous suspension and b) swallowed nebulized budesonide (Dellon et al., 2012)

However, in another oesophageal transit study comparing formulations of budesonide consisting of xanthan gum, honey and a sucralose slurry, the residence of xanthan gum was superior (3 minutes) to honey and sucralose (Hefner et al., 2016)

Gamma scintigraphy studies have also been conducted on orally dissolving tablets (ODTs). Here the Zydis formulation is referenced whereby the total transit time from mouth to stomach was 5 minutes (Seager, 1998). However, the majority of the administered dose seemed to reach the stomach similar to the viscous suspension. However, owing to slightly longer transit (total 5 minutes) than the budesonide suspension, chances are that it may allow sufficient time for buccal absorption to take place. This may be giving the ODT an advantage over BVS for a drug like budesonide; which can avoid the first pass gastrointestinal metabolism thereby being able to achieve systemic levels.

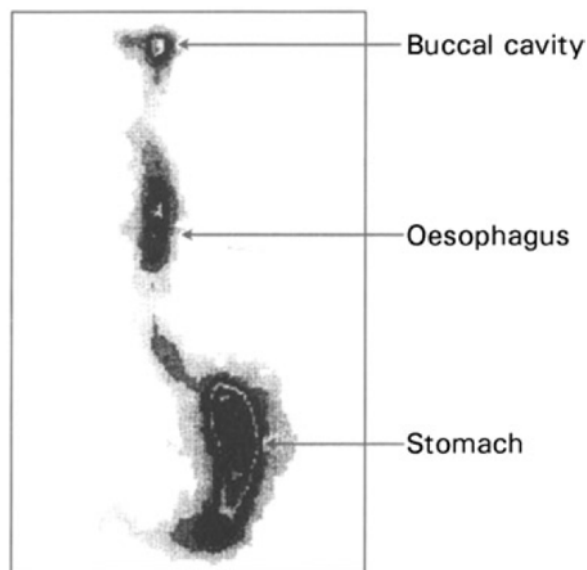


Figure 2-2: Gamma scintigraphy of the Zydys orally dissolving tablet transiting from the buccal cavity to the stomach (Seager, 1998)

Budesonide has been claimed to act locally for the treatment of eosinophilic esophagitis. Based on the above transit times for the viscous suspension and orally disintegrating tablets it warrants an investigation of whether the drug even actually accumulates in the esophageal epithelium given that the residence time is very short.

2.3 Efficacy of budesonide viscous suspension (BVS) versus budesonide orally dissolving tablet (BET)

As discussed in Chapter 1, the budesonide oral suspension has shown a histologic response (a primary end-point to determine efficacy) of less than 50% versus 95% for the orally dissolving tablet. The orally dissolving tablet (ODT) referenced herein is the budesonide effervescent tablet (BET) marketed in EU under the brand name Jorveza[®]. Both formulations are claimed to act locally to reduce the inflammation in the oesophagus, associated with eosinophilic esophagitis. While the dose is the same i.e 1mg twice daily, it is intriguing why the histological response is significantly different between the two formulations.

Budesonide has a high local to systemic affinity ratio and therefore, it may be able to permeate the epithelium in a short time span. Once permeated in the epithelium, it can traverse the multiple cell layers found in the oesophageal epithelium. If this is the absorption mechanism for a moderately lipophilic drug like budesonide, then it can be hypothesized that the same would apply for a highly lipophilic drug like tacrolimus.

2.4 Rationale for local delivery of tacrolimus for treatment of EoE

The permeation of drugs such as fentanyl citrate log P 3.8 (Drugbank, 2020a) has been studied across both fresh and frozen (-20°C) porcine oesophageal mucosa. It was observed that the permeability was in the order of $23.4 \pm 3.4 \text{ } \mu\text{g}/\text{cm}^2/\text{h}$ supporting the fact that lipophilic molecules can easily cross the oesophageal mucosa (Diaz del Consuelo et al., 2005, Diaz-del Consuelo et al., 2005). Furthermore, the flux of fentanyl citrate in pig buccal and oesophageal mucosa was found to be similar indicating that learnings from the buccal mucosal absorption could be extrapolated to oesophageal mucosa.

Tacrolimus when formulated as an ointment (Protopic 0.1%) has shown to permeate pig skin both in vitro, using Franz diffusion tests, and in vivo (Pople and Singh, 2010, Ma et al., 2013). Furthermore, in humans, plasma levels $< 1 \text{ ng/ml}$ have been detected in patients suffering from skin conditions such as atopic dermatitis (Cheer and Plosker, 2001). The ability of tacrolimus to cross the epidermal and dermal barrier and to achieve quantifiable plasma concentrations may either be attributed to its lipophilicity or the impaired barrier in atopic dermatitis.

Tacrolimus has been applied buccally to paediatric kidney transplant patients and it was found to penetrate these epithelia and achieve blood levels (Goorhuis et al., 2006). However, when applied sublingually to healthy adults, systemic levels of tacrolimus were not observed (Stift et al., 2014).

Therefore, herein we set out to understand the tissue accumulation and/or permeability collectively termed permeation/permeability of tacrolimus and budesonide (as a control) using porcine oesophageal mucosa. Tissue accumulation is herein defined as

the amount of drug quantified exclusively in the tissue constituting the epithelium and submucosa whereas permeability is the amount of drug that can pass through the oesophageal tissue and accumulate in the basolateral side of the Ussing chamber.

2.5 Materials and methods

2.5.1 Materials

Budesonide and tacrolimus were purchased from Sigma Adrich, UK. Water and Acetonitrile from VWR, UK. Cremophor RH60 was a gift from BASF. Ussing chambers (Harvard Apparatus, Cambridge, UK). The ingredients used for Krebs bicarbonate ringer (KBR) solution were as follows; D-glucose, sodium chloride, calcium chloride, magnesium chloride, sodium bicarbonate (Sigma Adrich, UK), potassium hydrogen phosphate and dipotassium hydrogen phosphate (VWR, UK). Sarstedt reagent tubes (Order-No. Sarstedt: 72.690.478).

Hypersil gold C18 column was used 150 x 2.1 mm, 5 μ m (Thermo Fisher) for tacrolimus and Phenomenex, Kinetex 2.6 μ phenylhexyl 100 $\text{\AA}$, 4.6mm $\times$ 50mm column (Phenomenex®, Cheshire, UK) for budesonide. HPLC agilent 1260 infinity was used for analysis.

2.5.2 Pigs

Male and female cross breed of Landrace and Large White pigs, 9-12 months old, were used as animal models to study permeation. The oesophagus was obtained from an abattoir (Cheale meats, UK) and the tissues were received in the lab within 3 hours of slaughter. The tissues were stored in ice-cold Kreb's bicarbonate ringers solution. The oesophageal mucosa (epithelium and submucosa) were separated from the muscle layer carefully using surgical scissors. These tissues were then stored at -80°C and thawed at 4°C when used for Ussing chamber permeability experiments. The tissues were separated into 3 sections namely proximal, mid and distal with each one being between 7-9 centimetres depending on the length of the oesophagus.

Porcine oesophageal mucosa has been used as a model to the human oesophageal mucosa as it is impossible to obtain human oesophageal tissue (Diaz del Consuelo et al., 2005). Porcine instead of human oesophageal mucosa has been used in permeation studies of drugs (Diaz del Consuelo et al., 2005, Diaz-del Consuelo et al., 2005, Caon et al., 2014, Caon and Simões, 2011). The porcine and human oesophageal mucosa both have non-keratinized epithelium and have submucosal glands which secrete bicarbonate. Furthermore, the gastrointestinal tract of pig is similar to humans in terms of anatomy, biology and biochemistry (Hatton et al., 2015) supporting the use of the pig oesophagus instead of human. Rodent oesophagus is keratinized epithelium and therefore not representative of the human epithelium which is non-keratinized (Wang, 2017).

2.5.3 Characterization of budesonide and tacrolimus in surfactant and co-solvent

Budesonide 5mg/ml was prepared by dissolving it in a mixture of 0.2g Cremophor RH60 and 0.84ml ethanol. This was added to artificial saliva pH 7.4 and Krebs's bicarbonate ringer's (KBR) solution pH 7.4 to give a final concentration of 0.33 mg/ml in each case. The solution was vortexed for 30 seconds and the particle size and poly dispersity index (PDI) was measured using a Malvern instruments dynamic light scattering (DLS) at 0 and 7 hours using polypropylene cuvettes with path length of 10mm and compared against water for injection as control. The solution was kept at 37°C during this time period.

2.5.4 Ussing chamber studies

The NaviCyte vertical Ussing system (Harvard Apparatus, Cambridge, UK) was used to measure drug transport across epithelial membranes which are polar structures possessing an apical (mucosal) and basolateral (serosal) side. The chambers are made of solid acrylic and the ones used henceforth are either low volume (1.5 ml) or large volume (5 ml). The working system consists of a unit to fit a maximum of six vertical chambers, a gas manifold for carbogen purging (95% O₂, 5% CO₂) and a heater block to maintain the temperature of the chambers at 37°C during the experiments. The

chambers are two piece assemblies held together by a high spring-tension retaining ring to ensure leak-free operation during the experiments.

The excised tissue segments are sandwiched between the two chambers by mounting on pins that surround the oblong shape opening. The Warner instrument's EC800 and Ag/AgCl electrodes were used to measure the transepithelial resistance (TEER) of the tissue samples. The resistance is determined by measuring the voltage across the tissue while supplying a constant current across the bicarbonate ringers solution. TEER monitors the presence of functional tight junctions, which are responsible for the barrier function (paracellular permeation). Tissues were considered viable if the TEER was greater than $500 \Omega \cdot \text{cm}^2$. A decrease in resistance will be detected as a result of greater flow of current across the paracellular spaces of the epithelium (Srinivasan et al., 2015). TEER did not decrease below $500 \Omega \cdot \text{cm}^2$ in any of the performed experiments.

Krebs-Bicarbonate Ringer's buffer (KBR) was prepared with 10mM (1.8g/L) D-glucose, 1.2mM (0.133 g/L) Calcium chloride, 1.2mM (0.114 g/L) Magnesium chloride, 115mM (6.7 g/L) Sodium chloride, 25mM (2.1 g/L) Sodium bicarbonate, 0.4mM (0.054 g/L) potassium dihydrogen phosphate, 2.4mM (0.418 g/L) dipotassium hydrogen phosphate in distilled water, and the pH was adjusted to 7.4 with 1M sodium hydroxide /hydrochloric acid (Clarke, 2009).

Artificial saliva added to the apical side after stabilization with Bicarbonate Ringer's solution (KBR) for 20 minutes was composed of the following ingredients: sodium nitrite 0.03 g/L, potassium carbonate 0.2 g/L, sodium chloride 0.5 g/L and sodium bicarbonate 4.2 g/L (Pind'áková et al., 2017). The osmolality of artificial saliva measured was about 150 mOsm/kg which is similar to human saliva which is hypotonic (75-125 mOsm/kg) and is principally bicarbonate ions (Smith et al., 2012).

2.5.5 Measurement of osmolality

The osmolality was measured by freezing point depression using the Hermann Roebling D-14129 osmometer. Briefly, it was first calibrated with distilled water to give a reading of 0 mOsm/kg. 100 μ l of the sample was pipetted into the Sarstedt

reagent tubes (Order-No. Sarstedt: 72.690.478) and it was attached to the measuring head which was depressed so that it enters the cooling aperture. Exactly at freezing point, a freezing needle was inserted into the sample for about one second and returned to its original position. During this point of inflection, the digital display showed sinking values. The minimum value was the result. The measuring head was raised and cleaned with a soft tissue.

2.5.6 Permeation studies of budesonide and tacrolimus in porcine oesophageal mucosa

For the tissue permeation studies, the tissues were thawed at 4°C and then mounted on the Ussing chamber with the epithelial side facing upwards. The exposed tissue area was 0.10 cm² for 2 x 10 mm opening chambers and 0.29 cm² for 4 x 8 mm opening chambers.

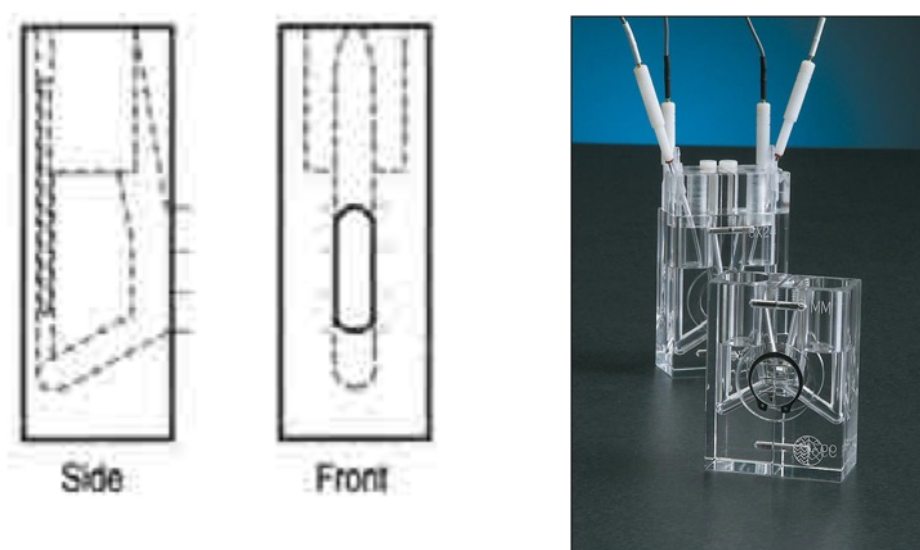


Figure 2-3: A representation of the Ussing chamber system used for testing the permeability of tacrolimus and budesonide across pig oesophageal tissue (Warner Instruments Catalogue).

Since both, budesonide and tacrolimus are insoluble in water, with the former having a saturation solubility of 40µg/ml and the latter 4µg/ml; a surfactant, co-solvent

approach was used to solubilise them. This is based on the injection formulation of tacrolimus which is completely soluble when added to saline solution for infusion.

Briefly, 0.2 g of cremophor RH60 and 0.84 ml of ethanol (density 0.758 g/mL) were added to 5 milligrams of tacrolimus to make a 5 mg/ml solution of tacrolimus. Same approach was used for budesonide.

The permeation study was conducted using 3ml of 0.33mg/ml tacrolimus or budesonide (5 mg/ml stock solution diluted in artificial saliva, pH 7.4) in the apical side and KBR pH 7.4 in the basal side. These concentrations were selected on the basis that 0.1% tacrolimus ointment and 1 mg budesonide viscous suspension and budesonide effervescent tablets have proven to be effective in atopic dermatitis and eosinophilic esophagitis, respectively (Miehlke et al., 2016, Reitamo et al., 2005). Apical samples 100 µl, were withdrawn at each time point and replaced with fresh artificial saliva buffer and basal samples were only taken at the last time point and analysed by HPLC.

2.5.7 HPLC/UV analysis for tacrolimus and budesonide

2.5.7.1 Tacrolimus

Briefly, a hypersil gold C18 column was used 150 x 2.1 mm, 5µm (Thermo Fisher) using a gradient method of mobile phase consisting of phosphate buffer pH 2.5 (A) and acetonitrile (B), where i) 0 min 30%B, ii) 30 min 80%B, iii) 32 min 80%B, iv) 32.5 min 30%B and v) 37min 30%B, column temp. 55 °C and flow rate 1 ml/min and detection wavelength 220nm.

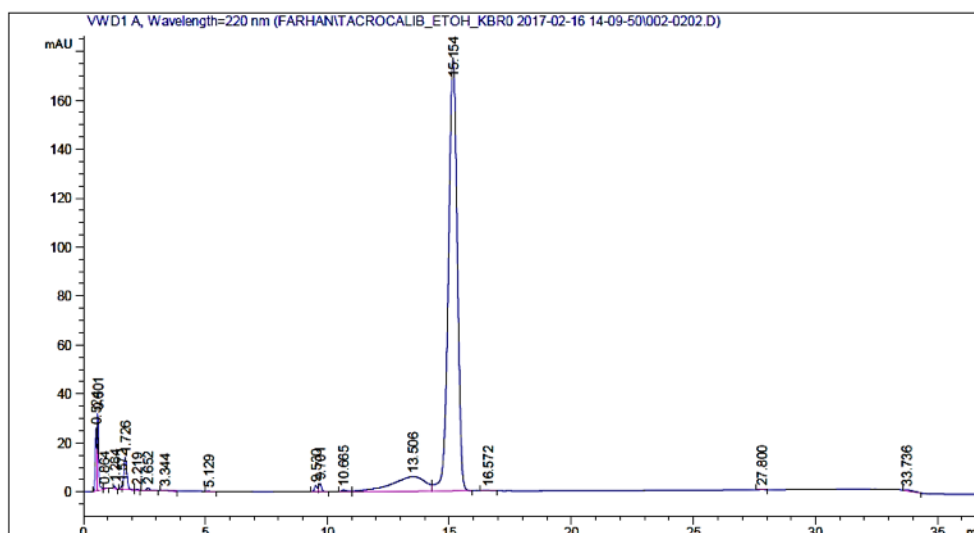


Figure 2-4: Tacrolimus chromatogram with retention time of 13.5 minutes for the 19-S epimer and 15.1 minutes for tacrolimus

2.5.7.2 Budesonide

For the analysis of budesonide, a Kinetex 2.6 μ phenylhexyl 100 $\text{\AA}$, 4.6mm $\times$ 50mm column (Phenomenex®, Cheshire, UK) was used at 40 °C and detected at 254 nm. Budesonide eluted using a gradient system of water (A) and acetonitrile (B) flowing at 1mL/min. For budesonide the following gradient programme was followed: i) 0–10 min, ii) 20–60% B; iii) 10–14 min, 60–20% B (Yadav et al., 2013)

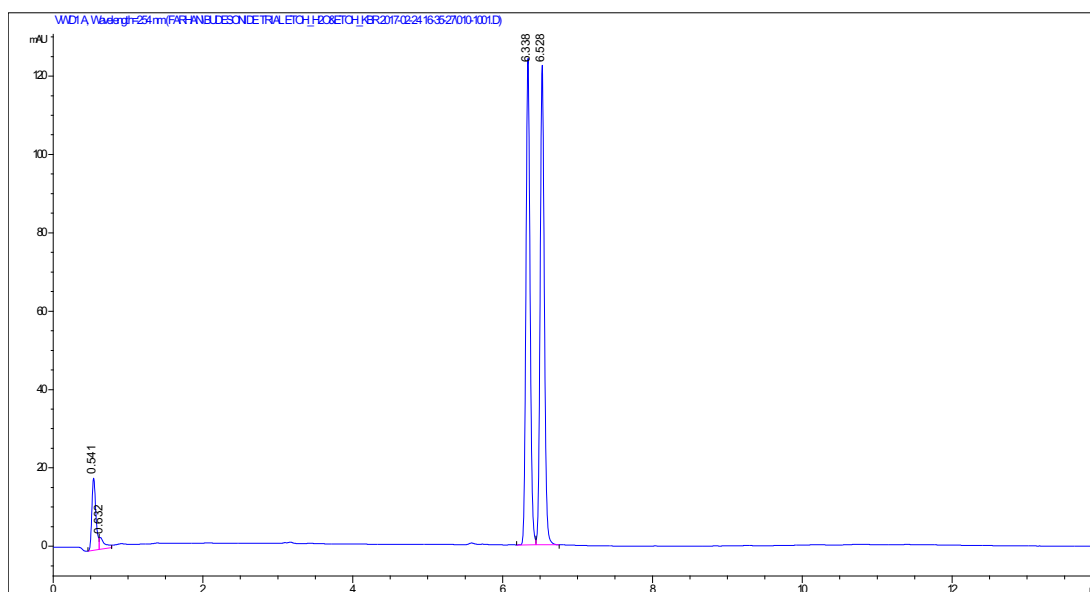


Figure 2-5: Budesonide chromatogram showing separation of both R and S enantiomers. Retention time was 6.3 and 6.5 minutes

2.5.8 Liquid chromatography tandem mass spectrometry (LC-MS/MS) method for quantification of budesonide and tacrolimus in porcine oesophageal tissue (ex-vivo)

LC-MS/MS is a sensitive technique which measures a quantifier ion generated from the ionization of the parent molecule. It is much more sensitive and specific than HPLC and can measure a substance in ng/ml or pg/ml levels. LC-MS/MS was used herein to quantify tacrolimus and budesonide in pig oesophageal tissues as HPLC did not give an indication of the/permeation into the porcine oesophageal tissue.

2.5.9 LC-MS/MS method for tacrolimus and budesonide

2.5.9.1 Tacrolimus

An Agilent triple quadrupole LC-MS/MS 6460 was used to determine the mass of the precursor ion for tacrolimus followed by its product ions and finally a MRM (multiple reaction monitoring) to obtain peak areas of the most abundant product ion. A linear calibration curve for the most abundant product ion was plotted. The chromatographic system included an autosampler, pump, a Waters Atlantis C18, 3 μ m, 2.1 x 100mm column maintained at a temperature of 40°C. The gradient elution was performed with a mobile phase composed of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was 0.6 ml/min and was programmed as follows: i) equilibration at initial conditions with 30% of B between 0 and 2 minutes, ii) increase to 90% of B between 2 and 5 minutes, iii) maintain 90% B between 5 and 7 minutes and iv) reversion to 30% B between 7 and 10 minutes and v) re-equilibration at 30% B between 10 and 12 minutes. Detection of tacrolimus dissolved in acetonitrile was performed by MS2 scan using electrospray ionization (ESI) mass spectrometry and the positively charged sodium adduct of tacrolimus, m/z 826 was obtained as the precursor ions (Figure 2-6: A MRM (multiple reaction monitoring) chromatogram for 50ng/ml tacrolimus. The red peak is isolated for m/z 616 (the most abundant product ion). The adduct transitions m/z 826 to m/z 616, m/z 459 and m/z 415 for tacrolimus (collision energy at 34, 46 and 54 eV and fragmentor 300).

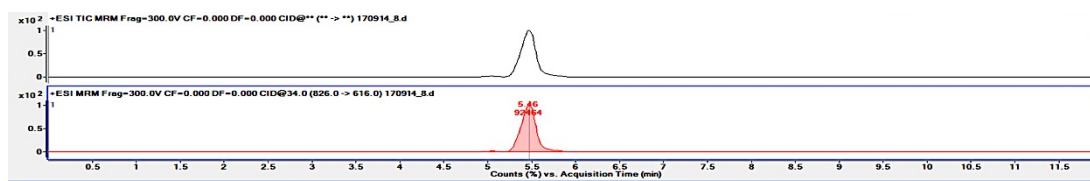


Figure 2-6: A MRM (multiple reaction monitoring) chromatogram for 50ng/ml tacrolimus. The red peak is isolated for m/z 616 (the most abundant product ion).

2.5.9.2 Budesonide

An Agilent triple quadrupole LC-MS/MS 6460 was used to determine the mass of the precursor ion for budesonide followed by its product ions and finally a MRM (multiple reaction monitoring) to obtain peak areas of the most abundant product ion. A linear calibration curve for the most abundant product ion was plotted. The chromatographic system included an autosampler, pump, a Kinetex 2.6 μ m Phenyl hexyl column, 4.6 x 50mm column maintained at a temperature of 40°C. The gradient elution was performed with a mobile phase composed of water (A) and acetonitrile (B). The flow rate was 1.0 ml/min and was programmed as follows: i) starting at 40% of B, ii) increasing to 95% of B at 3 minutes and iii) decreasing to 40% B between 3 and 6 minutes. Detection of budesonide dissolved in acetonitrile was performed by MS2 scan using electrospray ionization (ESI) mass spectrometry and the positively charged product ion of budesonide, m/z 431.1 was obtained. The product ion transitions were m/z 431.1 to m/z 413.2, m/z 173 and m/z 147 for budesonide at collision energy at 10, 26 and 34 eV respectively and fragmentor 95.

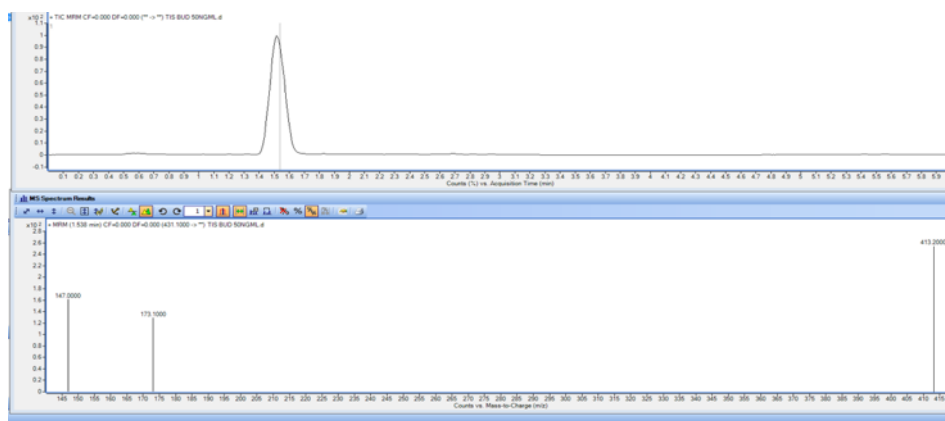


Figure 2-7: Budesonide 50 ng/ml chromatogram showing spectral peaks for all fragments

2.5.9.3 Sample preparation

Calibration curves ranging from 5 ng/ml to 100 ng/ml were done by spiking the porcine oesophageal tissue with known concentrations of tacrolimus or budesonide. Calibration curve samples and tissue samples collected from the Ussing chamber were prepared by addition of extraction solvent (acetonitrile) in a ratio of 30mg tissue to 1ml acetonitrile. This was followed by cutting into small pieces and sonication for 15 minutes in cold water. An OASIS prime HLB cartridge (Waters, USA) was used to remove any salts, proteins and lipids from the sample when trying to quantify drug in the porcine oesophageal tissue. The samples were analysed by LC-MS/MS and ion transition ratios checked to confirm that there was no interference from tissue components. The limit of quantification (LOQ) was 1 ng/ml for budesonide and 5 ng/ml for tacrolimus.

2.5.10 Thermal analysis of Prograf capsule and tacrolimus API

Differential scanning calorimetry (DSC) was used to characterise the pure tacrolimus active pharmaceutical ingredient (API) and Prograf capsule. The material (average sample mass of 8-10 mg) was spread on TA aluminium pans to ensure it covered the pan's surface evenly. The pan was placed in a Q2000 DSC (TA instruments, Waters,

LLC, USA) and measurements began at a heating rate of 10°C/minute. Nitrogen was used as a purge gas with a flow rate of 50ml/minute. Data were collected with TA advantage software (version 2.8.394).

2.5.11 Hematoxylin and Eosin (H & E) staining

Porcine mid oesophageal tissue after 20 minutes stabilization in KBR pH 7.4, was exposed on the apical side to KBR pH 1.5 and pepsin from porcine gastric mucosa (3.2 mg/ml corresponding to 3200 U/ml) for 60 minutes. The tissue was removed from the Ussing chamber and stored in 4% paraformaldehyde at 4°C until sectioning and hematoxylin and eosin staining which was conducted at Institute of neurology, UCL, London, UK. Briefly, the tissues were dehydrated, paraffinized and sectioned to a thickness of 10 µm after which they were deparaffinized and stained with hematoxylin and eosin stain and observed under a light microscope at 40x magnification.

2.5.12 Statistical analysis

The results generated in the study were expressed as mean $\pm$ standard deviation (SD) (n = 3). The data were analysed by Mann Whitney for non-parametric tests comparing sex (male and female) differences and one-way analysis of variance (ANOVA) for tissue accumulation at 90 seconds, 10 and 30 minutes, followed by a Tukey post-hoc analysis with a 95% confidence interval (for time point studies) using IBM SPSS Statistics 16 (SPSS Inc., Chicago, IL, USA).

2.5.13 Experimental controls

Budesonide and tacrolimus comparisons for the same region (proximal, mid or distal) and formulation (micellar versus prograf capsule powder/pulmicort respule) were not conducted on the same day. This is because experiments were conducted as HPLC/LC-MS/MS methods were optimized. It was not possible to repeat experiments for this pilot chapter owing to lack of time.

2.6 Results and discussion

2.6.1 Stability of budesonide and tacrolimus micellar solutions in artificial saliva pH 7.4 and KBR pH 7.4

Cremophor RH60 is a non-ionic surfactant with the ability to solubilise poorly soluble compounds. Cremophor type surfactants have produced smaller nano sized droplets and better size distribution along with lesser propensity towards Ostwald ripening (Zeng et al., 2017). In Table 2-1, the stability of budesonide/cremophor RH60/ethanol was studied by adding 0.2 ml of 5 mg/ml solution to 2.8 ml of artificial saliva or Kreb's bicarbonate ringers buffer. This was to ascertain that the budesonide did not precipitate out of the solution while the permeation experiments were ongoing. Therefore, the solution upon reconstitution and vortexing, was left for 2 and 12 hours at 37°C after which it was centrifuged at 10x g and the supernatant was analysed by HPLC.

After 2 hours, 97.1 and 99.9% of budesonide was recovered in the supernatant of the artificial saliva and bicarbonate ringer's solution pH 7.4, respectively. A slight decrease was observed after 12 hours, where 95.2 and 96.3% of budesonide were recovered from artificial saliva and bicarbonate ringer's solution, respectively. This indicates that budesonide is stable in the surfactant, co-solvent system. Dynamic light scattering (DLS) analysis show that micelles of 14.7 ± 0.2 nm are formed when the budesonide concentrate is reconstituted with the buffers (Figure 2-8).

Table 2-1: 2 and 12 hour stability of budesonide/cremophor RH60/ethanol 0.33mg/ml in artificial saliva and Kreb's bicarbonate ringer's solution pH 7.4

Time (h)	% Budesonide remaining in artificial saliva pH 7.4	% Budesonide remaining in bicarbonate ringer's solution pH 7.4
0	100	100
2	97.1	99.9
12	95.2	96.3

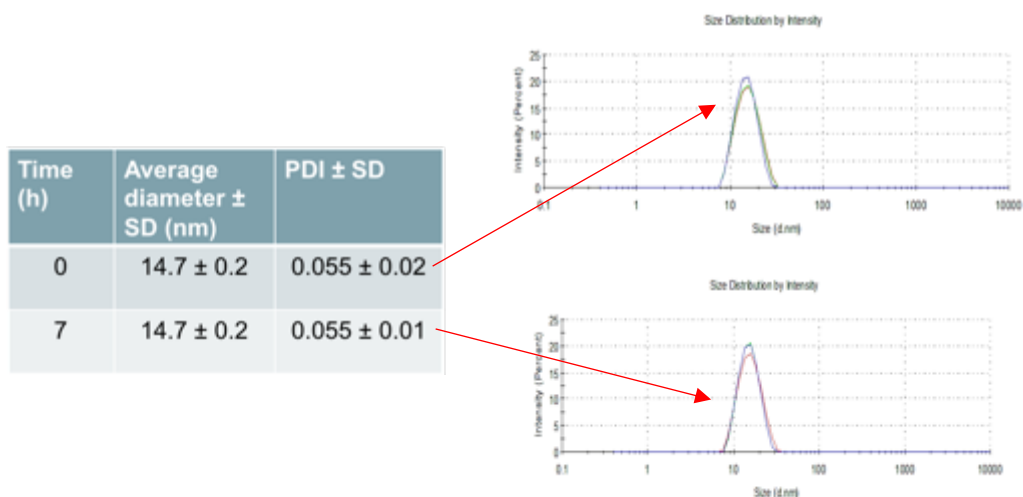


Figure 2-8: Particle size of budesonide/cremophor RH60/ethanol micelles when diluted with artificial saliva and Kreb's bicarbonate ringer solution pH 7.4

Similarly, the same experiment was performed for tacrolimus/cremophor RH60/ethanol as presented in Table 2-2. Tacrolimus concentration over a period of 12 hours was reduced by about 10.5% in artificial saliva but was quite stable (98.4%) in Krebs-Ringer' bicarbonate solution. It seems as though tacrolimus due to its low solubility is precipitating out of the buffers. Tacrolimus is probably affected because of its 10 times lower solubility compared to budesonide. DLS results show with ~15.8 nm diameter a similar micellar size to that of budesonide/cremophor RH60/ethanol (Figure 2-9).

Table 2-2: 2 and 12 hour stability of tacrolimus/cremophor RH60/ethanol 0.33mg/ml in artificial saliva and Kreb's bicarbonate ringers solution pH 7.4

Time (h)	% Tacrolimus remaining in artificial saliva pH 7.4	% Tacrolimus remaining in bicarbonate ringer's solution pH 7.4
0	100.0	100.0
2	94.7	-
12	89.5	98.4

Prograf 0.33 mg/ml		
Time (h)	Average diameter (nm) $\pm$ SD	PDI $\pm$ SD
0	15.7 $\pm$ 0.2	0.074 $\pm$ 0.01
7	15.85 $\pm$ 0.03	0.053 $\pm$ 0.02

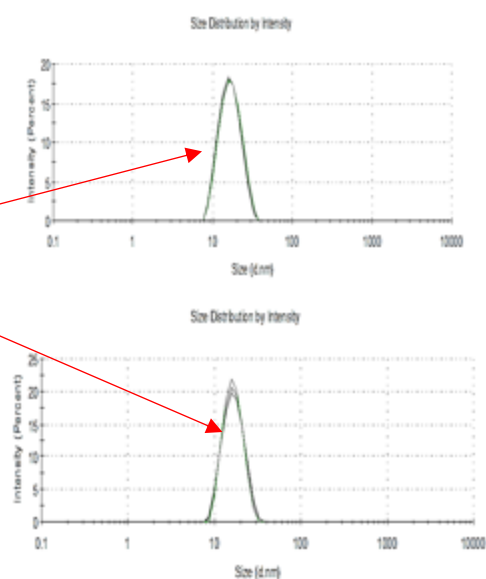


Figure 2-9: Particle size of tacrolimus/cremophor RH60/ethanol micelles when diluted with artificial saliva and Krebs bicarbonate ringers solution pH 7.4

2.6.2 Permeation studies of budesonide and tacrolimus using porcine oesophageal tissues – indirect quantification of tissue permeation by HPLC

2.6.2.1 HPLC Method development for budesonide and tacrolimus

As discussed in section 2.5.7, calibration curves were developed for both tacrolimus and budesonide. For both tacrolimus (Figure 2-10) and budesonide (Figure 2-11), the linearity was $r^2 > 0.99$ and % coefficient of variation for the lowest, middle and highest concentration were $\leq 10\%$ indicating intra method precision and reproducibility.

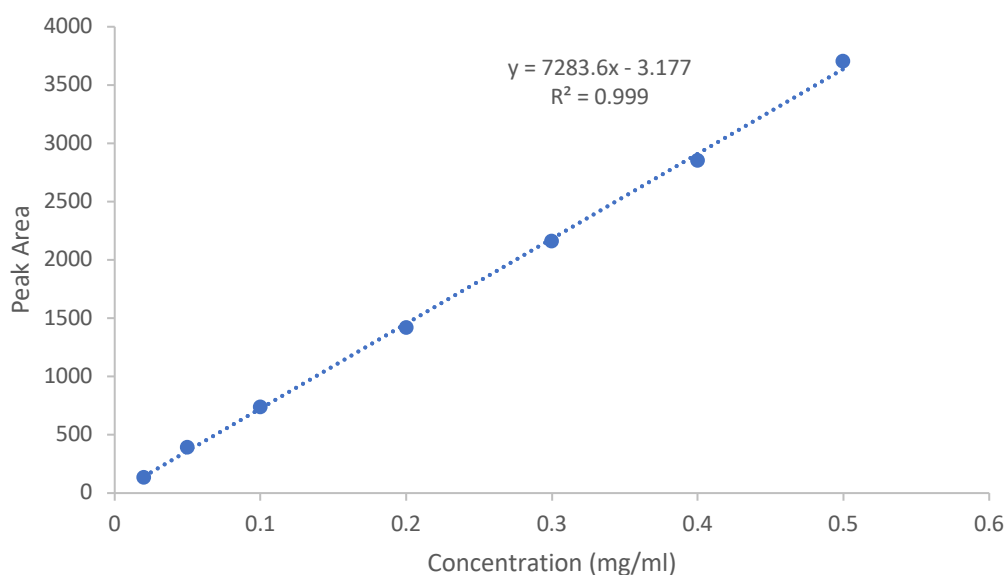


Figure 2-10: Calibration of tacrolimus in ethanol and cremophor RH60 added to Krebs-Ringer bicarbonate solution to make the calibrator concentrations

For budesonide, the linearity was also >0.99 and the % coefficient of variation for the lowest, middle and highest concentration were $\leq 10\%$ indicating intra method precision and reproducibility.

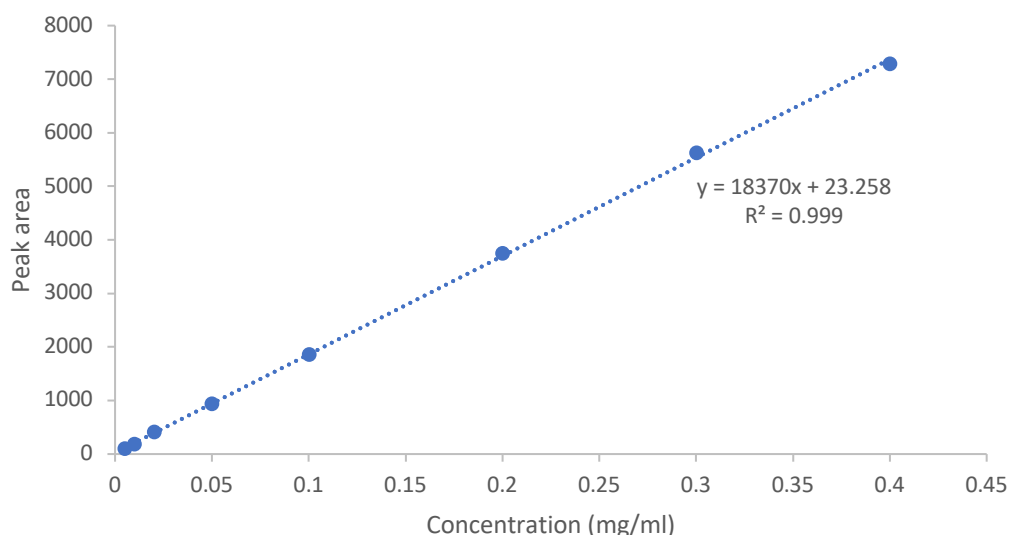


Figure 2-11: Calibration of budesonide in ethanol and cremophor RH60 added to bicarbonate ringer's solution to make the calibrator concentration

2.6.2.2 Quantification of permeation of budesonide and tacrolimus using HPLC

Permeation studies to determine the amount of budesonide or tacrolimus that has permeated across the porcine oesophageal mucosa were conducted. The amount of budesonide or tacrolimus was measured on the apical and basal side by taking samples at initial, 10 minute and 20 minute time points. These time points reflect the short transit times of formulations in vivo (discussed previously) and in vitro studies done (Smart et al., 2013, Smart et al., 2015, Richardson et al., 2005, Richardson et al., 2004) on porcine mucosal flow model. In the porcine flow model, oesophageal tissues were kept on a 45 degrees incline at 37°C and 75% RH and with an artificial saliva flow of 1ml/minute, reflecting saliva secretion rates. However, the studies herein were done in vertical Ussing chambers so tissue was mounted vertically and permeation was conducted while in vertical position and in constant contact with the artificial saliva pH 7.4 containing drug.

As seen in Figure 2-12, the initial concentration of budesonide on addition into the artificial saliva was 100% and gave varying results at 10 and 20 minutes for all the different regions of the oesophagus. The proximal oesophagus showed no change in apical concentrations, mid oesophagus showed a decrease to 97.6% and then an increase to 109.7% and the distal part first showed an increase followed by a decrease at 10 and 20 minutes respectively. No budesonide was detected on the basal side. Therefore, the results of this experiment were inconclusive as to whether the budesonide was actually getting permeated. This basically, meant that either the budesonide was not uniformly distributed when sampling or it was precipitating and re-dissolving. However, it must be noted that these values were all within the acceptance range of the method which is $\pm 10\%$.

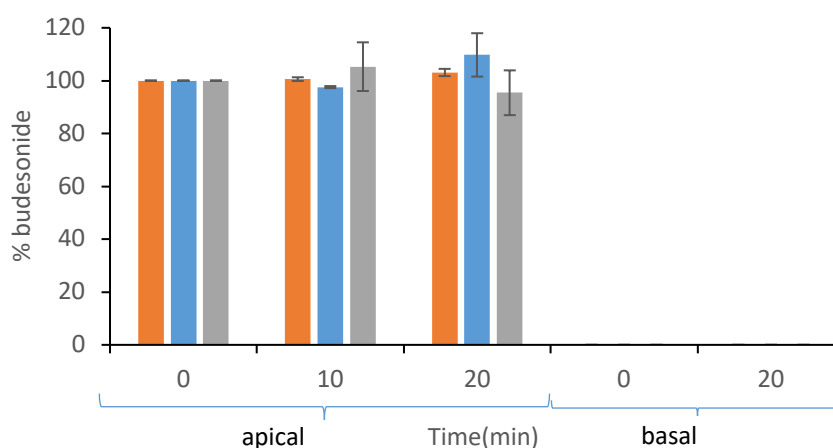


Figure 2-12: Budesonide/Cremophor RH60/ethanol permeation study, $t=20$ minutes at apical conc. 0.33mg/ml , $n=2$, mean $\pm$ SD in porcine proximal (orange bars), mid (blue bars) and distal (grey bars) oesophageal tissue sections

Similarly, the same was tried for tacrolimus, where the apical and basal concentrations of tacrolimus were measured at 0, 10 and 20 minutes as seen in Figure 2-13, Tacrolimus, on the other hand showed a steady decrease in the apical concentration from 100% to about 95% for proximal, mid and distal oesophageal tissues. However, no tacrolimus was detected on the basal side. This is probably because the decrease in apical concentrations corresponds to the decrease at the end of 2 hours most probably from precipitation or adhering to the walls of the Ussing chamber. Alternatively, it

could be that tacrolimus has permeated the lipophilic oesophageal tissue but has not been able to appear on the basal side owing to the thick oesophageal epithelium which is in the order of about 700 μm (Orlando, 2006). Tacrolimus owing to its high logP of 3.3 may prefer to partition into the oesophageal epithelium. However, it should also be noted that the acceptance range of the method is $\pm 10\%$ therefore inconclusive whether it is as a result of that.

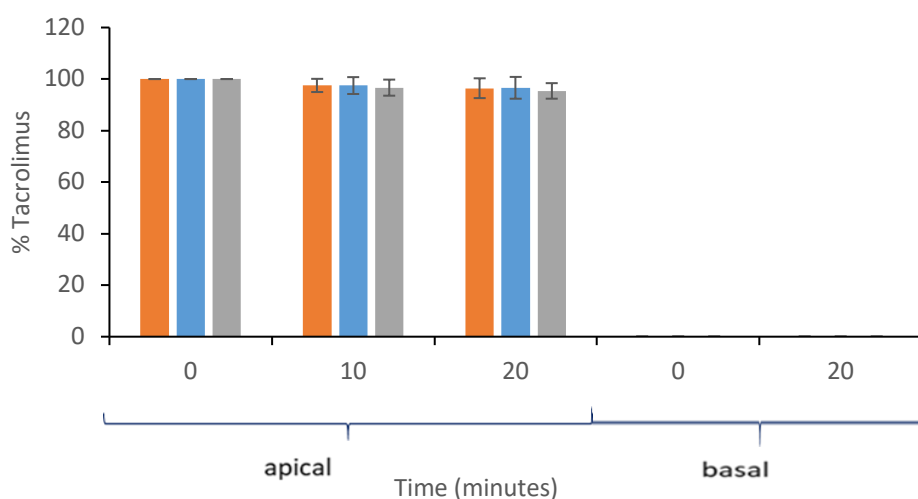


Figure 2-13: Tacrolimus/Cremophor RH60/ethanol permeation study, $t=20$ minutes at apical conc. 0.33mg/ml , $n=3$, mean $\pm$ SD in porcine proximal (orange bars), mid (blue bars) and distal (grey bars) oesophageal tissue sections

2.6.2.3 Permeation of budesonide and tacrolimus in injured oesophageal mucosa

In EoE the oesophageal mucosa is affected by the secretion of cytokines whereby especially IL-13 is implicated towards decreasing the filaggrin and desmoglein proteins of the epithelial tight junctions (Sherrill et al., 2014). This causes dilation of intercellular spaces (Ravelli et al., 2014, Furuta and Katzka, 2015) in the epithelium and could therefore serve as an opportunity for drugs to permeate deeper into the epithelium and in-turn reduce inflammation.

In gastro oesophageal reflux disease (GERD), there is reflux of acid and pepsin from the stomach (Kahrilas, 2008). This causes the formation of dilated intercellular

epithelial spaces up to the order of $> 2.4 \mu\text{m}$ (Tobey et al., 1996, Ravelli et al., 2006). Therefore, this rationale was used to develop a model of the injured oesophageal epithelium and therefore mimic the barrier disruption as seen in EoE. It was done by first stabilizing the tissue in Ussing chamber using Kreb's bicarbonate ringer's solution (KBR) pH 7.4 for 20 minutes (TEER values were between $900 - 1800 \Omega \cdot \text{cm}^2$) followed by incubation of the oesophageal mucosae in the Ussing chamber for 1 hour with 3 ml of KBR pH 1.5 and 3200 U/ml of pepsin from porcine stomach (Sigma Aldrich) on the apical side and KBR pH 7.4 on the basal side. This acidified pepsin composition is similar to that of simulated gastric fluid (SGF) (Wang et al., 2015) except that instead of water and sodium chloride, KBR and 3.2 g/L pepsin from porcine gastric mucosa was used. This led to a decrease in TEER which is expected when tissues are exposed to acidic conditions due to inhibition in the apical sodium channels in an environment with H^+ ions (Orlando, 2010). TEER values did not go below $400 \Omega \cdot \text{cm}^2$. Following this, the apical and basal side was rinsed with KBR pH 7.4 and permeation studies were conducted using a 0.33 mg/ml tacrolimus (prepared using Prograf 5 mg/ml injection solution) in artificial saliva at pH 7.4.

The TEER of the pig tissue in KBR pH 7.4 was compared to that exposed to pepsin 3.2 mg/ml in KBR pH 1.5 (Figure 2-14). A drop in TEER was observed for all the segments corresponding to observations made by other authors when rabbit oesophageal tissues were exposed to acid refluxate elements. Rabbit oesophagus is non-keratinized similar to human and pig (Tobey et al., 1996, Tobey et al., 2001).

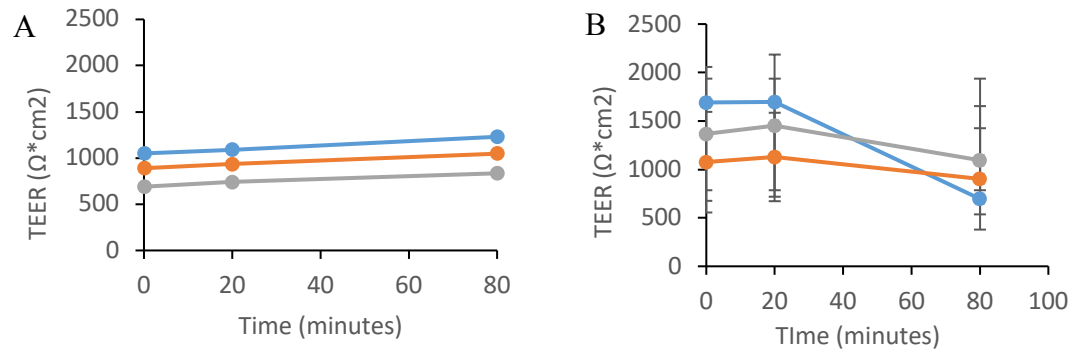


Figure 2-14: Transepithelial electrical resistance (TEER) on exposure to A) KBR pH 7.4 as control and B) SGF pH 1.5 (pepsin 3.2mg/ml or 3200U/ml) of proximal (blue), mid (orange) and distal (grey) sections of porcine oesophagus, n=3, mean $\pm$ SD.

Permeation studies with tacrolimus (Figure 2-15) and budesonide (Figure 2-16) were conducted using this injured tissue. Tacrolimus permeation after acidified pepsin exposure was studied in 3 pig oesophageal tissues and at the end of 20 minutes it was noted that the amount of tacrolimus remaining on the apical side was between 87.5% and 89%. Permeation amounting to about 30 μg into the tissue is very large for such a short exposure. Therefore, since this value correlates to the amount of tacrolimus in artificial saliva pH 7.4 after 12 hours ref Table 2-2, this could either be owing to precipitation of tacrolimus on the apical side or adsorption to the surface of the Ussing chambers. Tacrolimus is known to adsorb onto glass (Zeng et al., 2018).

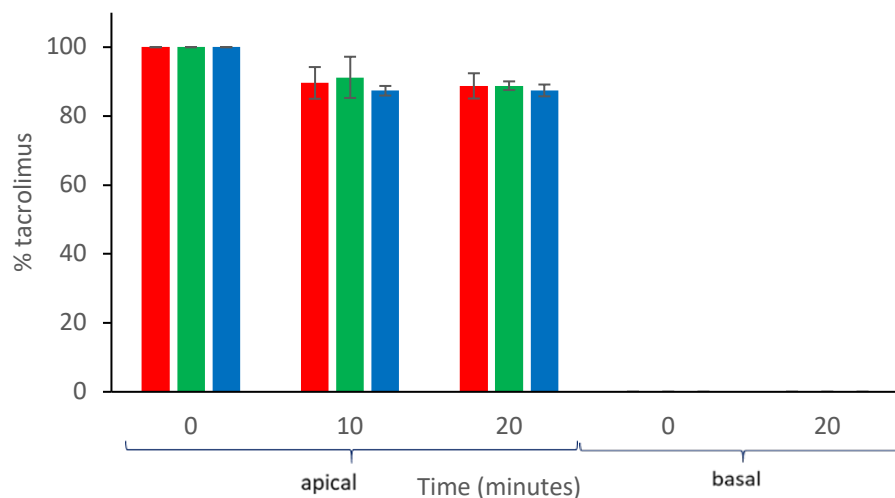


Figure 2-15: Tacrolimus/cremophor RH60/ethanol apical conc. 0.33mg/ml permeation study on pig oesophageal mucosa, proximal (red bars), mid (green bars) and distal (blue bars), exposed to SGF pH 1.5 3.2mg/ml (pepsin 3200 U/ml), n=3, t=20 minutes, mean $\pm$ SD,

The study of artificially injuring the pig oesophageal mucosa with acidified KBR and pepsin was repeated to study the permeation of budesonide. Budesonide mouthwash has been used in chronic graft versus host disease (cGVHD) patients and it was observed that blood concentrations after a single dose of the mouthwash kept in the mouth for 10 minutes were similar to that achieved from oral systemic absorption of a budesonide effervescent tablet dissolved in water (Dilger et al., 2009). This could be attributed to by-passing the first-pass hepatic metabolism, lower cytochrome 3A4 enzymes in the buccal mucosa of patients or leaky epithelium in the mucosa of cGVHD patients. Figure 2-16 shows that no permeation of budesonide took place even after 20 minutes. This is surprising considering the fact that budesonide (mol wt 430g/mol) is a molecule with a smaller molecular weight than tacrolimus (mol wt. 804 g/mol) and it would therefore be expected to better permeate through the injured oesophageal epithelium through the leaky tight junctions.

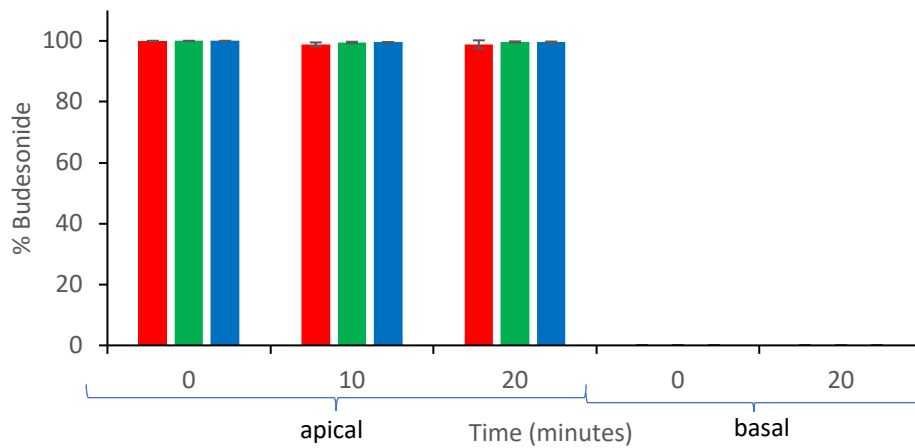


Figure 2-16: Budesonide/cremophor RH60/ethanol apical conc. 0.33mg/ml permeation study on pig oesophageal mucosa, proximal (red bars), mid (green bars) and distal (blue bars,) exposed to SGF pH 1.5 3.2mg/ml (pepsin 3200 U/ml), n=3, t=20 minutes, mean $\pm$ SD,

Hematoxylin and Eosin (H&E) staining was performed on the pig mid oesophageal tissues exposed to acidified KBR and pepsin. The results showed that there was mild spongiosis (oedema of the epithelium) in the epithelium. Spongiosis has been reported in gastroesophageal reflux disease as well as eosinophilic oesophagitis (Bejarano et al., 2004, Katzka et al., 2014).

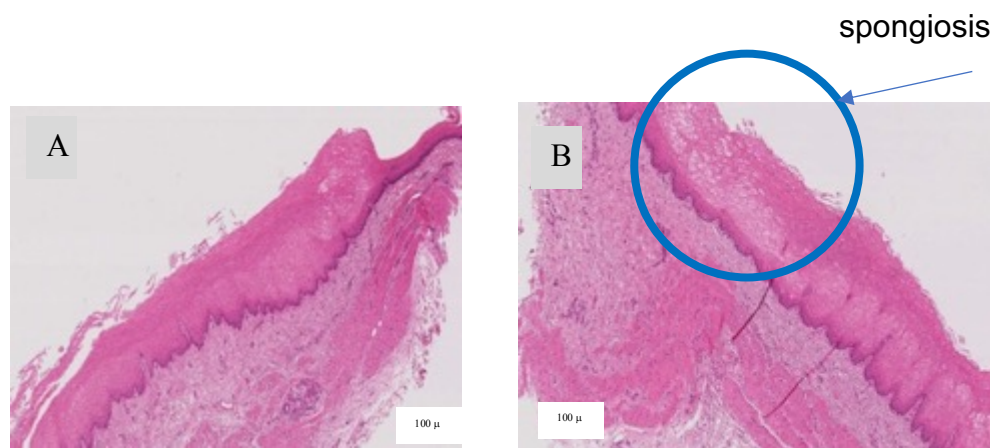


Figure 2-17 Hematoxylin & Eosin stain of porcine mid oesophageal mucosa exposed to a) KBR pH 7.4 and b) KBR pH 1.5 + pepsin 3.2mg/ml or 3200 U/ml (magnification x100)

It should be noted that the HPLC method is not a sensitive method and limit of quantification (LOQ) for budesonide was 5 µg/ml whereas that for tacrolimus was 10 µg/ml. The apical decrease owing to the permeation of budesonide or tacrolimus in the order of 20-30 µg/ml seems highly unlikely given the short exposure time. The results observed are either due to: i) the acceptance range of the method, ii) the adsorption of the drugs to the Ussing chambers, especially for tacrolimus which has been shown to adsorb on glass (Zeng et al., 2018) iii) or precipitation out of solution due to being below critical micelle concentration on dilution or as a result of salting out.

Therefore, a more sensitive method is required to directly determine accumulation of drug in the oesophageal tissue for local action; that specifically being the aim for delivery of these APIs to treat EoE. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a sensitive method and budesonide and tacrolimus in plasma samples have been quantified using LC-MS/MS at nanograms/ml levels (Lensmeyer and Poquette, 2001, Edsbäcker and Andersson, 2004). It was hypothesized that there may be accumulation of drugs at nanogram levels occurring in the porcine oesophageal tissue and therefore it was tried to quantify the drugs in the tissue homogenates.

2.6.2.4 LC-MS/MS method development for quantification of budesonide and tacrolimus in porcine oesophageal mucosa

2.6.2.4.1 Budesonide LC-MS/MS method development

As explained in the LC-MS/MS method, the budesonide parent ion of m/z 431.1 underwent optimizer over a range of collision energies to give a quantifier ion of m/z 413.3 and m/z 173. Calibration curves as seen in

Figure 2-18 and Figure 2-19 using calibrator concentrations of budesonide/cremophor RH60/ethanol stock solution prepared in artificial saliva pH 7.4 were spiked onto 30 mg sections of oesophageal mucosa. 1 ml of acetonitrile was added to and tissue chopped finely using scissors. The samples were then subjected to sonication in ice-cold water bath for 15 minutes, followed by centrifugation at 10 x g for 15 minutes. The supernatant was removed and sample clean-up (removal of phospholipids, salts and proteins) was done by passing through an OASIS Prime HLB extraction cartridge (Waters Corp., USA). An equivalent amount of acetonitrile was passed through the cartridge to improve the recovery of the budesonide. The sample was then analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The standard calibration concentrations of budesonide in acetonitrile were also prepared and subjected to similar conditions as the samples spiked on to porcine oesophageal tissue.

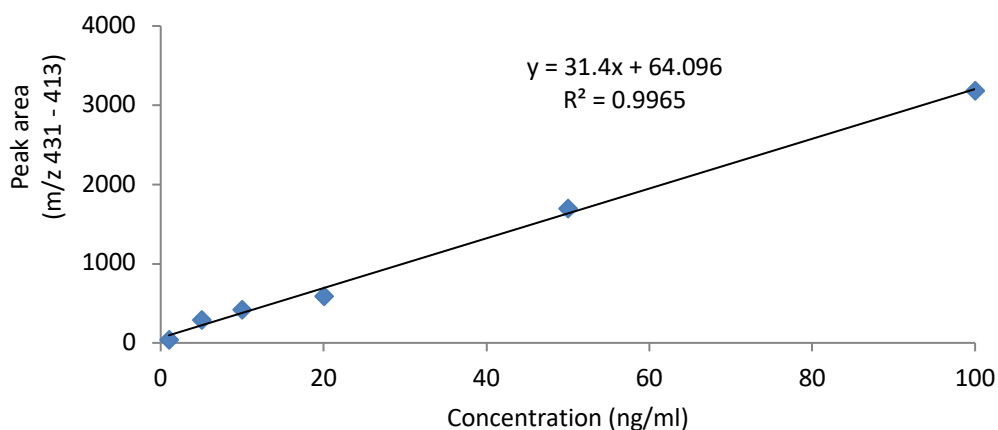


Figure 2-18: Calibration curve of Budesonide/Cremophor RH60/Ethanol in acetonitrile (ACN)

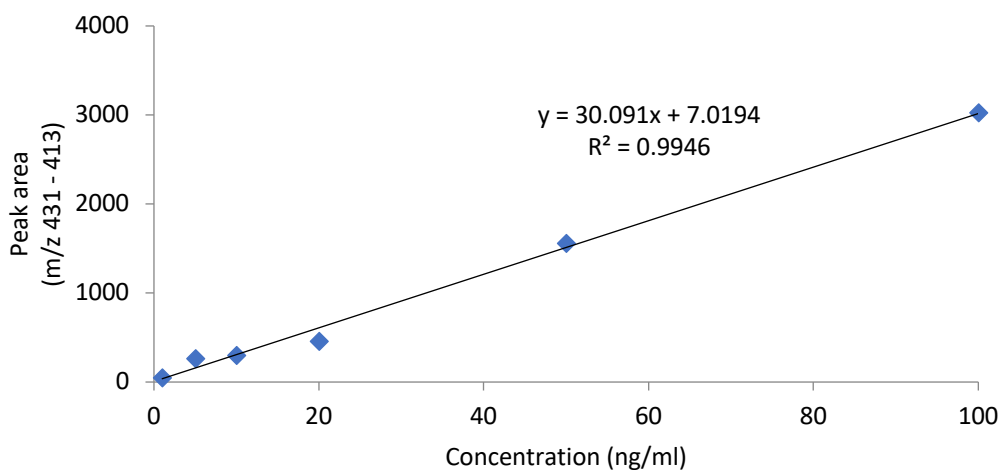


Figure 2-19: Calibration curve of Budesonide/Cremophor RH60/Ethanol in pH 7.4 artificial saliva spiked on pig oesophageal tissue

The percentage recovery from the tissue was calculated and was out of the 80-120% range for the 10 and 20 ng/ml concentrations. In order to determine if there was matrix interference from the tissue, ion ratios for the quantifier (m/z 413.2) and qualifier ion (m/z 173.1) were calculated and Tolerance was set in the range of $\pm 20\%$ which

indicates that there is no interference of these ions from the tissue matrix (Table 2-3) (Angeles and Aga, 2018, Communities, 2002).

$$\text{Tolerance} = \frac{(\text{ion ratio of budesonide in ACN} - \text{ion ratio of budesonide spiked on esophageal tissue}) \times 100}{\text{ion ratio of budesonide in ACN}}$$

Table 2-3: Percentage recovery values of budesonide spiked on porcine oesophageal tissue versus budesonide dissolved in acetonitrile only. Tolerance values of budesonide indicate no tissue matrix interference.

Budesonide Conc (ng/ml)	% recovery on spiking tissue (80-120%)	Ion transition ratio (calibration of budesonide in ACN)	Ion transition ratio (calibration of budesonide spiked on pig oesophageal tissue)	Tolerance (%)
1	120.0	0.9	0.88	2.2
5	89.0	0.79	0.69	12.7
10	70.9	0.68	0.76	-11.8
20	77.6	0.63	0.73	-15.9
50	91.7	0.52	0.52	0.0
100	94.8	0.53	0.56	-5.7

2.6.2.4.2 Budesonide quantification in intact and simulated gastric fluid injured porcine oesophageal mucosa

Budesonide permeation in intact tissue over a 20 minute period was assessed in all 3 oesophageal segments, proximal, mid and distal as seen in Figure 2-20. It was found that the greatest accumulation took place in the mid and distal (21.31 ± 11 ng/mg and 20.3 ± 1.2 ng/mg). This equated to a higher percent applied dose of ca. 0.20% in the distal segment as compared to the mid segment. The higher percentage applied dose in the distal segment is owing to a heavier tissue as compared to the mid segment. The percent applied dose is calculated by dividing total accumulation of drug in tissue by the total amount of drug exposed on the apical side.

Given that budesonide could be detected in oesophageal tissues upon permeation studies, the aim was then to determine if there would be greater permeation in an injured tissue due to increased paracellular space. The tissue was same as per that used in Figure 2-16 and was now being used to quantify the amount of budesonide permeated into the tissue. Briefly, it was exposed for 60 minutes to KBR pH 1.5 containing 3.2 mg/ml pepsin (simulated gastric fluid like). The simulated gastric fluid media was then removed and replaced with artificial saliva pH 7.4 and permeation conducted for 20 minutes with budesonide at an apical concentration of 0.33 mg/ml. As seen in Figure 2-20, the permeation of budesonide in the injured porcine oesophageal tissue is almost the same as the intact tissue except for the proximal segment where it is slightly higher (22 ± 1.6 ng/mg versus 12 ± 11 ng/mg). This shows that there is greater accumulation of budesonide occurring in the injured tissue relative to the non-injured tissue. However, these experiments (non-injured versus injured tissue) were not done at the same time and on the same day and therefore small inter-day variations although tissues form the same pig, may have influenced these results. Therefore, these experiments would need to be repeated comparing the control to the test on the same day and time. Also, the permeation exposure times will be conducted for 90 seconds, 10 minutes and 30 minutes which are representative of the in vivo gamma scintigraphy and ex-vivo bioadhesion studies mentioned above (Dellon et al., 2012, Collaud et al., 2007, Richardson et al., 2005).

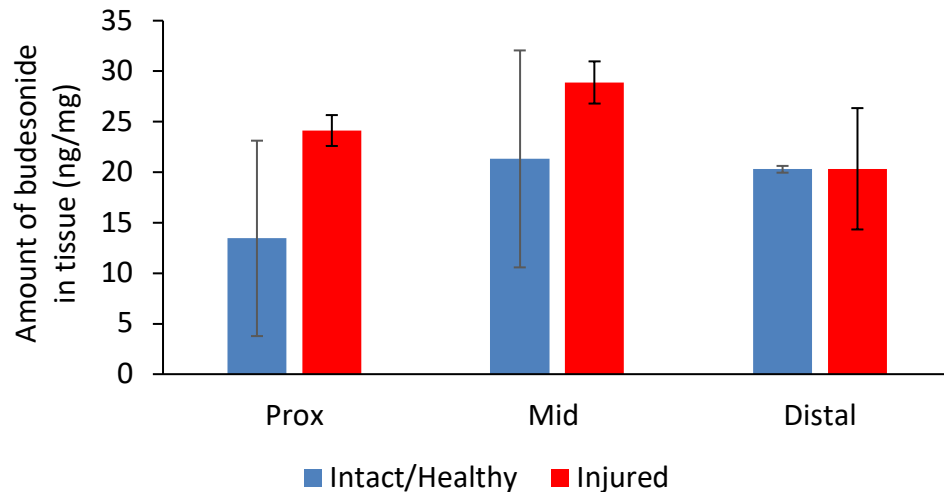


Figure 2-20: Permeation of budesonide/Cremophor RH60/ethanol formulation on intact/healthy (blue bars) and injured (red bars) pig proximal, mid and distal oesophageal tissue over 20 minutes, at apical conc. 0.33 mg/ml (n=2), expressed in ng/mg, mean $\pm$ SD

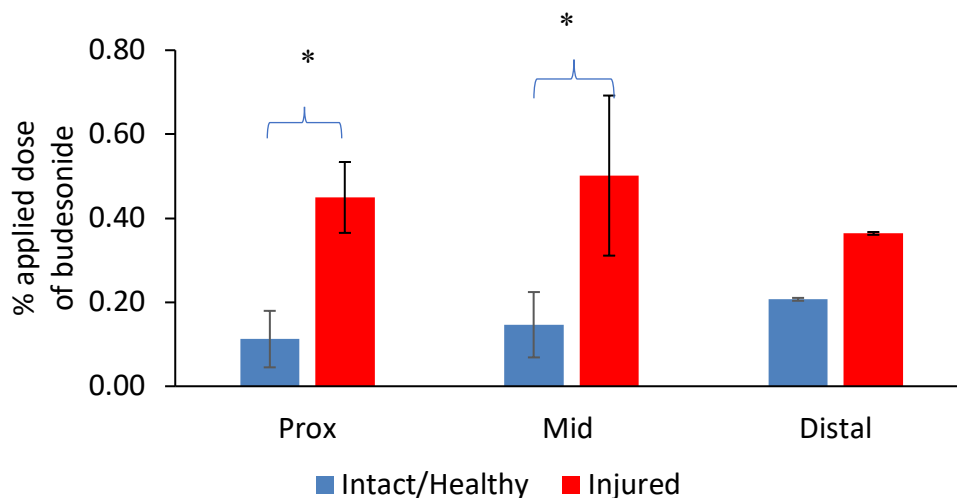


Figure 2-21: Permeation of budesonide/Cremophor RH60/ethanol formulation on intact/healthy (blue bars) and injured (red bars) pig proximal, mid and distal oesophageal tissue over 20 minutes, at apical conc. 0.33 mg/ml (n=2), expressed as percentage of applied dose, mean $\pm$ SD. * Denotes $p < 0.05$

The micellar form of budesonide was used in the above experiments. This affords complete solubilisation and therefore may be giving higher tissue accumulation. The current off-label use of budesonide is from suspensions mainly prepared from Pulmicort respules with sucralose in the form of a slurry. Therefore, the permeation of budesonide in intact porcine mid and distal oesophageal tissues as seen in Figure 2-22 was determined using Pulmicort respules whereby the solution was added to artificial saliva pH 7.4. Interestingly, the accumulation of budesonide in the mid and distal segments is lower with the suspension form as compared to the micellar form; 7.5 ± 2.4 mg/mg and 11.0 ± 8.1 ng/mg versus 21.31 ± 11.00 ng/mg and 20.3 ± 1.2 ng/mg respectively. However, it must be noted that this experiment was done on a different day but using tissues from the same pigs. The suspension form needs to first solubilize then gets absorbed. Therefore, the permeation is dissolution rate limited and lower as compared to the micellar form.

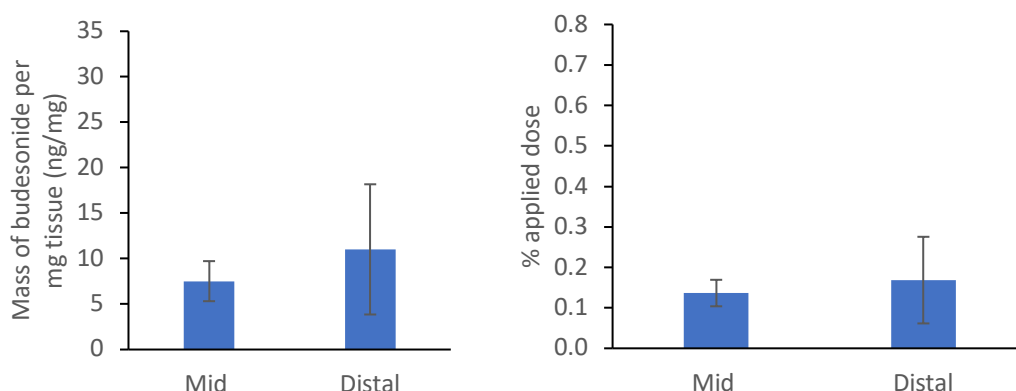


Figure 2-22: Permeation of budesonide using Pulmicort respules, apical conc. 0.33 mg/ml (n=3) over 20 minutes on pig mid and distal oesophagus, mean $\pm$ SD

2.6.2.4.3 Tacrolimus

As explained in the LC-MS/MS method, the sodium adduct of tacrolimus, m/z 826 underwent optimizer over a range of collision energies to give a quantifier ion of m/z 616 and qualifier ion of m/z 415. Calibration curves as seen in Figure 2-23 and Figure 2-24 using calibrator concentrations of tacrolimus/cremophor RH60/ethanol stock solution prepared in artificial saliva pH 7.4 were spiked onto 30 mg sections of

oesophageal mucosa. 1 ml of acetonitrile was added to and tissue chopped finely with scissors. The samples were then subjected to sonication in ice-cold water for 15 minutes, followed by centrifugation at 10 x g for 15 minutes. The supernatant was removed and sample clean-up (removal of phospholipids, salts and proteins) was done by passing through an OASIS Prime HLB extraction cartridge (Waters Corp., USA). An equivalent amount of acetonitrile was passed through the cartridge to improve the recovery of the tacrolimus. The sample was then analysed by LC-MS/MS. The standard calibration concentrations of tacrolimus in acetonitrile were also prepared and subjected to similar conditions as the samples spiked on to porcine oesophageal tissue.

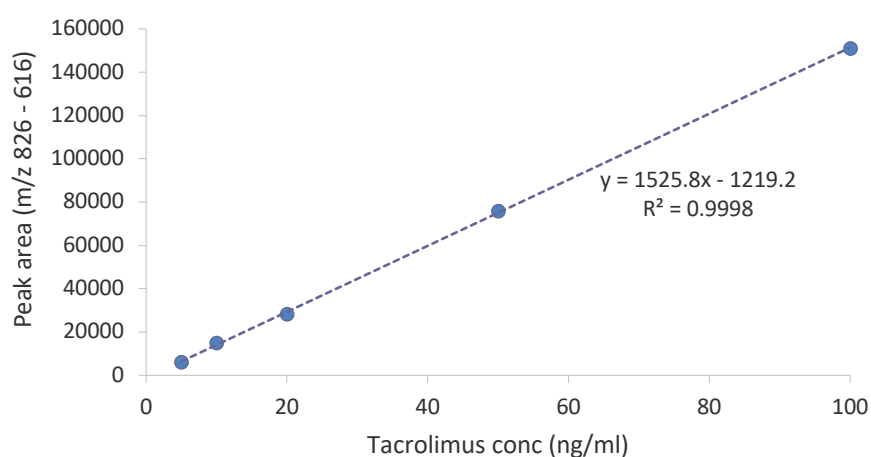


Figure 2-23: Calibration curve of tacrolimus/Cremophor RH60/ethanol in acetonitrile (ACN)

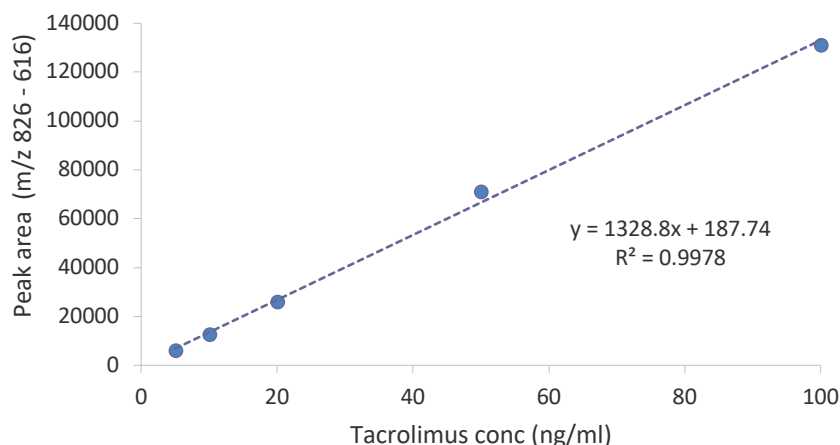


Figure 2-24: Calibration curve of tacrolimus/Cremophor RH60/ethanol in pH 7.4 artificial saliva spiked on pig oesophageal tissue

The percentage recovery from the tissue was calculated and all concentrations were within the 80-120% range. In order to determine if there was matrix interference from the tissue, ion ratios for the quantifier (m/z 616) and qualifier ion (m/z 415) were calculated and were in the range of $\pm 20\%$ tolerance, except for the 5ng/ml calibrator concentration, indicating that there is no interference of these ions from the tissue matrix (Table 2-4) (Communities, 2002, Angeles and Aga, 2018).

$$\text{Tolerance} = \frac{(\text{ion ratio of tacrolimus in ACN} - \text{ion ratio of tacrolimus spiked on esophageal tissue}) \times 100}{\text{ion ratio of tacrolimus in ACN}}$$

Table 2-4: Percentage recovery values of tacrolimus spiked on pig oesophageal tissue versus tacrolimus dissolved in acetonitrile only. Tolerance values of budesonide indicate no tissue matrix interference.

Tacrolimus Conc (ng/ml)	% recovery on spiking tissue (80- 120%)	Ion transition ratio (calibration of tacrolimus in ACN)	Ion transition ratio (calibration of tacrolimus spiked on pig oesophageal tissue)	Tolerance (%)
5	101.3	0.79	0.62	21.5
10	84.2	0.62	0.65	-4.8
20	91.8	0.58	0.68	-17.2
50	106.9	0.57	0.60	-5.3
100	86.8	0.55	0.60	-10.3

2.6.2.4.4 Tacrolimus quantification in intact and simulated gastric fluid injured porcine oesophageal mucosa

Next, the permeation of tacrolimus was studied in the proximal, mid and distal porcine oesophageal healthy tissue. Tacrolimus is also a lipophilic drug (BCS class II) similar to budesonide and owing to its higher lipophilicity (log P 3.3 versus 2.4), should achieve similar or even better permeation than budesonide. As seen in Figure 2-25 the permeation of tacrolimus is not different to budesonide when expressed as ng/mg. Furthermore, the percentage of applied dose (Figure 2-26) is up to 0.2% for the intact tissue, which shows a low local drug accumulation similar to budesonide.

In Figure 2-25 and Figure 2-26, the permeation of tacrolimus was tested in the tissues from the experiment in Figure 2-15. Briefly, it was exposed for 60 minutes to KBR pH 1.5 containing 3.2 mg/ml pepsin (simulated gastric fluid like). The simulated gastric fluid media was then removed and replaced with artificial saliva pH 7.4 in apical side and permeation conducted with tacrolimus at a concentration of 0.33 mg/ml. The aim was to understand whether injury mimicking gastro oesophageal reflux will increase the permeation as compared to non-injured tissue. The data shows that there is no difference in the tissue accumulation of tacrolimus after exposure to KBR pH 1.5 and pepsin 3.2 mg/ml.

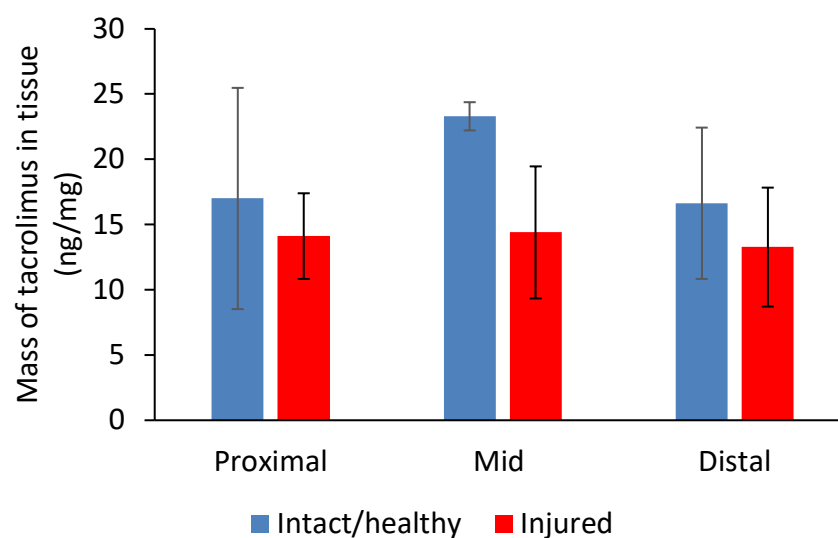


Figure 2-25: Permeation of tacrolimus/Cremophor RH60/ethanol formulation on intact/healthy (blue bars) and injured (red bars) pig proximal, mid and distal oesophageal tissue over 20 minutes, at apical conc. 0.33 mg/ml (n=3), expressed in ng/mg, mean $\pm$ SD

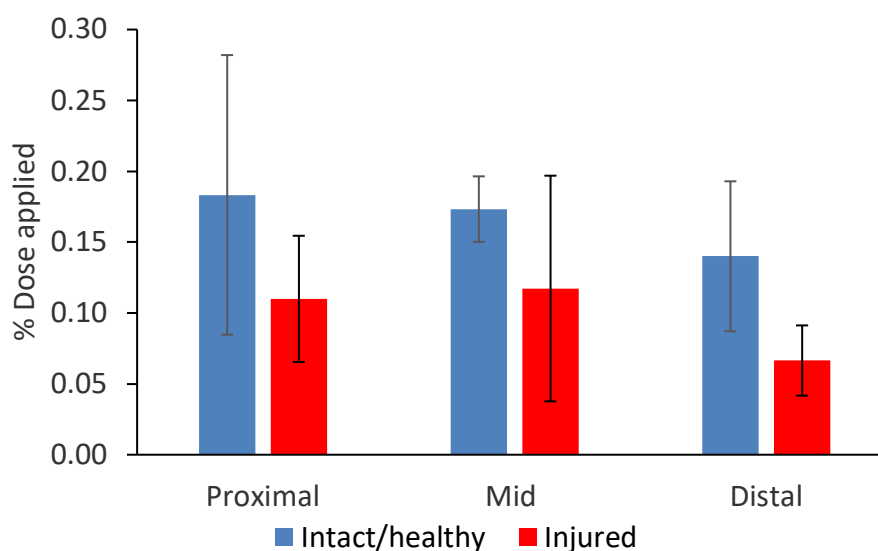


Figure 2-26: Permeation of tacrolimus/Cremophor RH60/ethanol formulation on intact/healthy (blue bars) and injured (red bars) pig proximal, mid and distal oesophageal tissue over 20 minutes, at apical conc. 0.33 mg/ml (n=3), expressed as percentage of applied dose, mean $\pm$ SD.

Lastly, similar to budesonide, tacrolimus in suspension form was studied as this would represent a capsule's content diluted with a sweetener, similar to budesonide's off label use. It is claimed that the tacrolimus in the Prograf[®] capsule is in a solid dispersion; this would mean that the tacrolimus is in an amorphous form and therefore dissolves quickly on coming in contact with the buffer in the donor compartment of the Ussing chamber. Performing differential scanning calorimetry (DSC) on the Prograf[®] capsule powder resulted in an endothermic peak at 146°C similar to the one obtained for the pure, crystalline powder. The slight shift between the two peaks may be due to the excipients contained in the Prograf capsule. The tacrolimus permeation is almost 5-fold lower as compared to the micellar form Figure 2-27. This indicates the need to use an enabling formulation such as the micellar solution of tacrolimus to understand its permeation in the absence of rate limiting dissolution. This is of critical importance given the fast transit time of formulations through the oesophagus not allowing a timely drug dissolution. Basolateral levels of tacrolimus are measurable which is an indication that the tacrolimus is permeating through the tissue and not adhering to the surface of the tissue.

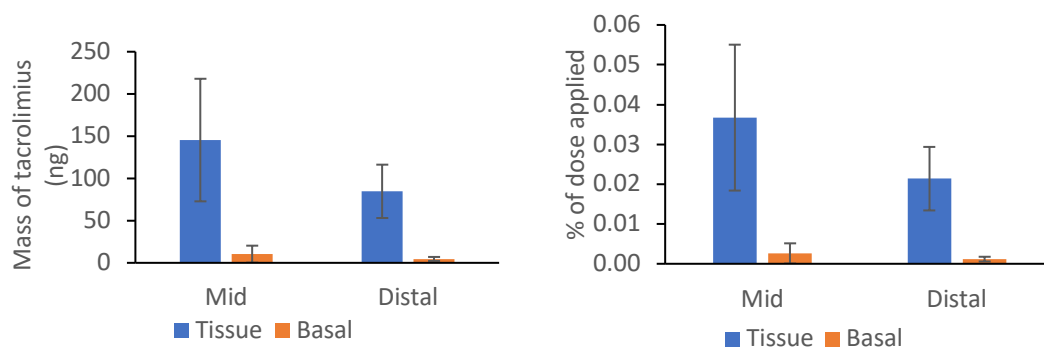


Figure 2-27: Permeation of tacrolimus using Prograf capsule powder, apical conc. 0.33 mg/ml (n=3) over 20 minutes on male pig mid and distal oesophagus (blue bars) and basal side (orange bars)

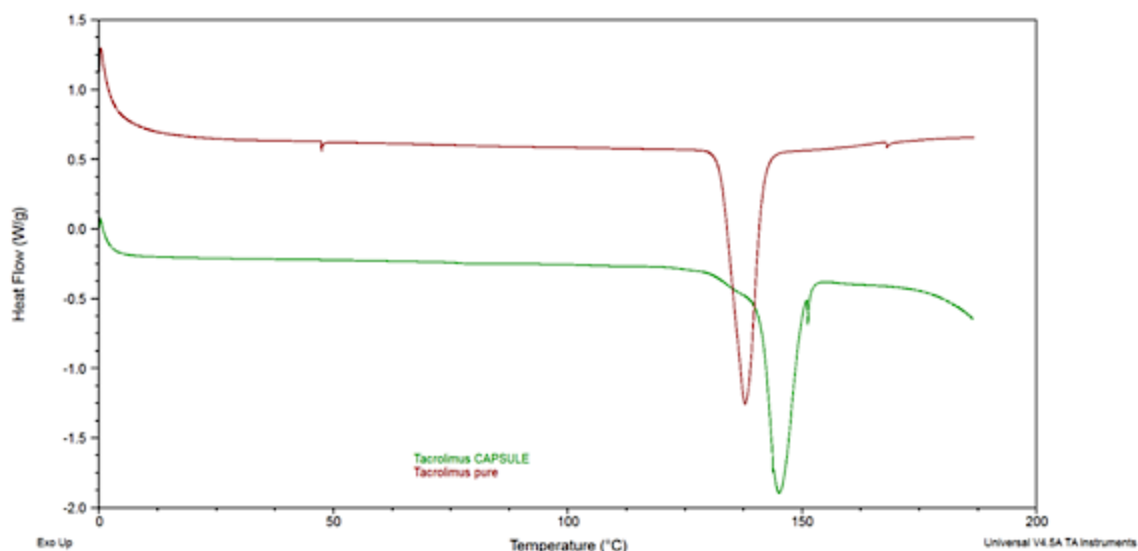


Figure 2-28: DSC thermogram of pure tacrolimus and the capsule contents of Prograf[®] capsule

2.6.2.5 Effect of sex (gender) and mucosal contact time on the permeation of budesonide and tacrolimus

EoE is a disease that predominantly affects males with a ratio of males to females being, 3:1 (Cianferoni et al., 2015). In Figure 2-29 and

Figure 2-30, the amount of budesonide accumulated in porcine mid oesophageal tissue is determined at different time points, 90 seconds, 10 and 30 minutes. Tissues from male and female pigs have been used to determine if there is a sex difference in the permeation. Budesonide and tacrolimus are both substrates of P-glycoprotein (P-gp) (Chen et al., 2017, Saeki et al., 1993). The porcine oesophagus has been reported to have P-gp levels in the oesophagus, with an increasing gradient observed distally (buccal to ileum) (Gao et al., 2014). However, it is unknown if P-gp is expressed in the oesophagus of humans. EoE is more prevalent in human males than females. Therefore, it was investigated if there are differences in the permeation of budesonide

and tacrolimus between male and female porcine oesophageal tissues, owing to potential differences in P-gp expression. This is because, P-gp has shown sex differences in its expression in the liver of humans (Bebawy and Chetty, 2009) and in the gastrointestinal tract (specifically the jejunum) of humans (Mai et al., 2020). Permeation studies in an Ussing chamber on rat intestinal tissues showed that both ganciclovir and ranitidine exhibited a sex difference in intestinal permeability in the fasted-state (Dou et al., 2018). Any sex differences in permeation of tacrolimus and budesonide in porcine oesophageal tissues could be attributed to P-gp thereby underpinning the importance of investigating sex differences in permeation of P-gp substrates.

Neither mucosal contact time nor sex had an influence on the accumulation of budesonide in oesophageal tissues after exposure for 90 seconds, 10 and 30 minutes. Interestingly, the amount of budesonide is similar in all tissues at all time points. This indicates that budesonide permeation starts taking place immediately. However, at 30 minutes some budesonide was detected in the basal side indicating that it takes longer to permeate through the tissue. It could also be the case that budesonide adheres to the unstirred water layer on the oesophageal epithelium in the first 90 seconds and over time gets absorbed into the tissue.

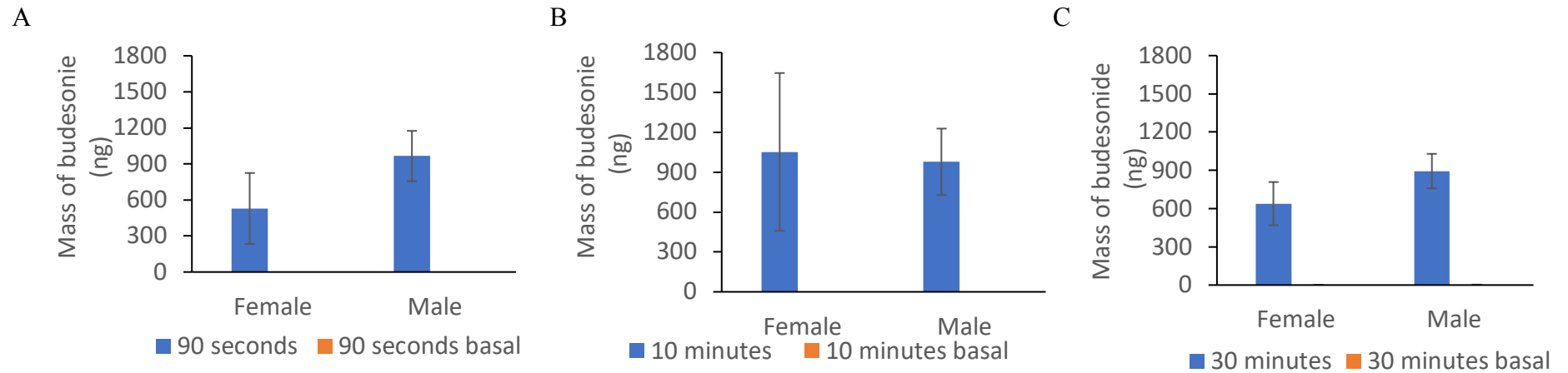


Figure 2-29: Effect of mucosal contact time and sex on porcine mid oesophageal mucosal permeability of budesonide at apical conc. 0.33mg/ml, n=3 mean±SD. Permeation of budesonide in males and female pig oesophageal tissues at A) 90 seconds, B) 10 minutes and C) 30 minutes represented as the mass of budesonide accumulated in nanograms in tissue (blue squares) and on the basolateral side (orange squares)

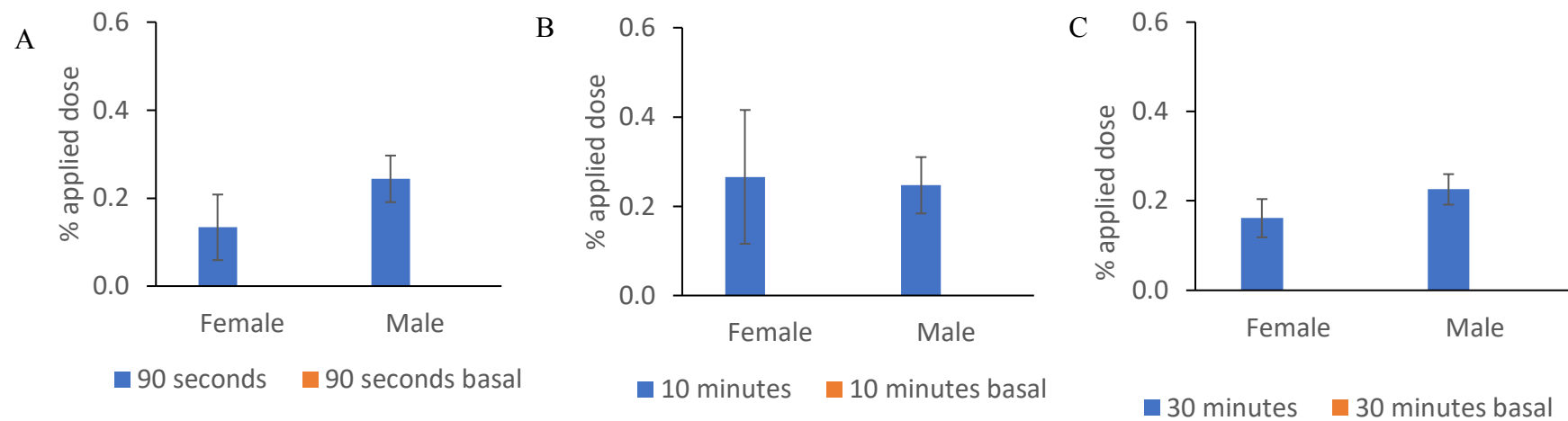


Figure 2-30: Effect of mucosal contact time and sex on porcine mid oesophageal mucosal permeability of budesonide, apical conc. 0.33mg/ml, n=3, mean±SD. A) Permeation of budesonide in males and female oesophageal tissues (blue squares) and onto the basal side (orange squares) at A) 90 seconds, B) 10 minutes and C) 30 minutes represented as the percentage of dose applied on apical side

In Figure 2-31 and Figure 2-32, the permeation of tacrolimus was determined in male and female mid pig oesophageal tissues. The aim was to determine mucosal accumulation of tacrolimus with contact times of 90 seconds, 10 and 30 minutes. Tacrolimus is also a P-gp substrate and the aim was to check whether there is a difference in accumulation of tacrolimus between male and female tissues. No gender difference was observed in tacrolimus accumulation up to 30 minutes of tissue exposure.

However, in the case of tacrolimus, the tissue accumulation increased with respect to exposure time. Basolateral accumulation of tacrolimus was observed at 90 seconds, ca. 6 ng increasing to almost 50 ng at 30 minutes. Tacrolimus is a lipophilic compound and it is not surprising that it can easily permeate the lipophilic oesophageal epithelium. However, its ability to reach the basolateral side in 90 seconds is remarkable.

It should be noted that the accumulation of tacrolimus is lower than budesonide. Permeation of budesonide in porcine mid oesophageal tissue was almost 10-fold greater than tacrolimus at 10 minutes. This is probably due to tacrolimus' lipophilic nature and ability to permeate quickly across the tissue and to the basolateral side as compared to budesonide which has a higher mucosal to plasma affinity ratio thereby remaining in tissue.

For tacrolimus expressed as a percentage of applied dose, no significant difference was observed between gender at same time point ($p>0.05$). However, permeation of tacrolimus at 30 minutes was significantly greater than 10 minutes and 90 seconds ($p<0.05$).

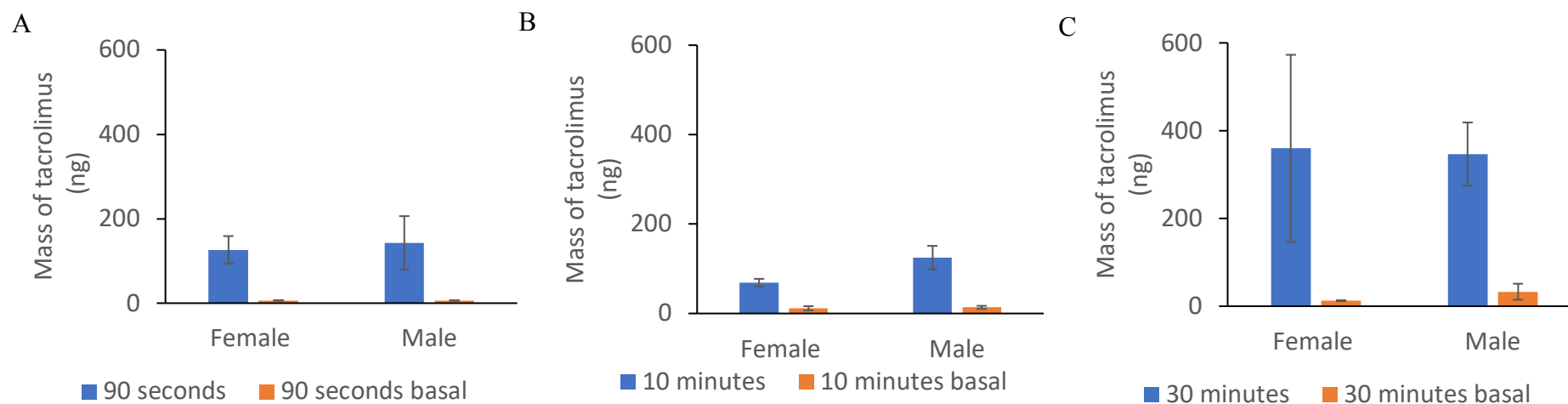


Figure 2-31: Effect of mucosal contact time and sex on porcine mid oesophageal mucosal permeability of tacrolimus, apical conc. 0.33mg/ml, n=3, mean±SD. Permeation of tacrolimus in males and female pig oesophageal tissues at A) 90 seconds, B) 10 minutes and C) 30 minutes represented as the mass of tacrolimus accumulated in nanograms in tissue (blue squares) and on the basolateral side (orange squares)

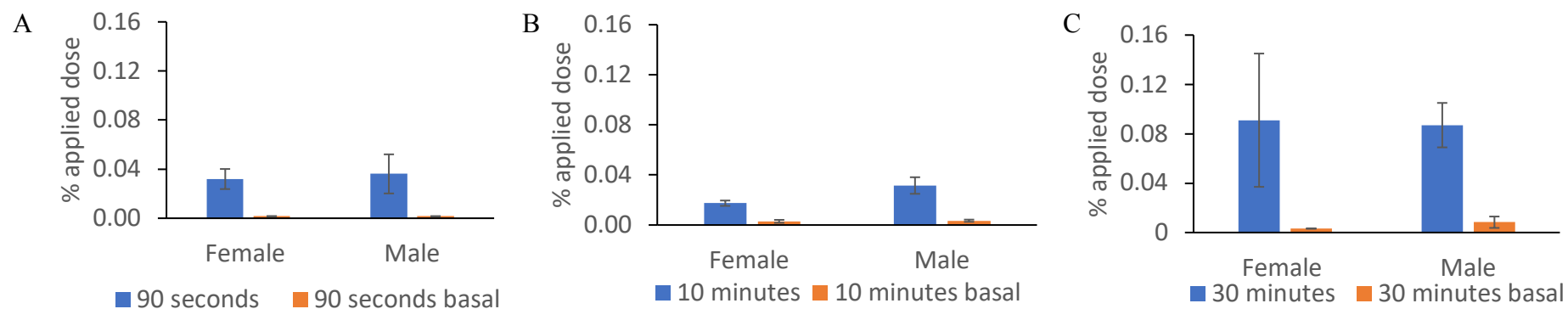


Figure 2-32: Effect of mucosal contact time and sex on porcine mid oesophageal mucosal permeability of tacrolimus, apical conc. 0.33mg/ml, n=3, mean±SD. Permeation of tacrolimus in male and female pig oesophageal tissues at A) 90 seconds, B) 10 minutes and C) 30 minutes represented as the percentage applied dose in tissue (blue squares) and on the basolateral side (orange squares).

2.7 Conclusion

Based on the above results, it can be concluded that the porcine oesophagus shows almost similar permeation across all the regions (intact proximal, mid and distal) for both, tacrolimus and budesonide. HPLC is not sensitive enough to indirectly detect the amount of budesonide or tacrolimus permeated by measuring the percentage reduced in the apical side owing to permeation into and through the tissue. However, LC-MS/MS due to its ability to detect in the nanogram range, serves to directly quantify the amount of drugs in the oesophageal tissue. The micellar solution offers superior permeation compared to the suspension form. Going forwards, drug in solubilised form was used to study the various permeation scenarios.

No data on accumulation of budesonide nor tacrolimus is available from oesophageal tissues of human EoE patients. It is therefore difficult to know what amount of drug in tissue is required to lead to induction and remission. However, if the budesonide slurry has an oesophageal contact time of 90 seconds to 3 minutes, it is possible that an even lower amount of budesonide accumulates than that observed in the ex vivo 30 minutes permeation study on porcine oesophageal tissues. Tacrolimus permeation was also observed at all contact time points, 90 seconds, 10 and 30 minutes with significantly greater permeation seen at 30 minutes. Furthermore, it also permeates on the basolateral side. Thus, the local application of tacrolimus to the oesophagus for the treatment of EoE is of promise.

However, due to the dilation of intracellular spaces (DIS), a hallmark feature of EoE, there may consequently be greater tissue accumulation of budesonide and tacrolimus. This was not observed in the preliminary experiments with simulated gastric fluid (SGF) probably because it was not able to create DIS as observed in gastroesophageal reflux disease. Furthermore, the experiments between intact and injured (SGF exposed) tissues were conducted on different days although the tissues were from the same pigs; thereby cannot quite compare the permeation of both these studies.

CHAPTER 3 : TISSUE ACCUMULATION AND/OR PERMEABILITY OF MONOCLONAL ANTIBODY INFLIXIMAB

3.1 Monoclonal antibodies (mAbs)

Monoclonal antibodies (mAbs) are a highly efficacious class of biotherapeutics with sales reaching about USD100 billion by 2020. They are used in a range of diseases spanning from cancers to inflammatory diseases and rare disorders. Seven of the 10 top selling drugs in 2019 were mAbs with Humira (adalimumab) being the highest selling mAb grossing sales approaching \$20 billion in 2018 (Lu et al., 2020).

Antibodies are of various types IgG, IgM, IgD, IgE and IgA. IgGs can be further classified into IgG1, IgG2, IgG3 and IgG4. Figure 3-1, shows the general structure of an immunoglobulin (IgG) antibody along with the hinge region, which is susceptible to proteolytic cleavage by trypsin, chymotrypsin and elastase, found predominantly in the small intestine (Mahato et al., 2003, Yadav et al., 2016a). The structure consists of two variable fragments (Fab), together called (Fab')₂ and the fragment crystallisable (Fc) which is the constant region. The mAb is comprised of two heavy chains of ~50kDa each and two light chains of ~25kDa each. The heavy chain consists of the three constant C_{H1}, C_{H2} and C_{H3} regions and one variable region (V_H). The light chain consists of one constant (C_L) and one variable domain (V_L). The Fab regions via complementarity determining regions (CDRs) binds to the antigen hindering its binding with the ligand. The ligands can be receptors, membrane bound or soluble proteins.

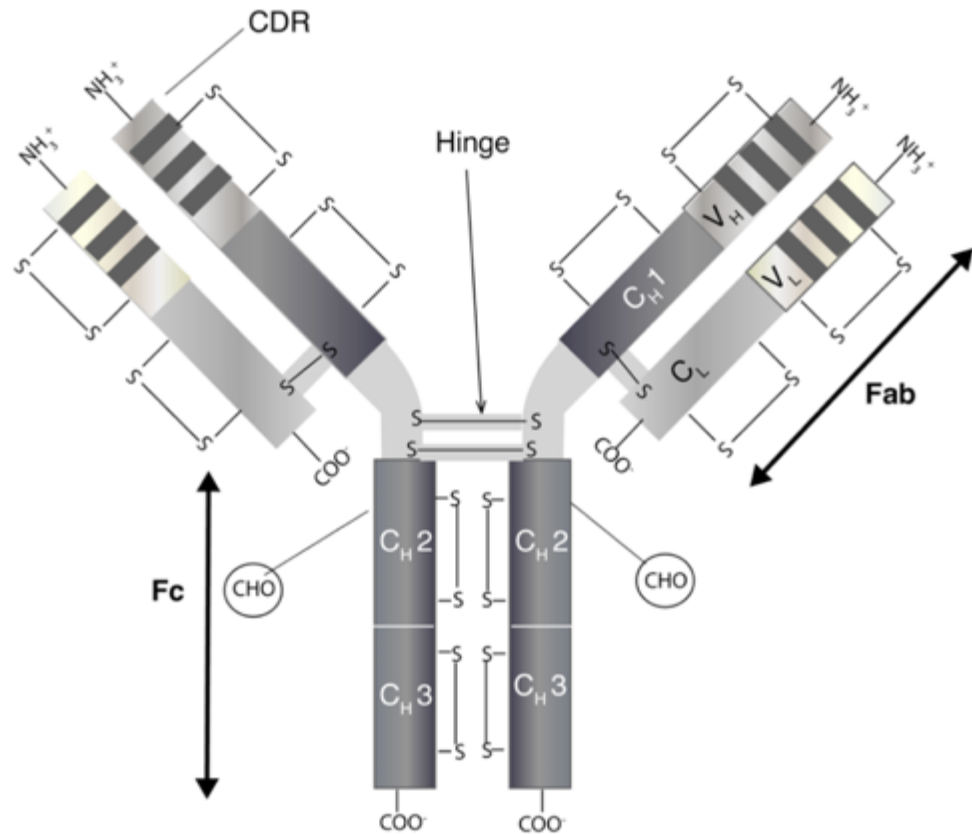


Figure 3-1: Graphical depiction of a monoclonal antibody (150 kDa). Fab regions are made up of variable light and heavy chains whereas Fc region is made up of constant heavy chains. Fab regions comprise of the complementarity determining region (CDR) which binds to the epitope on the antigen (Adapted from (Awwad and Angkawinitwong, 2018))

3.1.1 Advantages of using whole IgGs as compared to Fab fragments

As an example, clinical and experimental studies have shown that membrane bound TNF- α rather than soluble TNF- α play a major role in driving intestinal inflammation. The strategy of neutralizing membrane bound TNF- α , has suppressed inflammation in colitic mice (Perrier et al., 2013). Also, as Infliximab and Adalimumab neutralize both soluble and membrane bound TNF- α and have shown high clinical efficacy by inducing T-cell apoptosis *in vivo*, compared to etanercept, which preferentially blocks soluble TNF- α and has little or no therapeutic effect in the treatment of IBD (Van den Brande et al., 2003). Furthermore, antibodies also control TNF- α related inflammation cascade by indirect effects. Monoclonal antibodies, such as Infliximab and Adalimumab, that have the Fc region interact with the Fc receptors of immune cells

such as natural killer cells and exert an immune response via antibody dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and antibody-dependent cellular phagocytosis (ADCP) (Chames et al., 2009). Antibody fragments lacking the Fc region like certolizumab pegol (pegylated Fab only) permit improved tissue penetration compared to full length antibodies. They still bind to ligands but owing to the lack of Fc region, do not have the advantage of target cell destruction by ADCC, CDC or ADCP. Furthermore, the constant heavy chain domains (C_H2 and C_H3) in the Fc region also interacts with the neonatal Fc receptor (FcRn) for uptake into the cell and extension of their half-life while circulating within the blood (Yeung et al., 2009).

3.1.2 IgG stability in the gastrointestinal tract

These large (150kDa) proteins are labile and undergo degradation in the stomach and small intestine both acidic and /or proteolytically hostile environments (Yadav et al., 2016a). Proteolysis takes place at the hinge region which can be subdivided into three regions, the upper hinge region adjacent to the Fab fragment, the core hinge region which contains the disulphide bonds linking the two heavy chains, and the lower hinge region which reaches the Fc domain (Brezski et al., 2011). Salivary secretions are from pH 5.5 – 7.5 and do not contain the array of proteases found in pancreatic secretions (like trypsin, chymotrypsin and elastase) in gram quantities. However, protease enzymes like kallikrein in saliva originate from certain types of bacteria inhabiting the oral cavity (Mohamed et al., 2012). These proteolytic secretions have been easily inhibited by using anti-bacterial agents such as chlorhexidine and preservatives such as sodium benzoate (Gaudette, 1994). The fact that the oral cavity does not pose a harsh proteolytic environment like the small intestine, presents an opportunity for local delivery of monoclonal antibodies to the oesophagus to treat EoE without systemic side effects that would arise from oral absorption.

3.1.3 Tissue distribution of monoclonal antibodies

Monoclonal antibody distribution is based on the extravasation phenomenon which involves 3 basic processes; passive diffusion, convective transport and transcytosis through vascular epithelial cells (Figure 3-2). This theory could also extend to their distribution into gastrointestinal tissues. The major barrier to the local permeation of monoclonal antibodies is due to their large size (~150 kDa) and they have a hydrodynamic radius in the order of 10 nm as compared to small molecules in the order of <0.5nm. Furthermore, their hydrophilic nature does not make them highly permeable. The oesophageal epithelium is lipophilic, multi-layered and does not promote the paracellular diffusion of molecules >500 daltons. Therefore, passive diffusion may not play a significant role in the epithelial permeability of mAbs. Convection is determined by the flux of fluid from the vascular space to the tissue, which is driven by the blood-tissue hydrostatic gradient, as well as by the sieving effect of the paracellular pores in the vascular epithelium. The sieving effect is determined by the size, tortuosity and number of the pores, as well as the size, shape and charge of the mAb. The principle behind convection is that the differential between hydrostatic and oncotic (colloid osmotic) pressures, coupled with the sieving effect, contributes to the net driving force for the extravasation of the mAb (Ryman and Meibohm, 2017, Li et al., 2016).

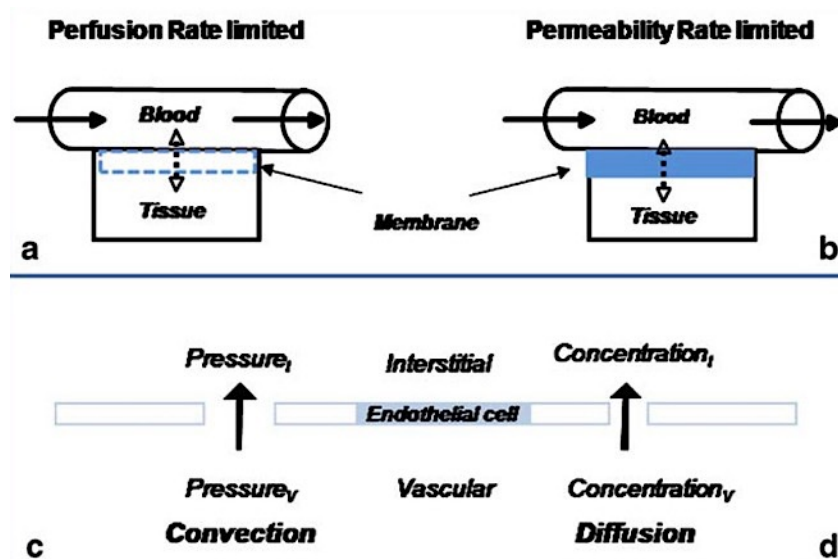


Figure 3-2: Movement of molecules from blood to body tissues. The rate of diffusion may be perfusion rate limited, (a), or membrane permeability rate limited (b), convection due to pressure gradient (c) and diffusion due to concentration gradient (d) (Tabrizi et al., 2010)

3.1.4 Gastrointestinal absorption of Monoclonal antibodies via neonatal Fc receptors (FcRn)

IgG has long been known to be the only class of antibody that is actively transferred from mother to offspring to confer short-term passive immunity. This specific transport of IgG is carried out by the neonatal Fc receptor (FcRn). FcRn transfers IgG from the mother's blood to the fetus across the placental capillaries and small intestine via milk. In rodents and humans, FcRn contributes to the transfer of IgGs from the mother's milk to the newborn across the intestinal epithelial cells by binding to the Fc domain (Roopenian and Akilesh, 2007, Cooper et al., 2014).

Stirling and co-workers have thoroughly investigated the porcine FcRn homology to humans, FcRn distribution and IgG pH dependent uptake and saturation in kidney cells lines (Stirling et al., 2005). In the pig, FcRn receptors were detected in the gut (duodenum, jejunum, ileum and colon), with an 80% homology to humans and 69% to rodents. IgG uptake studies conducted using pig IgG and bovine/human/mouse IgG

showed that IgG from these species can be taken up by the pig gut. Bovine IgG from colostrum was taken up after administration over a period of 7 days with maximum plasma levels at 7th day and then fell to control levels by 28th day indicating their absorption and slow elimination owing to endosomal recycling by FcRns. Similar to humans, pig FcRn is expressed in adult life as well as before weaning stage and has shown a broad species specificity just like mouse FcRn (Stirling et al., 2004).

FcRn has a MHC class I like structure and binds the C_H2-C_H3 Fc region of IgG. FcRn does not bind IgA, M or E. The Fc receptor binds to the Fc domain of the IgG antibody at acidic < pH 6.5 and dissociates at neutral pH 7.4 with two FcRn molecules binding one IgG (Rodewald, 1976, Raghavan et al., 1994). At acidic pH between 5.5 to 6.5, certain amino acids in the Fc region of IgG are protonated to form salt bridges with amino acid residues in the FcRn with hydrogen bonds and hydrophobic interactions also playing a role (Medesan et al., 1997).

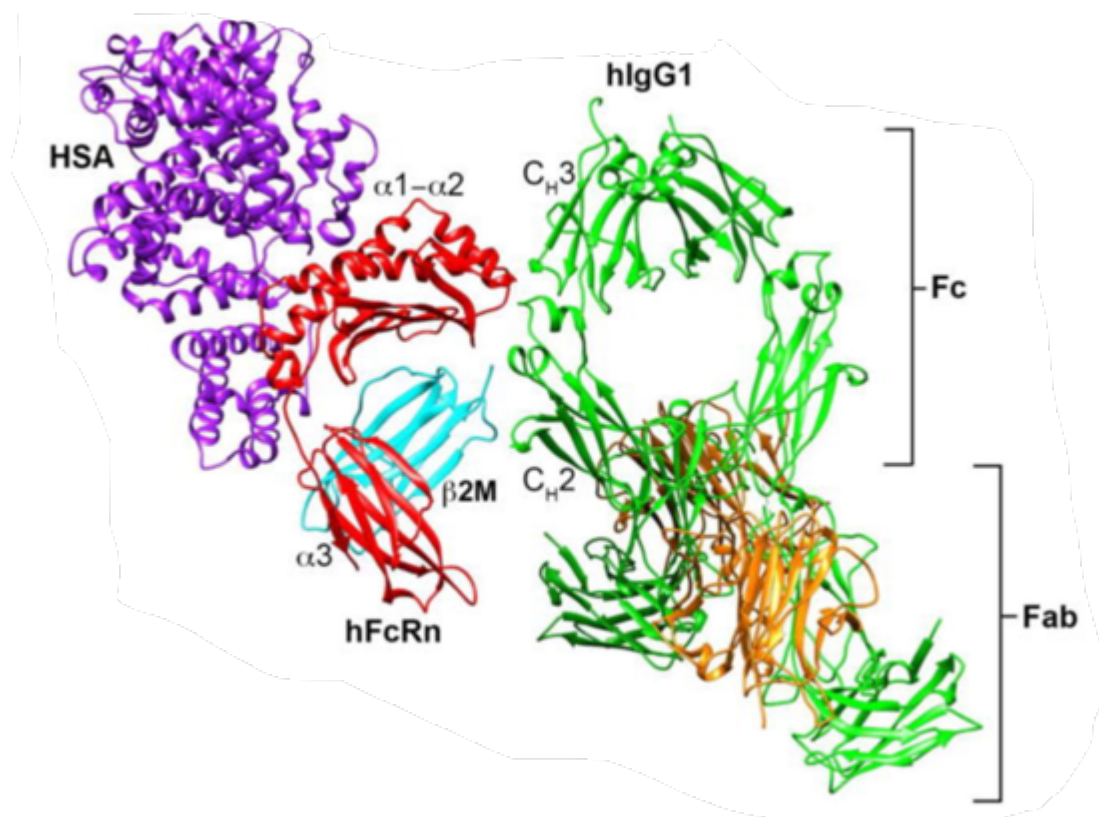


Figure 3-3: Structure of human FcRn in contact with hIgG1 and human serum albumin. Human IgG1 (green) contacts the $\alpha 2$ domain of human FcRn (red) and N-terminus of $\beta 2M$ (cyan) at the intersection of the $C_H 2$ - $C_H 3$ domains within the Fc portion of IgG. Human serum albumin (purple) contacts a distinctly different binding site that spans the $\alpha 1$ - $\alpha 2$ domains of FcRn and $\beta 2M$. (Sokolosky and Szoka, 2015).

The IgG is believed to enter the cells through fluid-phase pinocytosis. The IgG is endocytosed along the endosomal pathway and binds with FcRn in the early endosome where the acidic microenvironment pH 6 favours the FcRn-IgG interaction. In conditions where the luminal pH is acidic, the IgG binds to the epithelial cell surface FcRn via receptor mediated endocytosis and the FcRn-IgG complex is internalized and reaches the early endosome. The unbound IgG are degraded by lysozymes, while the FcRn-IgG complex is trafficked away from lysosomal degradative pathway and is transcytosed for release at the extracellular neutral pH environment (Sokolosky and Szoka, 2015).

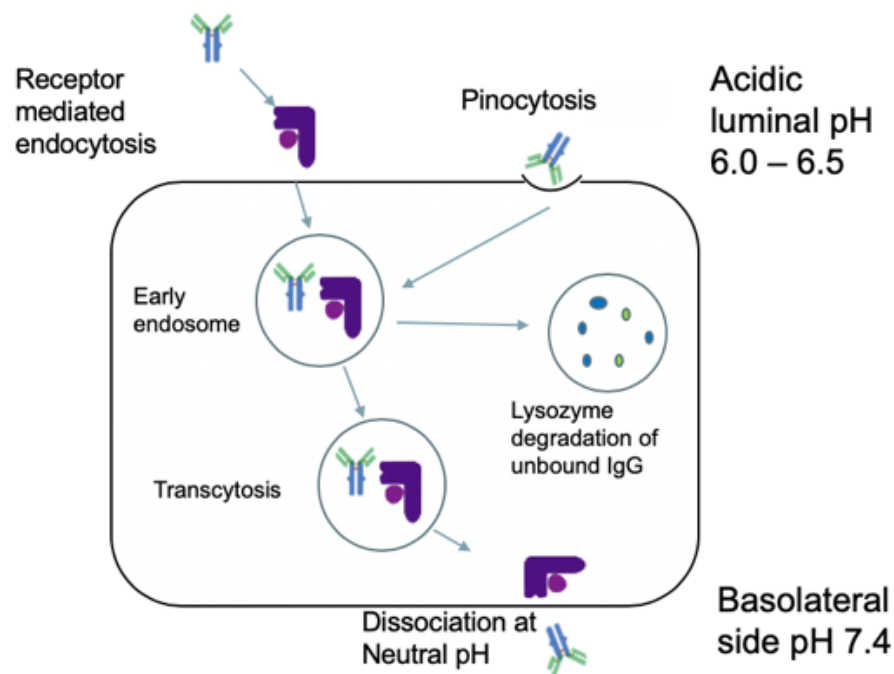


Figure 3-4: Graphic representation of the FcRn mediated recycling and transcytosis model in gastrointestinal epithelial cells. The IgG either binds to the FcRn (purple receptor) at the apical membrane if the luminal pH is acidic or is taken up by pinocytosis and binds with the FcRn at the acidic intracellular pH. This interaction protects it from lysosomal degradation allowing it to transcytose to the basolateral side where the IgG dissociates from the FcRn due to the neutral pH (Sokolosky and Szoka, 2015).

3.2 Rationale for local delivery of Monoclonal antibodies

Humans and non-human primates both have an increasing gradient of FcRn expression in the gastrointestinal tract, with the highest in the proximal colon (Hornby et al., 2014). IgG1 intestinal infusion acutely in anesthetized cynomolgus resulted in detectable serum monoclonal antibody (mAb) levels (0.3%). Although very low, this confirms transcytotic uptake of mAbs through the gastrointestinal epithelium. Muzammil and co-workers have investigated the intestinal uptake of IgG in cynomolgus monkeys and whether it could achieve blood levels above a desired concentration. Desired blood levels were not achieved, however there was some absorption into the blood indicating the IgG is absorbed by the FcRn mechanism (Muzammil et al., 2016). Furthermore, Stirling and co-workers have also shown uptake of IgG in pig cell lines (Stirling et al., 2005). This provides evidence to go ahead and explore whether therapeutic monoclonal antibodies can achieve sufficiently high tissue levels when administered orally for local action in the oesophagus. Furthermore, since mAbs would degrade on reaching the stomach, they could potentially only work locally sparing the body of all adverse systemic effects arising from systemic administration. Currently, a few companies are also investigating the local delivery of anti TNF- α and anti-integrin monoclonal antibodies via the oral route, for the treatment of inflammatory bowel disease, outlined in the review by (Durán-Lobato et al., 2020).

FcRn expression has also shown to be upregulated in inflammatory conditions and especially in the presence of pro-inflammatory cytokines such as TNF- α and NF- κ B activation (Liu et al., 2007) thereby posing an opportunity of greater local mAb uptake owing to inflammation in EoE.

3.3 Materials and methods

3.3.1 Materials

Remicade (Johnson and Johnson) and Remsima (Celltrion) 100mg vials were sourced by University College Hospital London (UCLH) pharmacy and were a gift from Tillotts Pharma Ag, Switzerland. The vials were reconstituted using phosphate buffered saline pH 7.4 (Sigma, Aldrich) and stored at 4°C for short term use and -20°C for long term use. Remicade and Remsima are freeze-dried products and contains sucrose, polysorbate 80, monobasic sodium phosphate and disodium hydrogen phosphate (EMC, 2019a, EMC, 2019b). Ussing chambers were purchased from Harvard Apparatus, Cambridge, UK. The ingredients used for Krebs bicarbonate ringer (KBR) solution were as follows; D-glucose, sodium chloride, calcium chloride, magnesium chloride, sodium bicarbonate which were obtained from Sigma Aldrich, UK, potassium hydrogen phosphate and dipotassium hydrogen phosphate were acquired from VWR, UK). Ingredients for extraction buffer include Sodium chloride, EDTA, Nonidet P40 substitute, sodium azide, sodium orthovanadate, phenyl methyl sulfonyl fluoride and protease inhibitor cocktail for general use were purchased from Sigma Aldrich, UK.

3.3.2 Methods

3.3.2.1 Pigs

Male and female Breed between Landrace and large white pigs 9-12 month old were used. The oesophagus was obtained from an abattoir (Cheale meats, UK) and the tissues were received in the lab within 3 hours of slaughter. The tissues were stored in ice-cold KBR. The oesophageal mucosa (epithelium and submucosa) were separated from the muscle layers carefully using surgical scissors. The tissues were stored at -80°C and thawed at 4°C when used for Ussing chamber experiments.

3.3.2.2 Immunohistochemistry to detect localization of neonatal Fc receptor (FcRn) in porcine oesophageal tissue

Cryosectioning

The oesophageal tissue section exposed to the drug was gently dismantled at the end of the experiment and immediately transferred to a cryostat (Leica CM3050, Leica Microsystems, Milton Keynes, UK) at -20°C. The tissue was allowed to freeze for 15-20 minutes. After the tissues were frozen, they were mounted onto a cryogel and thin sections of the tissue (10 µm) were sliced and mounted on adherent microscope slides (SuperFrost® Plus, VWR International, Leuven, Belgium). Up to 6 sections from the tissue exposed to the drug and control were sliced.

Immunohistochemical staining

The frozen tissues were sectioned at a thickness of 10µm in a cryostat described above. After allowing the sections to attain room temperature, the sections were incubated in anti-human FcRn antibody (rabbit polyclonal, Novus bio, NBP1-89128) at a dilution of 1:50 for 30 minutes and washed after using PBS pH 7.4. The control tissue was incubated in rabbit polyclonal antibody as isotype control (abcam, ab27478). Following this, the sections were fixed using 4% paraformaldehyde for 10 minutes and washed using PBS pH 7.4. Then the sections were exposed to 1% Bovine serum albumin (BSA) for 30 minutes after which they were washed with PBS pH 7.4. The sections were then incubated with secondary antibody (Goat Anti-Rabbit IgG, H&L, Alexa fluor 488, Abcam, ab150077) at a 1:50 dilution for 1 hour. This was followed by washing with PBS pH 7.4. Finally, the sections were stained using DAPI (Vectashield hardset mounting medium with DAPI).

3.3.2.3 Ussing chamber studies

Ussing chamber experiments were conducted in a similar way as described in section 2.5.4. The oesophageal tissues from different regions (proximal, mid and distal) of the oesophagus were stabilised in KBR pH 7.4 for 20 minutes and then the permeation study, using an apical concentration of 0.8 mg/ml infliximab, was conducted at apical pH 6.0 and 7.4 in KBR over a period of 30 minutes. Infliximab is given intravenously (i.v) at a dose of 5mg/kg which equates to 300mg for a 60 Kg adult. If this i.v dose is given orally as a solution i.e with 180ml of water it would amount to 1.67mg/ml. Owing to the fact that local delivery directly allows the whole dose to come in contact with the oesophageal mucosa, half the dose, 0.8 mg/ml was chosen as it is economical.

Different apical pH (6.0 and 7.4) was used to represent the pH range of saliva. Furthermore, it has been reported that majority of biotherapeutic mAbs exhibit isoelectric points (pIs) ≥ 8 , with infliximab at 7.7 and carry a positive charge under formulation conditions (typically pH 5-6) (Goyon et al., 2017, Yang et al., 2019). Therefore, the influence of the effect of charge on permeation was evaluated at slightly acidic pH 6.0 and physiological pH 7.4. The epithelium owing to proteoglycans, has a net negative charge (Orlando, 2006, Bennett KM, 2014). Therefore, the aim of performing the permeation study at pH 6.0, was to improve the interaction of the positively charged infliximab at pH 6.0 with the negatively charged epithelium so as to promote its uptake via endocytosis or pinocytosis.

Apical samples were taken at initial time point followed by every 10 minutes for up to 30 minutes. Basal samples were taken at initial time point and at 30 minutes to determine if any infliximab had permeated through the oesophageal tissue. The samples were centrifuged at 10000 xg and supernatant analysed by High Performance Size Exclusion Liquid Chromatography (HPLC-SEC).

3.3.2.4 HPLC-SEC (High Performance Size Exclusion Liquid Chromatography)

Sample analysis was performed using a high performance liquid chromatography (HPLC) system (Agilent Technologies, 1260 Infinity) equipped with a pump (model G1311C), autosampler (model G1329B) and a diode-array UV detector (model G1314B). A YMC pack diol 200 gel filtration column (YMC Japan) size exclusion (SE) chromatography column was used for sample separation. Infliximab was eluted using a pH 7.3 phosphate buffer as the mobile phase. The mobile phase was of the following composition; 40g sodium chloride, 1.0g potassium chloride and 7.2g of disodium hydrogen phosphate dihydrate and 1.0g of potassium dihydrogen phosphate were dissolved in 5 litres of distilled water. The pH was adjusted to 7.3. The analysis was operated at a flow rate of 1 ml/min at room temperature and UV detection wavelength was set at 280 nm. The retention time of infliximab was 14.8 minutes.

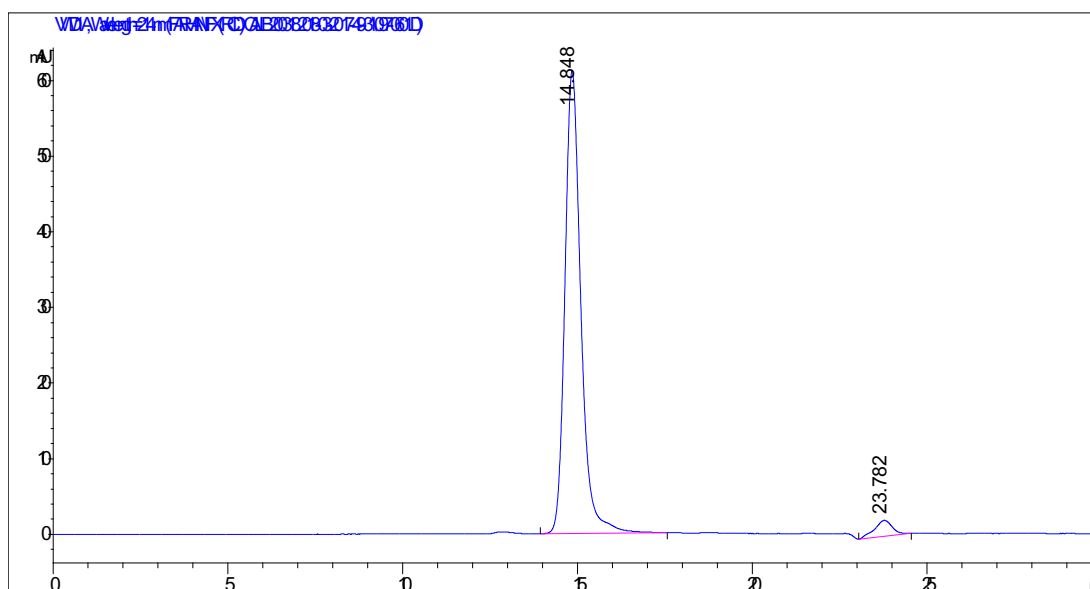


Figure 3-5: HPLC-SEC chromatogram of infliximab showing retention time of the peak at 14.8 minutes

3.3.2.5 Immunohistochemistry to detect localization of infliximab in porcine oesophageal tissue

Cryosectioning

The oesophageal tissue section exposed to the drug was gently dismantled at the end of the experiment and immediately transferred to a cryostat (Leica CM3050, Leica Microsystems, Milton Keynes, UK) at -20°C. The tissue was allowed to freeze for 15-20 minutes. After the tissues were frozen, they were mounted onto a cryogel and thin sections of the tissue (10 µm) were sliced and mounted on adherent microscope slides (SuperFrost® Plus, VWR International, Leuven, Belgium). Up to 6 sections from the tissue exposed to the drug and control were sliced.

Staining procedure

The slides were kept at room temperature for 15 minutes before starting the staining procedure. The tissue sections were fixed in 4% paraformaldehyde (Sigma-Aldrich, UK) for 10 minutes followed by incubation with 0.1% Triton X-100 (Sigma-Aldrich, UK) surfactant for 5 minutes to open up the tight junctions. The sections were then incubated with 1% bovine serum albumin (BSA) (Sigma-Aldrich, UK) for 30 minutes to avoid non-specific binding. Washing steps were included at every stage using PBS pH 7.4. The sections were then stained with secondary antibody, 10µg/ml, (Red) (anti-human IgG from goat, Alexa Flour® 633, Molecular Probes, UK) for 1 hour. The sections were then stained with vectashield Hard Set mounting medium with DAPI (Blue) (Vector Laboratories, Inc., Burlingame, CA, USA) to stain the cell nuclei. The slides were stored at 2-8°C in the dark until analysis by confocal laser-scanning microscopy (LSM 710, Zeiss, Cambridge, UK). The images were processed and analysed by Zen 2012 imaging software (Carl Zeiss Ltd., Cambridge, United Kingdom).

3.3.2.6 ELISA

This method was adapted from the work of Yadav (Yadav, 2016). The oesophageal tissue samples from the Ussing chamber experiments were stored at -80 °C and were thawed at 4°C for 30 minutes. The tissue samples were treated with extraction buffer at a ratio of 30 mg tissue:1ml extraction buffer (Table 3-1). The extraction buffer breaks down the lipid cell membrane barrier and dissolves the intracellular contents and the protease inhibitor cocktail has various inhibitors to prevent the degradation of the mAb.

Table 3-1: Composition of the extraction buffer

Ingredient	Function	Quantity for 10ml buffer
Tris buffer 10x	Buffering agent	2ml
Sodium chloride	Maintain ionic strength	146mg
EDTA	Matrix metalloprotease inhibitor	18mg
Nonidet TM P40 substitute	Lysis of cell membrane	100µl
Sodium azide	Bacterial growth inhibitor	2mg
Sodium orthovanadate	Tyrosine and alkaline phosphatase inhibitor	2mg
Phenyl methyl sulfonyl fluoride	Serine protease inhibitor	1.8mg
Protease inhibitor cocktail for general use	Mixture of protease inhibitors	8ml PBS per bottle

Table 3-2: Protease inhibitor cocktail composition

Ingredient	Class of inhibitor	Concentration (µg/ml)
4 – benzenesulfonyl fluoride hydrochloride	Serine protease inhibitor	48
Aprotonin	Serine protease inhibitor (except elastase)	0.2
Bestatin HCl	Aminopeptidases inhibitor	4
E-64	Cysteine protease inhibitor	0.5
EDTA	Matrix metalloprotease inhibitor	29.2
Leupeptin hemisulfate salt	Serine and cysteine protease inhibitor	0.005

After addition of the extraction buffer, the samples were homogenized using a DI 18 disperser S2 (IKA, Staufen, Germany) at 10,000rpm for 20 seconds on ice. The homogenate was incubated at 4 °C for 2 hours to allow efficient lysis and extraction of infliximab.

The samples were then centrifuged at 4 °C and at 10 x g for 20 minutes. The supernatant was then analysed by ELISA to quantify the amount of extracted infliximab.

In this study an anti-TNF- α antibody detection ELISA kit (Alpha Diagnostic International, Texas, USA) was used. The kit comprised of TNF- α coated 96 well plate, an anti-human Fc domain binding specific IgG labelled with HRP (secondary antibody), wash solution, dilution buffer and TMB substrate. The steps are as follows:

- 1) The appropriate number of microwell strips were removed from the pack while the unused strips were refrigerated. All wells and reagents were brought to room temperature (18-30 °C) for at least 30 minutes. The wells were filled with 300 µl wash buffer and left for 5 minutes after which the wash buffer was poured off.

2) Primary antibody incubation

100µl of the diluted test sample (supernatant obtained from the extraction and centrifugation step), infliximab positive control (mAb in PBS) and negative control (homogenate of oesophageal tissue without drug) or calibration curve samples (1000, 500, 100, 50, 25 and 10 ng/ml infliximab prepared by spiking oesophageal tissue) were added to the wells of the 96 well plate in triplicates and incubated at 70 rpm shaking for 1 hour at room temperature. Post incubation, the wells were washed with wash buffer 4 times for 5 minutes each.

3) Secondary antibody incubation

Horseradish peroxidase (HRP) conjugated anti-human IgG was diluted 1:100 and 100 µl of the solution was added to each well. The plate was incubated for 30 minutes at 70 rpm shaking at room temperature, followed by 5 washes for 5 minutes each with wash buffer.

4) Substrate incubation

To each well, 100 µl of TMB (tetramethyl benzidine) was added and incubated in the dark for 15 minutes. The wells began to turn blue due to the reaction between HRP and (TMB). Briefly, when a solution of tetramethylbenzidine and hydrogen peroxide is added to horse radish peroxidase, the tetramethyl benzidine undergoes oxidation to form a blue intermediate (Josephy PD, 1982).

5) Stop step

The enzyme substrate reaction was stopped by addition of 100 µl of stop solution (1% sulfuric acid). The wells turned yellow and the plate was read within 30 minutes of addition of stop solution at 450nm using a SpectraMax M2e microplate reader.

6) Data analysis

The Optical density (O.D) readings of the calibration curve were plotted against log concentration in a 4-parameter logistic regression curve fit (4-PL) using GraphPad Prism 7 (GraphPad Software Inc., San Diego, USA).

The following equation was used to calculate the concentration of the unknown.

$$\text{Log } X = \log IC50 - (\log ((T - Y)/(Y - B))/Hillslope)$$

Log X = log concentration, Y=O.D @ 450nm

T - Top, B - Bottom, Hillslope and R^2 were provided by the software. By fitting the OD values and other given values, the log concentration was determined and converted to concentration in ng/ml ($10^{\log \text{conc}}$) to calculate recovery of the calibration curve, which should be between 80-120% of the actual nominal concentration.

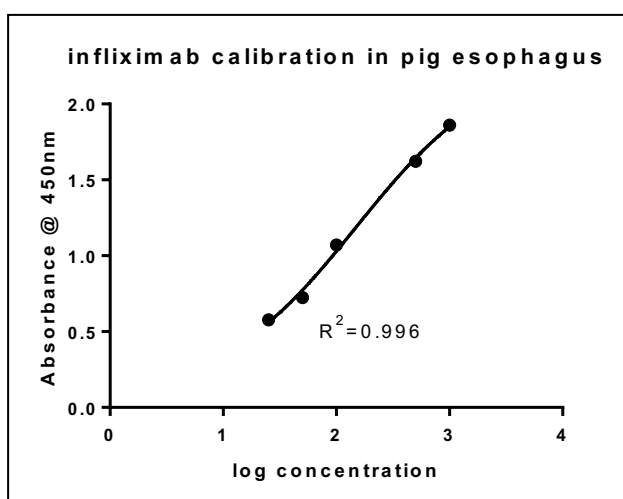


Figure 3-6: Calibration curve of Infliximab by spiking on porcine oesophageal tissue

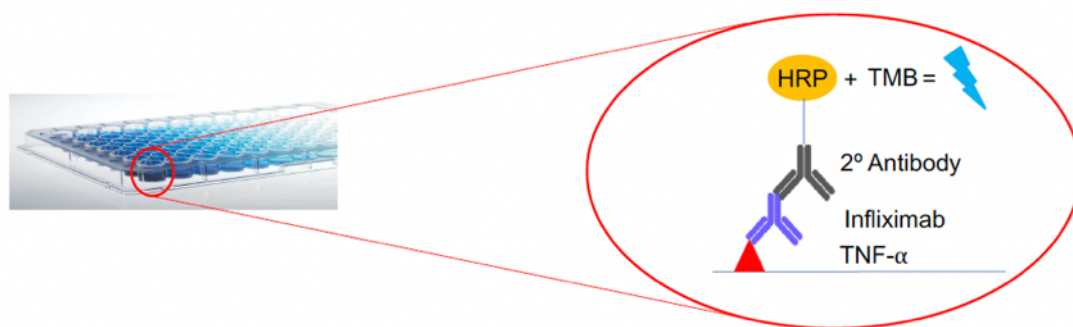


Figure 3-7: Graphical illustration of detection of infliximab in porcine oesophageal tissue by ELISA. 1) Addition and incubation of sample or calibrator into the 96 well plate coated with TNF- α , 2) Incubation with secondary antibody (anti-human IgG) labelled with HRP, 3) Addition of TMB substrate that reacts with HRP to give a blue colour

3.3.2.7 Stability of infliximab in porcine oesophageal tissue homogenates at 4°C and -20°C

The stability of the antibody during extraction and overnight storage of samples in 4°C and -20°C was determined. This was done by adding lysis buffer with and without protease inhibitor (protease inhibitor cocktail for general use, Sigma Aldrich) in ratio of 20 mg to 1 ml and 30 mg to 1 ml of tissue and then spiking the tissue with 1000 ng/ml of Remsima followed by homogenization and 2 hour incubation at 4°C. The samples, after 2 hours were centrifuged at 10 xg for 15 minutes and the supernatant left in 4°C and -20°C and analysed by ELISA (Alpha diagnostics Inc., TNF antigen kit) after 24 hours.

3.3.2.8 Comparing the permeation of Remicade to the biosimilar Remsima in porcine oesophageal tissue by ELISA

Porcine male proximal, mid and distal tissues were mounted in the vertical Ussing chambers described and permeation study conducted in the presence of Remicade or Remsima apical concentration 0.8 mg/ml and in the presence of KBR pH 6.0 and 7.4

on the apical side and KBR pH 7.4 on the basolateral side. The test was conducted from tissues obtained from 3 different pigs.

3.3.2.9 Permeation of infliximab (Remsima) in porcine male mid oesophageal tissue at 0.02, 0.2 and 2.0 mg/ml apical concentrations

Porcine male mid oesophageal tissues mounted in low volume Ussing chambers were stabilised in Kreb's bicarbonate ringer's (KBR) solution pH 7.4 at 37°C for 20 minutes. The exposure area was 0.1 cm². The KBR was then changed and new KBR pH 7.4 was added. Remsima (infliximab) solutions were added to the apical side to give concentration of 0.02, 0.2 and 2.0 mg/ml and permeation study conducted for 30 minutes. Remicade was no longer available from UCLH, hence the switch to Remsima, its biosimilar.

The amount of infliximab (Remsima) accumulated, was quantified in the tissue and on the basal side by ELISA.

3.4 Statistical analysis

One-way ANOVA was used to check for differences in tissue accumulation of infliximab at different apical concentrations followed by Tukey's post hoc analysis using IBM SPSS Statistics 16 (SPSS Inc., Chicago, IL, USA). The level of significance was kept at $p < 0.05$.

3.5 Results and discussion

3.5.1 Detection of FcRn receptors in porcine oesophageal mucosa

Neonatal Fc receptor expression was found in the gut, liver, kidney, spleen and aortic endothelial cells in pigs via mRNA analysis and Western Blotting. The pig expresses FcRn throughout young and adult life similar to humans. Furthermore, the pig FcRn is able to transcytose human, bovine and murine IgG with a pH dependent uptake

observed which can reach saturation at increasing concentrations (Stirling et al., 2005).

It is not known whether the pig oesophageal epithelium expresses FcRn receptors. Therefore, the aim was to detect whether the oesophageal epithelium expresses FcRn receptors. The antibody against porcine FcRn was not available hence the decision to use an anti-human FcRn antibody. The antibody against human FcRn was selected as there is an 82% homology between human and pig FcRn with maximal overlap between the amino acid residues in the part of the receptor embedded in the cytoplasmic tail. As seen in Figure 3-8, no FcRn receptors were observed in the porcine oesophageal epithelial mucosa. This is either due to the fact that the porcine oesophageal mucosa does not express FcRn or the anti-human FcRn antibody could not detect the FcRn.

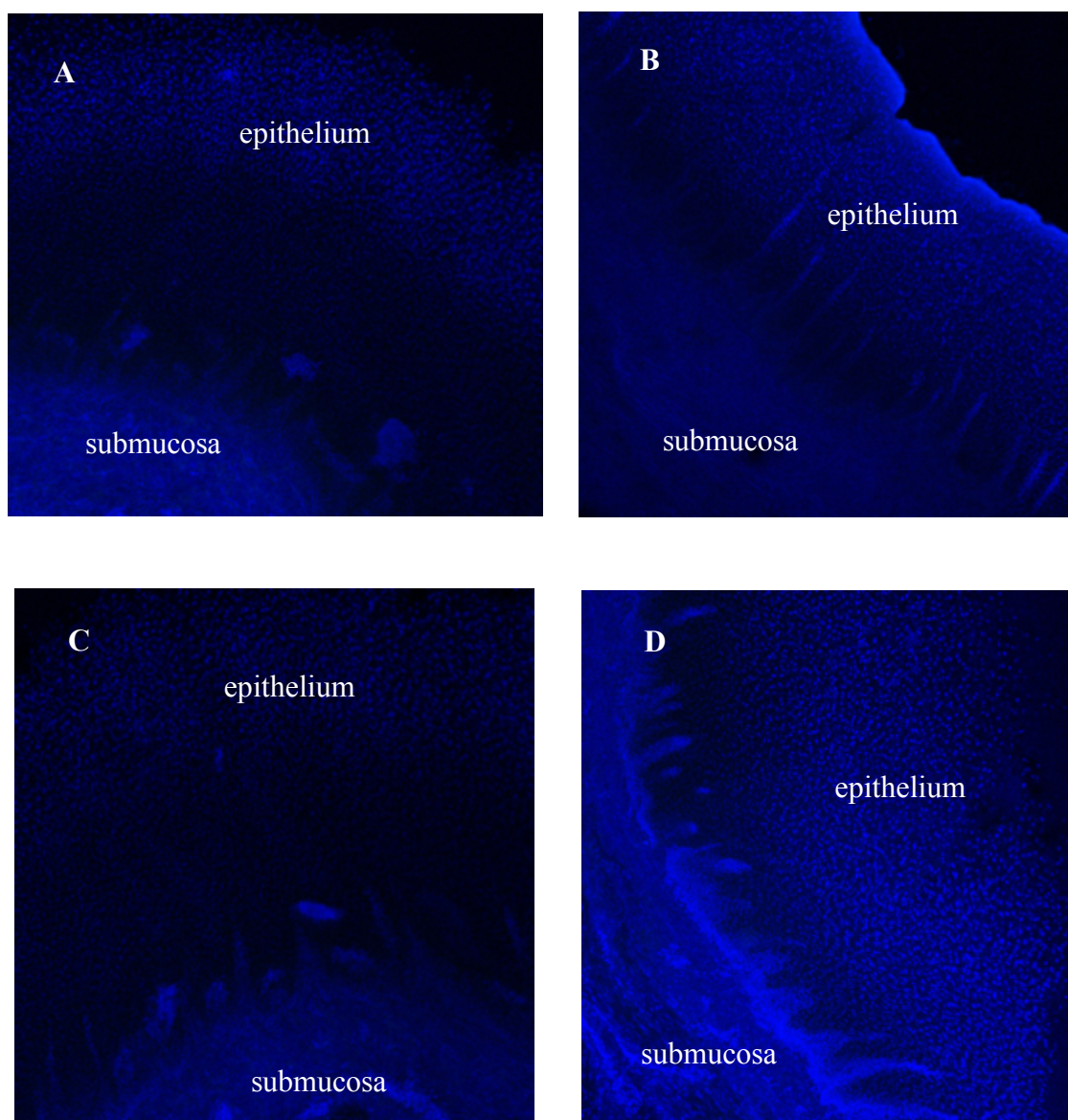


Figure 3-8: FcRn detection using anti-human FcRn antibody (rabbit polyclonal, Novus bio, NBP1-89128). Control (A), pig proximal (B), pig mid (C) and pig distal (D)

3.5.2 Permeation of Infliximab in porcine oesophageal tissue

Infliximab stability at 4 °C was studied in KBR and artificial saliva at both pH 6.0 and 7.4. An insignificant decrease of $\leq 1.2\%$ was observed over a 24 hour period (Table 3-3).

Table 3-3: Stability of infliximab (Remicade) in Bicarbonate ringer solution and artificial saliva by HPLC-SEC

Infliximab 0.2 mg/ml stability	Peak area		% remaining at 24 hours	% reduction
	T= 0h	T=24 h		
Infliximab 0.2 mg/ml in KBR pH 6.0	1355	1345	99.3	0.7
Infliximab 0.2 mg/ml in KBR pH 7.4	1368	1348	98.5	1.5
Infliximab 0.2 mg/ml in artificial saliva pH 6.0	1364	1355	99.3	0.7
Infliximab 0.2 mg/ml in artificial saliva pH 7.4	1356	1340	98.8	1.2

A first study was performed on porcine oesophageal mucosa whereby it was exposed to an apical concentration of 0.8 mg/ml of Remicade (Infliximab) over a period of 30 minutes. This time point was chosen as the best case scenario; based on adhesion studies performed ex vivo on porcine oesophageal tissues where hydrogels have shown to adhere upto 30 minutes (Richardson et al., 2005, Casiraghi et al., 2020, Batchelor et al., 2002). Furthermore, also wanted to ensure that a large molecule like monoclonal antibody can even permeate through the multi-layered oesophageal epithelium.

Samples were withdrawn every 10 minutes and any apical reduction in the peak area of infliximab, was correlated to an increase on the basal side at the end of 30 minutes (Figure 3-9). No change was observed for the proximal segment at both pH 6.0 and 7.4. However, reductions of up to 25% from the original apical concentration were noted for the mid region and distal regions with greater reductions observed for pH 6.0 as compared to pH 7.4. mAb accumulation was observed on the basal side amounting to less than 20% of total dose applied. However, this is not exactly the same as the reduction observed on the apical side in the order of >25%. This equates to about 0.2 mg of infliximab permeated through the tissue which is questionable considering lipophilic small molecules like tacrolimus and budesonide only

accumulate in the order of nanograms. Therefore, an experiment to determine the specificity of this method was conducted; porcine oesophageal tissue without infliximab exposure was sandwiched in Ussing chambers and apical and basal samples were collected. Peaks at similar retention time and area were observed on the basal side at the end of 30 minutes. This indicates that infliximab may not be translocating at all on the basal side but rather the peak observed was from endogenous IgG in the porcine oesophageal tissue.

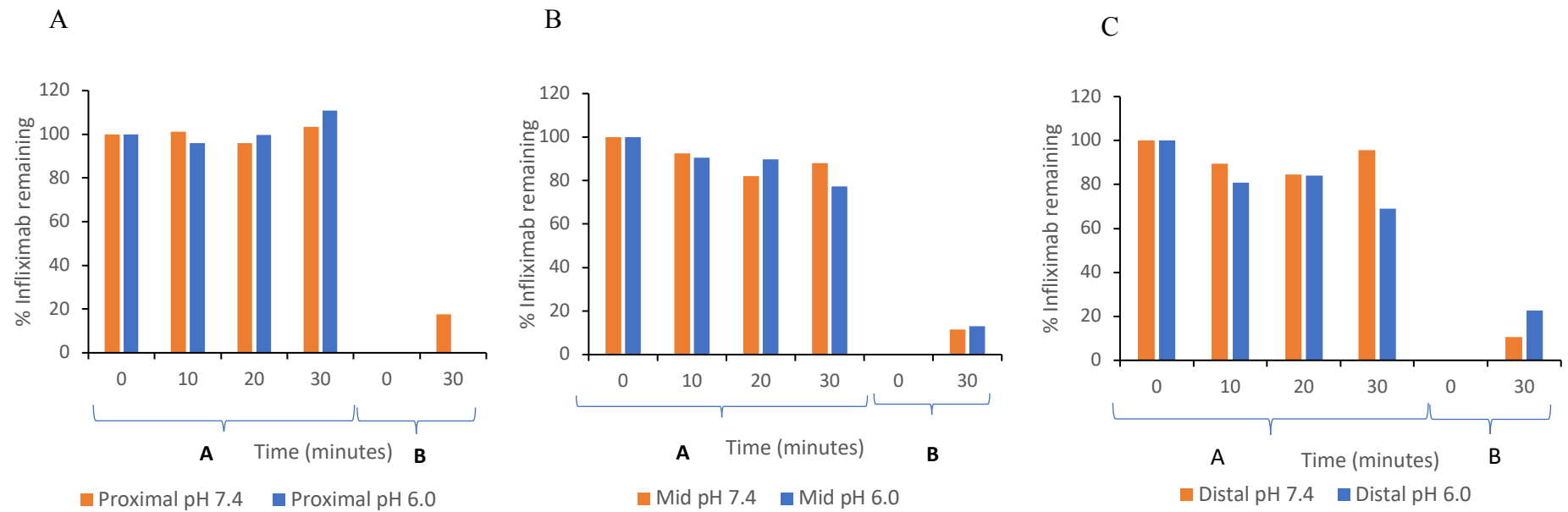


Figure 3-9: Percent apical infliximab remaining after permeation study on porcine A) proximal, B) mid and C) distal oesophageal tissue, infliximab apical conc. 0.8 mg/ml in artificial saliva pH 6.0 (blue bars) and 7.4 (orange bars), n=1; A – apical, B- basolateral side of the Ussing chamber

Therefore, the tissues were analysed for infliximab accumulation by ELISA to understand whether this large molecule undergoes transcytosis and accumulate in the oesophageal epithelium. As seen in Figure 3-10, the same tissues, which gave inconclusive evidence of permeability, have shown infliximab (Remicade) accumulation when quantified by a more specific and sensitive method like ELISA. No direct increase in infliximab accumulation is noted owing to the pH effect. Since this was only tested in tissues from one pig, it is difficult to infer whether there is a pH effect playing a role in the increased uptake of mAb, infliximab.

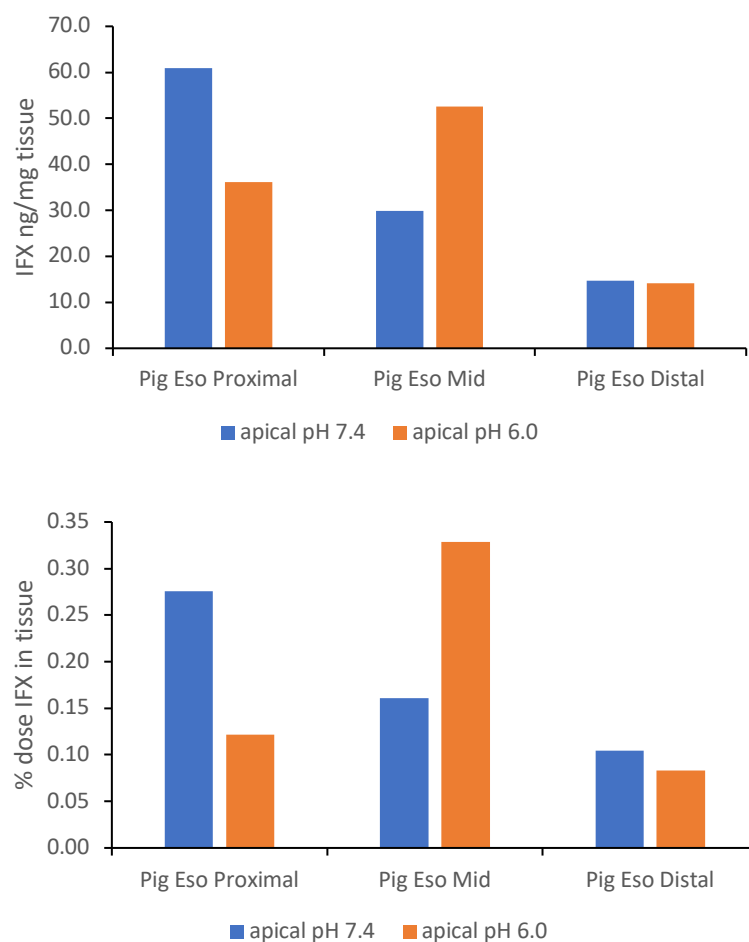


Figure 3-10: Quantification of infliximab (Remicade) using ELISA. Permeation in porcine oesophageal mucosa using KBR at apical pH 6.0 (orange bars) and 7.4 (blue bars) and apical concentration 0.8mg/ml, t=30 min, n=1

This experiment was repeated with a different set of oesophageal tissues from a single pig and confocal microscopy was used to image the different segments to confirm that Infliximab permeated the oesophageal epithelium and was not adhered to the top of the tissue. The images in Figure 3-12 show that the red fluorescence arising from the fluorophore alexa fluor 633 is from the interaction of the secondary antibody binding to the infliximab permeated into the epithelium. Interestingly, proximal and mid porcine oesophageal segments show the highest fluorescence at pH 6.0 relative to control (Figure 3-11) while this is not observed for distal oesophageal segments. This correlates to the ELISA findings whereby there was lower accumulation in the distal oesophageal segments as compared to the proximal and mid. In the ELISA, the maximal accumulation in the mid-section was observed at pH 6.0 and this is comparable to the depth of fluorescence observed in the mid segment exposed to infliximab at apical pH 6.0.

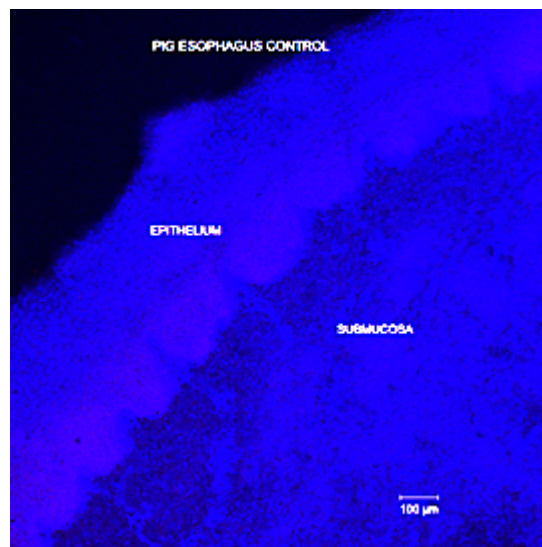


Figure 3-11: Confocal microscopy to determine permeation in pig oesophageal control tissue. The tissue was stained with secondary anti human Fc detection antibody conjugated to Alexa fluor 633

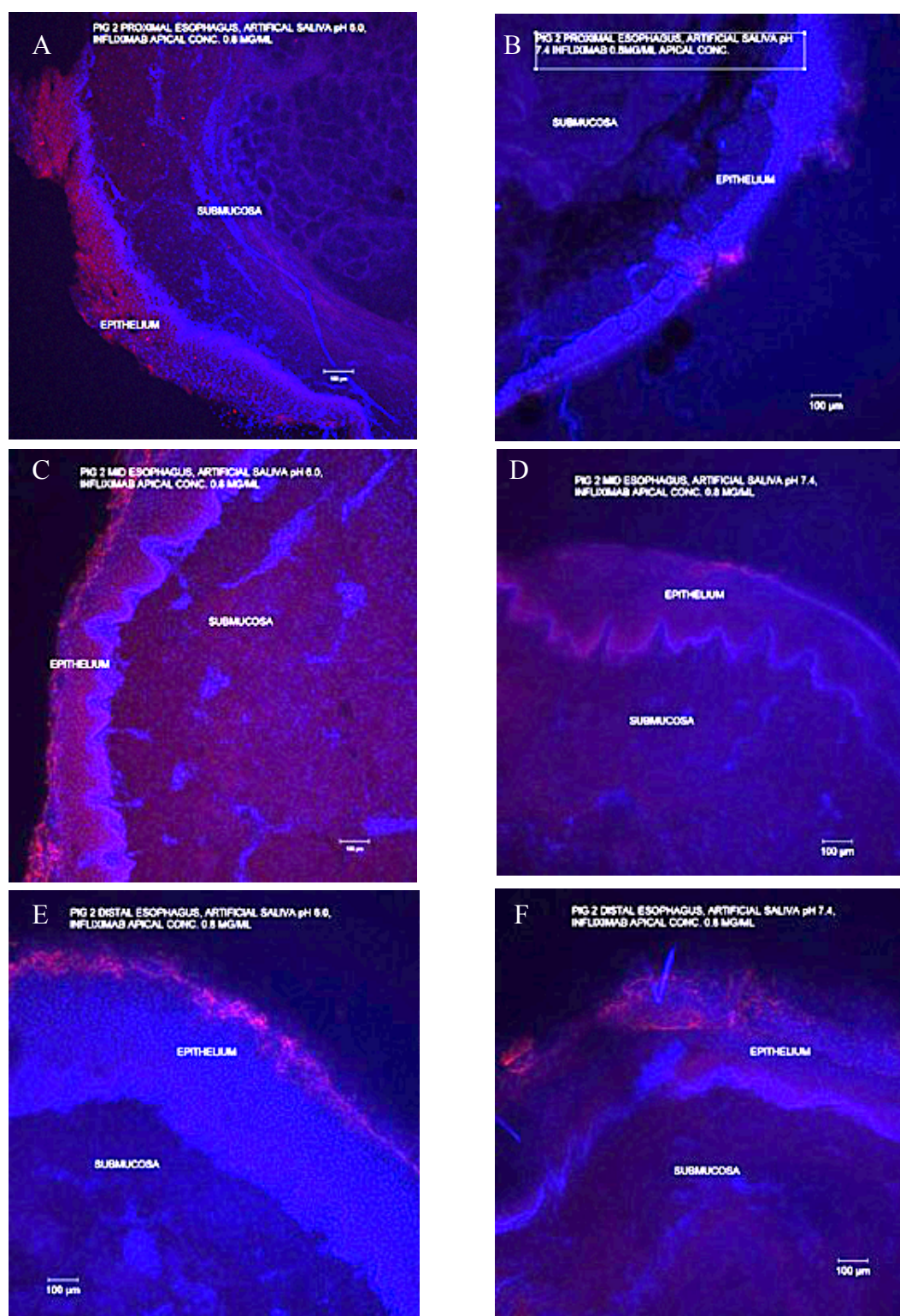


Figure 3-12: Confocal microscopy images of infliximab (Remicade) permeation through pig oesophageal mucosa at apical concentration 0.8 mg/ml in artificial saliva pH 6.0 (A - Proximal ,C - Mid and E - Distal) and pH 7.4 (B - Proximal, D - Mid and F - Distal)

3.5.3 Comparison between Remsima and Remicade

Biosimilars are drugs that are ‘generic’ versions of the innovator mAbs. However, it has been observed that since they are obtained from biological cells, they vary slightly in their structure from batch-to-batch. In the case of biosimilars these are produced from different cell lines to the innovator. However, their regulation states that there should be no clinically meaningful differences in terms of structure, function, pharmacodynamics and mechanism of action, pharmacokinetic properties, clinical efficacy and safety (Moore, 2017). No significant differences have been observed between the immunogenicity of biosimilars nor their ability to elicit the ADCC and ADCP response despite minor differences in the glycosylation pattern in their Fc regions. In a review by Ilias et al., they have concluded that it is safe to switch to the biosimilar of Remicade for the treatment of ulcerative colitis (Ilias et al., 2018). Furthermore, Chingcuanco et al. concluded in their systematic review that evidence supports the biosimilarity and interchangeability of biosimilar and reference TNF- α inhibitors, including infliximab (Chingcuanco et al., 2016).

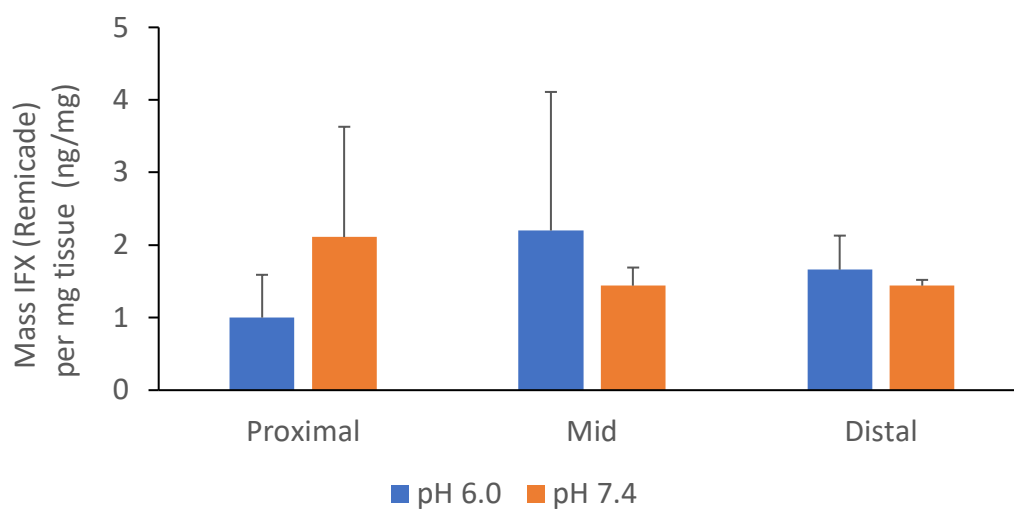
Infliximab innovator is Remicade and the biosimilar is Remsima. Therefore, as seen in Figure 3-13 and Figure 3-14, it was investigated whether there was a difference between Remicade and Remsima accumulation in the porcine oesophageal tissue. This was due to two reasons; firstly, to be able to interchangeably use the originator and biosimilar for formulation development and secondly to understand if it can be used for our studies going forward owing to its significantly lower cost compared to Remicade.

There was statistically no significant difference between the Remsima and Remicade permeation through oesophageal tissue for all segments. Moreover, no difference in antibody accumulation was noted due to different apical pH (6.0 versus 7.4). Interestingly, a greater accumulation was obtained at pH 6.0 in the mid segment of the oesophagus ($0.015 \pm 0.012\%$ Remicade versus $0.0075 \pm 0.0025\%$ Remsima).

Overall, Remicade shows slightly higher accumulation in the porcine oesophageal tissue as compared to Remsima. This comparison has been drawn since the tissues are used from the same pig. Remsima shows a similar amount of accumulation in all tissue

segments. Therefore, it can be inferred that going forward Remsima could be investigated for the oesophageal mucosal permeability of infliximab.

A



B

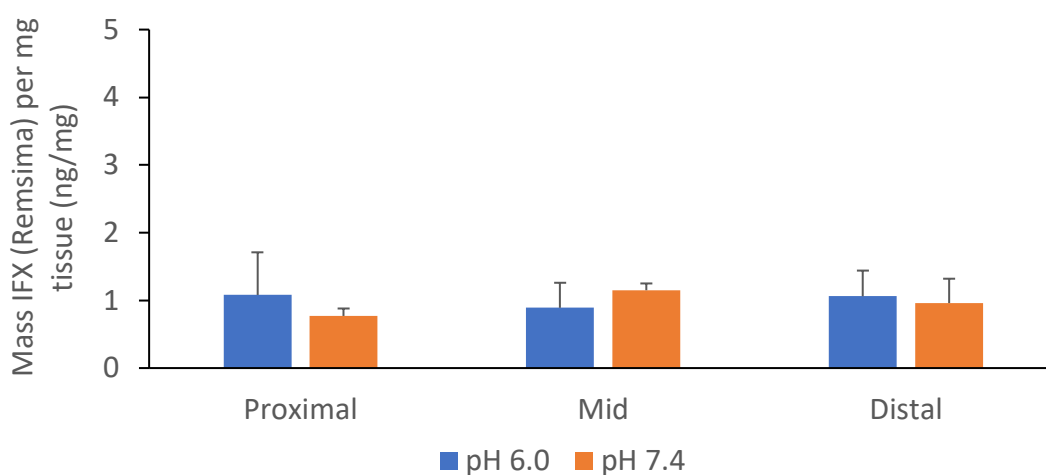


Figure 3-13: Accumulation of Remicade (A) and Remsima (B) in porcine proximal, mid and distal oesophageal tissue at apical pH 6.0 (blue bars) and 7.4 (orange bars) and apical concentration 0.8mg/ml over 30 minutes, n=3, mean $\pm$ SD

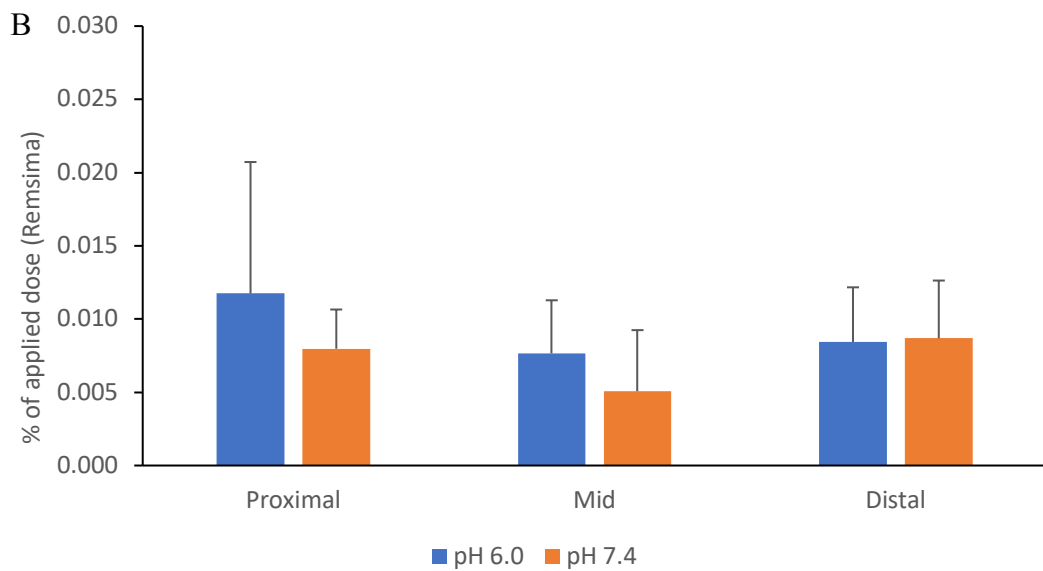
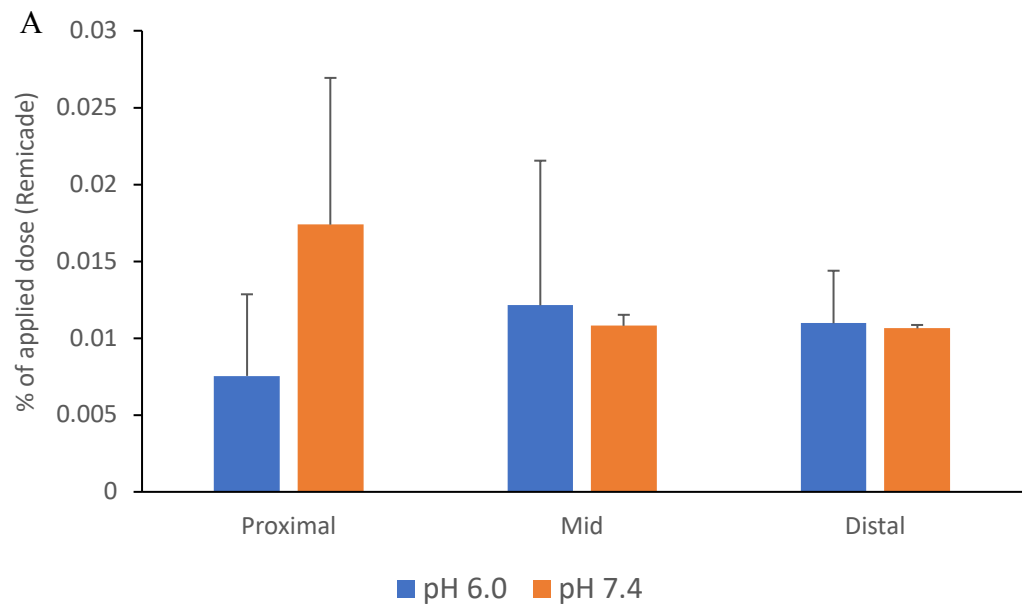


Figure 3-14: Accumulation of Remicade (A) and Remsima (B) as a percentage of total applied dose in porcine proximal, mid and distal oesophageal tissue at apical pH 6.0 (blue bars) and 7.4 (orange bars) and apical concentration 0.8mg/ml over 30 minutes, n=3, mean $\pm$ SD

3.5.4 Tissue accumulation of infliximab (Remsima) at different apical concentrations

However, since a pH dependent effect on tissue accumulation or permeability of infliximab was not conclusive, it was hypothesized that the permeation is either taking place as a result of pinocytotic transport or concentration gradient dependent passive diffusion.

Hornby and colleagues reported approximately 2.5 fold increase in basolateral concentrations across human intestinal segments when the IgG concentrations on the apical side were increased 10 fold (from 0.02 to 0.2 mg/ml) (Hornby et al., 2014). The 0.2mg/ml IgG apical concentration was well above the saturation concentration of surface FcRn receptors. Thus, non-receptor mediated endocytotic uptake can take place by mechanisms other than surface FcRn-mAb interaction at acidic luminal pH. In this study 0.02, 0.2 and 2.0mg/ml apical concentrations at apical pH 7.4 were used to determine mAb accumulation in porcine oesophageal tissue and transcytosis to the basolateral side. This gave an insight that mAb uptake is concentration gradient diffusion based especially because neonatal Fc receptors were not found to be located on the pig oesophageal mucosa by immunohistochemical staining.

As seen in Figure 3-15, there is a significant difference in the accumulation of infliximab in the porcine oesophageal tissue between 0.2 and 0.02 mg/ml and 2.0 and 0.2 mg/ml. At 2.0 mg/ml there is quite a high amount of infliximab permeated onto the basal side. Intracellular FcRn may interact with the internalized mAb in the acidic early endosome and therefore transcytose the mAb across the multi-layered epithelium and onto the basolateral side where it will dissociate at the pH 7.4.

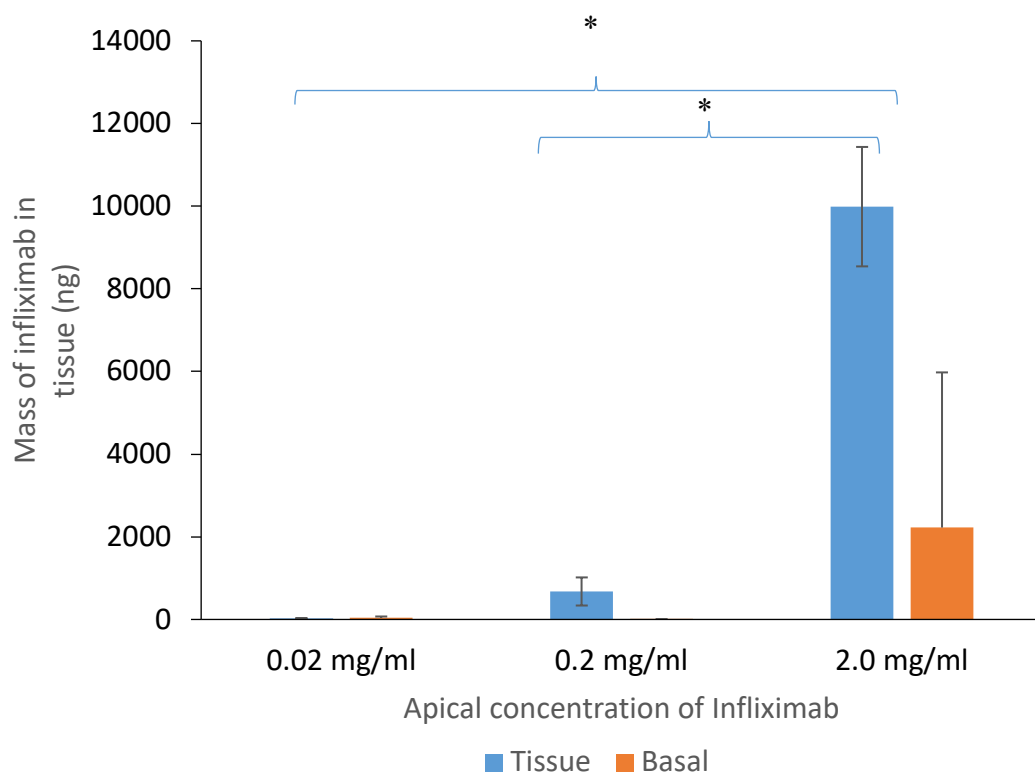


Figure 3-15: Permeation study of Remsima (infliximab) in pig male mid oesophageal tissue at apical conc. 0.02, 0.2 and 2.0 mg/ml. Accumulation in tissue (blue bars) and basolateral side (orange bars). * indicates $p < 0.05$ for 2.0mg/ml by OneWay Anova and post-hoc tukey analysis

Table 3-4: Total amount of infliximab in nanograms accumulated in porcine mid oesophageal tissue and basal side after 30 minutes, $n=3$, mean $\pm$ SD

Apical concentration (mg/ml)	Mass of infliximab in tissue (ng)	Mass of infliximab on basal side (ng)
0.02	25.5 $\pm$ 4.2	40.7 $\pm$ 31.1
0.2	677.2 $\pm$ 339.9	8.6 $\pm$ 0.46
2.0	9988.6 $\pm$ 1444.9	2223.3 $\pm$ 3754.6

However, in Figure 3-16, when plotted as a percentage accumulation of the applied dose, the significant difference in porcine oesophageal tissue accumulation of infliximab was only observed between 2.0 and 0.02 mg/ml. There was no difference between 0.2 mg/ml and 2.0 mg/ml.

In a study by Stirling and co-workers, they determined the uptake of porcine immunoglobulin G (IgG) by incubating porcine RS2 kidney cells with increasing concentrations of IgG. A receptor saturation uptake which was pH-dependent was observed with increasing concentration and plateauing at about 0.02mg/ml (Stirling et al., 2005). In the studies herein, a concentration gradient effect was evaluated. It may be a case whereby the accumulation of infliximab is taking place by diffusion since the highest concentration showed the greatest accumulation. Alternatively, a pinocytotic uptake mechanism may also be responsible especially considering that the accumulation has not shown a pH- dependence in previous experiments.

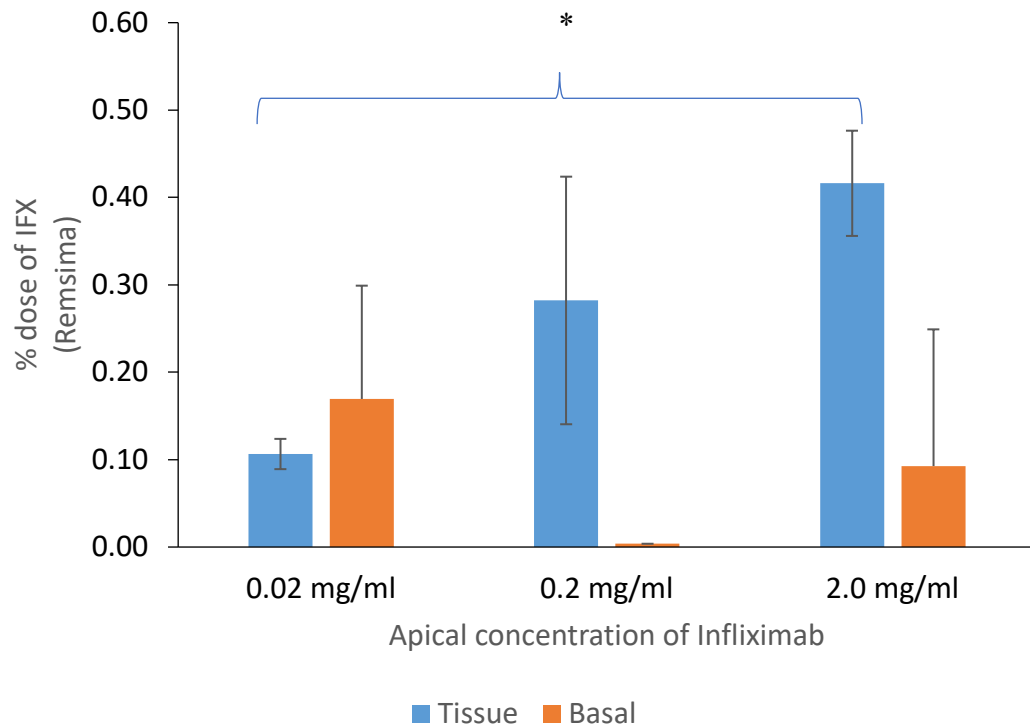


Figure 3-16: Permeation study of Remsima in pig male mid oesophageal tissue at apical conc. 0.02, 0.2 and 2.0 mg/ml, n=3, mean $\pm$ SD, * indicates $p < 0.05$ for 2.0mg/ml versus 0.02mg/ml by One Way Anova and post hoc Tukey

Table 3-5: Tissue and basal accumulation of infliximab as a percentage of the apical dose applied to porcine mid oesophageal tissue, n=3, mean $\pm$ SD

Apical concentration (mg/ml)	Percentage of applied dose in tissue	Percentage of applied dose on basal side
0.02	0.11 $\pm$ 0.02	0.17 $\pm$ 0.13
0.2	0.28 $\pm$ 0.14	0.004 $\pm$ 0.0002
2.0	0.42 $\pm$ 0.06	0.093 $\pm$ 0.16

3.5.5 Conclusion

We herein evaluated whether the monoclonal antibody infliximab permeates the porcine oesophageal epithelium. HPLC-SEC was used to determine the decrease in apical concentration so as to correlate with permeability. However, this method did not prove useful as it is not specific towards infliximab and endogenous tissue IgG. Therefore, an ELISA method specific to detect anti TNF α mAbs was used and this provided reliable interpretations of infliximab accumulation in tissue and basal side from Ussing chamber ex vivo experiments. The mucosal permeation of infliximab was confirmed using confocal microscopy. As such, there were no significant differences between the permeation of Remicade (original) and Remsima (biosimilar). Furthermore, the presence of FcRn receptors was not detected and this correlates well with the lack of pH-mediated antibody permeation. The accumulation of infliximab is dependent on concentration in the apical side, with greater accumulation observed on increasing apical concentration. However, there is very low level of tissue accumulation observed with respect the total dose applied. This means that local delivery of monoclonal antibodies could be very expensive owing to the high costs of mAbs required to achieve a small amount of permeation and consequently a therapeutic effect.

**CHAPTER 4 : DEVELOPMENT OF EX-VIVO INJURY MODEL AND
TISSUE ACCUMULATION AND/OR PERMEABILITY STUDIES OF
BUDESONIDE, TACROLIMUS AND INFLIXIMAB**

4.1 Introduction

Eosinophilic oesophagitis (EoE) is characterised by inflammation and fibrosis which features in the more severe stages of the disease. Studies were conducted on intact porcine oesophageal mucosa to understand whether budesonide (a drug currently used clinically), tacrolimus and infliximab permeate the oesophageal mucosa. All 3 drugs show to accumulate the oesophageal mucosa but we do not know whether these levels would achieve therapeutic efficacy. However, in EoE, budesonide has shown to be therapeutically effective (as seen from histological improvements and symptoms discussed in chapter 1) and is claimed to be due to higher accumulation of the drug in the oesophageal epithelium. Therefore, the aim in this chapter is to understand if the disease histopathology offers a greater opportunity towards permeation of budesonide in the oesophageal epithelium and whether this could be the main reason for budesonide's therapeutic efficacy from local application.

4.2 Absorption of drugs in inflammatory states

Currently, drugs when applied locally, are able to achieve higher local tissue concentration in inflammatory states for instance in inflammatory bowel disease. This has been attributed to increased mucosal permeability owing to disruption of epithelial integrity by cytokines and therefore opening of tight junctions between cells allowing greater passive permeability (Michielan and D'Incà, 2015). Also, reduction of CYP P450 enzymes have been attributed to this effect in that they reduce intestinal first-pass metabolism. Budesonide mouthwash has been given to chronic graft versus host disease (cGVHD) patients to alleviate or treat the mucositis. The systemic exposure to budesonide was markedly lower in healthy subjects after the mouthwash compared to oral dosing (mean relative bioavailability 18%–36%). However, the systemic concentrations thereafter in patients were as high as those after the identical dose of oral budesonide (Dilger et al., 2009). This has been attributed to higher permeability owing to buccal inflammation and low mucosal CYP enzymes in EoE and therefore a lower first-pass effect.

4.3 Histopathology of Eosinophilic Esophagitis versus healthy oesophageal epithelium/ Barrier function in healthy versus EoE oesophagus

The oesophageal epithelial cells similar to other epithelial structures are linked together by tight junctions. In a healthy oesophagus, these junctions are intact making the underlying submucosa impermeable to external stimuli such as microbes, aero and food allergens. Tight junctions (TJs) (Figure 4-1) are a group of transmembrane proteins that play critical role in maintaining epithelial adhesion; they are made of claudins, occludin, E-cadherin, and cytosolic proteins. Tissue integrity is affected by their dysregulation (Abdulnour-Nakhoul et al., 2015)Warners et al., 2017a). For instance, low levels of claudin-1 and E-cadherin have been correlated with increased gastrointestinal permeability in GERD (Björkman et al., 2013).

The adherens junction is important in stabilizing the cell-cell adhesion and regulation of the intracellular actin cytoskeleton. The adherens junction is made-up of the transmembrane protein epithelial-cadherin. The most basal structure involved in cell-cell adhesion is the desmosome, which provides mechanical support to the adherens junction complex. Given their role of maintaining cell-cell adhesion, both the adherens junction and desmosomes contribute to paracellular transit in an indirect fashion (Blevins et al., 2018)

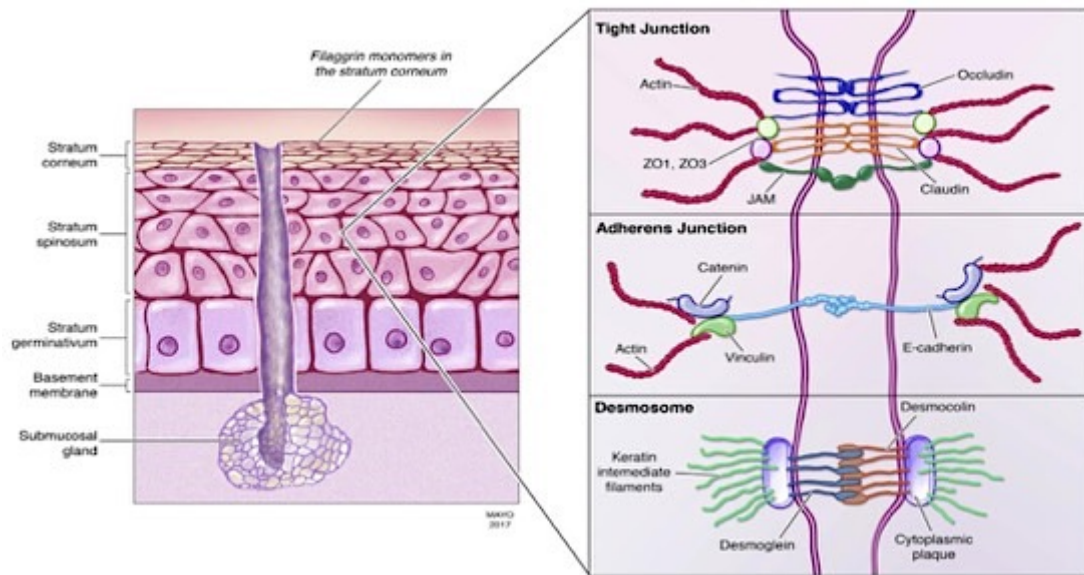


Figure 4-1: Stratified squamous epithelium of the oesophagus with magnification of the intercellular apical junction complex, showing the proteins involved in each of the 3 components of this complex. E-cadherin, epithelial-cadherin; ZO, zonulin (Blevins et al., 2018)

As described in Chapter 1, the pathophysiology of EoE involves a T-Helper 2 mediated immune response to allergens with the key involvement of IL-13 that upregulates the expression of certain proteins like periostin and downregulates the expression of others such as filaggrin and desmoglein (Figure 4-2). The latter proteins make up the epithelial tight junctions and loss of these causes disruption of epithelial tight junction integrity resulting in barrier defects. This leads to formation of dilated intercellular spaces and decrease in mucosal impedance along with increase in paracellular permeability allowing molecules of up to 40kDa (similar size to food allergens) to pass through the mucosa of patients with EoE and PPI responsive EoE (PPI-REE) (van Rhijn et al., 2014). Immunohistochemistry data showed that Desmoglein-1 was almost absent in oesophageal biopsies from EoE patients as compared to healthy controls (Figure 4-3) (Sherrill et al., 2014).

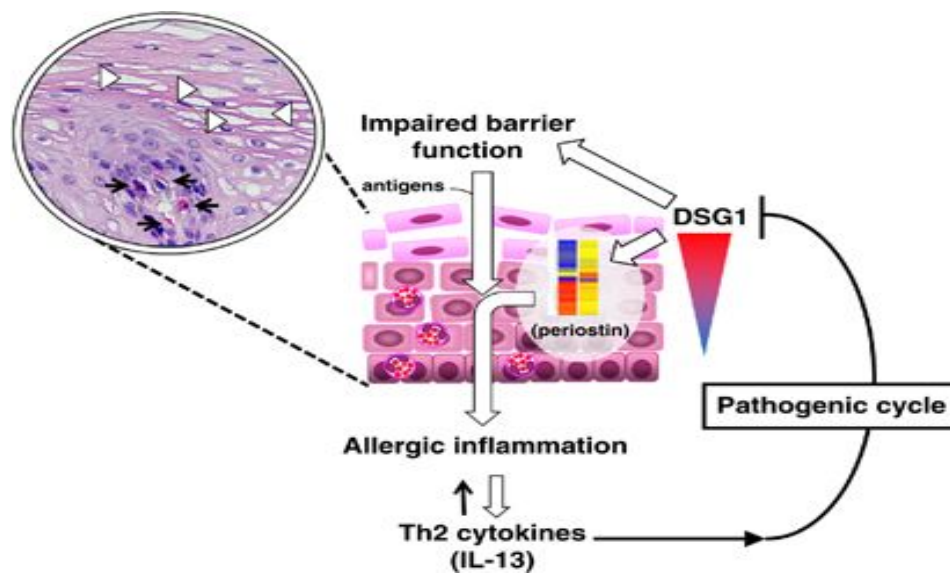


Figure 4-2: IL-13 effect on epithelial barrier function showing dilated intercellular spaces (arrow heads) and eosinophilic infiltration (arrows) (Sherrill et al., 2013).

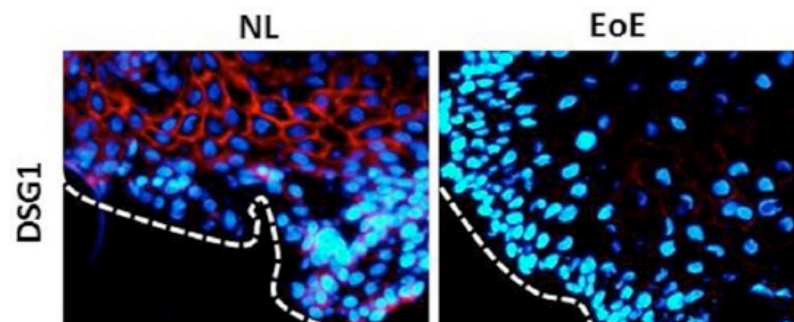


Figure 4-3: Confocal microscopy image showing absence of Desmoglein-1 (DSG-1) in EoE versus healthy controls (Sherrill et al 2014)

4.4 Seeking inspiration from gastroesophageal reflux disease (GERD): Towards development of an ex-vivo injury model to mimic dilated intercellular spaces in EoE

Gastroesophageal reflux disease (GERD), a disease of the oesophagus with similar histopathological features as EoE is herein used to develop a suitable model of EoE to allow systematic permeability testing of compounds. The main feature being DIS (dilated intercellular spaces) which may contribute to the accumulation of drugs like budesonide in the oesophageal epithelium, despite the short transit time of viscous 'adherent' formulations.

GERD is characterised by luminal reflux of either stomach contents i.e. acid and pepsin or the contents of the small intestine, gastroduodeno refluxate which in addition contains bile salts and pancreatic enzymes at a slightly higher pH of 4-7 (McQuaid et al., 2011). Bile salts are formed in the liver and these are primary bile acids chenodeoxycholic acid (CDCA) and cholic acid (CA) which can be conjugated by amino acids such as glycine and taurine to form glyco/tauro chenodeoxycholate and glyco/tauro cholic acid. Secondary bile acids such as deoxycholic acid and lithocholic acid are formed from cholic acid and chenodeoxycholic acid respectively. These are formed by deconjugation and dehydroxylation in the intestine by bacterial enzymes. Bile acids are found in the following decreasing order in the duodenum taurocholic acid>glycocholate>glycochenodeoxycholate>glycodeoxycholate>taurochenodeoxycholate>taurodeoxycholate (Moreno et al., 2006).

Oesophageal tissues from patients treated for GERD have shown DIS 1.0-1.7 μm (van Malenstein et al., 2008) and EoE 1.8-2.2 μm (Sherrill et al., 2014, Mueller et al., 2008, Ravelli et al., 2006). The dilation of intercellular spaces in both has been attributed to inflammatory cytokines, which regulate gene expression of tight junctions protein such as, E-cadherin and desmoglein-1 in GERD and EoE, respectively. Under light microscopy, histologic evidence of GERD in the oesophageal mucosa has included basal cell hyperplasia. Using TEM, investigators have also documented dilated intercellular spaces as a sensitive marker in patients with GERD. The inflammation observed in GERD also involves loss of E-cadherin and originates from the basal

epithelium owing to exposure to either acids or pancreatic enzymes and bile salts (Jovov et al., 2011, Souza et al., 2009).

Human mucosa tissues affected by GERD and EoE have shown similarities whereby there is decrease in transepithelial electrical resistance (TEER), a marker of paracellular and transcellular integrity, and consequently an increase in the flux of paracellular markers, fluorescent labelled dextrans (Weijenborg et al., 2013). Experiments with rabbit oesophageal mucosa exposed to acid pH, pepsin and bile salts also showed similar decrease in TEER (Tobey et al., 1996). This indicates an opening of paracellular spaces and possibility allowing higher tissue accumulation of drugs such as budesonide not only in the epithelial spaces, but also in the submucosa.

The aim of this work was therefore to develop an ex vivo injury model using porcine oesophageal tissue and which mimics primarily the dilated intercellular spaces. Therefore, inspiration from GERD has been sought to develop this model. The pig is susceptible to GERD and displays similar features like sloughing of the epithelium and dilation of intercellular spaces (Schopf et al., 2009). Hence the porcine oesophageal tissue was used to develop the injury model and understand the permeation of budesonide, tacrolimus and infliximab with the main aim being to understand if DIS offers greater opportunity for drug accumulation.

4.5 Permeation studies of budesonide, tacrolimus and mAb (infliximab) in ex-vivo injured tissue

4.5.1 Materials

Pepsin (800-1200 U/mg) from porcine gastric mucosa, sodium taurocholate and pancreatin from porcine pancreas (x8 USP activity) was obtained from Sigma Aldrich, UK and glycodeoxycholic acid sodium salt from Merck, Germany. Fluorescein sodium and fluorescein labelled dextran 4000 (FD4) was obtained from Sigma Aldrich, UK. The ingredients used for Krebs bicarbonate ringer (KBR) solution were as follows; D-glucose, sodium chloride, calcium chloride, magnesium chloride, sodium bicarbonate (Sigma Adrich, UK), potassium hydrogen phosphate and dipotassium hydrogen phosphate (VWR, UK).

A biorelevant buffer mHanks (Fadda et al., 2009) on the apical side of the Ussing chamber was used to represent the duodenal fluid and consisted of sodium chloride, potassium chloride, magnesium sulphate heptahydrate, disodium hydrogen orthophosphate dihydrate, monosodium phosphate, sodium bicarbonate and calcium chloride dihydrate from Sigma Aldrich UK, and VWR, UK.

4.5.2 Methods

4.5.2.1 Screening experiments towards developing an ex vivo model mimicking EoE

Ussing chamber experiments, as described in chapter 2, were conducted to simulate the conditions of gastroesophageal and gastroduodenal oesophageal reflux. Injury using different conditions (Table 4-1), was carried out in Ussing chambers with KBR pH 1.5 and pepsin 3.2mg/ml (SGF formula in USP NF), KBR pH 1.5 + pepsin 3.2mg/ml + 5mM sodium taurocholate and KBR pH 5.0 + pepsin 3.2mg/ml + 5mM sodium taurocholate + pancreatin (1.3 mg/ml) for 45 minutes. The concentration of pancreatin was based on the amount used in the SIF (simulated intestinal fluid) formula (USP NF). The permeation of fluorescein as a paracellular marker, tacrolimus and budesonide was compared against a control (no injury) in KBR pH 7.4.

After 45 minutes, the tissue was washed with KBR pH 7.4 twice and then drug permeation studies were conducted at a concentration of 0.33 mg/ml for budesonide or tacrolimus with cremophor RH60/ethanol and 0.5 mg/ml for fluorescein sodium.

The quantification of drug in tissue and basal (serosal) side was done by LC-MS/MS for tacrolimus and budesonide as described in previous chapters and fluorescein was quantified by a spectrofluorometer (BMG LABTECH PheraStar, Germany).

Samples were taken at 0, 15, 30, 45 and 60 minutes for the fluorescein sodium permeation in different injury conditions and controls and analysed in a fluorometer at excitation 460 nm and emission 520 nm.

Table 4-1: Exposure of pig mid oesophageal tissue to different injury conditions mimicking GERD

Formula	Kreb's bicarbonate Ringer's solution (KBR)	Pepsin (3.2mg/ml)	Taurocholic acid sodium salt hydrate (5mM)	Pancreatin from porcine pancreas (1.3 mg/ml)	Injury time (minutes)
1	pH 1.5	✓			45
2	pH 1.5	✓			45
3	pH 2	✓	✓		45
4	pH 5	✓	✓	✓	45

Table 4-2: Transepithelial resistance (TEER) values over 45 minutes exposure to different refluxate elements

TEER ($K\Omega \text{ cm}^2$)				
Time (minutes)	Tissues treated with KBR pH7.4	Tissues treated with KBR/SGF pH1.5, and pepsin	Tissues treated with KBR pH 1.5, pepsin, and 5mM sodium taurocholate	Tissues treated with KBR pH 5, pepsin, pancreatin and 5mM sodium taurocholate
0	3.2	3.4	3.7	3.1
10	2.5	1	2	2.3
20	2.4	0.9	2.3	2.0
30	2.3	0.8	2.1	1.6
45	2.2	0.7	1.8	1.2

4.5.2.2 Optimization and validation of ex vivo injury model using biorelevant gastroduodeno refluxate elements

The porcine mid oesophageal tissue was thawed at 4°C and mounted in 4x8mm (0.29 cm²) Ussing chambers and stabilized for 30 minutes in Krebs bicarbonate ringers (KBR) solution pH 7.4. After 30 minutes, the apical side was replaced with 3 ml of a mixture of the biorelevant mHanks solution pH 5 and containing 3.2 mg/ml pepsin, 1.25 mg/ml pancreatin and 10 mM GDC (Table 4-3). Freshly produced KBR pH 7.4 was added to the basal side. The duration of this stage was 60 minutes. This was lengthened compared to the previous study in 4.5.2.1 based on the fact that no significant difference in permeation was noted when tissues were injured for 45 minutes.

For the tacrolimus permeation study, tacrolimus/cremophor RH60/ethanol was added to the apical side at 90 minutes to give a concentration of 0.33 mg/ml and permeation was conducted over 30 minutes against a control (mHanks pH 5.0 only on apical side*). Transepithelial electrical resistance (TEER) measurements were taken every 15 minutes. At the end of 120 minutes, the tissue was collected for tacrolimus accumulation quantification using LC-MS/MS and basal samples were also collected.

For the study to determine paracellular permeability using FD4, after 30 minutes stabilization with KBR pH 7.4, the 10mM FD4 solution was co-applied with mHanks buffer pH 5 containing containing 3.2 mg/ml pepsin, 1.25 mg/ml pancreatin (Table 4-3) and 10mM GDC against a control (mHanks pH 5.0 only*). Basal samples were taken every 15 minutes and measured using a fluorometer (BMGLabtech, Germany) against a standard calibration curve. Transepithelial electrical resistance (TEER) measurements were taken every 15 minutes.

*Control solution in both cases, 10mM FD4 and 0.33mg/ml tacrolimus/cremophor RH60/ethanol was prepared in mHanks buffer pH 5.0 on the apical side.

Flux was calculated by the following formula =
$$\frac{\text{Conc of FD4 (nmol)}}{\text{Area (cm}^2\text{) * Time (min)}}$$

Table 4-3: mHanks buffer recipe representing a biorelevant duodenal fluid (Fadda et al., 2009)

Ingredient	g/L
Sodium chloride	8.0
Potassium chloride	0.4
Magnesium sulphate heptahydrate	0.2
Disodium hydrogen orthophosphate dihydrate	0.06
Monosodium phosphate	0.06
Sodium bicarbonate	0.35
Calcium chloride dihydrate	0.19

To make the mHanks suitable to induce the injury, pepsin 3.2 mg/ml, pancreatin 1.25 mg/ml and GDC 4.71 mg/ml (10mM) was added.

4.5.2.3 Transmission electron microscopy (TEM) to check dilated intercellular spaces formation

The porcine oesophageal tissues after exposure to the conditions described in section 4.5.2.1 and 4.5.2.2, were stored in a fixative (2% glutaraldehyde and 2% cacodylate) for TEM. Tissues were post fixed with osmium tetroxide and dehydrated through a graded alcohol series then embedded in epoxy resin. Ultrathin sections were post stained with uranyl acetate. The specimens were examined and images of the inner and outer epithelium were taken using a x 5000 magnification. This work was done by a TEM expert at UCL anatomy building.

4.5.3 Statistical analysis

All data are presented as means $\pm$ SD. Comparisons were performed by non-parametric Mann Whitney U-test. The effect of the different conditions on permeability of tacrolimus and budesonide was analysed using one-way ANOVA followed by Tukey post-hoc test using IBM SPSS Statistics 16 (SPSS Inc., Illinois, USA). The significance level was set at $p < 0.05$.

4.5.4 Results and discussion

4.5.4.1 Screening experiments towards developing an ex vivo model mimicking EoE using simulated gastric fluids and gastroduodeno refluxate elements

4.5.4.2

Figure 4-4, shows the permeation of fluorescein sodium in different injury conditions. The intention was to determine whether we can achieve the similar flux as observed when compared against an injury inflicted in vitro to rabbit oesophageal mucosa using 5mM sodium taurocholate at pH 2 and 392U/mg of pepsin (Tobey et al., 2001). Interestingly, the flux in the control (non-injured tissues) was similar compared to the study by Farre and colleagues (Farré et al., 2008) , except that they had those values for tissues exposed to 5 mM sodium taurocholate at pH 2 and 392 U/mg of pepsin for 2 hours. However, in Figure 4-4, reduction in permeation of fluorescein sodium was observed for the injury conditions. The results obtained are also not comparable and therefore cannot be validated against the results obtained by Warners et al (Warners et al., 2017b) and Weijenborg et al (Weijenborg et al., 2013) who performed permeation of fluorescein in EoE human tissues and in human GERD patient tissues. This means that these conditions used have not been successful at causing injury to the tissue so as to form dilated intercellular spaces.

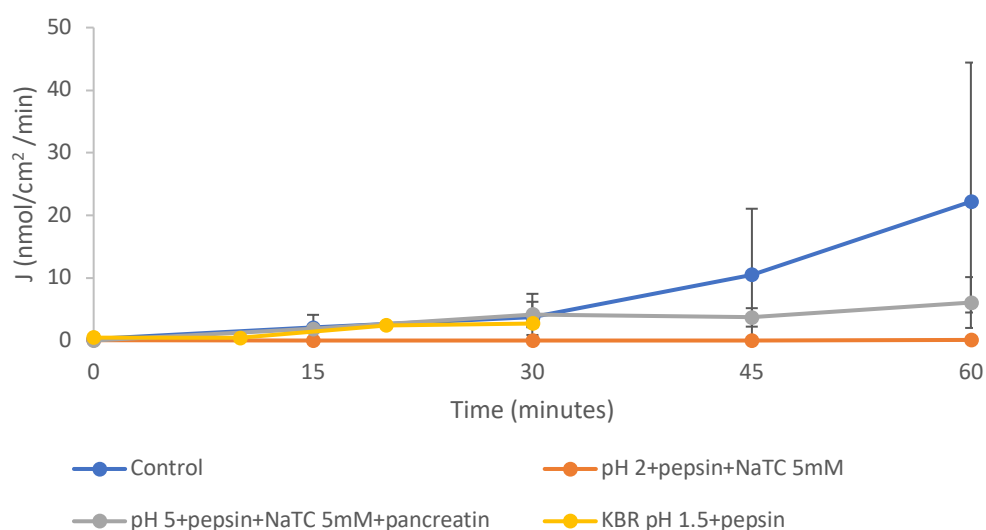


Figure 4-4: Fluorescein sodium permeability through porcine mid oesophagus in different injury conditions (n=3). Mean $\pm$ SD

Preliminary injury experiments as seen in chapter 2, indicated a marginal increase in tissue accumulation of budesonide when the tissue was exposed to SGF pH 1.2 and pepsin 3.2 mg/ml. However, since these experiments were not compared to a control on the same day and time, it is important to ascertain whether the accumulation of budesonide or tacrolimus is higher as compared to control experiments. In Figure 4-5 and Figure 4-6, it was observed that the maximum accumulation of budesonide was at 10 minutes as compared to 90 seconds and 30 minutes. However, it was not different to the control (non-injured tissue), $p > 0.05$. This probably explains that despite achieving decrease in TEER (Table 4-2) on exposure to the acidic pH and pepsin, the TEER goes back to normal when the injury elements are removed i.e. during drug permeation studies. Acid promotes increase in the formation of membrane coating granules which may be hindering the permeation of budesonide a moderately lipophilic drug despite decrease in TEER (Bateson et al., 1981).

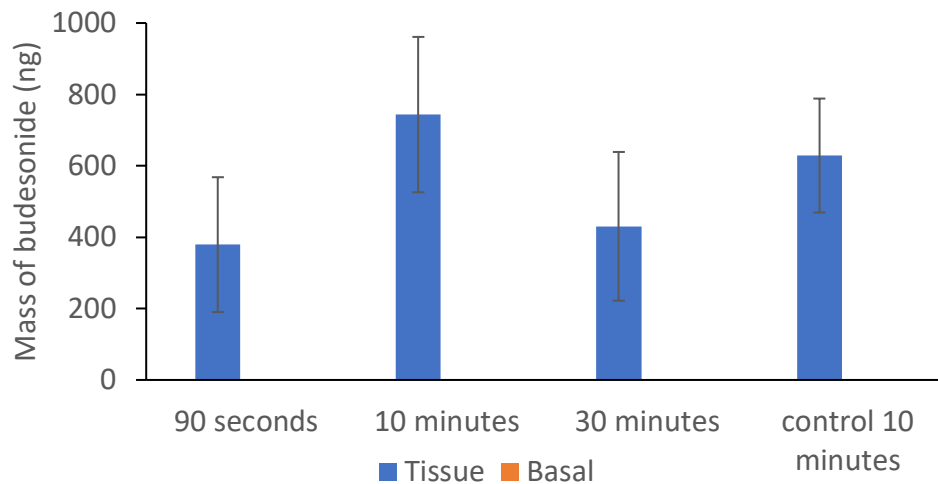


Figure 4-5: Permeation of budesonide/cremophor RH60/Ethanol in nanograms in tissue (blue square) and basolateral side (orange square) in male mid pig oesophageal tissues at apical conc. 0.33mg/ml using SGF pH 1.5 + pepsin 3.2mg/ml, t=90 seconds, 10 minutes and 30 min, n=3, mean $\pm$ SD.

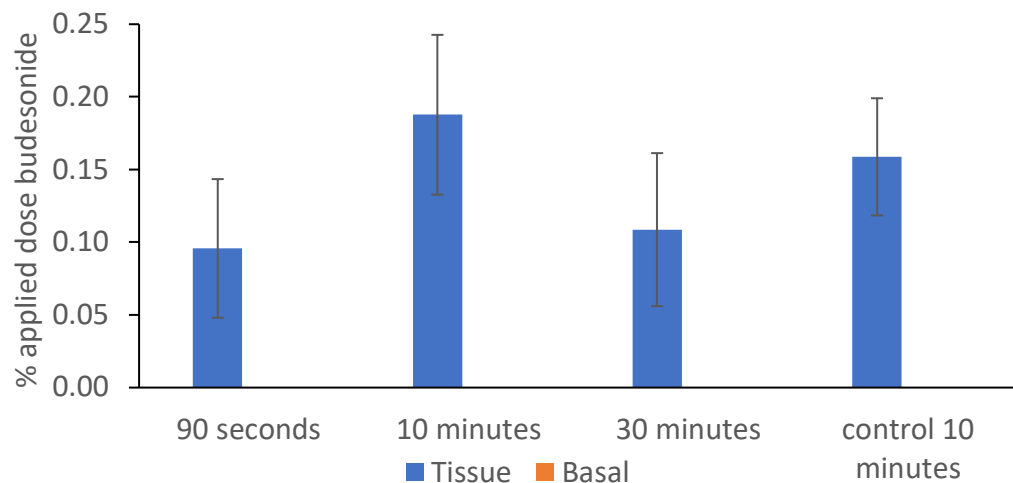


Figure 4-6: Permeation of budesonide/cremophor RH60/Ethanol as percentage of total applied dose in tissue (blue square) and basolateral side (orange square) in male mid pig oesophageal tissues at apical conc. 0.33mg/ml using SGF pH 1.5 + pepsin 3.2mg/ml, t=90 seconds, 10 minutes and 30 min, n=3, mean $\pm$ SD

The same procedure was also repeated for tacrolimus to understand whether a significant increase in tissue accumulation would be observed when compared to control experiments at 10 minutes. The permeation study was conducted for different length; 90 seconds, 10 and 30 minutes similar to budesonide. As seen in Figure 4-7 and Figure 4-8, there is no difference between the tissue accumulation of tacrolimus and control at 10 minutes. This is probably owing to the fact that although temporary opening of tight junctions took place as indicated by reduction in TEER (Table 4-2), it went back to normal after the exposure of acid pH and pepsin were removed and therefore tacrolimus accumulation was not greater than control.

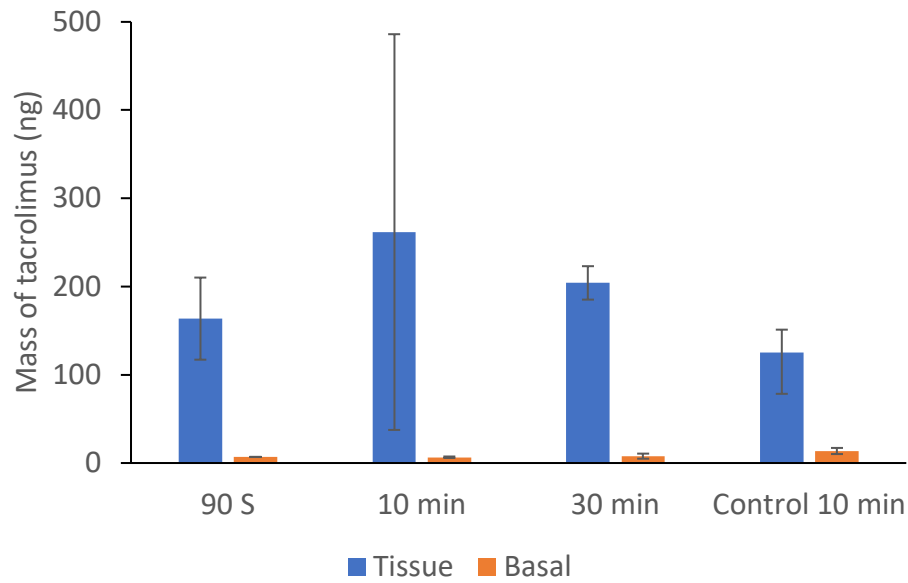


Figure 4-7: Permeation of tacrolimus/cremophor RH60/Ethanol 0.33mg/ml in nanograms in tissue (blue square) and basolateral side (orange square) in male mid pig oesophageal tissues at apical conc. 0.33mg/ml using SGF pH 1.5 + pepsin 3.2mg/ml, t=90 seconds, 10 and 30 min, n=3, mean $\pm$ SD

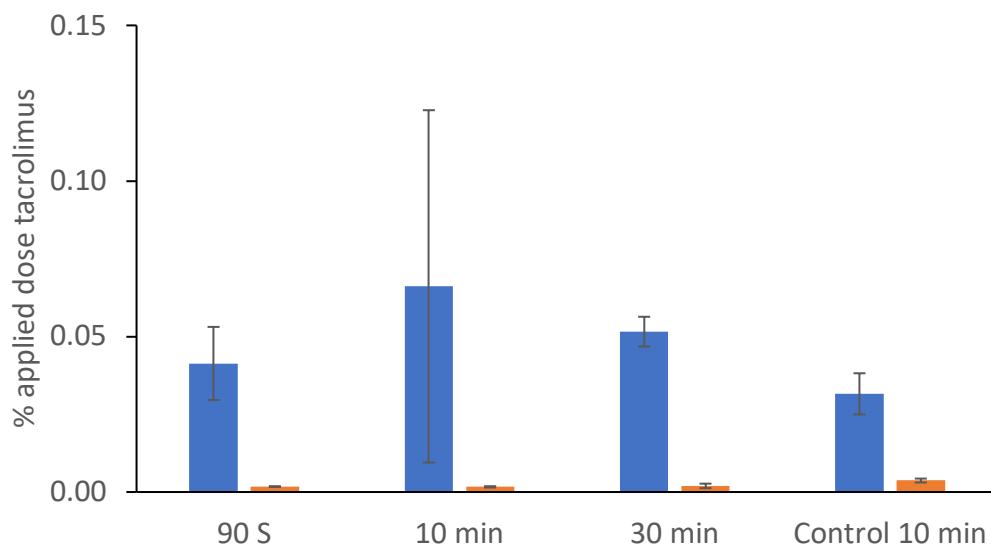


Figure 4-8: Permeation of tacrolimus/cremophor RH60/Ethanol 0.33mg/ml as percentage of total applied dose in tissue (blue square) and basolateral side (orange square) in male mid pig oesophageal tissues at apical conc. 0.33mg/ml using SGF pH 1.5 + pepsin 3.2mg/ml, t=90 seconds, 10 and 30 min, n=3 mean $\pm$ SD

It was observed in Figure 4-5 and Figure 4-6, that there was no budesonide detected on the basal side. Studies were undertaken to determine the reason for this. Budesonide/Cremophor RH60/Ethanol stock solution in acetonitrile were added to artificial saliva pH 7.4, KBR pH 7.4, artificial saliva pH 2.0, KBR pH 2.0 to give concentrations between 5-20 ng/ml. It was observed that the budesonide concentration was retrievable when added to acidified artificial saliva or KBR. Going forwards, budesonide samples taken from the basal compartment were immediately acidified using 1M hydrochloric acid before analysis.

In Figure 4-9 and Figure 4-10, budesonide was quantified in porcine mid oesophageal tissues after exposure to bile salt sodium taurocholate, pepsin and KBR pH 2 or pH 5. There was no significant difference observed between the various injury conditions and controls. Farre et al., observed greatest flux of fluorescein when exposing rabbit porcine oesophageal tissue to 5 mmol/L of sodium taurocholate with greatest reduction in TEER observed in Ussing chambers (Farré et al., 2008). Interestingly, no difference in tissue accumulation of budesonide was observed between the test and controls. Additionally, no increased influx of fluorescein sodium was observed between similar test and control. This is probably because budesonide and fluorescein sodium are small molecules of less than 500 daltons and increased paracellular permeability does not make a difference to their accumulation in tissue nor flux to the basal side.

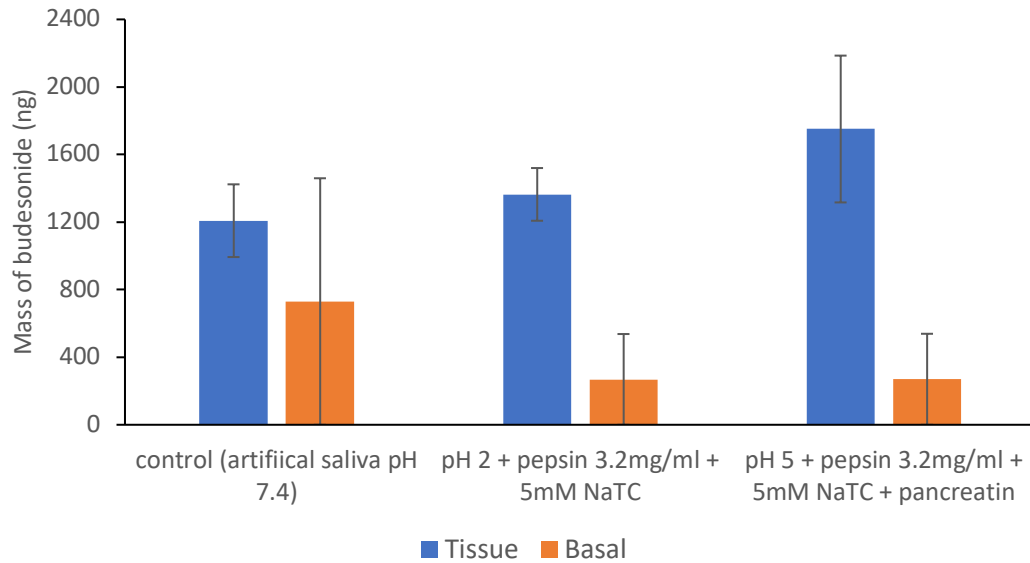


Figure 4-9: Budesonide/cremophor RH60/ethanol 0.33mg/ml permeation expressed as total accumulation in nanograms in male mid pig oesophageal tissues (blue bars) and basolateral side (orange bars) in different injury conditions versus control, t=30 min, n=3, mean $\pm$ SD

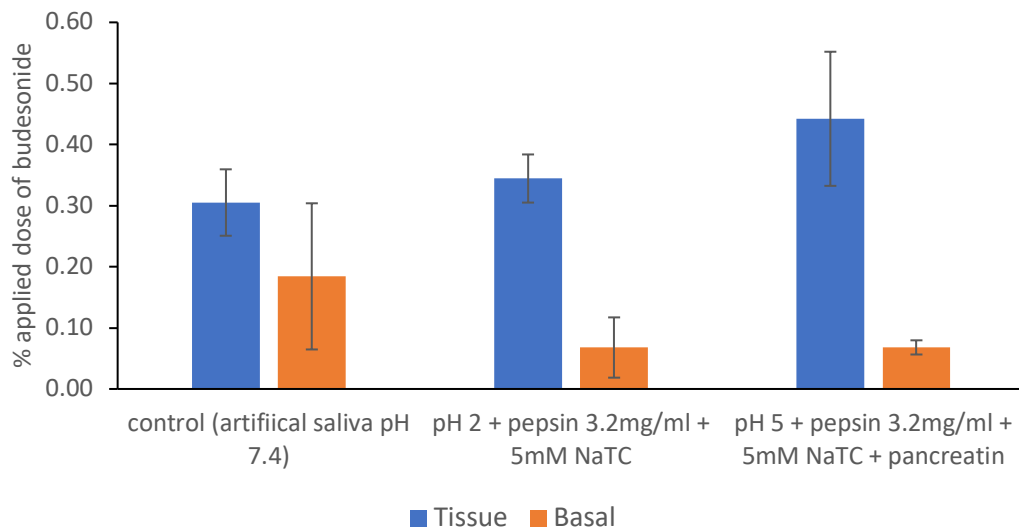


Figure 4-10: Budesonide/cremophor RH60/ethanol 0.33mg/ml permeation in male mid pig oesophageal tissues (blue bars) and basolateral (orange bars) expressed as a percentage of applied dose in different injury conditions versus control, t=30 min, n=3, mean $\pm$ SD

However, in the case of tacrolimus, as seen in Figure 4-11, there was significant tissue accumulation between the injury condition where it was exposed to KBR pH 5, 3.2 mg/ml pepsin, 5 mM sodium taurocholate and 1.3 mg/ml pancreatin versus KBR pH 2, 3.2 mg/ml pepsin and 5 mM sodium taurocholate and control (KBR pH 7.4). Tacrolimus, unlike budesonide, has a molecular weight higher than 500 Da and therefore it is less likely to diffuse passively via the paracellular spaces. Therefore, the higher accumulation observed may be owing to greater uptake into the epithelial layer due to removal of the outermost layer of the epithelium which is a tendency of bile salts and increased fluidity of the membrane (Bateson et al., 1981).

When the concentration of bile salts is above the critical micellar concentration (CMC), bile salts are known to remove the intercellular polar lipids of the epithelium thereby increasing paracellular permeability. In our study however, the bile salts may have not been able to do so at pH 2 (bile acids owing to their pKa may be ionized and therefore unable to dissolve the intercellular lipids at pH 2 compared to pH 5).

At pH 5 the bile salts would be in unionized state and would therefore be able to dissolve the intercellular lipids. In addition, the pancreatin which is active at pH 5, also leads to digestion of tight junction proteins thereby increasing paracellular permeability, as evidenced by a drop in TEER Table 4-2. In sum, this would help achieve the formation of dilated intercellular spaces.

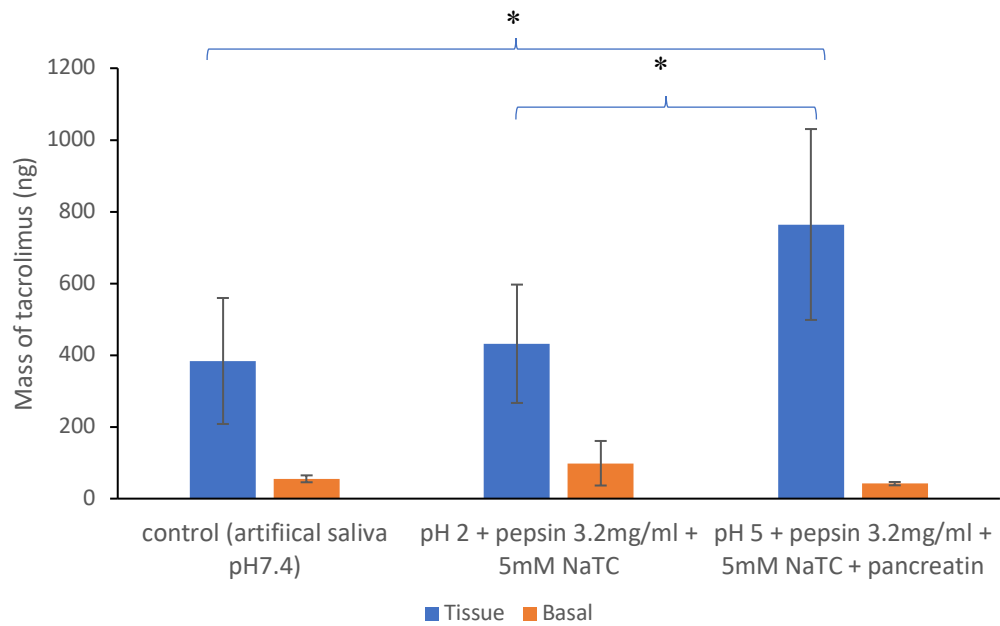


Figure 4-11: Tacrolimus/Cremophor RH60/ethanol permeation expressed as total accumulation in nanograms in male mid pig oesophageal tissue permeation in different injury conditions versus control, t=30 min, n=3. Mean $\pm$ SD * denotes a significant difference $p < 0.05$

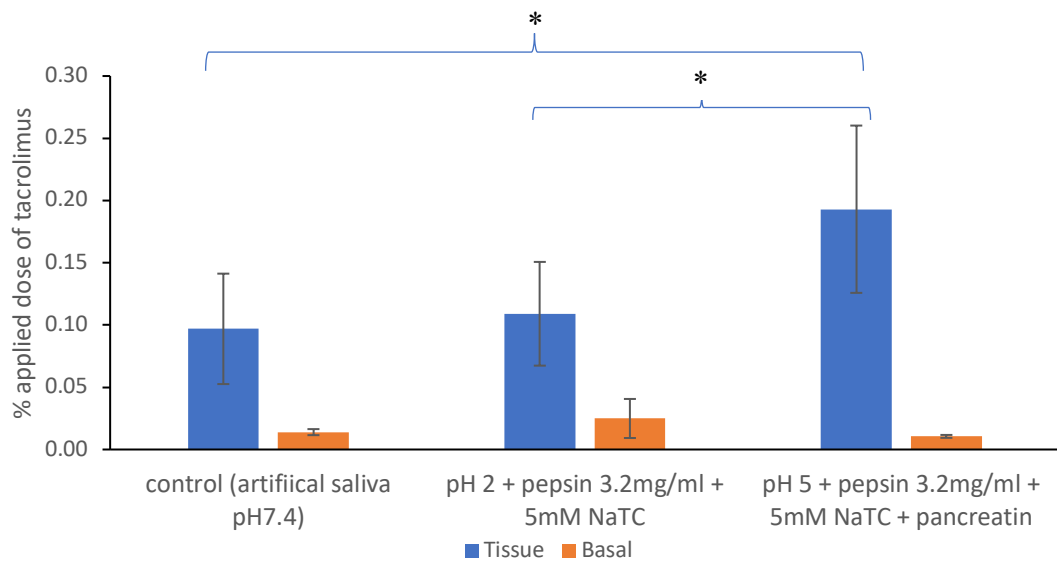


Figure 4-12: Tacrolimus in male mid pig oesophageal tissues, expressed as a percentage of applied dose in different injury conditions versus control, t=30 min, n=3. Mean $\pm$ SD * denotes a significant difference $p < 0.05$

Transmission electron microscopy (TEM) has been used to observe the dilation of intercellular spaces (DIS) in oesophageal biopsy specimens from EoE and GERD patients. TEM images of porcine oesophageal mucosa exposed to only KBR pH 7.4 as control and different injury conditions; a) KBR pH 2.0 and 3.2mg/ml pepsin b) KBR pH 2.0 + 3.2mg/ml pepsin + 5mM sodium taurocholate in the

Figure 4-13 below show the dilation of intercellular spaces and shortening or loss of desmosomes versus the control especially in the presence of sodium taurocholate. This has been observed in tissues from EoE and GERD patients (van Malenstein et al., 2008, Ravelli et al., 2014).

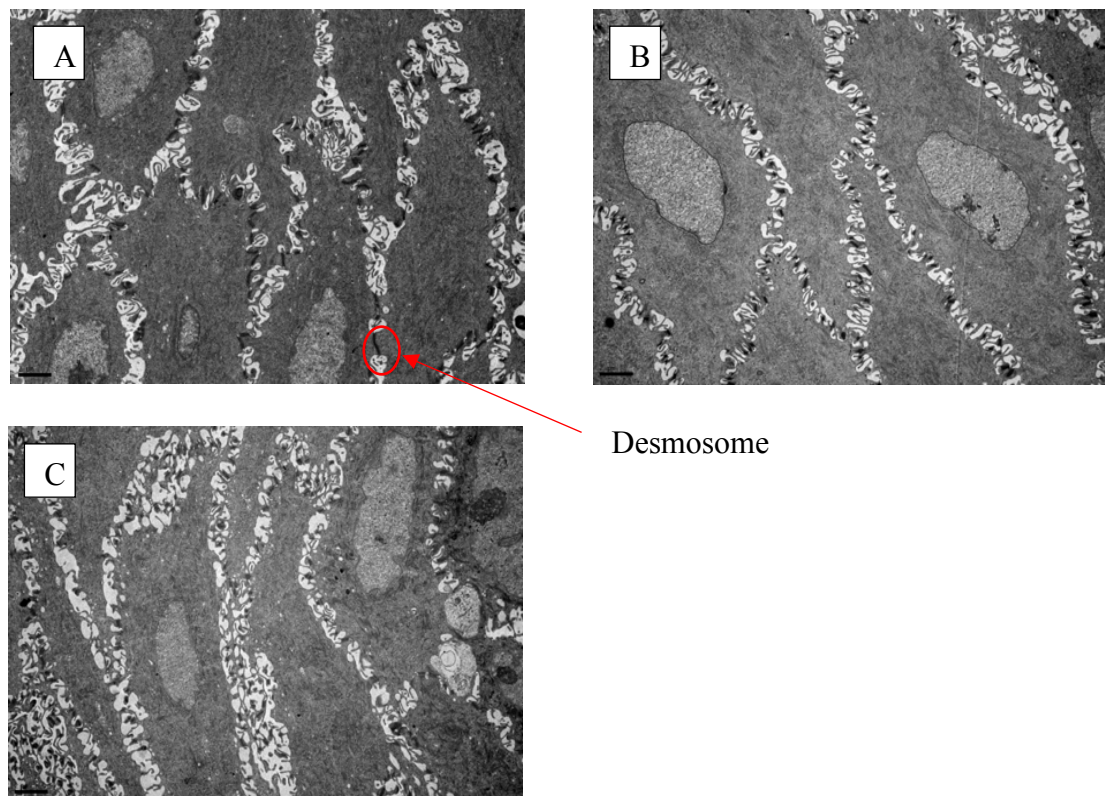


Figure 4-13: TEM images of basal layer of porcine oesophagus in a) KBR pH 7.4, b) KBR pH 2.0 and 3.2mg/ml pepsin c) KBR pH 2.0 + 3.2mg/ml pepsin + 5mM sodium taurocholate. All images at x 5000 magnification.

4.5.4.3 Optimization and validation of ex vivo injury model using biorelevant gastroduodeno refluxate elements

Hoogstrate and colleagues (Hoogstrate et al 1996 a and b) intended to improve the bioavailability of a peptide drug, buserelin. Among several permeation enhancers, they decided to use bile salts for the buccal permeation of buserelin in pigs. They found that sodium salt of glycodeoxycholic acid (GDC) improved the permeation of a paracellular marker fluorescein isothiocyanate dextran 4400 (FD4) by about 6-fold and when buserelin was concomitantly applied with GDC, it improved the bioavailability by 3-fold in pigs. Therefore, in the studies going forward, towards the development of an injury model, sodium glycodeoxycholate was used. This is on the basis that greater permeation of tacrolimus with sodium taurocholate and pancreatin was observed in the experiments conducted previously. Basically, sodium taurocholate is a primary bile acid whereas the glycodeoxycholate is a secondary bile acid. Secondary bile acids have shown to increase permeability of colon epithelium via decrease in TEER probably indicating their greater potential to open tight junctions (Sarathy et al., 2017).

The next step was to use sodium glycodeoxycholate (GDC) and make the apical buffer more biorelevant to represent the ionic and buffer composition of the small intestine more specifically the duodenum to mimic the gastroduodenorefluxate. In order to do this the buffer was changed from bicarbonate ringers' solution (KBR) to mHanks. This is a biorelevant buffer and its ionic composition simulates that of the proximal small intestine (Fadda et al., 2009). Therefore, the studies to follow were conducted in mHanks to which 3.2 mg/ml pepsin, 10 mM GDC and 1.25mg/ml pancreatin was added.

The porcine male mid oesophageal tissue was stabilised in the Ussing chamber for 30 minutes with KBR pH 7.4. This was then replaced with biorelevant mHanks solution pH 5 and containing 3.2 mg/ml pepsin, 1.25 mg/ml pancreatin and 10mM GDC. Fresh KBR pH 7.4 was added to the basal side and TEER was measured every 15 minutes. 10 mM FD4 was added to the apical side and the permeation study was conducted for a period of 90 minutes and compared against a control (FD4 in mHanks pH 5.0). TEER

values were recorded every 15 minutes. As explained in Figure 4-14, the exposure time of the gastroduodenorefluxate elements was increased from 45 minutes (previous experiments) to 90 minutes and 10 mM FD4 was concomitantly applied to the apical side of the Ussing chamber showed greater flux of FD4 ($p < 0.05$) at all time points over a 90 minute period as compared to control. The increased flux of FD4 in this instance is probably owing to opening of tight junctions as seen by an approximately 25% decrease in TEER between end and start of exposure. However, since the FD4 was concomitantly applied with the pancreatin, the proteases in pancreatin may have broken the isothiocyanate bond and therefore the increase in FD4 could be purely due to FD4 permeation or fluorescein.

Flux was calculated by the following formula =
$$\frac{\text{Conc of FD4 (nmol)}}{\text{Area (cm}^2\text{) * Time (min)}}$$

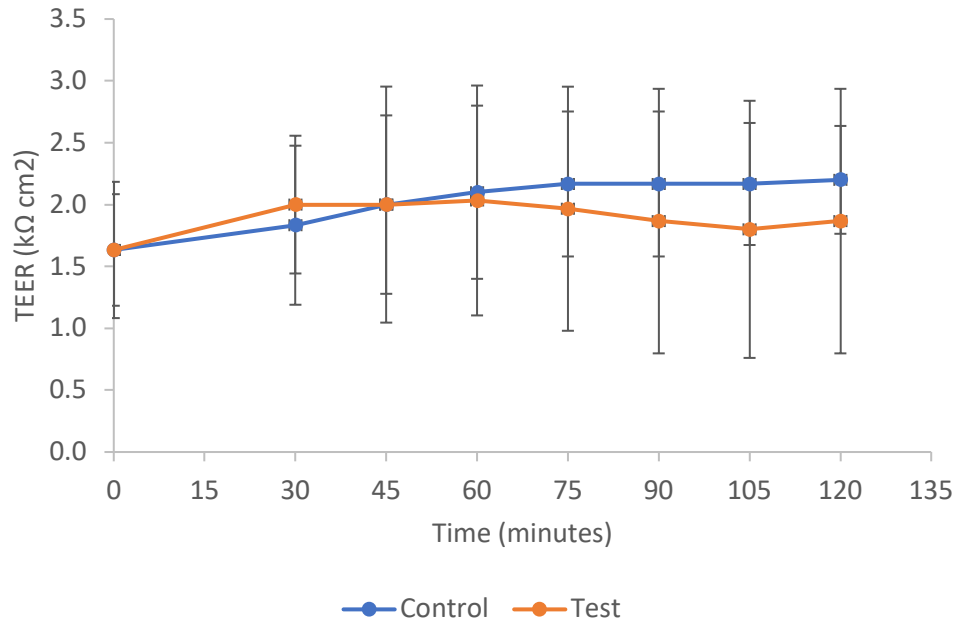


Figure 4-14: TEER measurements of pig mid oesophageal mucosa for first 30 minutes (stabilization in KBR pH 7.4) followed by exposure to mHanks pH 5, GDC 10mM, pancreatin 1.25mg/ml and pepsin 3.2mg/ml (test) for 90 minutes and mHanks pH 5 (control) for 90 minutes. n=3, mean $\pm$ SD

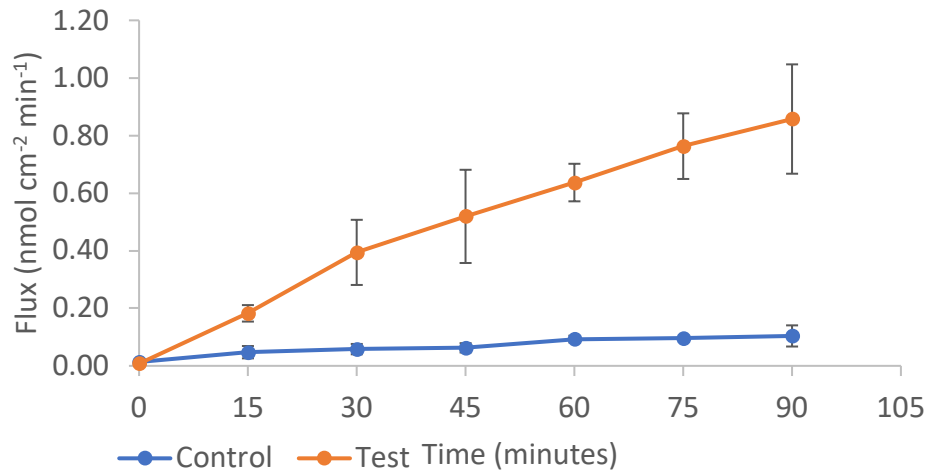


Figure 4-15: Flux of 10mM FD4 across pig mid oesophageal mucosa exposed to mHanks buffer pH5 (control) and mHanks pH5 + pepsin 3.2mg/ml + pancreatin 1.25mg/ml + 10mM GDC (test), n=3, mean $\pm$ SD, $p < 0.05$ for all time points by Mann Whitney U-test

Based on the above results, tacrolimus permeation was studied again by co-applying the tacrolimus 0.33 mg/ml to mHanks pH 5 containing 3.2mg/ml pepsin, 10mM GDC and 1.25mg/ml pancreatin. The permeation study was conducted over a 30 minute period. The Figure 4-16 and Figure 4-17 did not show a difference between tacrolimus accumulation in the tissue between test and control. This phenomenon has been previously observed whereby concomitant application of a bile acid, Azone (Caon et al., 2014) or laurocapram (Handler et al., 2018, Meng-Lund et al., 2014) as permeation enhancer has shown no difference or even decreased the absorption of the drug. The concentration of GDC used in this study is above critical micelle concentration (CMC) which is 4mM (Xiang et al., 2002, Dawson et al., 2006) and therefore, the decreased permeability of tacrolimus could be owing to the fact that the micelles formed in the apical side prevent absorption of tacrolimus.

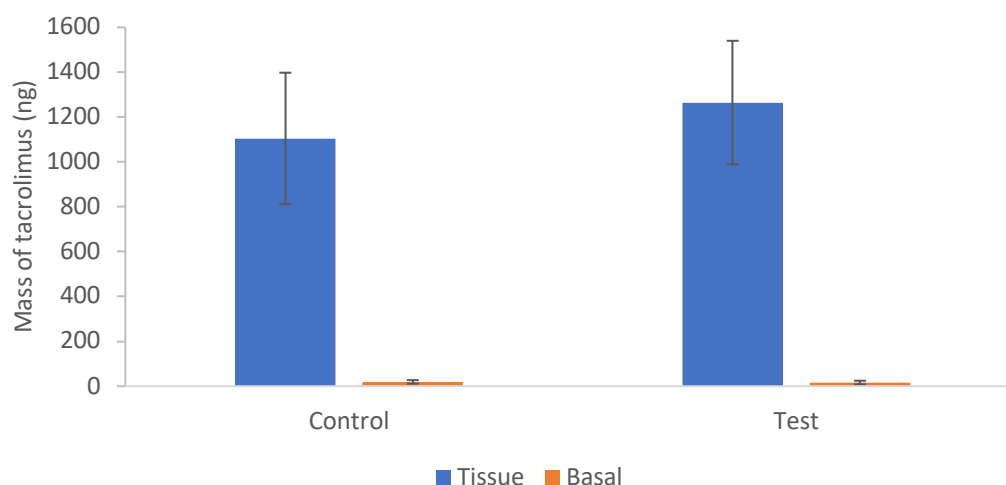


Figure 4-16: Permeation of tacrolimus apical conc. 0.33mg/ml over 30 minutes in pig mid oesophageal tissue in the presence of pepsin 3.2mg/ml, pancreatin 1.25mg/ml and 10mM GDC (test) and mHanks pH 5 (control), n=3, mean $\pm$ SD, Mann Whitney U-test, $p>0.05$ between test and control

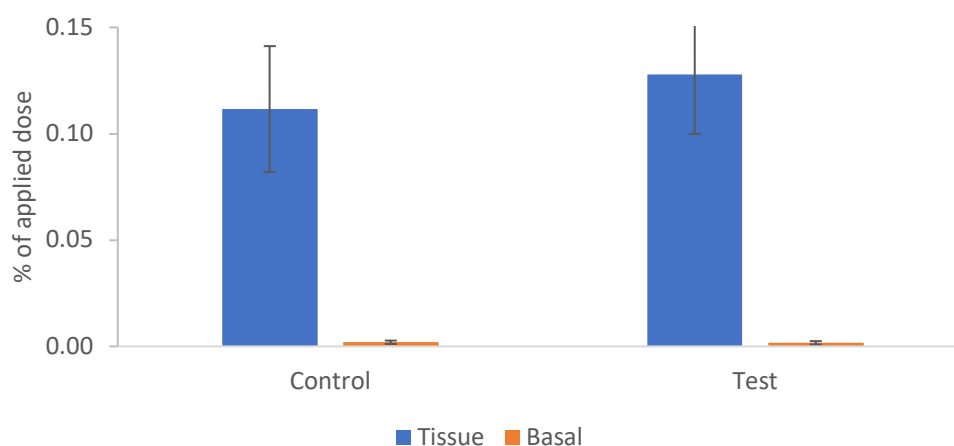


Figure 4-17: Permeation of tacrolimus apical conc. 0.33mg/ml over 30 minutes in pig mid oesophageal tissue in the presence of pepsin 3.2mg/ml, pancreatin 1.25mg/ml and 10mM GDC (test) and mHanks pH 5 (control). N=3, mean $\pm$ SD, Mann Whitney U-test, $p>0.05$ between test and control

Based on the above results, it was determined that the exposure to the gastroduodeno refluxate elements for a longer time period improved the permeation of the paracellular marker indicating removal or solubilization of the intercellular polar lipids and perhaps opening of the tight junctions thereby facilitating the permeation of the paracellular marker.

The next step from these preliminary experiments was to develop an injury model mimicking the opening of the tight junctions. TEER is a marker of paracellular permeability as previously discussed. Basically, the idea going forward, was to decrease the TEER to a point where it becomes irreversible even after removal of the gastroduodenal refluxate elements. This would give assurance that the opening of tight junctions is irreversible.

4.5.4.4 Optimization and validation of the injury model mimicking GERD. Permeation of budesonide, tacrolimus and infliximab

Pig (male) mid oesophageal tissue was stabilised with KBR pH 7.4 on both sides (apical and basal) for 30 minutes in an Ussing chamber. A screening experiment was performed with mHanks pH 5.0 + 3.2mg/ml pepsin + 10mM GDC + 1.25mg/ml Pancreatin (referred to as gastroduodeno refluxate elements), n=1. Here, the tissue was exposed to the gastroduodeno refluxate elements for 60, 90, 120, 150 and 180 minutes. TEER readings were taken at 30-minute intervals.

Following each exposure period, the tissue while still mounted in the Ussing chamber, was washed twice with KBR pH 7.4 and then exposed to artificial saliva pH 7.4 on the apical side and KBR pH 7.4 on basal side. TEER readings were taken every 30 minutes. The aim was to observe at which exposure time period the TEER reading drops irreversibly i.e cannot increase even after the injury causing agents are removed. This observation was conducted for a minimum period of 1 hour. In Figure 4-18, it was observed that unlike the control tissue and 60 minutes exposure tissue, the TEER did not recover from the 90 and 120 minutes exposures onwards. However, to prevent the tissues from becoming non-viable, 90 and 120 minutes timepoints were chosen for

the experiments going forward as it indicates irreversible opening of the tight junctions.

The optimal exposure period determined, i.e irreversible change in TEER on removal of injurious agent exposure was used for the tacrolimus permeation study. Briefly, male pig mid oesophageal tissues were stabilized with KBR pH 7.4 on apical and basal sides for 60 minutes. Then they were exposed to a mixture of 3ml of mHanks pH 5.0 + 3.2mg/ml pepsin + 10mM GDC + 1.25mg/ml pancreatin for 90 and 120 minutes on apical side and KBR pH 7.4 on basal side to mimic gastroduodenal reflux. At the end of the exposure periods, the tissues were washed twice with KBR pH 7.4 and stabilized for 30 minutes (to ensure that the drop in TEER is not reversing) with artificial saliva pH 7.4 on apical side and KBR pH 7.4 on basal side. Following this, 0.2 ml of artificial saliva pH 7.4 was removed from the apical side and 5 mg/ml of tacrolimus in cremophor RH60/Ethanol was added to give an apical concentration of 0.33mg/ml. The permeation study for both conditions was conducted for 30 minutes with n=3 tissues for each.

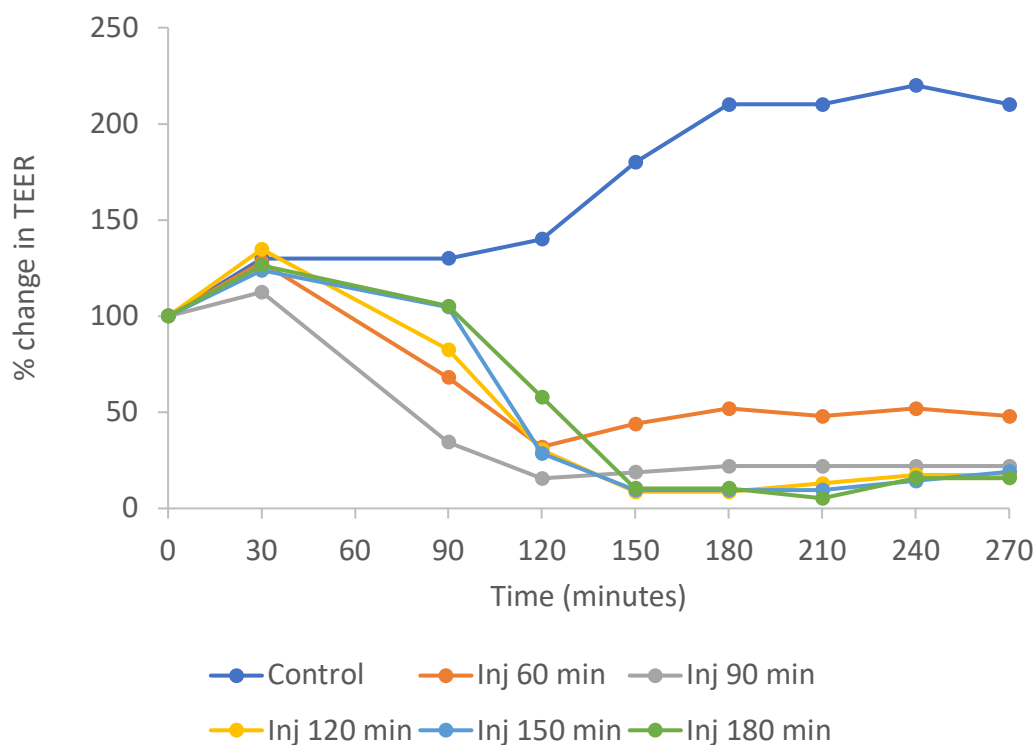


Figure 4-18: TEER measurements of oesophageal mucosa for different exposure times of mHanks pH 5.0 + 3.2mg/ml pepsin + 10mM GDC + 1.25mg/ml Pancreatin (gastroduodenorefluxate elements), n=1. 30 minutes stabilization in KBR pH 7.4, exposure to gastroduodenorefluxate elements for said time and then removal of the gastroduodenorefluxate elements and addition of artificial saliva pH 7.4 on apical side and KBR pH 7.4 on basal side to determine recovery

Based on this, the first aim was to test with a paracellular marker like FITC Dextran 4000 (FD4) whether there is increased accumulation of the paracellular marker from the tissues exposed to gastroduodeno refluxate compared to controls. As seen in Figure 4-19, it was observed that the TEER from exposing the mid oesophageal porcine tissue decreased to almost 25% of the original value and did not increase over the duration of the 2 hour permeation study.

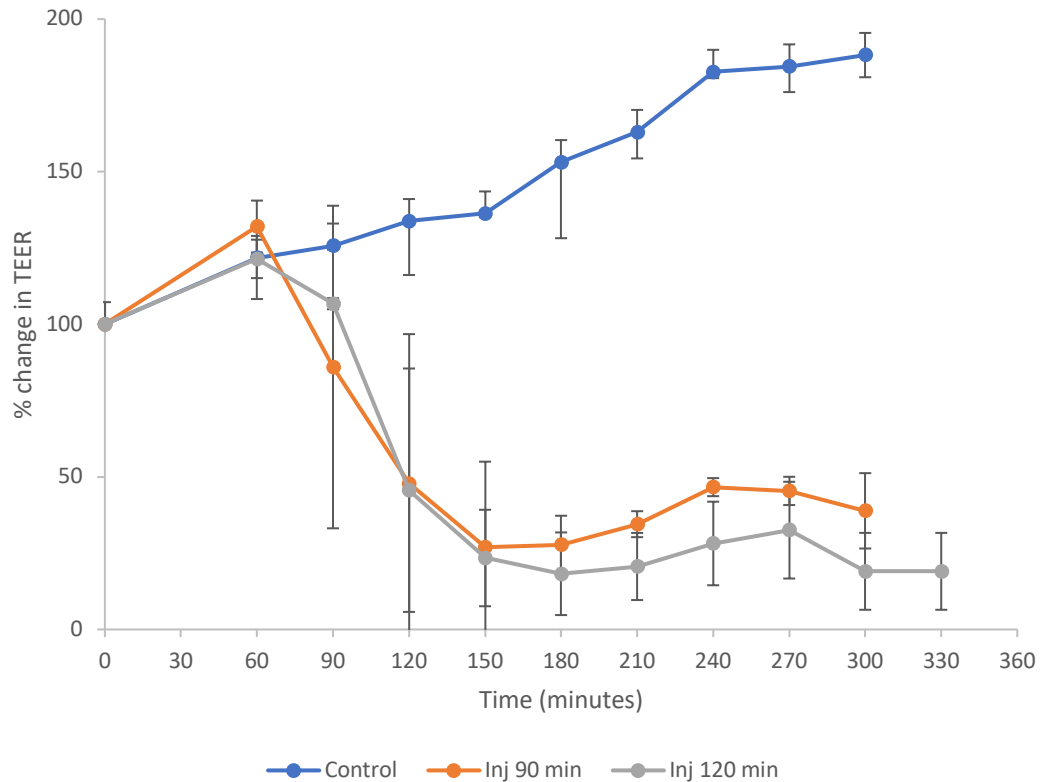


Figure 4-19: TEER measurements for Permeation of FITC dextran 4000 (FD4) after exposure of pig mid oesophageal tissue to mHanks pH 5.0 + 3.2mg/ml pepsin + 10mM GDC + 1.25mg/ml Pancreatin (gastroduodenorefluxate elements) for 90 (orange) and 120 minutes (grey) against control (blue), n=3 , mean $\pm$ SD

The permeation study was conducted with 10 mM FITC dextran 4000 (FD4) which is the concentration based on the work from Hoogstraate and co-workers with busserelin and sodium glycodeoxycholate (Hoogstraate et al., 1996b, Hoogstraate et al., 1996a). This time the permeation study of FD4 was done in the absence of the gastroduodenorefluxate elements i.e tissue was exposed to the gastroduodenorefluxate elements and then washed and subjected to permeation with FD4. Permeation at all time points between the injured tissues was not significantly different from control although the trend as seen in Figure 4-20 shows an almost 2 fold greater permeability of FD4 at all time points compared to the control. However, the differences are not statistically significant compared to control for both the injury conditions. In previous studies it has been observed that at very high concentrations of bile salts, both paracellular and transcellular routes have been affected (Hoogstraate et al., 1996b). Such an observation was made to visualize various fluorescently labelled dextrans in

porcine buccal mucosa in the presence and absence of bile salts. At low concentrations of the bile salts, the amount of dextran present in the intercellular spaces was increased, suggesting that the bile salts possibly solubilized the intercellular lipids and thus enhanced dextran diffusivity via the paracellular route. At higher concentrations of bile salts, dextrans began to appear in the epithelial cells indicating that these concentrations of bile salt were able to increase permeability through the cell membranes, possibly due to disruption of cell membrane lipids. This was verified in another study where sodium glycodeoxycholate applied to the surface of the porcine buccal epithelium resulted in a significant reduction in the tissue levels of polar lipids (intercellular) and cholesterol (cell membrane) (Hoogstraate et al., 1997).

These results show that injury to the tissue leads to solubilization of the intercellular lipids and opening of the paracellular spaces between the oesophageal epithelial cells as a result of injury from the bile salts and pancreatic proteases and lipases. However, the high variability indicates that there was varying degree of opening of the tight junctions as a result of injury. Another possibility is that the high concentration of bile salts has increased the accumulation of the FD4 in the tissue as well. In a recent study it was observed that biopsy grade 2 (inflammation + fibrosis) tissues from ulcerative colitis patients showed an increased permeation as compared to control. This was explained by the fact that grade 2 tissues have more fibrosis and therefore allow greater accumulation of the paracellular marker FD4 (Nakai et al., 2020). Therefore, it could be a case that the high concentrations of bile salt above CMC (4 mM) and pancreatic enzymes, have increased the tissue accumulation of FD4 owing to increased fluidity of the cell membrane or vacuolization of the cells.

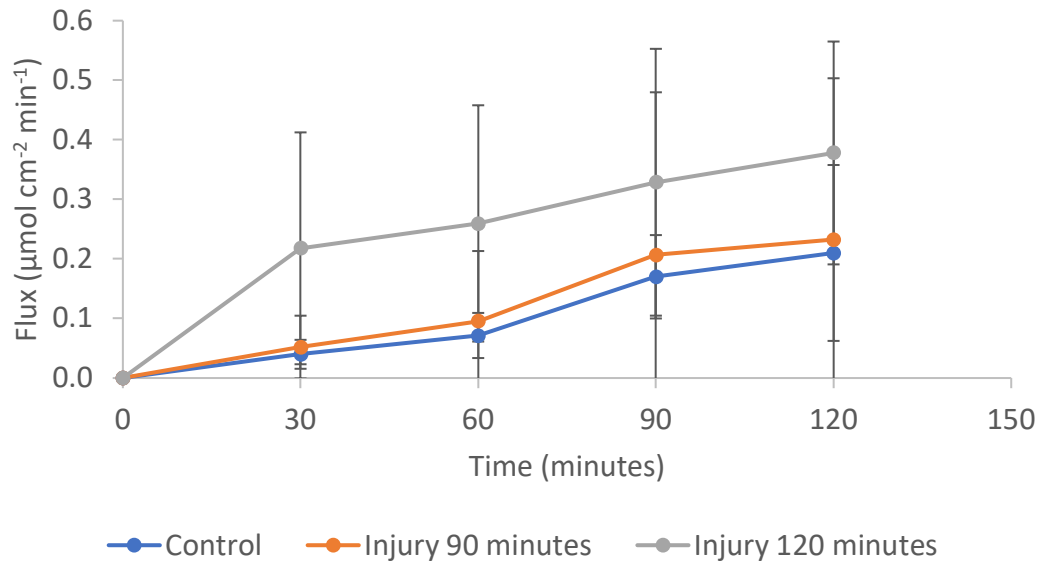


Figure 4-20: Permeation of 10mM FITC dextran 4000 (FD4) apical conc. after exposure of pig mid oesophageal tissue to mHanks pH 5.0 + 3.2mg/ml pepsin + 10mM GDC + 1.25mg/ml Pancreatin (gastroduodenorefluxate elements) for 90 minutes (orange line) and 120 minutes (grey line) against control (blue line), $n=3$, mean $\pm$ SD, One-way ANOVA for each time point, $p>0.05$

Following permeation studies with the paracellular marker FD4, transmission electron microscopy was conducted to understand the different morphological changes that take place to the histology and ultrastructure of the oesophageal epithelium. The tissues were either exposed to KBR pH 7.4 for 120 minutes or the gastroduodenorefluxate elements (mHanks pH 5.0 + 3.2mg/ml pepsin + 10mM GDC + 1.25mg/ml Pancreatin) for 90 and 120 minutes respectively.

As observed in Figure 4-21, the histology of the inner and outer epithelium of the control tissue is similar to that observed in healthy human oesophageal tissues (Van Malenstein et al., 2008). The outer epithelium has the cells intact and tight junctional desmosomes can be seen. In the inner epithelium the paracellular spaces in the basal layer shown no opening with desmosomes being observed.

In Figure 4-22 and Figure 4-23, the epithelial morphology of the outer epithelium resembles that reported by Bateson and co-workers (Bateson et al., 1981). They reported that pepsin causes widespread damage to the superficial cells however it does

not cause internalization of desmosomes. Duodenal juice containing all the elements such as bile salts and pancreatic enzymes (proteases, lipases and amylases) elicited a number of changes. The surface cells became leaky and showed microvesiculation with large membrane bound vesicles observed at the basal layer including internalization of desmosomes. Trypsin has been reported to be responsible for the splitting and internalization of the desmosomes and hemidesmosomes. Bile acids caused the appearance of microvesicles in the plasma membrane with increase over time. In addition, specifically Borysenko et al 1997 had found that oesophageal desmosomes are sensitive to deoxycholate used in our injury model studies (Borysenko and Revel, 1973).

In this work 90- and 120-minutes injury (Figure 4-22 and Figure 4-23) shows thinning of the upper epithelium being more prominent in the tissues exposed for 120 minutes as compared to 90 minutes. Cell lysis and vacuolization were observed in the tissues injured for 120 minutes. There is loss of desmosomes in the epithelium from the tissues injured at 90 and 120 minutes and consequently opening of paracellular spaces in the order of approximately 2 μ m in the inner epithelium.

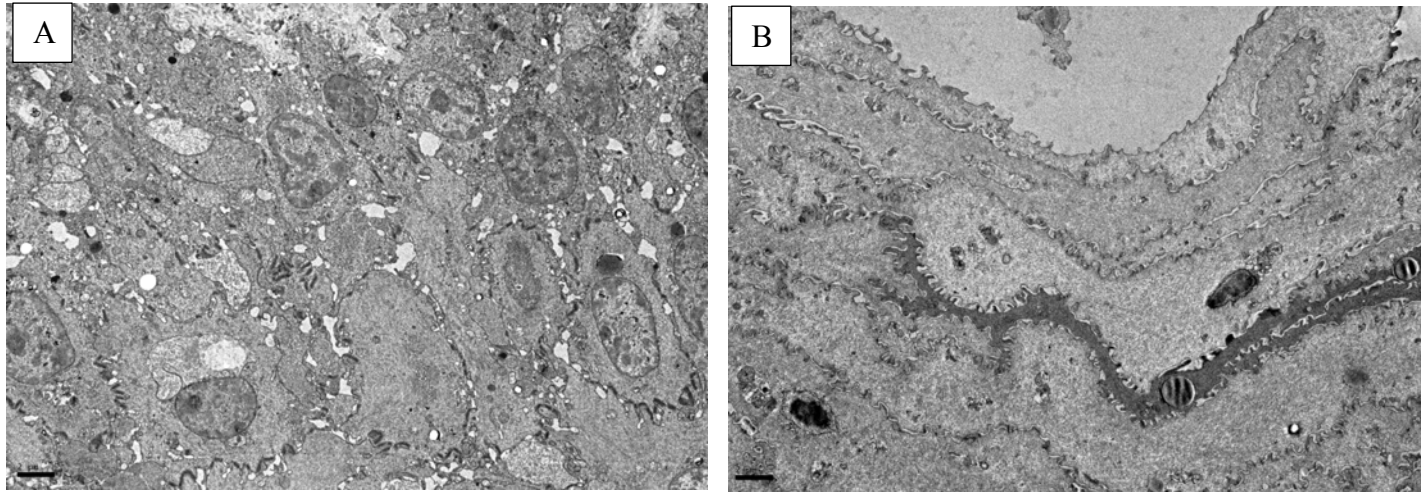


Figure 4-21: Transmission electron microscopy (TEM) study of porcine mid oesophageal tissues exposed to KBR pH 7.4 (control), mag x 5000, Scale 2 μ m, a) inner epithelium b) outer epithelium

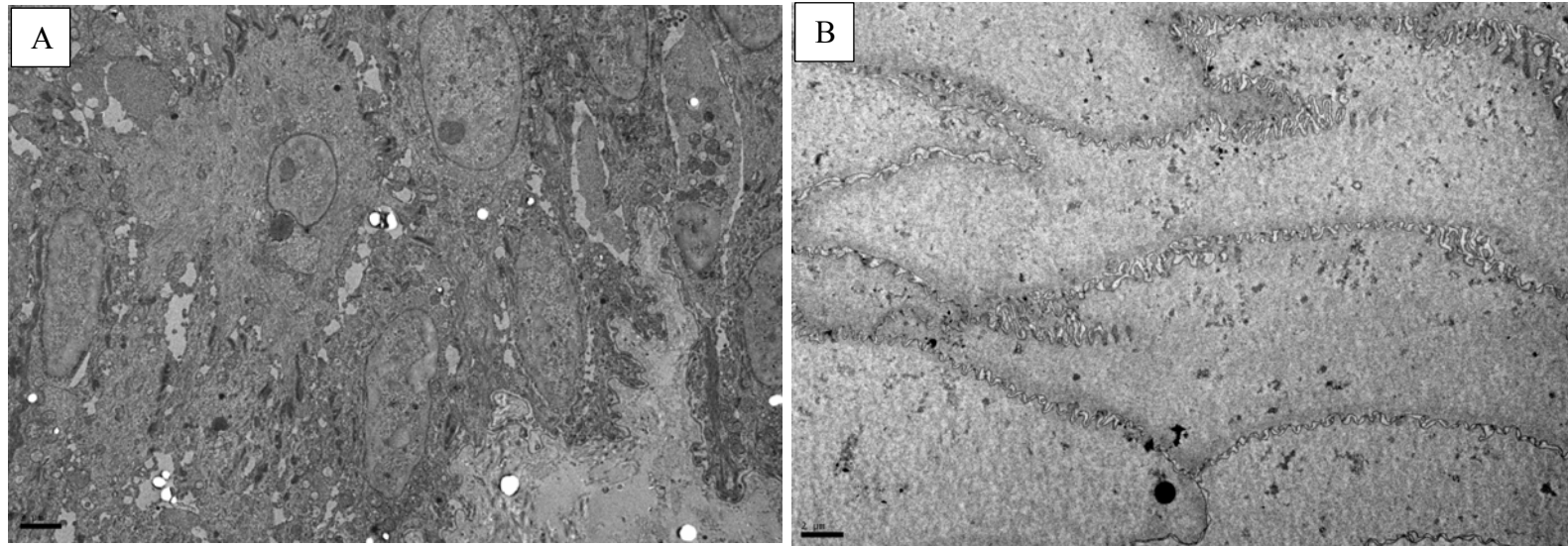


Figure 4-22: Transmission electron microscopy (TEM) study of porcine mid oesophageal tissues exposed to gastroduodeno refluxate elements (mHanks pH 5.0 + 3.2mg/ml pepsin + 10mM GDC + 1.25mg/ml Pancreatin) for 90 minutes, mag x 5000, Scale 2 µm a) inner epithelium b) outer epithelium

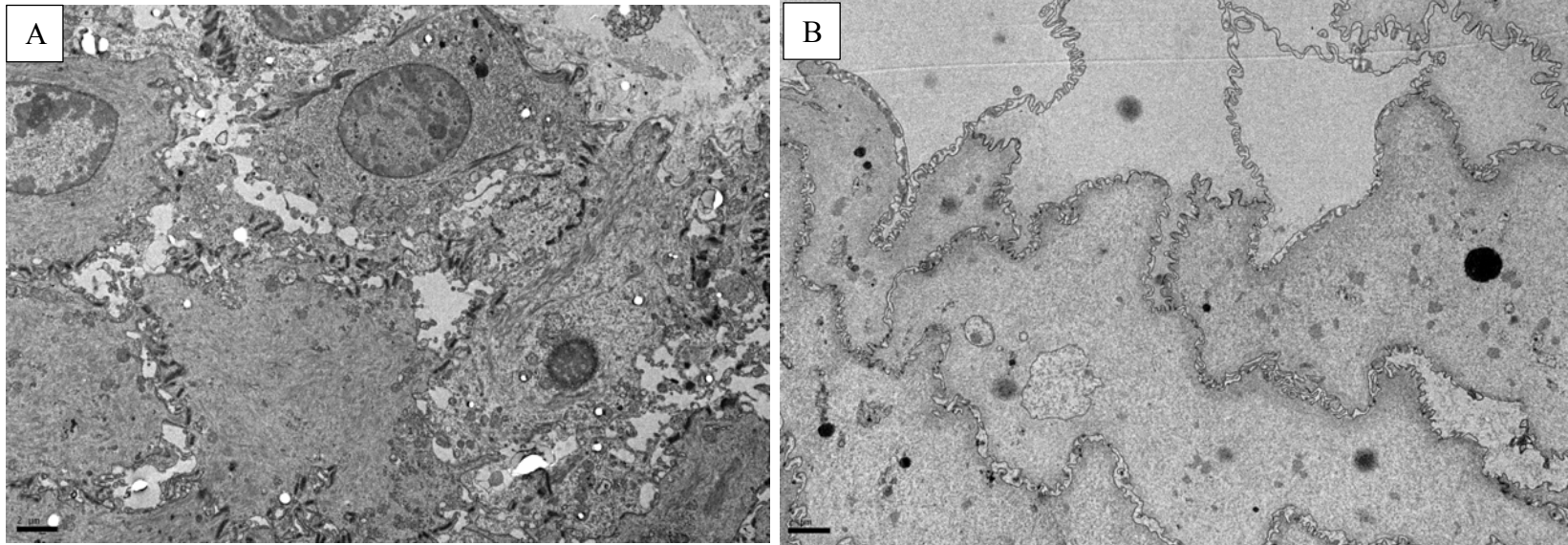


Figure 4-23: Transmission electron microscopy (TEM) study of porcine mid oesophageal tissues exposed to gastroduodenorefluxate elements (mHanks pH 5.0 + 3.2mg/ml pepsin + 10mM GDC + 1.25mg/ml Pancreatin) for 120 minutes, mag x 5000, Scale 2 µm, a) inner epithelium b) outer epithelium

4.5.4.4.1 Permeation of budesonide

In Figure 4-24, the tissues exposed to the gastroduodeno refluxate elements for 90 and 120 minutes have both shown irrecoverable TEER. This would mean that tight junctions have opened permanently as a result of the injury to the oesophageal epithelium. The TEER pattern observed here is similar to that in Figure 4-18.

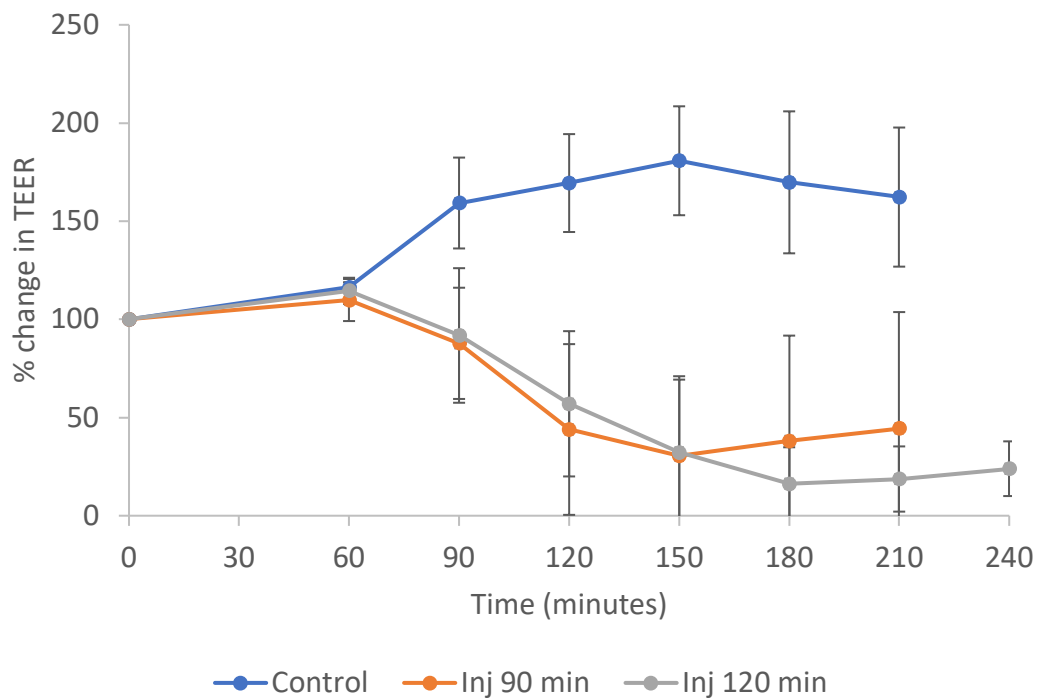


Figure 4-24: TEER measurements for 90 minutes and 120 minutes exposure to mHanks pH 5.0 + 3.2mg/ml pepsin + 10mM GDC + 1.25mg/ml Pancreatin (gastroduodeno refluxate). 0-60 minutes stabilization with KBR pH 7.4 followed by exposure to gastroduodenorefluxate for 90 (orange) and 120 minutes (grey) against control (blue) followed by washing with KBR pH 7.4 and stabilization with artificial saliva pH 7.4 for 30 minutes and finally permeation with budesonide 0.33mg/ml apical conc. for 30 minutes, n=3, mean $\pm$ SD

As seen in Figure 4-25 and Figure 4-26, budesonide permeation over a 30 minute period did indeed show a more than 2-fold increase in tissue accumulation from the injury at 90 minutes but not 120 minutes. The basal accumulation of budesonide, is greater for 120 minutes as compared to 90 minutes exposure to the gastroduodeno refluxate elements. Therefore, it seems as though there is increased permeation at 120 minutes owing to increased paracellular permeability allowing higher flux of budesonide through the oesophageal mucosa. In comparison to 90 minutes injury, where the tight junctions have not opened up all the way to the basal epithelium thereby promoting greater tissue accumulation in tissue injured for 90 minutes. Budesonide has a log P 2.4 and therefore it has been observed in previous experiments that surfactants like bile salts enhance the permeation of moderately lipophilic compounds which traverse the paracellular route (Deneer et al., 2002). This means that budesonide would permeate across the epithelium and into the blood at larger amounts owing to its high paracellular permeability while also achieving a higher tissue accumulation than in control experiments. However, the severity of the disease would dictate the paracellular permeability of budesonide and therefore its ability to accumulate in the epithelium and submucosa while also achieving systemic levels.

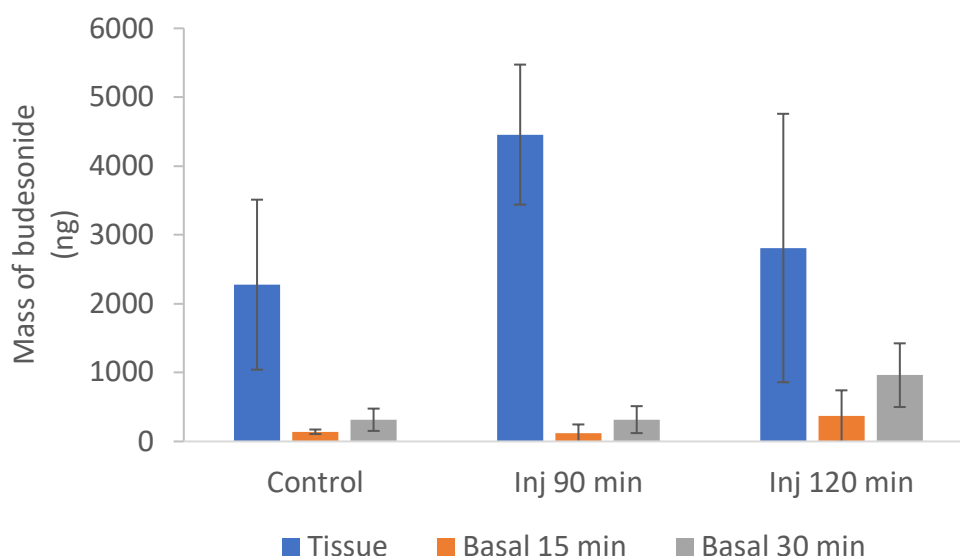


Figure 4-25: Mass of budesonide in porcine mid oesophageal tissue (blue bars) and basal side (orange and grey bars) for 90 and 120 minutes injury exposure at apical conc. 0.33mg/ml, n=3, mean $\pm$ SD, *p>0.05 for injury 90 and 120 minutes versus control for tissue accumulation

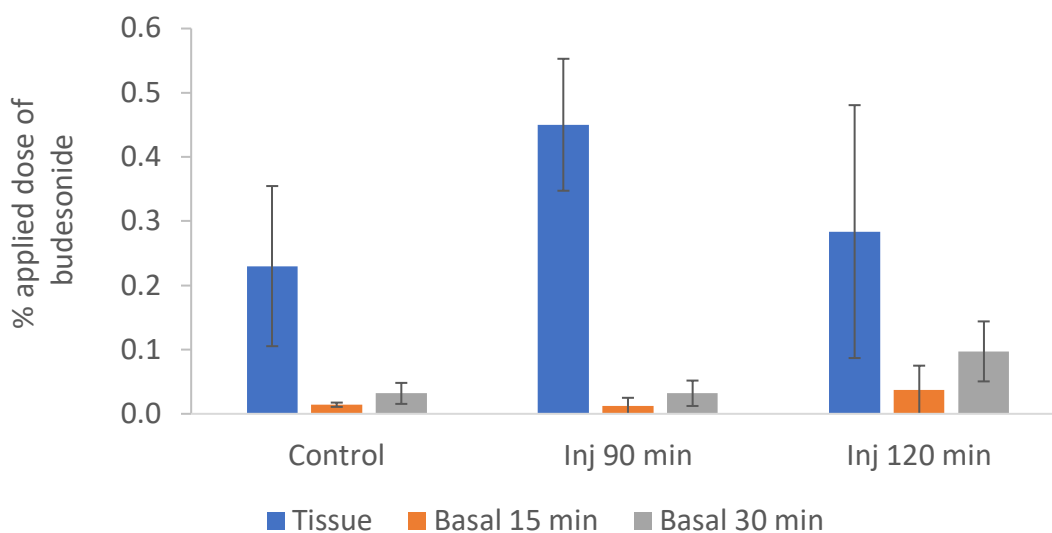


Figure 4-26: Percentage of applied dose of budesonide in porcine mid oesophageal tissue (blue bars) and basal side (orange and grey bars) for 90 and 120 minutes injury exposure at apical conc. 0.33mg/ml, n=3, mean $\pm$ SD, p>0.05 for injury 90 and 120 minutes versus control for tissue accumulation

4.5.4.4.2 Permeation of tacrolimus

The results for tacrolimus in Figure 4-27 also show that the TEER after exposure to 90 and 120 minutes is irrecoverable as compared to control; up to and including the duration of the permeation experiment.

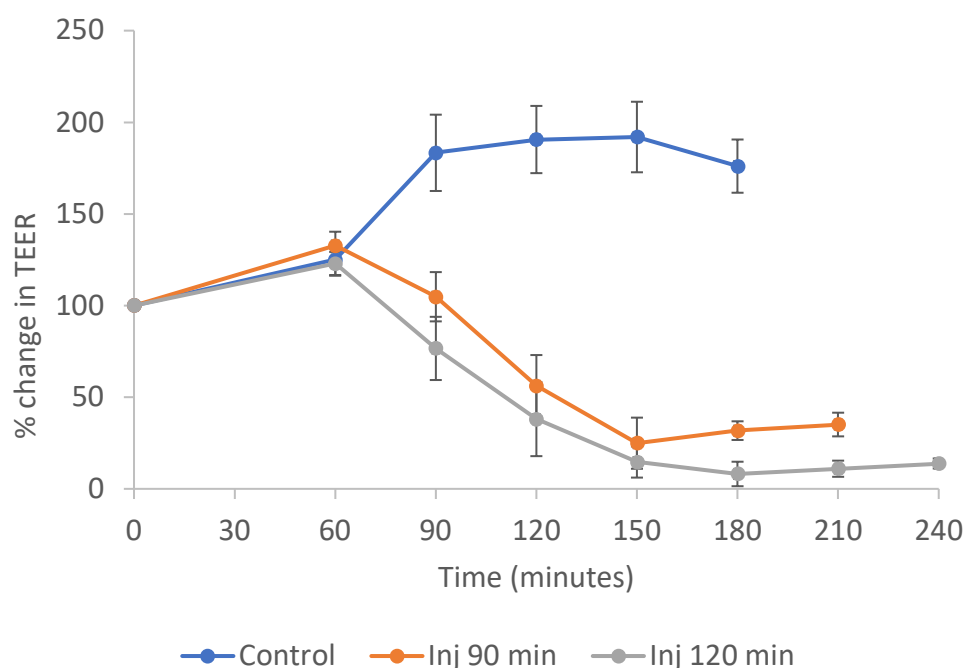


Figure 4-27: TEER measurements for 90 minutes and 120 minutes exposure to mHanks pH 5.0 + 3.2mg/ml pepsin + 10mM GDC + 1.25mg/ml Pancreatin (gastroduodeno refluxate). 0-60 minutes stabilization with KBR pH 7.4 followed by exposure to gastroduodeno refluxate for 90 (orange) and 120 minutes (grey) against control (blue) followed by washing with KBR pH 7.4 and stabilization with artificial saliva pH 7.4 for 30 minutes and finally permeation with tacrolimus 0.33mg/ml apical conc. for 30 minutes, n=3, mean $\pm$ SD

In Figure 4-28 and Figure 4-29, we have observed that the accumulation of tacrolimus is greater in the oesophageal mucosa for both injury conditions, 90 and 120 minutes relative to control, $p < 0.05$. The 90 and 120 minutes exposure periods were chosen for

the permeation study outlined in the methods section. It can be observed that unlike the control tissue the TEER did not recover at the end of the 90 and 120 minutes indicating irreversible tight junction disruption. Furthermore, there is greater accumulation of tacrolimus in the tissue exposed to injury elements for 90 minutes (2158 ± 156 ng) as compared to tissue injured for 120 minutes (1573 ± 229 ng) and 899 ± 214 ng for control. However, the basal accumulation at 30 minutes of tacrolimus tissue exposed to injury for 90 and 120 minutes is 56 ± 19 ng and 105 ± 39 ng respectively, as compared to control 42.1 ± 13 ng. This probably indicates that the depth of injury at 90 minutes exposure is probably less than at 120 minutes; allowing greater paracellular permeability at 120 minutes. In eosinophilic oesophagitis, the depth of the dilation of intercellular spaces (DIS) in the oesophageal epithelium has been reported to be up to the basal layer (Sherrill et al., 2014, Warners et al., 2017a, Ravelli et al., 2006)

The top third layer of the buccal epithelium is a barrier to hydrophilic compounds like mannitol (Hansen et al., 2018). The epithelium of the porcine buccal mucosa is similar to human oesophagus (Abdulnour-Nakhoul et al., 2007, Orlando, 2006). Therefore, it would be expected that a lipophilic drug like tacrolimus is readily absorbed in the control tissue with all layers intact. However, as seen in previous transmission electron microscopy (TEM) images from preliminary experiments, exposing to the gastroduodenorefluxate elements causes loss of the top third layer and internalization of desmosomes leading to increase in paracellular space. This means that the increased tissue accumulation of tacrolimus from the injury at 90 and 120 minutes has to be owing to increase in DIS and perhaps also increased fluidization of the cell membrane caused by the bile salt and GDC (Nicolazzo et al., 2005, Meng-Lund et al., 2014).

Lipophilic drugs like oestradiol which have a $\log P > 3$ have shown to partition in the lipophilic epithelium in the presence of certain surfactants which have a tendency to dissolve the lipids in the intercellular spaces. Tacrolimus has a $\log P > 3$ and therefore it may have preferentially partitioned into the lipophilic cell membrane. This basically means that the epithelium fluidity may have increased as a result of bile salts action and enzyme lipase (in pancreatin). Therefore, the chances are that tacrolimus has accumulated in greater amounts in the epithelium rather than traversed through the paracellular spaces. In a study from biopsy tissues of UC patients, it was observed that

those with grade 2 level of inflammation along with fibrosis showed greatest accumulation of a transcellular marker (metoprolol) in the tissue as compared to drug permeation on the basal side (Nakai et al., 2020). This is was attributed to greater fibrosis on the grade 2 level UC tissues as compared to less inflamed tissue and control. In EoE, there is fibrosis and therefore tacrolimus poses an advantage to being given locally as it would allow greater accumulation of drug in the tissue allowing it to act locally on the inflammation and possibly sparing from systemic effects.

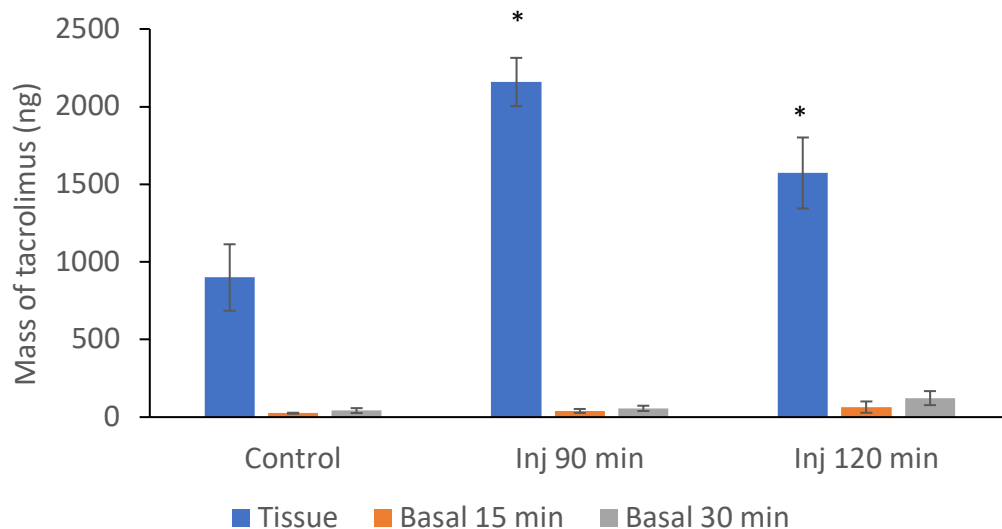


Figure 4-28: Mass of tacrolimus in porcine mid oesophageal tissue (blue bars) and basal side (orange and grey bars) for 90 and 120 minutes injury exposure at apical conc. 0.33mg/ml, n=3, mean $\pm$ SD, * denotes $p < 0.05$ for injury 90 and 120 minutes versus control for tissue accumulation

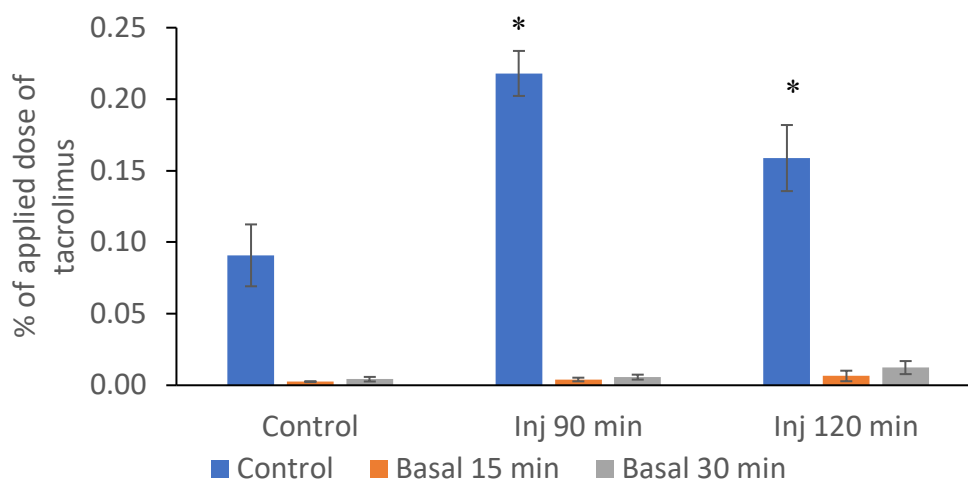


Figure 4-29: Percentage of applied dose of tacrolimus in porcine mid oesophageal tissue (blue bars) and basal side (orange and grey bars) for 90 and 120 minutes injury exposure at apical conc. 0.33mg/ml, n=3, mean $\pm$ SD, *denotes $p < 0.05$ for injury 90 and 120 minutes versus control for tissue accumulation

4.5.4.4.3 Permeation of monoclonal antibody, infliximab (Remsima)

Monoclonal antibodies are a class of compounds that have shown high therapeutic efficacy. Radiolabelled bevacizumab, when injected, achieved gastrointestinal low tissue concentrations; ca. 1.8% in the gastric mucosa, 3 and 2% in the small intestine and colon, respectively (Nagengast et al., 2007). This is probably owing to their hydrophilicity causing them to remain in the plasma and as a result only transcytosis into the tissues via uptake pathways or convective transport in highly perfused organs occurs. Anti-TNF monoclonal antibodies are highly efficacious, especially for moderate to severe forms of inflammatory bowel disease (ulcerative colitis) (Yadav et al., 2016b) When administered intravenously, the tissue accumulation of anti-TNF drugs like infliximab have shown to decrease in inflamed tissue from IBD patients relative to the healthy uninflamed tissues (Yarur et al., 2015).

Monoclonal antibodies have been investigated for the treatment of EoE and some like TNF- α inhibitors (infliximab) when administered parenterally did not show positive symptom and histological outcomes (Straumann et al., 2008b). Although, as mentioned earlier, this study was conducted in only 3 patients and therefore the results are inconclusive, Other monoclonal antibodies, more specific to the pathophysiology of EoE like anti IL-13 and IL-4, have shown greater efficacy, both in terms of histological and symptoms score. Therefore, it is important to understand whether the disease histopathology (dilation of intercellular spaces) offers greater tissue accumulation of antibodies when applied locally from the luminal side, so that they can block the pro-inflammatory cytokines in the epithelium and submucosa.

The intercellular junctions which consist of gap junctions have been shown to be separated by a gap of 2-5 nm and a gap of this size would probably permit the passage of molecules of molecular weight up to several thousand daltons (Harris and Robinson, 1992). It has been observed, in in vitro studies, that fluorescently labelled dextrans of molecular weight greater than 20,000 Da, could not permeate intact porcine buccal mucosa (Junginger et al., 1999).

A full length IgG monoclonal antibody is 150,000 Da and therefore it would be practically impossible for it to permeate through the paracellular spaces. As seen in chapter 3, monoclonal antibodies permeate the oesophageal epithelium probably owing to transcellular permeability either as a result of pinocytotic, endocytotic uptake or convective transport due to a higher concentration gradient between the apical and basal side.

In light of the low tissue accumulation in inflammation of mAbs given intravenously for IBD, it was rationalized to assess the permeability of the monoclonal antibody infliximab in the injury model of the oesophageal tissue. The aim was to assess whether higher tissue accumulation of the monoclonal antibody infliximab can be achieved when applied from the luminal/mucosal side.

In Figure 4-30, it was again observed that the TEER of the tissues exposed to the gastroduodeno refluxate elements was irrecoverable after 90 and 120 minutes of injury. Therefore, permeation studies from the tissue injured for 90 and 120 minutes were compared against the control for the permeation of Remsima.

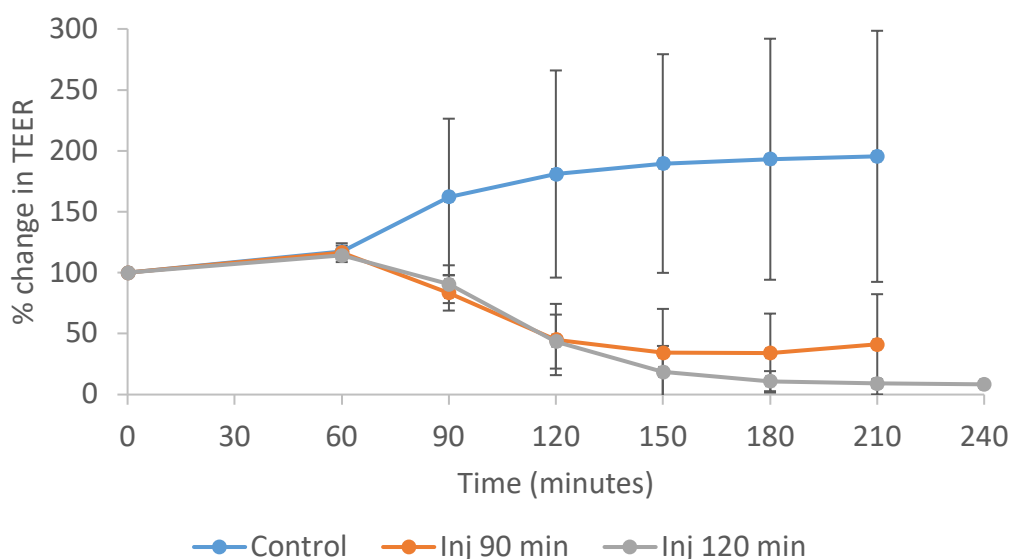


Figure 4-30: TEER measurements of pig mid oesophageal tissue for 90 minutes and 120 minutes exposure to mHanks pH 5.0 + 3.2mg/ml pepsin + 10mM GDC + 1.25mg/ml Pancreatin (gastroduodeno refluxate). 0-60 minutes stabilization with KBR pH 7.4 followed by exposure to gastroduodeno refluxate for 90 (orange) and 120 minutes (grey) against a control (blue) followed by washing with KBR pH 7.4 and stabilization with artificial saliva pH 7.4 for 30 minutes and finally permeation with infliximab 2.0 mg/ml apical conc. for 30 minutes

Permeation studies in injured and non-injured oesophageal tissues (Figure 4-31) were compared against the control experiment. It was observed that the accumulation of Remsima was significantly ($p < 0.05$) higher from tissue injured for 120 minutes as compared to control. However, the same was not observed for 90 minutes injured oesophageal tissue. The accumulation of infliximab from the tissue injured for 120 minutes was 76854 ± 21294 ng and 90 minutes was 58776 ± 12006 ng and control was 9817 ± 13276 ng (the high SD was because of very high variability owing to the biological nature of the tissues). The accumulation of infliximab on the basal side at 30 minutes was 2674 ± 2680 ng for the tissue injured for 120 minutes and 8220 ± 10133 ng for the tissue injured for 90 minutes and 1177 ± 156 ng for the control. In the case of 120 minutes injured tissue, the accumulation is 7-fold greater than the control and 1.5-fold greater than that of 90 minutes injured tissue. This shows that there is probably loss of the top third of the epithelial barrier and disruption of the lipids and protein domains of the cell membranes leading to micro-vesiculation and

vacuolization and consequently allowing greater accumulation of infliximab. The lower basal accumulation from 120 minutes injured tissue relative to 90 minutes can be explained by preferential accumulation of the antibody within the tissue as compared to the paracellular route.

Interestingly, when plotted as percentage of applied dose (Figure 4-32), there was no significant difference between the tissue injured for 120 minutes versus 90 minutes and control. This is probably owing to the very high apical dose of 2.0mg/ml versus the very low tissue accumulation in the order of a few micrograms throughout the section of tissue exposed to infliximab.

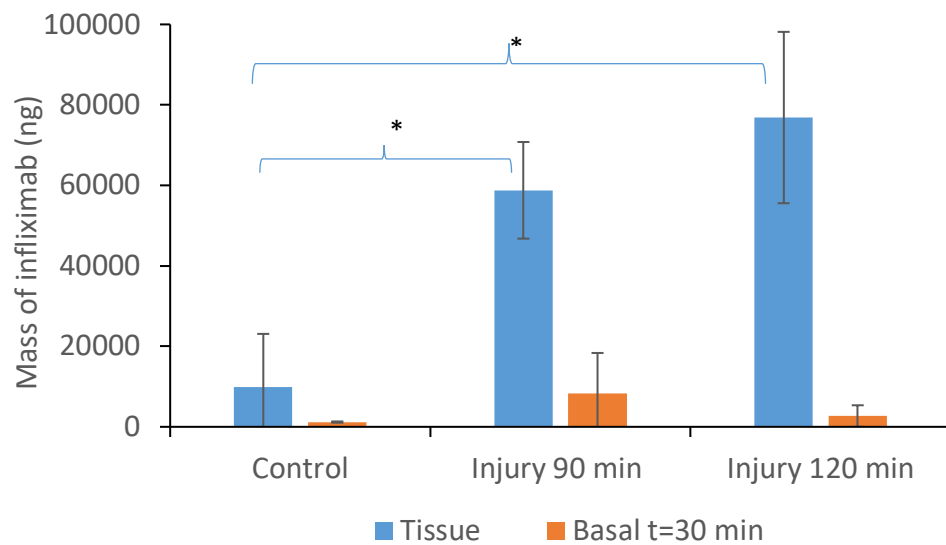


Figure 4-31: Mass of infliximab (Remsima) in tissue (blue bars) and basal side (orange bars) side for 90 and 120 minutes injury exposure at apical conc. 2.0 mg/ml, n=3, mean±SD. * denotes $p < 0.05$ for injury 90 and 120 minutes versus control for tissue accumulation

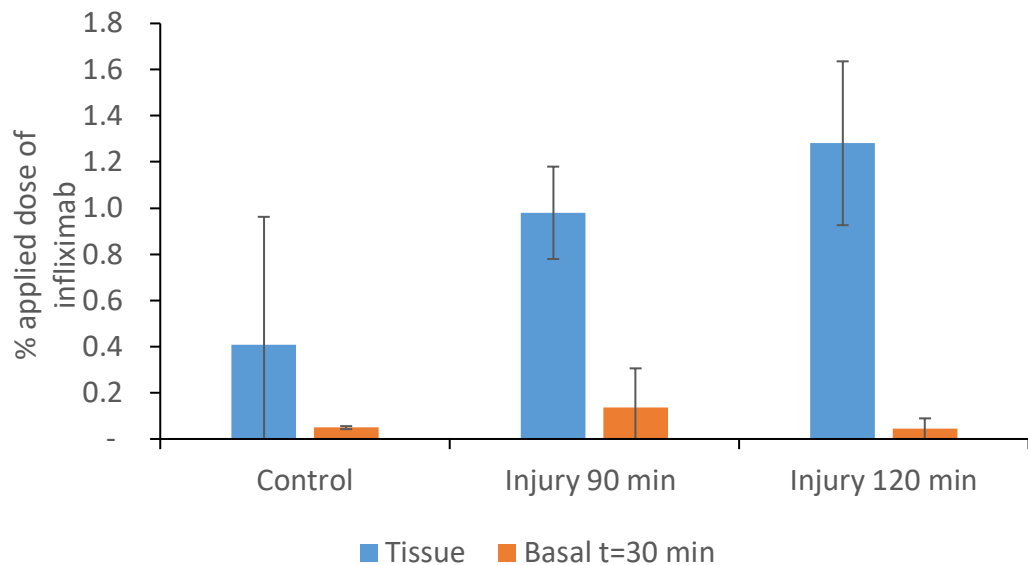


Figure 4-32: Percentage applied dose of infliximab (Remsima) in tissue (blue bars) and basal side (orange bars) for 90 and 120 minutes injury exposure at apical conc. 2.0 mg/ml, n=3, mean±SD. $p > 0.05$ for injury 90 and 120 minutes versus control for tissue accumulation

4.5.5 Conclusion

An injury model based on the principles of gastroesophageal reflux disease was developed with the main aim to mimic the dilated intercellular spaces in EoE. This model was validated against a paracellular marker FD4 whereby it showed an increase in paracellular permeability as a result of injury at 120 minutes especially but not significantly probably owing to the large variability in the results. Tacrolimus showed greater tissue permeation from tissue injured at 90 and 120 minutes whereas infliximab permeated in higher extent only after 120 minutes injury in comparison to control. This indicates that the model in addition to the opening of the paracellular spaces may also allow accumulation of drugs in the tissue probably as a result of increased cellular fluidity or cellular leakage forming voids which act as depots for the drug in tissue. Budesonide showed higher accumulation on the basal side as compared to tacrolimus from tissues injured for 120 minutes; indicating that increased paracellular permeability as a consequence of DIS allows greater flux of budesonide. In EoE, if budesonide is acting locally as claimed, it is probable that the DIS allows systemic exposure and therefore adrenal suppression as a side effect, as reported in studies requiring long term administration. Since we have not seen a significantly greater permeation of budesonide in injured tissues relative to control, it can be hypothesized that DIS in EoE is not offering greater tissue accumulation of budesonide as compared to healthy state. Thus, from these results, it is not clear, whether the improvement in EoE from local application of budesonide is entirely as a result of a local effect. In sum, tacrolimus has shown most promise of achieving greater tissue accumulation.

CHAPTER 5 : DOES BUDESONIDE ACT LOCALLY, SYSTEMICALLY OR BOTH WHEN ORALLY ADMINISTERED FOR THE TREATMENT OF EOSINOPHILIC OESOPHAGITIS?

5.1 Introduction

As observed from the *ex vivo* injury model, budesonide does not significantly accumulate in the oesophageal epithelium in the injured tissue mimicking dilated intercellular spaces (DIS), which is also a feature of EoE. However, there is a slightly greater permeation of budesonide into the basal compartment indicating that it is likely getting into the blood, owing to DIS.

Budesonide effervescent tablet (BET) and budesonide viscous suspension (BVS) have been used for treating EoE with the claim that budesonide is acting topically. BVS has a transit time of less than 2 minutes as observed by gamma scintigraphy with majority of the formulation going to the stomach after a swallow (Dellon et al., 2012). On the other hand, the BET formulation is aimed to increase the salivary levels of budesonide and consequently coat the oesophageal mucosa for local exposure (Miehlke et al., 2016).

However, based on the *ex vivo* permeation results in chapter 4, it is probable that budesonide is not preferentially accumulating more in the inflamed oesophageal mucosa in EoE as compared to intact (healthy) mucosa. The BET formulation in a phase 3 clinical study has shown superior efficacy compared to BVS in a phase 2 study with 95% (Lucendo et al., 2019) and 50% (Dellon et al., 2019) histological response, respectively. Budesonide from BET formulation probably gets absorbed through the highly vascularized tongue and buccal mucosa and into the blood (Hellekant, 1976). Since absorption via the buccal route helps to avoid the first-pass metabolism effect, it may achieve higher blood levels. Jorveza[®] is a budesonide orally dissolving effervescent tablet registered in the EU for the treatment of EoE and the pharmacokinetic data states that the lag time is 0.19 hours (<15 minutes) and T_{max} is 1 hour (Agency, 2017). The C_{max} is 35% higher and bioavailability is higher by 60% between patients suffering from EoE versus healthy (EMC, 2018). This high bioavailability has been attributed to the low CYP enzymes in EoE (Dilger et al., 2013).

An oral lyophilizate tablet (ODT) of selegiline (log P 3.0 and solubility 0.025 mg/ml) with a dose of 1.25mg has shown to achieve plasma levels within less than 10 minutes and is bioequivalent to the 10 mg dose given orally which takes over 30 minutes before it is detected in blood (Drugbank, 2020b). The 8-fold higher bioavailability and >85% reduction in metabolites from the ODT compared to the conventional oral tablet of selegiline has been explained by the fact that the selegiline from the ODT is absorbed pre-gastrically (from the buccal space), a route known to avoid first-pass metabolism (Clarke et al., 2003).

Therefore, budesonide showing greater efficacy from BET may be a combination of achieving higher blood levels owing to avoidance of the first-pass effect and lower CYP 3A4 levels in EoE. Budesonide low efficacy from BVS can be explained by the following. Budesonide has a low systemic availability of 10% owing to the first-pass metabolism accounting for 80-90% and its high tissue to blood affinity prolonging its residence in the GI tract (Edsbäcker and Andersson, 2004). Therefore, in a normal situation, it is unlikely that BVS can allow sufficient accumulation in the blood after reaching the stomach and lower GI tract but since the CYP enzymes in EoE are low, it can be argued that there is greater systemic exposure from budesonide getting swallowed into the stomach. The fact that budesonide is getting absorbed systemically, can also be justified by reports of adrenal insufficiency in patients suffering from EoE especially on long-term treatment lasting >12 weeks (Harel et al., 2015, Golekoh et al., 2016). While the doses given may be low, budesonide may achieve a steady state concentration in plasma when dosed over a prolonged period of about 2 weeks.

However, even if the budesonide achieves plasma levels, it is highly unlikely and therefore questionable that it can accumulate into the oesophageal tissue. This is because the blood perfusion to the oesophagus in man is low when collectively measured for the stomach and oesophagus relative to other regions such as the small intestine. The oesophagus and stomach collectively receives 1% of the cardiac output whereas the small intestine 10% (Valentin, 2002). This in terms of blood flow equates to the 0.26 ml/min/g for the oesophagus and 0.8 ml/min/g for the small intestine. Therefore, based on these facts, it is highly unlikely that although budesonide may achieve systemic levels, it may not be able to accumulate in therapeutic concentrations in the oesophagus, via the systemic route.

The rationale for this *in vivo* study was therefore to use the rat which is a good pre-clinical model to humans for oral absorption of drugs (Hatton et al., 2015). However, in this study, we are not investigating the absorption of budesonide from the lumen of the rat oesophagus and therefore the keratinization of the rat's oesophageal epithelium is not relevant for the purpose of the study. Furthermore, the Sprague Dawley rat also shows similar patterns in gastrointestinal blood perfusion to humans; with the oesophagus (0.6 ± 0.14 ml/min/g) being poorly perfused relative to the small intestine (2.7 ± 0.28 ml/g/min) (Steiner and Mueller, 1961) and same observed for other strains of rats (Stott et al., 1983). The rat and human are similar in terms of proportion of cardiac output to the stomach and small intestine being 1% and 10% respectively (Williams and Leggett, 1989). The aim was therefore to confirm whether budesonide accumulates in the oesophagus of the rat after oral and intravenous administration. These two routes of administration would mimic the absorption of budesonide occurring from the gastrointestinal tract as a result of the dose of BVS reaching the stomach and the intravenous route mimics the buccal or lingual absorption of budesonide with avoidance of the first-pass intestinal and hepatic metabolism.

5.2 Materials and methods

5.2.1 Materials

Male Sprague Dawley rats (Envigo, UK), Lithium heparin coated 200 μ l (Sarstedt), Budesonide (Sigma Aldrich), Cremophor RH40 was a gift from BASF, Germany. Kinetex 2.6 μ m Phenyl hexyl column (Phenomenex), 4.6 x 50mm column, Agilent triple quadrupole LC-MS/MS 6460.

5.2.2 Methods

5.2.2.1 Preparation of calibration curves of budesonide in Sprague Dawley rat oesophagus, stomach, small intestine and colon tissue of rats

An Agilent triple quadrupole LC-MS/MS 6460 was used to determine the mass of the precursor ion for budesonide followed by its product ions and finally a MRM (multiple reaction monitoring) to obtain peak areas of the most abundant product ion. A linear calibration curve for the most abundant product ion was plotted. The chromatographic system included an autosampler, pump, a Kinetex 2.6 μ m Phenyl hexyl column (Phenomenex), 4.6 x 50mm column maintained at a temperature of 40°C. The gradient elution was performed with a mobile phase composed of water (A) and acetonitrile (B). The flow rate was 1.0 ml/min and was programmed as follows: starting at 40% of B and increasing to 95% of B at 3 minutes and decreasing to 40% B between 3 and 6 minutes. Detection of budesonide dissolved in acetonitrile was by MS2 scan using electrospray ionization (ESI) mass spectrometry and the positively charged product ion of budesonide, m/z 431.1 was obtained. The product ion transitions were m/z 431.1 to m/z 413.2, m/z 173.2 and m/z 147.0 for budesonide at collision energy at 10, 26 and 34 eV respectively and fragmentor 95.

Calibration curves were done as follows:

Standard concentrations of 0.0004, 0.002, 0.004, 0.008, 0.020 and 0.040 mg/ml were prepared in LC-MS grade water. For calibration concentrations in acetonitrile only, 2.5 µl of the respective standard stock solution was added to 997.5 µl of LC-MS grade acetonitrile and vortexed for 10s. This resulted in standards of the following concentrations of 1, 5, 10, 20, 50 and 100 ng/ml. respectively.

Tissue calibration curves were prepared by spiking 30 mg of tissue with 2.5 µl of the respective stock solution and 997.5 µl of LC-MS grade acetonitrile was added. The tissue was chopped into small pieces. The vials were sonicated for 15 minutes and then the contents were centrifuged at 10 x g for 15 minutes at 4°C. The supernatant was collected and sample clean-up was done by passing it through an OASIS Prime HLB light cartridge (Waters Corp., USA) and washed with an equal volume of acetonitrile to improve recovery of budesonide. The sample was then analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS).

The recovery and tolerance was calculated as described previously. The recovery of budesonide spiked on rat gastrointestinal tissue (oesophagus, stomach, jejunum and colon) was calculated and was mostly in the range (80-120%) for all concentrations except the lower 1 and 5 ng/ml where these outliers are expected.

The ion ratio of qualifier (m/z 147.0) to the quantifier ion (m/z 413.2) was first calculated. The tolerance was calculated using the formula:

$$\text{Tolerance} = \frac{(\text{Ion ratio of budesonide} - \text{ion ratio of budesonide spiked on tissue})}{\text{ion ratio of budesonide}} \times 100$$

The tolerance for all the concentrations and for all the different segments was determined and was within $\pm 20\%$ range. Therefore, it means that there is no interference from endogenous tissue components for the qualifier and quantifier ion of interest.

5.2.2.2 Preparation of calibration curve in Sprague Dawley rat plasma

Standard concentrations 0.04, 0.2, 0.4, 0.8, 2.0 and 4.0 mg/ml were prepared in acetonitrile (ACN) or water. 5 µl of each was added to 195 µl of male Sprague Dawley rat plasma and vortexed for 10 seconds to give concentrations ranging from 1, 5, 10, 20, 50 and 100 ng/ml. 400 µl of ACN was added and vortexed for 5 seconds. The eppendorfs were then centrifuged at 10 x g for 10 minutes at 4°C to obtain a clear supernatant. The supernatant was analysed by LC-MS/MS.

The recovery and tolerance calculated as described. The recovery of budesonide spiked on rat plasma was calculated and was poor (<30% for all concentrations).

The ion ratio of qualifier (m/z 147.0) to the quantifier ion (m/z 413.2) was first calculated. The tolerance was calculated using the formula:

$$\text{Tolerance} = \frac{(\text{Ion ratio of budesonide} - \text{ion ratio of budesonide spiked on tissue}) \times 100}{\text{ion ratio of budesonide}}$$

While the recovery is poor probably due to ion suppression and binding of drug to the proteins in plasma, it is possible to obtain calibration curves ($r^2 = 0.997$) between the range of interest. However, the tolerance for the ion transition ratios for 5, 20 and 50 ng/ml is <20%, indicating that there are no matrix interferences for the fragment ion m/z 413.2 being quantified.

5.2.2.3 Preparation of budesonide solution for administration

The budesonide solution was prepared by dissolving 5mg budesonide in 0.2g cremophor RH60 and 0.84ml ethanol to give 5mg/ml. This was then diluted in sterile 0.9% normal saline to give a solution of 0.1mg/ml.

5.2.2.4 Dosing of rats for screening study 1, 4 and 24 hours and quantification of budesonide in plasma and gastrointestinal tissues

All the animal work was conducted in accordance with the Home Office standards under the Animals (Scientific Procedures) Act, 1986. The rats were housed at room temperature (25 °C) and in a light-dark cycle of 12 h.

a) Male Sprague Dawley rats (Envigo, UK) weighing between 225-250 grams were fasted overnight for 12 hours (maximum 5 per cage on a wire grid without bedding and separated from the faeces to prevent coprophagy) and then administered a solution of 0.1mg/ml budesonide at a dose of 1mg/kg by oral gavage and 0.33 mg/kg intravenously via the tail vein. The intravenous dose was decided based on a study by Raje and co-workers (Raje et al., 2018) where they used one-third of the oral dose. The oral dose of 1mg/kg was calculated using the conversion of the human oral dose to rat dose. The dose extrapolation from human to rat was based on species body surface area (BSA). In order to extrapolate the animal dose from a human equivalent dose (HED), Reagan Shaw et al. explained that BSA would enable the most appropriate conversion as it provides acceptable correlations across different biological parameters, including “oxygen utilization, caloric expenditure, basal metabolism, blood volume, circulating plasma proteins, and renal function” (Reagan-Shaw et al., 2008). The following equation was applied for the conversion:

$$HED \left(\frac{mg}{kg} \right) = AD \left(\frac{mg}{kg} \right) * \frac{Animal\ Km}{Human\ Km}$$

where HED is the human equivalent dose, AD the animal dose, Km is the BSA conversion factor which is 37 for humans and 6 for rats. The HED for budesonide was 0.15 mg/kg/day taken orally and calculated by dividing a 9 mg dose by the weight of a 60 kg adult.

Control rats were intravenously administered with the vehicle (cremophor RH60 and ethanol) diluted 50 times in 0.9% sterile normal saline. Food was resumed 1 hour after either administration.

The blood was sampled from the tail vein in lithium heparin coated microtainer tubes and centrifuged at 10 x g for 2 minutes @ 16°C to obtain the plasma. The rats (n=1) were culled by carbon dioxide asphyxiation at 1 hour, 4 hour and 24 hours and the tissues immediately extracted, rinsed in distilled water and stored on dry ice to prevent degradation of the drug.

Budesonide was quantified in the plasma by precipitating the plasma with 2x volume of acetonitrile (ACN) and centrifuged at 10 x g for 10 minutes @ 4°C. The supernatant was analysed by LC-MS/MS.

The budesonide in the tissue was quantified by weighing oesophagus, jejunum or ascending colon tissue between 80-120 mg and then adding acetonitrile at a ratio of 30 mg tissue to 1 ml ACN. The tissues were finely chopped and sonicated for 10 minutes in glass vials. The water in the sonication bath was kept cold. The contents of each sample were centrifuged at 10 x g for 10 minutes at 4°C and 0.5 ml of supernatant was passed through the OASIS Prime HLB cartridge (Waters Inc.). An equal volume of ACN was passed through to improve efficiency of extraction. This was then analysed by LC-MS/MS and quantified against the calibration curves of budesonide in plasma and rat gastrointestinal tissues (oesophagus, jejunum and ascending colon, $r^2 > 0.98$ for all).

5.2.2.5 Intravenous dosing in rats and gastrointestinal tissue harvesting at 30 minutes post administration of budesonide and quantification in tissues by LC-MS/MS

To simulate immediate absorption from the buccal space, budesonide 1mg/kg (same as oral dose) was injected via the tail vein of overnight fasted 280 - 300 grams male Sprague Dawley rats. Blood was taken via the tail vein and the animals were culled 30 minutes after injection. The gastrointestinal regions, namely oesophagus, stomach, jejunum and colon were harvested and washed externally and lumenally with fixed volume of distilled water. Budesonide was quantified in the tissues by LC-MS/MS.

5.2.2.6 Administration by oral gavage of budesonide with and without ketoconazole to rats followed by tissue harvesting at 2.5 hours. Quantification in tissues by LC-MS/MS

An experiment was conducted whereby, in one arm budesonide (1 mg/kg) and in the other arm ketoconazole (1.65 mg/kg) and budesonide (1mg/kg) was dosed orally by gavage to 200g (8-10 week old) male Sprague Dawley rats (n=3 in each arm). Ketoconazole dose was derived from Seidegard and co-workers study where there was increased budesonide bioavailability from CYP inhibition when using increasing doses of ketoconazole (Seidegård et al., 2012). Blood samples were withdrawn at 135 minutes and animals were culled at 150 minutes and gastrointestinal regions (oesophagus, jejunum and colon) harvested and stored in dry ice. Tissue harvesting at 150 minutes was done based on the fact that budesonide solution would have a gastric emptying time of 30 minutes and the lag time to T_{max} in plasma is 90 minutes totalling 120 minutes with an additional 30 minutes given for distribution into the gastrointestinal tissues. The tissues were then cleaned by washing externally and lumenally in fixed volume of distilled water. About 100 mg of the tissues were homogenized in extraction solvent to extract budesonide. Budesonide levels were quantified by LC-MS/MS.

5.3 Statistical Analysis

The results generated in the study were expressed as mean $\pm$ SD ($n = 6$). The data was analysed by a one-way ANOVA in each segment, followed by a Tukey post-hoc analysis with a 95% confidence interval using IBM SPSS Statistics 16 (SPSS Inc., Illinois, USA).

5.4 Results and discussion

5.4.1 Oral and intravenous dosing of rats for screening study 1, 4 and 24 hours

In the preliminary study to determine the accumulation of budesonide in gastrointestinal tissues from oral and intravenous dosing, tissue levels of 0.04 ng/mg budesonide were achieved in the oesophagus of the rat dosed orally and culled after 1 hour and 0.02 ng/mg in the jejunum of the rat dosed intravenously and culled at 1 hour (Table 5-1). No budesonide was observed in rat gastrointestinal tissues at other time points. The amount measured in the oesophagus at 1 hour from the rat dosed orally, was probably due to the stomach contents refluxing (when removing tissues) as gastric emptying of the dosed liquid did not seem to have taken place. The amount, 0.02 ng/mg, observed in the jejunum and not the oesophagus from the rat dosed intravenously at 1 hour gives more assurance to the fact that the jejunum is more perfused relative to the oesophagus and therefore achieved levels of budesonide.

In the rat dosed intravenously, plasma concentration was 72.4 ng/ml at 1 hour and 0.25 ng/ml at 4 hour. A similar reduction in plasma levels of budesonide over a 4 hour period, in male Sprague Dawley rats, has been observed in the study by Raje and coworkers (Raje et al., 2018).

Table 5-1: Tissue concentration of budesonide achieved following 1, 4- and 24-hour administration of budesonide 1mg/kg by oral gavage and 0.33 mg/kg intravenously

Accumulation budesonide (ng/mg)							
Rat #	Time (h)	Oral			Intravenous		
		Oesophagus	Jejunum	Colon	Oesophagus	Jejunum	Colon
1	1	0.04	0	0			
2	4	0	0	0			
3	24	0	0	0			
4	1				0	0.02	0
5	4				0	0	0
6	24				0	0	0
7 (control)	24				0	0	0

5.4.2 Intravenous dosing in rats and gastrointestinal tissue harvesting at 30 minutes post administration of budesonide and quantification in tissues by LC-MS/MS

In a study where mice were injected intravenously with radioactive ^3H budesonide (Andersson P Fau - Appelgren et al., 1986), budesonide radioactivity reduced significantly in the stomach in the first 30 minutes. In another study determining budesonide distribution in mice (Mårs et al., 2012) it was observed that radioactive ^3H -budesonide injected subcutaneously distributed to the gastric mucosa (generalized and not specified for each gastrointestinal region). The accumulation in the gastric mucosa was dependent on the number of doses with 14 daily doses registering higher concentrations as compared to 3 daily doses or single dose. There was a drop-in radioactivity with time. ^3H labelled budesonide given intravenously to the rat had a plasma half life ($t_{1/2}$) of 4.7 hours and about 7 hours in the trachea. However, this has been attributed to the ability of budesonide to form budesonide esters thereby prolonging its residence in the tracheal tissue (Jendbro et al., 2001). Budesonide also has a high mucosal affinity as evidenced by its high mucosal to systemic ratio (Chanoine et al., 1991, Miller-Larsson et al., 2002). However, in the preliminary study, no budesonide was detected in any other tissue except the jejunum at 1 hour. That could be as a result of it getting metabolized within less than an hour in other regions of the GI tract or not accumulating at all owing to low blood perfusion.

Despite the oesophagus being a poorly perfused organ, there are reports of drugs accumulating in the oesophagus. 5- aminolevulinic acid (5-ALA) is a photosensitizer with a log P of -1.5 and molecular weight 131 g/mol. It has shown to accumulate in the oesophagus of humans with fluorescence being measured in oesophageal tumour and normal mucosa up to 6 hours after a bolus or 6 divided oral doses (Regula et al., 1995). Furthermore, a moderately lipophilic compound N-methyl-N-nitroso benzylamine is used to induce oesophageal carcinoma in rats. Rats intravenously injected with labelled N-methyl[U- ^3H]-N-nitroso benzylamine were killed 10 minutes, 1 hour and 24 hours after administration and it was observed that the radioactivity was detectable in the oesophagus after 10 minutes, 1 hour and 24 hours with no differences between the time points (Iizuka T Fau - Ichmura et al., 1978).

Budesonide effervescent tablet, Jorveza® and selegiline ODT (Zelapar®) have shown to achieve blood levels within a lag time of 10-15 minutes and T_{max} in about 1 hour. As observed for selegiline, the ODT dose is 8-fold lower than the oral immediate release tablet with over 85% reduction in plasma metabolites. This therefore means that selegiline gets absorbed pre-gastrically/buccally from the ODT. Due to similarity in pharmacokinetics between the budesonide effervescent ODT and Zelapar, it was hypothesized that budesonide gets absorbed pre-gastrically from the ODT formulation and therefore the high efficacy observed from BET is owing to a systemic effect with the advantage of avoiding first-pass metabolism. Budesonide would therefore reach the blood and then be distributed into the oesophagus.

Therefore, going forward, the rationale for the next experiments was to dose the rats intravenously to mimic the buccal absorption of budesonide and orally by gavage and quantify the levels of budesonide in the gastrointestinal tissues (oesophagus, stomach, jejunum and colon) so as to determine whether budesonide can actually accumulate in the oesophagus as a result of achieving plasma levels.

On the basis of the results in Figure 5-1 below, it can be concluded that budesonide accumulates in all regions of the gastrointestinal tract with no real correlation with blood perfusion to the regions. The small intestine of rat receives ca. 10% as a proportion of the cardiac output whereas the stomach receives 1% (Steiner and Mueller, 1961, Williams and Leggett, 1989). In terms of blood flow rate, the oesophagus of a Sprague Dawley rat receives 0.6 ± 0.14 ml/min/g whereas the jejunum receives 0.9 ± 0.4 ml/min/g (Steiner and Mueller, 1961). Therefore, it is expected that the jejunal levels will be higher than that of the oesophagus. In this case the levels of budesonide in the oesophagus were 0.08 ± 0.02 ng/mg and those in the jejunum were 0.12 ± 0.01 ng/mg but $p > 0.05$ therefore was no difference. No difference in accumulation is probably owing to the fact that budesonide gets metabolised faster in the jejunum owing to higher CYP levels or gets effluxed into the lumen owing to the higher P-glycoprotein levels as compared to the oesophagus. Significant differences were however observed between stomach (0.17 ± 0.01 ng/mg) and oesophagus (0.08

± 0.02 ng/mg). This shows that the oesophagus accumulates similar amount of budesonide as compared to the jejunum irrespective of the amount/rate of perfusion.

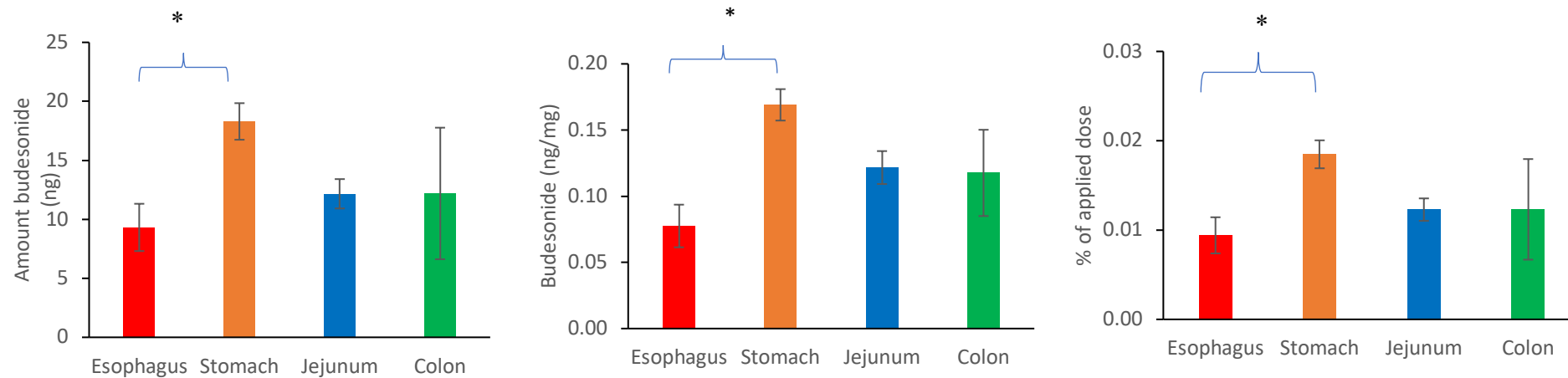


Figure 5-1: Distribution of budesonide in rat gastrointestinal tissues (oesophagus, stomach, jejunum and colon) 30 minutes after a 1mg/kg intravenous dose, n=3, mean±SD, * denotes $p < 0.05$ by One-way ANOVA followed by Tukey's Post Hoc analysis

5.4.3 Administration by oral gavage of budesonide with and without ketoconazole to rats followed by tissue harvesting at 2.5 hours. Quantification in tissues by LC-MS/MS

After culling the rats, the dissection was carefully conducted so as to ensure that there is no rupture of blood vessels and therefore no contamination of tissues (oesophagus, stomach, jejunum and colon) by blood. Moreover, the tissues after dissection, were washed using distilled water, before being stored at -80°C for quantification of budesonide. This careful step gives assurance that budesonide can actually reach and accumulate in the oesophagus. The next step was to understand whether it can achieve higher plasma levels after oral administration with a CYP 3A4 inhibitor (ketoconazole). No budesonide was detected in the plasma nor rat gastrointestinal tissues (jejunum and colon) after oral gavage (ref Table 5-1). It was assumed that budesonide may have undergone intestinal first pass metabolism and therefore did not achieve plasma levels for it to accumulate in the oesophagus. Higher bioavailability of budesonide has been reported in EoE owing to reduced CYP enzymes. Ketoconazole is a CYP3A4 inhibitor and therefore has been co-administered orally with budesonide to inhibit mucosal and hepatic CYP enzymes so as to simulate the reduced CYP enzymes in EoE. This is to understand if the budesonide taken via the viscous formulation or ODT will achieve sufficient plasma levels so as to be able to accumulate in the oesophagus of EoE patients. This would add to the view that budesonide via the formulations taken orally perhaps does not entirely act locally as it can accumulate in the oesophagus after systemic exposure.

To evaluate this concept, rats were orally gavaged a budesonide solution whereby 3 rats were dosed only budesonide and 3 rats were dosed budesonide with a CYP 3A4 inhibitor, ketoconazole. In a study by Seidegard et al., the aim was to evaluate increased budesonide bioavailability (BA) from CYP inhibition using increasing doses of ketoconazole. Baseline budesonide BA was 27% (12–42%) and increased to 45% on concurrent administration of 16 mg of ketoconazole (Seidegård et al., 2012). In EoE, there is increased bioavailability of budesonide and this has been attributed to lower CYP 3A4 activity in the disease state (Dilger et al., 2013), consequently affecting metabolism of budesonide, increasing bioavailability.

The considerations for harvesting the gastrointestinal organs at 2.5 hours was on the following basis; gastric emptying time in rats is about 80% at 30 minutes in 3-4 month old rats (Smits and Lefebvre, 1996) and the C_{max} of budesonide has been documented to be 1 hour in some cases with a range of 60 and 90 minutes (Raje et al., 2018, Chanoine et al., 1991). Therefore, it was determined that gastric emptying time of 30 minutes plus time taken to reach C_{max} 90 minutes and accumulate in the oesophagus by 30 minutes would deem appropriate to harvest the organs at 150 minutes.

The results as per Figure 5-2, below were quite variable but also surprising. About 0.02 ng/mg of budesonide was detected in the oesophagus of one rat given only budesonide but no budesonide was detected in the oesophagus given budesonide + ketoconazole. It has been observed that the blood levels of budesonide are higher in EoE subjects and this has been attributed to reduced CYP levels. Jejunal levels of budesonide in both cases was similar at a mean of 0.01 ± 0.01 ng/mg which indicates that the budesonide had reached the jejunum and was getting absorbed and also accumulated owing to its high tissue binding affinity to blood ratio (Miller-Larsson et al., 2002).

The fact that we could not detect blood levels nor oesophageal levels successfully at 150 minutes shows that the gastric emptying and absorption has either taken place earlier than predicted and hence accumulation and metabolism too in the oesophageal tissue.

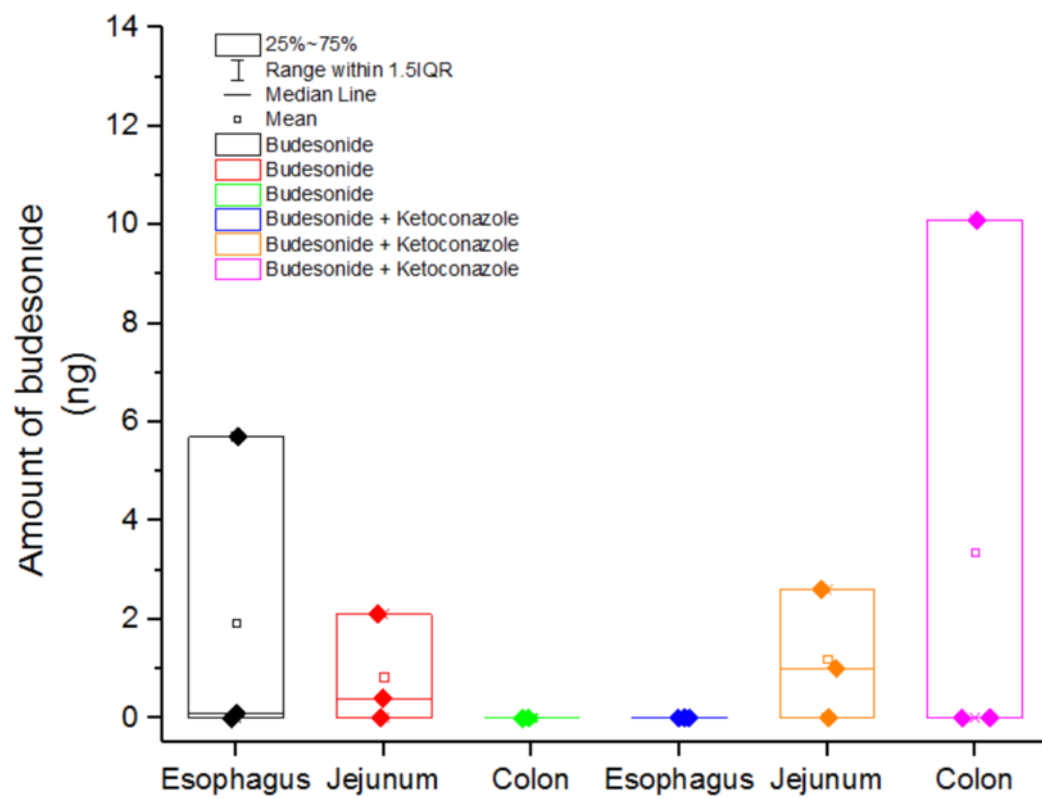


Figure 5-2: Box plot showing accumulation of budesonide in rat oesophageal, jejunal and colonic tissue at 2.5 hours on administration by oral gavage with and without ketoconazole, n=3 each arm.

5.5 Conclusion

Based on the above results from intravenous studies in rats, it is clear that budesonide distributes and accumulates in the poorly perfused oesophagus with not much difference compared to the other highly perfused organs such as the small intestine. Therefore, the work from these experiments when correlated with the pharmacokinetics of the budesonide orally dissolving tablet, Jorveza[®], indicate that if blood budesonide levels are reached in EoE patients, the ODT must be acting majorly via a systemic effect and partially locally owing to short transit time of swallowed saliva through the oesophagus (Cassiani et al., 2015). Same explanation applies for budesonide viscous suspension.

Drugs that get absorbed buccally have a greater bioavailability at lower doses compared to those taken orally especially if they are substrates of mucosal and hepatic CYP enzymes. This supports our assumption that the budesonide tablet (BET) is affording blood levels of budesonide perhaps higher than conventional oral formulations and therefore acting via a systemic effect.

CHAPTER 6 : THESIS CONCLUSIONS

The aim of this work was to understand the absorption of small molecules <1000Da (budesonide and tacrolimus) and infliximab (a large 150kDa protein) into the porcine oesophageal mucosa (used as a substitute to human oesophageal mucosa) with the aim to deliver these molecules for local treatment of eosinophilic esophagitis. Additionally, based on clinical trial results for various formulations of budesonide claimed to act locally, we set out to investigate whether this effect was entirely local or a combination of local and systemic owing to extremely short transit time of viscous liquids through the oesophagus. The knowledge generated would be useful in a rational design of effective formulations for the treatment of EoE.

The permeation of budesonide and tacrolimus in ex vivo Ussing chamber diffusion models using intact porcine oesophageal tissue with respect to time (90 seconds, 10 and 30 minutes) was investigated and was measurable at 90 seconds exposure. Interestingly, no statistically significant increase was observed over time. Additionally, no sex differences in terms of drug accumulation in the oesophagus was observed. s. Therefore, while the drug may be adhering or permeating into the tissue after such short exposure, a large proportion may also get washed away by flow of saliva and therefore get swallowed.

Monoclonal antibodies are very large molecules ~180 fold larger than tacrolimus and ~350 fold greater than budesonide. They are normally given by injection and have not shown to be very efficacious in most instances for treatment of EoE. Whether that is owing to poor distribution into the oesophagus as is observed for other tissues or not targeting the key cytokine is yet in question. Therefore, the potential for local delivery to the oesophagus was evaluated by means of permeation studies. Surprisingly, Infliximab is able to permeate the depth of the oesophageal mucosa in some extent. However, no difference in permeation as a result of apical pH 6.0 or 7.4 was observed quantitatively by ELISA. However, confocal microscopy results indicate that infliximab (Remicade) permeate across the mucosa and submucosa when exposed to apical pH 6.0. However, since no FcRn is detected, the permeation cannot be really linked to any FcRn mediated uptake but rather concentration dependent.

The hallmark feature of EoE is the inflammation and dilated intercellular spaces (DIS). Inflammation in the buccal epithelium in cGVHD patients has increased the bioavailability of a budesonide mouthwash. The fact that budesonide formulations show improvement in EoE, may be owing to the leaky epithelium as a result of DIS in EoE and therefore allowing greater accumulation of budesonide. An ex-vivo injury model simulating these DIS was developed using the principles of gastroesophageal reflux disease (GERD). Tacrolimus and infliximab showed greater accumulation in the tissues exposed to gastroduodenorefluxate elements whereas surprisingly budesonide did not. This indicates that DIS in EoE does not offer greater accumulation of budesonide and therefore the differences in efficacy from the currently used formulations, the budesonide effervescent tablet (BET) and the viscous suspension (BVS) may be arising from majorly a systemic effect combined with a local effect with huge variability from person to person.

Reports state that swallowed budesonide from prolonged use (up to 6 months or more) meant for local action in the oesophagus to treat EoE, has led to the development of adrenal suppression (Golekoh et al., 2016, Harel et al., 2015). Furthermore, budesonide pharmacokinetics from Jorveza[®], the ODT formulation of budesonide, have compelling pharmacokinetic profiles with lag times of absorption in 15 minutes and T_{max} of about 1 hour indicating pre-gastric (buccal) or absorption post swallow (EMC, 2018). However, while systemic absorption of budesonide is observed, the poor perfusion of blood to the oesophagus challenges the fact that budesonide could accumulate in the oesophagus. Rats and humans have similarity in the proportion of cardiac output between the poorly perfused stomach and oesophagus and highly perfused small intestine. A study in rats whereby budesonide was dosed intravenously to simulate buccal absorption and avoidance of the first-pass effect showed that budesonide accumulated in the oesophagus in almost similar amounts to the jejunum. However, no accumulation of budesonide at the dose tried by oral gavage was observed therefore further explaining the lower efficacy in terms of histological response for the viscous suspension versus the budesonide effervescent tablet.

In summary, the work in this thesis has contributed to the understanding of the tissue accumulation of budesonide, tacrolimus and infliximab in a porcine ex vivo model.

Budesonide does not increasingly accumulate as a result of prolonged contact with the injured tissue nor does the disease morphology offer significantly greater opportunity for it to accumulate in the oesophageal mucosa. These observations support the argument that it could be acting both locally and systemically. On the other hand, tacrolimus and infliximab showed significantly greater accumulation in the injured porcine oesophageal tissue as compared to control and therefore are better candidates to target for local delivery to the oesophagus in EoE.

CHAPTER 7 : FUTURE WORK

i) Permeation results in healthy human oesophageal tissues to compare against the pig. Furthermore, it is important to confirm the permeation results of the ex vivo injury model against tissues from human subjects suffering from eosinophilic oesophagitis. This can be done using the Ussing chamber diffusion system.

ii) A study by Collaud and co-workers has shown that polymer solutions of chitosan at concentrations of 1.5 and 1.7 %w/v adhere to the human oesophagus for 10 minutes when observed under an endoscope (Collaud et al., 2007). However, this is not a sufficient period of time as it may take sometime for the drugs to release from the polymer matrix and subsequently permeate. There was a good correlation between residence time of chitosan on rat tissues when these polymer solutions were applied ex-vivo. Therefore, rat can be used a pre-clinical model for a start to evaluate the length of time polymers such as Gantrez MS-955, chitosan 80/500 and 80/1000, sodium alginate, pluronic F127 (poloxamer 407) and sodium carboxy methyl cellulose can adhere in the oesophagus. This can be done by either using imaging techniques such as PET/CT using a tracer such as ^{18}F -FDG (Fluorodeoxyglucose (^{18}F)). This has previously been used to determine the residence time of ophthalmic formulations on the rat eye (Luaces-Rodríguez et al., 2018). If the drug can be labelled with the PET tracer, then one can also determine its absorption in the tissue and distribution across the body following oral administration.

iii) Mouse models of EoE have been developed; intranasal challenge using *Aspergillus fumigatus* extract and intra tracheal administration of interleukin 13 (IL-13) so far most resembles the pathophysiology of EoE in humans (Mishra, 2013). Seoane-Viano I et al., have investigated the progression of inflammation in TNBS rat model of colitis using [^{18}F] FDG PET/CT. The rats that were not given methylprednisolone showed greater uptake of the tracer whereas those given methylprednisolone after the TNBS, showed reduction in the uptake of the tracer (Seoane-Viaño et al., 2018). This shows that [^{18}F] FDG tracer can be used to track the progression or amelioration of inflammation in the gastrointestinal mucosa. If the polymers as explained in (ii) can adhere for a prolonged period in the mouse oesophagus as well, the budesonide/tacrolimus/monoclonal antibody can be incorporated in the polymer

solution and uptake of the tracer compared between the drug loaded formulation and control (no drug in polymer). Furthermore, inflammation targeting hydrogels (ITH) like ascorbyl palmitate (Zhang et al., 2015) shown to adhere to the inflamed colon in mouse models of colitis can also be investigated to understand whether they adhere for a prolonged period of time to the inflamed oesophagus in mouse models of EoE. PET/CT can be used to assess their residence time in the oesophagus.

iv) Ex vivo permeation results for tacrolimus and infliximab show a greater tendency to accumulate in the oesophagus when mimicking the histopathology of EoE i.e dilated intercellular spaces. It has been observed that lipophilic drugs $\log P > 3$ have a tendency to accumulate in the buccal tissue for longer periods (Itin et al., 2020). Therefore, if polymer solutions are not able to reside in the oesophagus for a prolonged period, then a different approach can be tried whereby the oesophagus is continuously bathed with a device containing a propellant driven pump. This has been inspired by a technology developed by Synagile (Figure 7-1) to deliver levodopa/carbidopa so as to prevent patients from having drug free periods in the blood and hence control symptoms of parkinson's disease.

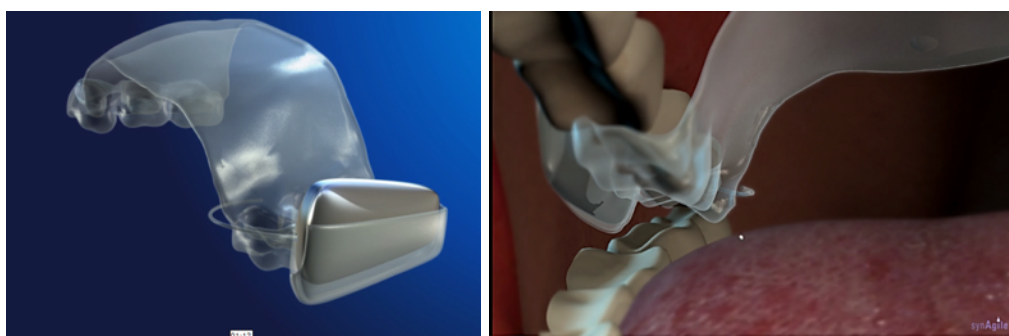


Figure 7-1: ORAFUSE™ – used to deliver carbidopa/levodopa to parkinsons disease patients (Synagile, 2017)

The ORAFUSE technology principle can be used to deliver tacrolimus or another monoclonal antibody intended to act locally on the oesophagus of EoE patients

especially while they are asleep. Studies in healthy persons suggest that during sleep there are longer swallow free periods (Lichter I Fau - Muir and Muir, 1975). This could be exploited in that it would prolong the residence time in the oesophagus and therefore offer greater chances of drug accumulation in the oesophagus. This slow trickling of the drug solution in very low volumes may allow greater contact time with the oesophagus and therefore drug accumulation for a local effect to prevent absorption.

Nanosized (200nm) model fluorescent particles when coated with 2% pluronic F127 showed more uniform colonic distribution in the mucosa of mice with TNBS colitis and appeared to penetrate the colonic tissue. Whereas 2µm particles coated in PVP were largely present in the colorectal lumen. This reflected in the in vivo study results whereby budesonide nanomilled and coated with 2% pluronic F127 showed significant reduction in macroscopic and microscopic symptoms and number of viable inflammatory macrophages as compared to the micronized budesonide (2µm) coated with 1% PVP (Date et al., 2018). This nanosized approach can be used to deliver tacrolimus using ORAFUSE as it would promote greater mucosal adherence and surface area coverage improving the chances of permeation and hence local delivery to the oesophagus.

v) Other, large hydrophilic molecules, such as nucleic acid based therapeutics have been tried in mouse models of EoE. RNA TRAIL (TNF related apoptosis inducing ligand) is released by structural airway cells in response to allergen stimulation resulting in upregulation of the E3 ubiquitin ligase Midline-1 (MID1) leading to increased NF-κB activation. A small interfering (si)RNA which inhibits MID1 has been tried intranasally in an *Aspergillus fumigatus* mouse model of EoE. MID1 silencing employing siRNA reduced oesophageal eosinophil and mast cell numbers (Collison et al., 2015). siRNA are large hydrophilic molecules which may be posed with similar uptake challenges as mAbs when administered locally. Therefore, the permeation of siRNA through the injured oesophagus should be compared to that in the intact oesophagus.

vi) Recently, there has been a publication on a targeted delivery system to the oesophagus, EsoCap (Krause et al., 2020). This has not been discussed previously in

the thesis as I came across it when about to submit. It consists of a drug containing mucoadhesive polymeric film housed in a weighted size 00 capsule. The film rolls out into the oesophagus when the capsule placed in an applicator is ingested with water. The visible contact length as per MRI (magnetic resonance imaging) images, ranges between 5 and 22 cm with the polymer film visible for more than 15 minutes in 69% of all administrations.

Potent drugs like tacrolimus and monoclonal antibodies have shown significantly greater permeation especially in oesophageal epithelium with dilation of intercellular spaces. This technology could be investigated for the delivery of these therapeutics to the oesophagus for the local treatment of EoE.

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