

THE ACTIONS OF CANNABIDIOL AND PALMITOYLETHANOLAMIDE ON INFLAMMATION AND PERMEABILITY OF THE GUT

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Statement of Authenticity

I declare that this thesis has been completed by myself under the supervision of Dr Saoirse O'Sullivan (PhD), Associate Professor, and Mr Jonathan Lund (PhD), Associate Professor, from the School of Graduate Entry Medicine, University of Nottingham, Royal Derby Hospital. This thesis is a record of work that has not been submitted previously for a higher degree.

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Pharmacology. London, December 2016.

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Abstract

In health, the gut provides a barrier between the external and internal environment. This selectively permeable barrier allows absorption of nutrients and water from the gastrointestinal contents, whilst preventing the transfer of noxious material such as bacteria. During episodes of inflammation, this barrier becomes compromised, allowing transfer of noxious material into the systemic circulation, leading to disease states such as inflammatory bowel disease and septic shock. There are no clinically available compounds to combat this increase in permeability directly.

The endocannabinoid system is a group of endogenous lipid signalling molecules which activate membrane-bound receptors. Plant-derived and synthetic compounds also act at these receptors, generating a wide variety of secondary effects. The aims of this study were to identify compounds with action on the endocannabinoid system which could be used clinically to treat inflammation and hyperpermeability of the gastrointestinal tract, exploring mechanisms of action.

Systematic review and meta-analysis of existing literature revealed 51 preclinical studies, and 2 clinical studies examining the effect of cannabinoid compounds. In preclinical studies, cannabinoid drugs reduced myeloperoxidase activity in the gastrointestinal mucosa within mouse and rodent models of colitis (standard mean difference -1.26, 95% confidence interval (CI)-1.54 to -0.97, I^2=48.1%) and macroscopic disease activity scores (standard mean difference -1.36, 95% CI -1.62 to -1.09, I^2=61%). Clinical trials found no overall benefit of cannabinoid drugs in Crohn's disease (mean difference -74.97, 95% CI –229 to 0.79, I^2=75%). Two compounds, cannabidiol and palmitoylethanolamide, possessing positive outcomes and preferable side effect profiles, were put forward for further study to examine potential clinical benefit.

The mechanism of action of palmitoylethanolamide and cannabidiol were explored further by examination of their effects on the immune response, permeability of cultured cell monolayers, intracellular signalling pathways, expression of membrane-bound proteins governing permeability and receptors of the cannabinoid system. We found that these agents were anti-inflammatory in both cultured Caco-2 cells and explant human colonic tissue, prevented increases in permeability secondary to inflammation, and were likely to act through adenylyl cyclase, protein kinase A and extracellular signal-regulated kinases. The downstream effects of these compounds prevented down-regulation of the TRPV1 receptor, upregulation of aquaporin 3 expression, and prevention of downregulation of claudin-3.

The effects of palmitoylethanolamide and cannabidiol were then examined on permeability in the human colon *in vivo* by means of a double blinded, randomised controlled trial. This study demonstrated that aspirin increased the permeability of the human gut, determined by increases in urinary concentrations of lactulose and D-mannitol, quantified by mass spectrometry. Groups receiving oral cannabidiol or palmitoylethanolamide demonstrated lower urinary concentrations of lactulose and D-mannitol, suggesting that these two drugs could be used clinically to prevent disease-induced hyperpermeability.

In conclusion, cannabidiol and palmitoylethanolamide have shown consistent anti-inflammatory actions in colonic *ex vivo* and *in vitro* models, and also prevented increases in intestinal permeability *in vitro* and also *in vivo* in a randomised, double blind, placebo-controlled trial. Their clinical use in IBD should now be assessed in phase II clinical trials.

Table of Contents

Table of Figures 14

List of Tables 19

Abbreviations 20

Glossary of cannabinoid compounds 24

1 Introduction 27

1.1 Colonic inflammation 27

1.1.1 Epidemiology 27

1.1.2 Autoimmune inflammatory bowel disease 28

1.1.3 The gut barrier in autoimmune colitides 28

1.1.4 Current clinical therapies to prevent pathologically increased mucosal permeability 40

1.2 The endocannabinoid system (ECS) 41

1.2.1 Receptors of the ECS 41

1.2.2 Ligands of the ECS 44

1.2.3 Phytocannabinoid ligands 51

1.2.4 Cannabinoids as potential therapies for IBD 54

1.3 Project aims 57

2 The use of cannabinoids in colitis: a systematic review and meta-analysis 58

2.1 Introduction 58

2.2 Methods 59

2.2.1 Search strategy 59

2.2.2 Data acquisition 59

2.2.3 Quality and risk of bias 59

2.2.4 Data analysis 60

2.3 Results 61

2.3.1 Search results 61

Figure 2.1 PRISMA flow chart of the record identification process. 61

2.3.2 Study characteristics 62

2.3.3 Meta-analyses....69
2.4 Discussion....83
2.4.1 Preclinical studies....83
2.4.2 Clinical studies....84
2.4.3 Suggested compounds for further study....85
2.4.4 Secondary endpoints....85
2.4.5 Limiting factors....87
2.4.6 Summary....87
3 Investigating the effect of CBD and PEA on the inflammatory response of the human colon....88
3.1 Introduction....88
3.1.1 PEA and CBD....88
3.1.2 Study aims....89
3.2 Methods....90
3.2.1 Ethical considerations....90
3.2.2 Caco-2 cell culture....90
3.2.3 Caco-2 experimentation....90
3.2.4 Human colon culture....90
3.2.5 Human colon experimentation....91
3.2.6 Intracellular signalling pathways....91
3.2.7 Cytokine production....92
3.2.8 Target sites of action of PEA & CBD....92
3.2.9 Statistical analysis....92
3.3 Results....93
3.3.1 Caco-2 intracellular response to inflammation....93
3.3.2 Human colon intracellular response to inflammation....95
3.3.3 Effects of PEA and CBD on cytokine production in response to inflammation....97
3.3.4 Antagonist studies....103
3.3.5 Effects of PEA and CBD on cytokine production in response to inflammation in IBD and appendicitis explants....106

3.4 Discussion 111
3.4.1 Caco-2 cell cultures 111
3.4.2 Explant colonic Tissue 112
3.4.3 PEA and CBD on permeability 113
3.4.4 Receptor sites 115
3.4.5 IBD and appendicitis tissue 116
3.4.6 Limitations 117
3.4.7 Summary and reviewed hypotheses 117
4 Investigating the effects of PEA and CBD on colonic permeability 118
4.1 Introduction 118
4.2 Methods 119
4.2.1 Caco-2 cell culture 119
4.2.2 Human colonic culture 120
4.2.3 Transport studies 120
4.2.4 Tight junction protein and receptor quantification 121
4.2.5 Aquaporin quantification 121
4.2.6 Statistical analysis 121
4.3 Results 123
4.3.1 Caco-2 cell culture 123
4.3.2 Human colonic tissue 133
4.4 Discussion 136
5 The Sweet PEA study – a randomised, double-blind controlled study investigating the effects of CBD and PEA on the permeability of the human gastrointestinal tract 141
5.1 Introduction 141
5.2 Methods 144
5.2.1 Participant recruitment 144
5.2.2 Sample size 144
5.2.3 Materials 144
5.2.4 Randomisation 145
5.2.5 Experimental Protocol 145

5.2.6 Determination of urinary concentrations of lactulose and D-mannitol 145
5.2.7 Statistical analysis 149
5.3 Results 150
5.4 Discussion 154
6 Discussion and future directions 157
7 References 163
8 Appendices 206
8.1 Appendix 1 – PRISMA statement 206
8.2 Appendix 2 - NHS Ethics 208
8.2.1 NHS participant consent form 208
8.2.2 NHS participant information sheet 209
8.2.3 NHS ethics approval 212
8.3 Appendix 3 – University of Nottingham permeability study ethics 213
8.3.1 Participant consent form 213
8.3.2 Participant information sheet 215
8.3.3 University of Nottingham Ethical approval 220

Table of Figures

Figure 1.1 Structure of the colonic wall. 29
Figure 1.2 Simplified representation of the tight junction complex. ZO-1; zona occludin 1, ZO-2; zona occludin 2, ZO-3; zona occludin 3, AF-6; ALL-1 fusion partner at chromosome 6. [Adapted from Anderson 2009 (26)].....31
Figure 1.3 Method of quantifying charged ion transfer across cultured epithelial monolayers using TEER. Two electrodes, one within each compartment, measure changes in electrical resistance across the cellular semi-permeable monolayer as a marker of paracellular permeability.....35
Figure 2.1 PRISMA flow chart of the record identification process.....61
Figure 2.2 Positive, negative and neutral outcomes of cannabinoid treatment across modes of inflammation (A). Incidence of endpoints across all experiments comparing cannabinoid treatment with control (B). The effect of cannabinoid drugs compared to control across all endpoints expressed as primary drug investigated (C). 67
Figure 2.3 Forest plot of the effects of cannabinoid treatment on Crohn's Disease, assessed by reduction in CDAI in human studies. 69
Figure 2.4 - Forest plot of the effects of cannabinoid treatment on Disease Activity Score subdivided by drug type. Time of administration in relation to onset of colitis is given where 'p' represents prophylactic administration, and 't' represents therapeutic administration. 73
Figure 2.5 Forest plot of the effects of cannabinoid treatment on MPO activity subdivided by drug type. Time of administration in relation to onset of colitis is given where 'p' represents prophylactic administration, and 't' represents therapeutic administration. 78
Figure 2.6 The effect of cannabinoid treatment on experimentally induced colitis in animals, determined by DAI (A) and MPO activity (B), predicted by timing of drug administration in relation to colitis onset. The effect of study quality, determined by mSTAIR score and SYRCLE score, on effect size in DAI (C, E) and MPO activity (D, F). Study weights are represented by diameter of circles, with larger circles representing studies with largest weight in the analysis.....81
Figure 2.7 Funnel plots evaluating for publication bias in (A) MPO activity and (B) DAI. Standard error of the standardized mean difference (SE (SMD), y axes) for each study is plotted against its effect size (SMD, x axes). 82
Figure 3.1 The effects of PEA and CBD on the intracellular levels of phosphorylated nuclear signalling proteins in response to an inflammatory protocol in cultured Caco-2 monolayers, measured by multiplex. Data are presented as percentage change from vehicle per plate +/- SEM, n=8 per condition. Data were analysed by one-

way ANOVA comparing against the vehicle control or inflammation where indicated (*<0.05, **<0.01 and ***<0.001). 94
Figure 3.2 The effects of PEA and CBD on the intracellular levels of phosphorylated signalling proteins in response to 24hr exposure to TNFα and IFNγ in cultured human colonic explants, measured by multiplex. Data are presented as percentage change from vehicle +/- SEM, n=13 per condition. Data were analysed by one-way ANOVA comparing against the vehicle control or inflammation where indicated (*<0.05, **<0.01). 96
Figure 3.3 The effects of PEA and CBD on the secreted cytokine response to an inflammatory protocol in cultured Caco-2 monolayers (A, C and E, column 1, n=8) and human colonic tissue (B, D and F, column 2, n=7), measured by ELISA. Error bars represent +/- SEM per condition. Data were analysed by one-way ANOVA comparing against the vehicle control or inflammation where indicated (*<0.05, **<0.01 and ***<0.001). 98
Figure 3.4 The effects of PEA and CBD on granulocyte macrophage colony stimulation (A, C) and monocyte chemotaxis (B, D) in response to an inflammatory protocol in cultured Caco-2 monolayers (column 1, n=8) and human colonic tissue (column 2, n=7), measured by ELISA. Error bars represent +/- SEM per condition. Data were analysed by one-way ANOVA comparing against the vehicle control or inflammation where indicated (*<0.05, **<0.01, ***<0.001 and ****<0.0001). 100
Figure 3.5 The effects of PEA and CBD on intracellular adhesion (A,D) and matrix metalloproteinase secretion (B,D) response to an inflammatory protocol in cultured Caco-2 monolayers (column 1, n=7) and human colonic tissue (column 2, n=7), measured by ELISA. Error bars represent +/- SEM per condition. Data were analysed by one-way ANOVA comparing against the vehicle control (*<0.05, ***<0.001 and ****<0.0001). 102
Figure 3.6 The effects of PEA (A,C,E) and CBD (B,D,F) on the secretion of IL-8, MCP-1 and IL-6 in response to an inflammatory protocol in explant human colonic tissue in the presence of receptor antagonists, measured by ELISA (n=10). Error bars represent +/- SEM per condition. Data were analysed by one-way ANOVA comparing against the vehicle control (*<0.05, **<0.01).....104
Figure 3.7 The effects of the receptor antagonists AM630, GW6471 & SB366791 on the secretion of MCP-1 of explant human colonic tissue, contrasted with the effect of an inflammatory protocol, measured by ELISA. Error bars represent +/- SEM per condition (n=7). Data were analysed by one-way ANOVA comparing against the vehicle control (*<0.05). 105

Figure 3.8 The effect of an inflammatory protocol on the secretion of inflammatory cytokines on normal colon, sections of IBD tissue and samples of appendicitis, measured by ELISA. Darkly shaded columns represent vehicle treated sections of tissue, lightly shaded bars represent tissue sections treated with an inflammatory protocol. Vehicle-treated normal colonic sections are compared with vehicle-treated IBD and appendicitis samples with two-way ANOVA (* represent significant difference from vehicle-treated healthy colon), inflammatory-treated normal colonic sections are also compared with inflammatory-treated IBD and appendicitis samples (significant differences represented by †). Error bars represent +/- SEM per condition. * or † <p0.05, ** or †† p<0.01, *** or ††† p>0.0001, **** or †††† p<0.00001. 107
Figure 3.9 The effects of PEA and CBD on the secreted inflammatory response in IBD colonic explants treated with an inflammatory protocol, measured by ELISA (n=6). Error bars represent +/- SEM per condition. Data were analysed by one-way ANOVA comparing against the vehicle control or inflammation where indicated (*<0.05). 108
Figure 3.10 The effects of PEA and CBD on the secreted inflammatory response in appendicitis explants treated with an inflammatory protocol, measured by ELISA (n=6). Error bars represent +/- SEM per condition. Data were analysed by one-way ANOVA comparing against the vehicle control (*<0.05, **<0.01) 110
Figure 3.11 Cell model suggesting putative sites of action of CBD indicated by asterisks..... 114
Figure 3.12 Cell model suggesting putative sites of action of PEA, indicated by starred sites..... 115
Figure 4.1 Culture of Caco-2 cells on membrane inserts. Membrane inserts are semi-permeable polyester platforms which allow cell culture between an apical and basolateral compartment. When fully confluent permeability of these cultured cells may be measured by placing fluorescent dextrans in the apical compartment, and measuring the fluorescence of the basolateral media at regular time points. 119
Figure 4.2 The effects of PEA, applied basolaterally, and CBD, applied apically, on the permeability of Caco-2 monolayers in response to 24hr exposure to TNFα and IFNγ measured by transfer of fluorescent dextrans (A:FD4 B: FD10 C: FD10 PEA & CBD). Raw data are expressed as the mean fluorescence per group +/- S.E.M. n=8 per condition. Data were analysed by two-way ANOVA using Dunnett's post hoc test comparing against the vehicle control (****p<0.0001)..... 124
Figure 4.3 The effects of PEA (basolateral, A) and CBD (apical, B) on the permeability of Caco-2 monolayers in response to 24hr exposure to TNFα and IFNγ in the presence of multiple receptor antagonists, measured by transfer of

fluorescent dextrans (FD10). Raw data is expressed as the mean fluorescence per group +/- SEM, n=8 per condition. Data was analysed by two-way ANOVA using Dunnett's post hoc test comparing against the vehicle control (*), ***<0.001, ****<0.0001).....125
Figure 4.4 The effects of CBD, applied apically, on the permeability of Caco-2 monolayers in response to 24hr exposure to TNFα and IFNγ in the presence of multiple signalling protein inhibitors measured by transfer of fluorescent dextrans (FD10). Raw data are given as mean fluorescence per group +/- S.E.M. n=8 per condition. Data were analysed using two-way ANOVA with Dunnett's post hoc analysis of selected pairs comparing against vehicle (****p<0.0001). 126
Figure 4.5 The effects of PEA, applied basolaterally, on the permeability of Caco-2 monolayers in response to 24hr exposure to TNFα and IFNγ in the presence of multiple signalling protein inhibitors measured by transfer of fluorescent dextrans (FD10). Raw data are given as mean fluorescence per group +/- S.E.M. n=8 per condition. Data were analysed using two-way ANOVA with Dunnett's post hoc analysis of selected pairs comparing against vehicle (****p<0.0001). 127
Figure 4.6 The effects of PEA and CBD on the production of mRNA levels for tight junction proteins in response to an inflammatory protocol in cultured Caco-2 cells; A=claudin-1, B=claudin-2, C=claudin-3, D=claudin-4, E=claudin-5, F=caludin-7, G=claudin-8 measured by multiplex. Data are presented as percentage change from vehicle per plate +/- SEM n=8 per condition. Data were analysed by one-way ANOVA comparing against vehicle.....129
Figure 4.7 The effects of PEA and CBD on the production of mRNA levels for multiple receptors in response to an inflammatory protocol in cultured Caco-2 cells; A=PPARδ, B=PPARγ, C=PPARα, D=TRPV1, E=GPR55, F=GPR119, G=CB1, H=CB2 measured by multiplex. Data are presented as percentage change from vehicle per plate +/- S.E.M. n=8 per condition. Data were analysed by one-way ANOVA comparing against vehicle (****p<0.0001). 130
Figure 4.8 The effects of PEA and CBD on the levels of membrane-bound AQP3 in Caco-2 (A) and human colonic mucosa (C) and AQP4 in Caco-2 cultures (B) and human colonic mucosa (D) in response to an inflammatory protocol in cultured Caco-2 monolayers measured by ELISA. Raw data are are expressed as mean protein levels per group +/- S.E.M. n=6-8 per condition. Data were analysed by one-way ANOVA comparing against vehicle (* p<0.05, ** p<0.01, *** p<0.0001, ****p<0.00001) or inflammation (†† p<0.01, †††† p<0.00001). 132
Figure 4.9 The effects of PEA and CBD on the production of mRNA levels for tight junction proteins in response to an inflammatory protocol in cultured human colonic tissue homogenates; A=claudin-1, B=claudin-2, C=claudin-3, D=claudin-4,

E=claudin-5, F=caludin-7, G=claudin-8 measured by multiplex. Data are presented as mean percentage change from vehicle +/- S.E.M. n=7 per condition. Data were analysed by one-way ANOVA and Dunnett's post hoc test comparing against vehicle (* p<0.05, **<0.01). 134
Figure 4.10 The effects of PEA and CBD on the production of mRNA levels for multiple receptors in response to an inflammatory protocol in cultured human colonic tissue homogenates; A=PPARδ, B=PPARγ, C=PPARα, D=TRPV1, E=GPR55, F=GPR119, G=CB1, H=CB2 (n=7) measured by multiplex. Data are presented as mean percentage change from vehicle +/- S.E.M. n=7 per condition. Data were analysed by one-way ANOVA and Dunnett's post hoc test comparing against vehicle (* p<0.05) or inflammation († p<0.05)..... 135
Figure 5.1 Study protocol..... 147
Figure 5.2 The effect of CBD, PEA and placebo on aspirin-induced intestinal absorption and urinary secretion of lactulose in healthy human subjects (n=10). Results are expressed as mean concentrations +/- SEM. Concentrations were compared to baseline sample using repeated measures ANOVA and Dunnett's multiple comparison test (*<0.05, **<0.01, ***<0.001, ****<0.00001)..... 151
Figure 5.3 The effect of CBD, PEA and placebo on aspirin-induced intestinal absorption and urinary secretion of lactulose in healthy human subjects (n=10). Results are expressed as mean concentrations +/- SEM. Concentrations were compared to baseline sample using repeated measures ANOVA and Dunnett's multiple comparison test (*<0.05, **<0.01, ***<0.001)..... 151
Figure 5.4 The concentration ratios of urinary lactulose and mannitol over time in healthy participants treated with aspirin and either placebo (n=10), CBD (n=10) or PEA (n=4), measured by LC MS. Results are expressed as mean ratios +/- SEM. Time points between groups were compared using two-way ANOVA using Dunnett's multiple comparisons test comparing to placebo at the same time point (*p<0.05, **p<0.01, ***p<0.001). 153

List of Tables

Table 1-1 Modes of colonic inflammation and incidences. *IBD - Inflammatory bowel disease. 27

Table 1-2 Members of the claudin family and function relating to monolayer permeability to water and charged ions (CL – claudin).**Error! Bookmark not defined.**

Table 1-3 Summary actions of the endo- and phytocannabinoids 46

Table 2-1 Characteristics of studies included for systematic review. 66

Table 2-2 The effects of cannabinoids on disease activity score caused by experimental colitis, grouped by compound (NS – non significant). 74

Table 2-3 The effects of cannabinoids on MPO activity caused by experimental colitis, grouped by drug (NS – non significant). 79

Table 5-1 Commonly used probes in measuring gastro-intestinal permeability. 142

Table 5-2 LC MS parameters for each compound monitored. 148

Table 5-3 Urinary concentrations of lactulose and mannitol in healthy volunteers receiving aspirin therapy (n=10). Results are expressed as mean concentrations +/- standard deviation. Concentrations were compared to baseline using repeated measures ANOVA and Dunnett's multiple comparison test (*<0.05, **<0.01, ***<0.001, ****<0.00001). 150

Table 5-4 Urinary concentrations of lactulose and mannitol in healthy volunteers receiving aspirin and CBD therapy (n=10). Results are expressed as mean concentrations +/- standard deviation. Concentrations were compared to baseline using repeated measures ANOVA and Dunnett's multiple comparison test (*<0.05, **<0.01, ****<0.00001). 152

Table 5-5 - Urinary concentrations of lactulose and mannitol in healthy volunteers receiving aspirin and PEA therapy (n=4-10). Results are expressed as mean concentrations +/- standard deviation. Concentrations were compared to baseline using repeated measures ANOVA and Dunnett's multiple comparison test (*<0.05, **<0.01, ****<0.00001). 152

Abbreviations

ABC	Adenosine-tri phosphate binding cassette
Ab-CBD	Abnormal cannabidiol
ACBG2/BCRP	ATP-binding cassette sub-family G member 2
ACEA	Arachidonyl-2'-chloroethylamide
AEA	Anandamide
Akt kinase B, PKB	Alpha serine/threonine-protein kinase, also known as protein
ANOVA	Analysis of variance
2-AG	2-Arachidonoyl glycerol
AQP	Aquaporin
BCA	Bicinchoninic acid
cAMP	Cyclic adenosine monophosphate
CB_1	Cannabinoid receptor 1
CB_2	Cannabinoid receptor 2
CBC	Cannabichromene
CBD	Cannabidiol
CBG	Cannabigerol
CBN	Cannabinol
CDAI	Crohn's disease activity index
CGRP	Calcitonin gene related peptide
CI	Confidence interval
CO	Croton oil
COX	Cyclooxygenase
CREB	cAMP response element-binding protein
C. sativa	*Cannabis sativa*
DAGL	Diacylglycerol lipase
DAI	Disease activity index
DNBS	Dinitrobenzene sulphonic acid
DSS	Dextran sulphate sodium
ECS	Endocannabinoid system

ELISA	Enzyme-linked immunosorbent assay
Erk	Extra-cellular signal regulated kinase
FAAH	Fatty acid amide hydrolase
F-Actin	Filamentous actin
G-Actin	Globular actin
GM-CSF	Granulocyte macrophage colony stimulating factor
GPCR	G protein-coupled receptor
IBD	Inflammatory bowel disease
I.C.	Intracolonic
ICAM-1	Intercellular adhesion molecule-1
IFNγ	Interferon gamma
IL-1β	Interleukin– 1 beta
IL-6	Interleukin- 6
IL-8	Interleukin- 8
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-17	Interleukin-17
I.V.	Intravenous
JAM	Junctional adhesional molecule
JNK	c-Jun N-terminal kinase
KO	Knock out
LC	Liquid chromatography
LPS	Lipopolysaccharide
MAGL	Monoacyl-glycerol lipase
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemotactic factor-1
MD	Mean difference
MDR1	Multi-drug resistance 1, ABCB1
MDR2	Multi-drug resistance 2, ABCB4
MMJ	Medicinal Cannabis
MMP-3	Matrix metallopeptidase-3
MPO	Myeloperoxidase

MS	Mass spectrometry
NAAA	N-acylethanolamine acid amide hydrolase
NAE	N-acylethanolamine
NAPE	N-acyl phosphatidylethanolamine
NAPE-PLD	N-acyl phosphatidylethanolamine phospholipase D
NF-κB	Nuclear factor-kappa of activated B cells
OATPs	Organic anion transport polypeptides
OATs	Organic anion transporters
OCTs	Organic cation transporters
OEA	Oleoylethanolamide
OM	Oil of mustard
PBS	Phosphate buffered saline
PEA	Palmitoylethanolamide
PKA	Protein kinase A
PKC	Protein kinase C
P.O.	Oral
PPAR	Peroxisome proliferator-activated receptor
P.R.	Per rectum
P70 S6K	p70-S6 Kinase 1
RhoA	Ras Homolog gene family A
S.C.	Subcutaneous
SEM	Standard error of the mean
SMD	Standard mean difference
STAT3	Signal transducer and activator of transcription-3
STAT5	Signal transducer and activator of transcription-5
TEER	Transepithelial electrical resistance
TGF-β	Transforming growth factor beta
THC	Δ^9-Tetrahydrocannabinol
TNBS	2,4,6-Trinitrobenzene sulfonic acid
TNFα	Tumour necrosis factor alpha
TRPV1	Transient receptor potential vanilloid-1
ZO-1	Zona occludin-1

ZO-2	Zona occludin-2
ZO-3	Zona occludin-3

Glossary of cannabinoid compounds

Phytocannabinoids

CBC	Cannibichromene
CBD	Cannabidiol
CBG	Cannabigerol
CBN	Cannabinol
THC	Tetrahydrocannabinol
MMJ	Medicinal cannabis

Endocannabinoids

2-AG	2-Arachidonoyl glycerol
AEA	Anandamide
OEA	Oleoylethanolamide
PEA	Palmitoylethanolamide
	Virodhamine

Synthetic Cannabinoids

αβ Amyrin	CB_1 and CB_2 agonist
ACEA	CB_1 selective agonist
Adelmidrol	PEA analogue
AM1241	CB_2 full agonist, partial CB_1 agonist
AM841	Peripherally restricted CB_1 agonist
βCaryophyllene	CB_2 agonist
CID16020046	GPR55 inverse agonist
Compound 26	Selective CB_2 agonist

CP55,940	Non-selective CB_1 and CB_2 agonist
HU210	THC analogue, non-selective CB_1 and CB_2 agonist
HU308	Selective CB_2 agonist
JHW133	Selective CB_2 full agonist, weak CB_1 agonist
JTE-907	CB_2 inverse agonist
JWH015	CB_2 full agonist, weak CB_1 agonist
O-1602	GPR18 and GPR55 agonist
SAB378	Peripherally restricted CB_1 and CB_2 agonist
WIN55,212-2	non-selective CB_1 and CB_2 full agonist

Enzyme Inhibitors

AM3506	FAAH inhibitor
AM9053	NAAA inhibitor
ARN2508	FAAH inhibitor
ARN272	AEA reuptake inhibitor
Compound 39	FAAH inhibitor
JZL184	MAGL inhibitor
PF-3845	FAAH inhibitor
URB597	FAAH inhibitor
URB602	MAGL inhibitor
VDM11	AEA reuptake and FAAH inhibitor

Receptor Antagonists

AM251	CB_1 antagonist
AM630	CB_2 antagonist
GW6471	PPARα antagonist
GW9662	PPARγ antagonist
SB366791	TRPV1 antagonist
CID16020046	GPR55 antagonist

1 Introduction

1.1 Colonic inflammation

1.1.1 Epidemiology

Disorders of the gastrointestinal tract represent around 14% of all inpatient hospital admissions in the UK (Elixhauser & Owens 2006). Of these disorders, inflammation of the colon represents a significant disease burden. Inflammation of the colon may be classified into autoimmune inflammatory conditions; infective conditions, such as diverticulitis and appendicitis; disorders of oxygenation, such as ischaemic colitis; and colitis secondary to radiation exposure. Incidences for these groups are displayed in Table 1.1. Of these groups, autoimmune colitis remains a substantial clinical challenge, due to their chronic course and limited treatment options.

Mode of inflammation	**Common examples**	**Incidence /100,000 per year**	**Treatment options**
Autoimmune/ IBD*	Crohn's disease	150-199 (Loftus 2004)	Immunosuppression
	Ulcerative colitis	211-246 (Loftus 2004)	Surgery
	Indeterminate	9-12 (Fine et al. 2000)	
Infective	Diverticulitis	49-299 (Warner et al. 2007)	Surgery
			Antibiotics
	Bacterial/infective	930 (Tam et al. 2012)	
Deoxygenation	Ischaemic colitis	44 (Higgins et al. 2004)	Surgery
			Endovascular intervention
Radiation exposure	Post-radiotherapy colitis	3-5 (Miller et al. 1999)	Local immunosuppression

Table 1-1 Modes of colonic inflammation and incidences. *IBD - Inflammatory bowel disease.

1.1.2 Autoimmune inflammatory bowel disease

Autoimmune inflammatory diseases of the colon may be divided into ulcerative colitis, Crohn's disease and indeterminate causes of colitis. In addition, the microscopic colitides may be further histologically sub-divided according to their histological appearance into microcytic colitis, lymphocytic colitis, and collagenous colitis among others.

Ulcerative colitis and Crohn's disease account for the majority of cases of IBD. Although the aetiology of these two diseases are currently unknown, several risk factors have been identified for both, such as familial predisposition, smoking, obesity, poor diet and physical inactivity (Cosnes 2010). Crohn's disease and ulcerative colitis are pathologically distinct, based on anatomical and histological differences. Anatomically, Crohn's disease may affect any part of the gastrointestinal tract from mouth to anus, whereas ulcerative colitis is strictly limited to the colon. At histology Crohn's disease may be characterised by non caseating granuloma formation, submucosal inflammation and patchy lymphocytic infiltration, whereas ulcerative colitis typically forms crypt abscesses and causes smooth muscle thickening with distortion of crypt architecture without the formation of granulomata. Despite these differences, ulcerative colitis and Crohn's disease may be difficult to differentiate clinically, as they share several signs and symptoms such as abdominal pain, passage of blood *per rectum*, weight loss, anorexia, fever, diarrhoea, urgency and frequency of stool and malaise.

1.1.3 The gut barrier in autoimmune colitides

An underlying characteristic of both Crohn's disease and ulcerative colitis is impairment of the gut barrier. In health, through a complex system of epithelial transport pathways and immune tolerance, the gut actively moves water and nutrients from the intestinal lumen into the submucosa (and then peripheral and lymphatic circulation) without noxious translocation of bacteria and lipopolysaccharide. During states of inflammation, this barrier function becomes compromised. Active absorption of water, sodium and macronutrients becomes diminished though dysregulation of active transport mechanisms leading to diarrhoea. Second, pathological breakdown of this barrier allows luminal bacteria and lipopolysaccharide to translocate into the submucosa of the gut wall and then serosa (Rojo et al. 2007; Raetz & Whitfield 2002). Locally, this leads to inflammation of the submucosa or serosa, causing inflammation of the neighbouring peritoneum (peritonitis), manifesting as abdominal pain, abscess or fistula formation. Systemic consequences of this translocation into the peripheral circulation cause endotoxaemia or septic shock, manifesting as fever, progressive

hypotension, confusion, malaise, tachycardia and ultimately death (Nikitenko et al. 2011).

This selectively absorptive mucosal gut barrier is maintained by three mechanisms (van der Flier & Clevers 2009). First, a monolayer of mucosal epithelial cells forms a continuous barrier between the gut lumen and the submucosal space (figure 1.1). Within and between these epithelial cells, a group of intercellular and transcellular proteins seal the luminal environment from the basolateral (submucosal) compartment whilst selectively absorbing water and macronutrients. Second, epithelial goblet cells secrete mucin, rich in glycoproteins and bioactive molecules, which limit bacterial contact with the epithelial cells of the mucosa (Kim & Ho 2010). Last, epithelial cells are able to generate an immune response which, in turn, activates sub-adjacent innate and adaptive immune cells such as macrophages, antigen-presenting dendritic cells and T cells. This allows for balance between tolerance of the luminal contents with asepsis of the submucosal space, and uptake of nutrients without infection (Coskun 2014; Peterson & Artis 2014; Kim & Ho 2010). Understanding the components of this barrier and how they undergo change in inflammation may generate novel strategies in the treatment of IBD.

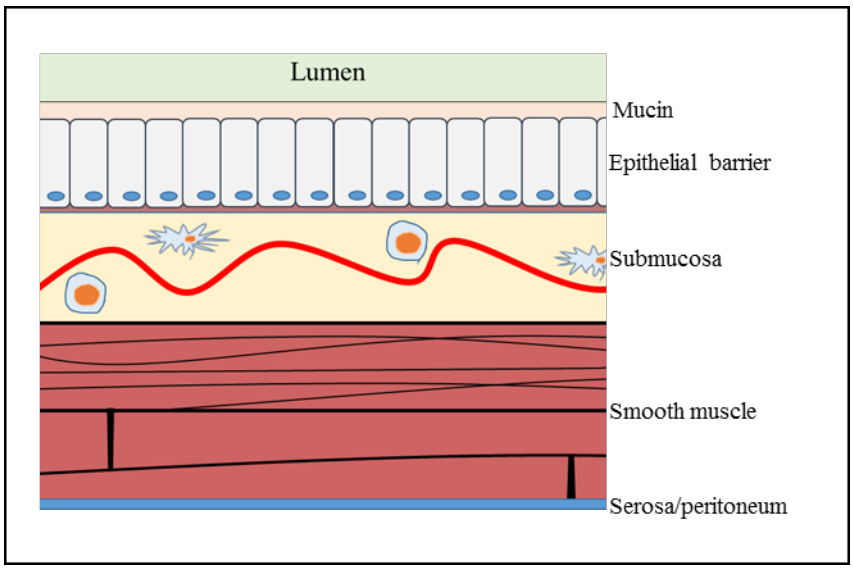


Figure 1.1 Structure of the colonic wall.

Epithelial barrier transport

Ions, food particles or bacteria may enter from the environment across the epithelium via two routes. Firstly, luminal contents may pass in-between cell-to-cell contacts along a paracellular route through pore-like structures known as tight junctions. Secondly, contents may pass directly through the cell membrane, along a transcellular route. The paracellular route is predominantly passive, whereas the transcellular route is largely active through energy dependant transporters, pinocytosis, and phagocytosis, although passive transfer may occur via membrane-bound pores.

The tight junction complex

The tight junction complex, or zona occludens, is a continuous band-like structure encircling the apical border of vertebrate epithelial cells. Tight junction complexes can be seen at apical cell to cell contact points on scanning electron microscopy, appearing as bands of protein continuous with the actin cytoskeleton (Pinto da Silva & Kachar 1982). Tight junctions are composed of two principle transmembrane proteins; occludin and claudin, with a third adjacent protein within the intercellular space: the junctional adhesion molecule (JAM) (figure 1.2) (Furuse et al. 1993; Furuse 1998; Niessen 2007). These proteins are fused to identical molecules on neighbouring epithelial cells at which point the intercellular space is sealed, preventing unregulated paracellular transport (Tsukita et al. 2001; Martin-Padura 1998). More than forty cytoplasmic proteins (such as zona-occludens 1 (ZO-1), ZO-2 and ZO-3) link the actin cytoskeleton to claudin and occludin proteins (Itoh et al. 1999; Anderson 2001; Van Itallie & Anderson 2006). As different epithelial tissues vary in their barrier function (for example the permeability properties of the kidney are vastly different from that of the retina), the density and composition of tight junctions at these sites also varies (Van Itallie et al. 2008).

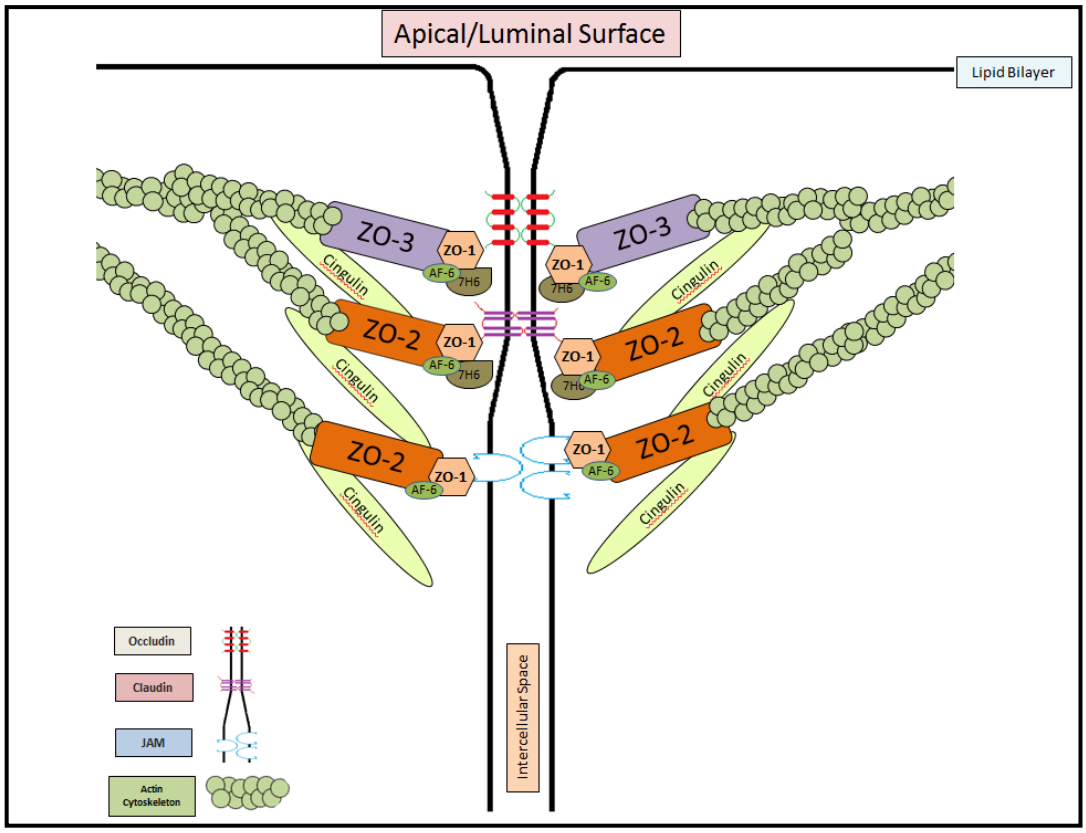


Figure 1.2 Simplified representation of the tight junction complex. ZO-1; zona occludin 1, ZO-2; zona occludin 2, ZO-3; zona occludin 3, AF-6; ALL-1 fusion partner at chromosome 6. [Adapted from Anderson 2009 (26)].

Claudins

Claudins, from the Latin *claudere* to close, are a family of twenty-four structurally similar proteins sharing 13-70% homology (Furuse 1998; Van Itallie & Anderson 2006; Tsukita et al. 2001; Van Itallie & Anderson 2004). The size of individual claudin proteins range from 20-26 kDa, each possessing four transmembrane domains. The two extracellular domains are of differing size and are densely polar. This allows for charged pore formation between opposing claudin proteins at adjacent cell membranes, allowing selective transport of ions across the paracellular space (Furuse 1998). The nature of these pores governs the degree of paracellular permeability between cells. Expression of different claudins between tissue types, with their differently charged extracellular loops, contributes to the different permeability properties across vertebrate epithelia (González-Mariscal et al. 2003). The permeability properties of different claudins and common sites are described in Table 1.2. For example claudin-2, a cation-selective pore-forming claudin, causes an increase in epithelial permeability, and is highly expressed in the permeable epithelia of the proximal renal tubule (Reyes et al. 2002). The less permeable

claudin-4 is expressed in epithelia of low permeability such as in the distal nephron (Kitajiri et al. 2004; Kiuchi-Saishin et al. 2002). The expression of claudins changes along the length of the gastrointestinal tract, reflective of the changing permeability needs of different regions. The mRNA encoding Claudin-8, a low permeability claudin, is found throughout the colon and ileum, but not in the duodenum and jejunum for example. Claudin-15 is highly expressed in the duodenum and jejunum, but expression decreases steeply in the ileum and colon (Fujita et al. 2006). In addition to anatomical variations in claudin expression, the type of claudin expression may change dynamically in response to the varying requirements of epithelia (Weber 2012). The changes across all claudin populations from mouth to anus are complex, and have been well summarized by Lu *et al*. (Lu et al. 2013).

Cl	Site	Effect on Permeability	Other Functions	
1	Duodenum, jejunum, nephron	Decreases permeability		(Furuse *et al.*, 2001; Rahner, Mitic and Anderson, 2001; Luettig *et al.*, 2015)
2	Duodenum, distal nephron, jejunum	Increases permeability		(Wolburg et al. 2003; Lu et al. 2013)
3	Duodenum, blood-brain barrier	Decreases permeability		(Van Itallie, Fanning and Anderson, 2003; Li, Huey and Yu, 2004;)
4	Duodenum, nephron	Decreases permeability		(Morita *et al.*, 1999; Nitta *et al.*, 2003; Li, Zeissig *et al.*, 2007)
5	Duodenum, blood-brain barrier, nephron	Decreases permeability		(Abuazza et al. 2006)
6	Nephron		Detected in foetal nephrons, but absent in adult kidney	(Li, Huey and Yu, 2004; Alexandre *et al.*, 2007)
7	Duodenum, nephron, jejunum	Decreases flow of Cl^- into the cell, increases flow of Na^+ into the cell		(Yu *et al.*, 2003; Zeissig *et al.*, 2007)
8	Nephron, duodenum, jejunum	Decreases permeability		(Abuazza et al. 2006)
9	Nephron		Detected in foetal nephrons, but absent in adult kidney	(Van Itallie & Anderson 2006; Breiderhoff et al. 2012)
10	Nephron, inner ear	Decreases flow of anions (10a) or cations (10b) into the cell		(Van Itallie et al. 2003)
11	Duodenum, jejunum, ileum, colon	Increases permeability		(Fujita et al. 2008)
12	Duodenum	Forms high resistance barriers to other positive ions in the intestine		(Abuazza et al. 2006)
13	Duodenum, jejunum, ileum, colon		Detected in foetal nephrons, but absent in adult kidney	(Miyamoto et al. 2005; Kitajiri et al. 2004)
14	Inner ear	Decreases permeability		(Itoh et al. 1999; Van Itallie et al. 2003)
15	Duodenum	Decreases permeability		(Hou & Goodenough 2010)
16	Nephron	Increases permeability		(Conrad et al. 2015)
17	Nephron	Anion selective increase in permeability		(Kitajiri *et al.*, 2004; Kim *et al.*, 2013)
18	Oesophagus, stomach, lung, osteoclasts	Decreases permeability		(Niimi *et al.*, 2001; Alanne *et al.*, 2009)
19	Nephron, Schwann cells	Increases permeability in the nephron & Schwann cells		(Krug, Schulzke and Fromm, 2014)
20	Bronchus, retina, skin	Anion selective increase in permeability		(Katoh & Katoh 2003)
21	Stomach	N/a		(Katoh & Katoh 2003)
22	Stomach	N/a		(Katoh & Katoh 2003)
23	Colon, stomach, placenta, skin	Decreases permeability		(Katoh & Katoh 2003)
24	Stomach	N/a		(Katoh & Katoh 2003)

Table 1-2 Members of the claudin family and function relating to monolayer permeability to water and charged ions (CL – claudin).

Occludin

Occludin is a 65 kDa protein composed of four transmembrane domains (Furuse 1998; Matter & Balda 1999). Its two extracellular domains are similar in size (Fujita et al. 2006). The presence of a rich tyrosine structure within occludin allows the formation of highly polar paracellular pores, but also dynamic interaction with neighbouring components of the paracellular apparatus through phosphorylation. Occludin contributes to the electrical resistance of the tight junction complex, as a loss of occludin density occurs alongside an increase in membrane permeability (Mankertz et al. 2000). Aside from tight junction resistance, occludin plays a role in tight junction construction. Fibroblasts transfected with occludin, which do not normally form tight junctions, form tight junction-like strands suggesting that occludin spontaneously takes part in the formation of tight junctions (Furuse 1998). However occludin knock out mice are able to form structurally normal tight junctions, but demonstrate growth retardation, gastritis and sterility (Saitou et al. 2000). This suggests that occludin is essential for maintaining membrane resistance as these pathologies may all be explained by increased permeability. Further evidence suggests that occludin, in addition to providing membrane stability, also plays a key role in cell signalling and immune cell trafficking (Barrios-Rodiles et al. 2005).

The junctional adhesion molecule

Junctional adhesion molecules (JAMs) are 43 kDa molecules with two extracellular and one cytoplasmic domain located immediately adjacent to the tight junction (Hartsock & Nelson 2008). Like claudins, several JAM subtypes have been described (Garrido-Urbani et al. 2014). JAM is responsible for maintaining monolayer resistance in cell lines; a concurrent increase in permeability, measured by transepithelial electrical resistance (TEER), is observed in cell cultures when JAM is knocked down (Mandell et al. 2005). TEER is a commonly used dynamic measure of monolayer permeability, quantifying the change in electrical potential across an epithelial barrier as a surrogate marker for permeability (Martinez-Palomo et al. 1980). This is achieved by placing probes in the apical and basolateral compartments (figure 1.3). JAM is the first transmembrane protein to link cell to cell contacts to the actin cytoskeleton in juvenile cultures, encouraging intercellular adhesion and cellular polarity (Garrido-Urbani et al. 2014). The precise method by which JAM establishes cellular polarity is unknown, but does not occur in the absence of occludin, suggesting dependence. To further support its need for occludin, JAM was shown to be unable to induce formation of tight junction stands or polarity when transfected into fibroblasts; cells that do not possess occludin (Martin-Padura 1998).

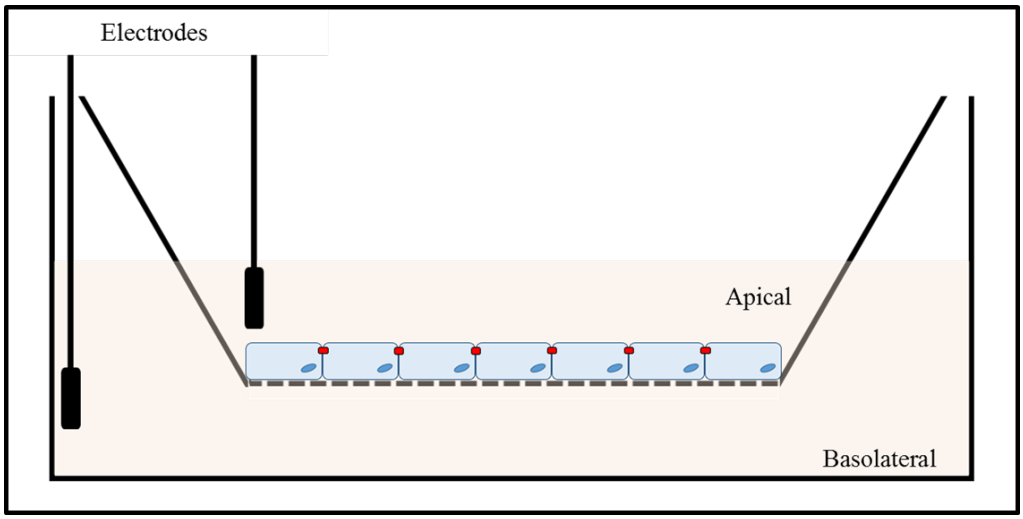


Figure 1.3 Method of quantifying charged ion transfer across cultured epithelial monolayers using TEER. Two electrodes, one within each compartment, measure changes in electrical resistance across the cellular semi-permeable monolayer as a marker of paracellular permeability.

Zona occludens 1, 2 and 3, intermediate proteins and the actin cytoskeleton

A myriad of intermediate proteins link claudin, occludin and JAM to the actin cytoskeleton (Figure 1.2) (Hartsock & Nelson 2008; Stevenson 1986; Fanning et al. 1998). The actin cytoskeleton is a network of actin subunits which are polymerised to form long chains known as filamentous-actin (F-actin) and provide cell morphology and allow movement. Single actin units are called globular actin (G-actin). An increased proportion of F- to G-actin increases cell tension, decreasing paracellular permeability. For example, the toxin phalloidin, produced by the death cap mushroom, inhibits F-actin depolymerisation preventing homeostatic physiological changes in paracellular permeability (Graeme 2014). Another mycotoxin, cytochalasin, induces rapid depolymerisation of F-actin, resulting in catastrophic increases in paracellular permeability (Graeme 2014). Both toxins are rapidly fatal.

The zona occludin proteins (ZO), cingulin, tetraspanins, coxsackievirus and adenovirus receptor, and ALL-1 fusion partner at chromosome 6 play critical roles in the interface between the actin cytoskeleton and the extracellular-facing occludin and claudins. A loss of these intermediary proteins, particularly ZO-1, causes a loss of cell tension, with subsequent increases in paracellular permeability (Van Itallie et al. 2009). The complex roles of these proteins are not discussed further here, a comprehensive summary can be found in the work by Gonzales-Mariscal *et al.* (González-Mariscal et al. 2003).

Inflammatory modulation of the paracellular apparatus

Once considered to be a static organelle, the composition and interaction of the tight junction complex with other cellular structures is dynamic and complex, and undergoes significant changes during inflammatory states (González-Mariscal et al. 2003; Arrieta et al. 2006; Sasaki et al. 2003; Weber 2012). The pro-inflammatory mediators interferon-gamma (IFNγ), tumour necrosis factor-alpha (TNFα), interleukin 1-beta (IL-1β), interleukin-6 (IL-6), and interleukin-17 (IL-17) cause increased paracellular permeability measured by TEER in cultured cell lines, and in human and murine tissue (Bruewer et al. 2005; Ma et al. 2005; Heller et al. 2005; Al-Sadi et al. 2008). Decreased activity of the anti-inflammatory factor interleukin-10 (IL-10) during episodes of acute inflammation also increases paracellular permeability (Kusugami et al. 1995; Kinugasa et al. 2000; Madsen et al. 1999; Howe et al. 2005). These cytokine changes have been shown to act at all three major components of the tight junction complex. For example, occludin levels fall in cultured H29 monolayers, an immortal gastrointestinal cell line originally derived from a colorectal cancer specimen in 1964 (Fogh et al. 1977), when exposed to a TNFα inflammatory protocol which is associated with a drop in permeability measured by TEER (Poritz et al. 2011; Mankertz et al. 2000). Furthermore, colonic biopsies taken from patients with active IBD display 50% lower occludin levels, specifically at mucosal areas of increased neutrophil migration (Kucharzik et al. 2001). This suggests that occludin has influence on maintaining the resistance of the paracellular channel in health, but may be downregulated during periods of inflammation to allow immune cell translocation, although not yet proven.

Claudin expression also alters under inflammatory conditions, in association with permeability changes (Weber & Turner 2007). Experiments in mice have demonstrated that enteric salmonella infection (noted for its permeability-increasing effects on the gut and profound diarrhoea) up-regulates intestinal claudin-2 expression over 6 hours, (Zhang et al. 2013). TNFα, via Nuclear factor-kappa beta (NF-κB) induction, decreases the expression of the permeability-lowering claudins 1, 3, 4, 5, 7, while increasing claudin-2 in inflamed explant human colonic mucosa (Günzel & Fromm 2012; Prasad et al. 2005). Furthermore, the pro-inflammatory cytokine transforming growth factor β (TGF β), Specificity Protein-1, and Grainy Head-like 2 directly influence claudin levels *in vitro* (Günzel & Yu 2013). Taken together, this suggests that claudins are particularly sensitive to experimental inflammatory stimuli. Clinically, claudin expression is not measured routinely, although colonic biopsies from patients with active ulcerative colitis also display changes in claudin expression (Prasad et al. 2005). Colonic biopsies from ulcerative colitis patients treated with IL-13 show dose dependent increases in

mucosal permeability, with increases in claudin-2 levels (Heller et al. 2005). Patients with quiescent Crohn's disease and ulcerative colitis express normal levels of the permeability-decreasing claudins-5 and -8, but show increased production of the permeability-increasing claudin-2 compared to healthy controls. During inflammatory exacerbations of Crohn's disease, these patients saw a fall in levels of claudin-5 and -8, alongside a sharp increase in claudin-2 levels (Zeissig et al. 2007). These changes were associated with increased mucosal permeability. Based on these observations it has been suggested that a complex genetic predisposition to early transcription of claudin-2 in response to normal, rather than acute inflammatory conditions may underlie the aetiology of Crohn's disease and ulcerative colitis. In support of this hypothesis, claudin-2 has been further shown to be increased IBD mucosa within areas of increased permeability in biopsies taken from patients during episodes of acute inflammation without an obvious stimulus (Weber et al. 2010).

JAM also contributes to permeability in inflammation. JAM deficient mice display normal gut permeability in health, but under inflammatory conditions display increased leucocyte proliferation, but not increased leucocyte translocation (Khounlotham et al. 2012). In this set of experiments, increased bacterial translocation across mucosal barriers was found in the JAM deficient mice, but with less overall colonic inflammation compared to healthy controls. Permeability *per se* was not measured. A further experiment on vascular tissue found that TNFα and IFNγ cause migration of JAM from the intercellular space to the luminal surface with a concurrent increase in endothelial permeability (Ostermann et al. 2002). These two studies together suggest that JAM may relocate to the cell surface under inflammatory conditions in order for polymorphonucleocyte transmigration to occur via interaction with JAM. The transmigration of polymorphonucleocytes in this manner may contribute to the increases in mucosal permeability found in colitis.

Inflammatory states also affect the presence and function of the accessory tight junction proteins, and the tension of the actin cytoskeleton. Changes in the presence or phosphorylation of many of these intermediate tight junction proteins and actin has previously been summarised, and linked to changes in mucosal permeability (Lee 2015).

In addition to cytokine action on paracellular permeability, luminal salt directly affects the permeability of the tight junction, associated with increased Th17-cell proliferation (Vojdani 2014). Increasing luminal glucose concentrations have also been shown to increase enteric tight junction permeability in mice by an unidentified pathway (Turner et al. 2000). Other factors such as presence of luminal dietary

peptides, lectins and yeasts affect tight junction integrity acting through various T-cell mediated mechanisms via release of anti- or pro-inflammatory mediators (Jeon et al. 2013).

Membrane-bound transcellular transport proteins

A collection of passive and active transport proteins control the passage of luminal contents via the trans-cytoplasmic route.

Aquaporins

Aquaporins (AQPs) are passive protein channels that allow the passage of water and small solutes across the epithelial barrier along osmotic gradients. They are ubiquitous in mammalian epithelial tissues, and play a crucial role in maintaining water uptake and reabsorption (Ishibashi et al. 2009). Thirteen AQP types have been described in humans (Ishibashi et al. 2009), which play multiple roles across different epithelia. For example, AQP2 deficient mice die rapidly from dehydration, through diabetes insipidus (a failure of the kidney to concentrate urine), suggesting a role in water resorption in the nephron. AQP9 plays a vital role in glycerol metabolism in intestinal goblet cells (Spegel et al. 2015), and AQP10 maintains optic lens transparency (Varadaraj et al. 2010). Within the intestinal epithelium, AQP3 is expressed at the basal membrane, and plays a role in glycerol transport (Thiagarajah et al. 2007). This study showed that AQP3 was also more prevalent at colonic ulcer sites, and that AQP3 deficient mice were more susceptible to colonic inflammation. This suggests that AQP3 plays a role in the recovery of intestinal epithelia during inflammation. In similar models of murine colitis, caustic inflammation caused a decrease in the expression of colonic AQP4, AQP7 and AQP8, which correlated with an inability to dehydrate luminal contents. This may suggest, therefore, that these pores transport luminal water across the epithelium in health, but are disabled or are downregulated by inflammation. Similar changes in aquaporin expression were found in colonic biopsies from patients with Crohn's disease and ulcerative colitis which was shown to correlate with disease activity (Hardin et al. 2004). The roles and distribution of aquaporins in the gut has been well summarized elsewhere by Zhu (Zhu et al. 2016)

Adenosine-tri phosphate binding cassette (ABC) proteins

ABC proteins are a family of trans-membrane bound proteins which are conserved from plants through to mammals. Within the intestine, key ABC proteins are Multi-drug resistance-1 (MDR1), Multi-drug resistance-associated-1 (MRP2), ATP-binding cassette sub-family G member 2 (ACBG2, also known as BCRP) and p-glycoprotein (Borst et al. 2000). These proteins are energy dependent active transporters which perform several roles such as movement of exogenous toxins, such as drugs (Dean

et al. 2001), bacterial particles (Mercado-Lubo & McCormick 2010), and food components back into the gut lumen (Dietrich et al. 2003), excretion of endogenous waste products, such as in the movement of bile salts in hepatic sinusoids in the production of bile (Meier & Stieger 2002), and trafficking of peptides and lipids from the cytosolic to extracellular compartment.

Deficiencies in ABC protein expression have been implicated in the development of IBD (Noble et al. 2008). For example, mutation in the gene coding for MDR1 has been associated with ulcerative colitis, but not Crohn's disease (Schwab et al. 2003). This was demonstrated in MDR1 knock out mice which developed colitis spontaneously, suggesting that ABC proteins may play a role in the intestinal barrier. Similarly, polymorphisms in the MRP2 gene, and reductions in expression of BCRP have been associated with both Crohn's disease and ulcerative colitis (Pazos et al. 2008; Savić Mlakar et al. 2015). Taken together this suggests that these proteins contribute to membrane permeability, and that inflammation may downregulate their production, or their absence contributes to intestinal inflammation.

Solute carrier transporters

Within this group are found three families of ion transporters expressed predominantly at the basolateral aspect of epithelial cells; organic anion transport polypeptides (OATPs), organic anion transporters (OATs), and organic cation transporters (OCTs). These active transporters are found throughout mammalian epithelia, and are involved in active drug and toxin excretion, nutrient absorption and bacterial debris elimination (Roth et al. 2012). Although the clinical literature focuses on the role of solute carrier proteins' importance in drug elimination and cancer chemotherapeutic resistance, decreased expression of OCTs, and increased expression of OATPs and OATs has been associated with ulcerative colitis (Noble et al. 2008; Kacper et al. n.d.). Specific variations in the expression of solute carrier proteins, and the effect on inflammatory cytokine expression and function has been well described by Ghishan (Ghishan & Kiela 2014).

Structural factors affecting epithelial permeability

Small particles may spontaneously translocate across cell lipid bilayers (Alberts et al. 2002). The permeability of the lipid bilayer is dependent on its viscosity (Bloom et al. 1991), which is dependent on factors such as temperature and membrane composition. The presence of glycoproteins and cholesterol is inversely proportional to membrane viscosity. In health, around 5% of the lipid bilayer is composed of these glycolipids (Alberts et al. 2002); however, patients with Crohn's disease have been found to have a decreased fraction of cholesterol and related glycolipids in the

lipid bilayer, leading to increased transcytosis of small particles (Aozaki 1989; Pravda 2011). However, these data are taken from erythrocyte analysis of patients with Crohn's disease, and has not yet been substantiated in the intestinal epithelia of Crohn's disease sufferers.

1.1.4 Current clinical therapies to prevent pathologically increased mucosal permeability

Clinical therapies used to prevent the consequences of increased mucosal permeability in the colon do so by limiting the inflammatory response, thereby reducing the effect of the inflammatory cascade upon the membrane-bound and paracellular apparatus involved in epithelial permeability. These therapies target inflammation either at a mucosal or systemic level and are limited by their side effects.

Corticosteroids

Corticosteroids such as hydrocortisone, prednisolone or budesonide are first line treatments for the induction of remission in Crohn's disease and ulcerative colitis (National Institute for Health and Care Excellence 2016a; National Institute for Health and Care Excellence 2016b). Corticosteroids cause immunosuppression through preventing the transcription of multiple pro-inflammatory cytokines (Lichtenstein et al. 2006). However down-regulation of the hypothalamic-pituitary-adrenal axis, and side effects such as moon-face, insomnia, psychosis, osteoporosis, diabetes, hypertension and increased risk of infection impede their continuous use (Yamada 2009).

Aminosalicylates

Aminosalicylates, such as mesalazine, are second line therapy for Crohn's disease and ulcerative colitis, and induce immunosuppression by inhibiting lymphocyte function and production of immunoglobulin through down-regulation of cyclooxygenase (COX), lipoxygenase and NF-κB (Greenfield et al. 1993; Desreumaux & Ghosh 2006; Wahl et al. 1998). Potential side effects are agranulocytosis, hepatitis, pneumonitis, hypospermia or pancreatitis (Walker et al. 1997; Stein & Hanauer 2000).

Immunomodulatory agents

Immunomodulators such as, azathioprine (and its metabolite 6-mercaptopurine) and ciclosporine are also potent suppressors of the immune response and may be used to induce or maintain remission in refractory cases of ulcerative colitis or

Crohn's disease. Side effects such as pancreatitis, liver toxicity and pancytopenia in the case of azathioprine, and renal failure, uncontrolled hypertension and subarachnoid haemorrhage in the case of ciclosporine limit their use (Lennard 1992; Dubinsky et al. 2003; Haber et al. 1986; Actis et al. 1998; Cohen et al. 1999).

Biological agents

Lastly, anti-TNFα antibodies such as infliximab are effective in reducing the inflammation of ulcerative colitis and Crohn's disease, though are limited in clinical practice by spontaneous opportunistic infections such as toxoplasmosis and aspergillosis, and secondly by their expense: a course of infliximab costs £12,584 (Salvana & Salata 2009; Dretzke et al. 2011; National Institute for Health and Care Excellence 2010).

1.2 The endocannabinoid system (ECS)

The colloquial use of *Cannabis sativa* for its analgesic effects have been well described both in historical and contemporary academic literature (Izzo & Camilleri 2008). The interest surrounding the psychoactive properties of *Cannabis sativa* lead to the characterisation of its active major compounds Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) (Turner et al.; Mechoulam & Hanus 2000). Discovery of the human receptors for these ligands followed, with the cloning of the cannabinoid receptor 1 (CB_1) and cannabinoid receptor 2 (CB_2) receptors (Matsuda et al. 1990; Munro et al. 1993). Endogenous ligands to these receptors were described shortly afterwards with anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Devane et al. 1992; Mechoulam et al. 1995; Sugiura et al. 1995), with the establishment of their metabolic pathways (Alhouayek & Muccioli 2012). Further endocannabinoid-like molecules with parallel metabolic pathways such as palmitoylethanolamide (PEA), oleoylethanolamide (OEA) and virodhamine have emerged which possess affinity for receptors such as the orphan receptors G Protein-coupled Receptor 55 (GPR55) and G Protein-coupled Receptor 119 (GPR119), and the nuclear receptor peroxisome proliferator-activated receptors PPARα (Porter et al. 2002; Borrelli et al. 2015; Petrosino et al. 2015). Since their discovery, these receptors and ligands have been identified throughout mammalian species.

1.2.1 Receptors of the ECS

CB_1 and CB_2

CB_1 and CB_2 are G protein-coupled receptors (Howlett et al. 2002). Expression of CB_1 has been well characterised in the brain, but is also found throughout the

autonomic nervous system and the gut mucosa (Witkamp 2015). Within neuronal tissue, CB_1 is located presynaptically and acts to modulate synaptic transmission by a retrograde signalling mechanism, however in epithelial tissues it is expressed at the apical and basolateral membranes (Ohno-Shosaku & Kano 2014; Wright et al. 2005). In the healthy gut, CB_1 is found on epithelial crypt cells, myenteric neural cells and submucosal nerve plexi, smooth muscle of submucosal blood vessels, smooth muscle in the bowel wall and the plasma cells of the lamina propria (Izzo, Fezza, et al. 2001; Massa et al. 2004). Activation of CB_1 via intracellular $G_{i/o}$ coupling, causes inhibition or activation of several downstream enzymes including adenylyl cyclase (Howlett et al. 1986), protein kinase A (PKA) and cyclic adenosine monophosphate (cAMP) (Felder et al. 1995). Activation also induces intracellular calcium release from intracellular reservoirs and potassium channel opening resulting in cell membrane hyperpolarisation (McAllister et al. 1999; Ross et al. 2008).

CB_2 is less widely expressed within the nervous system, though has dense expression on immune cells, which are upregulated during times of inflammation. In the normal gut, CB_2 is found at low levels at the base of mucosal crypts in the small intestine, and on the surface of sub-epithelial macrophages plasma cells, myenteric plexi, and muscularis mucosae of the small and large intestine (Wright et al. 2005; Wright et al. 2008). Activation of CB_2 also inhibits cAMP and adenylyl cyclase and has been shown to inhibit TNFα via the NF-κB and Ras Homolog gene family A (RhoA) pathways (Felder et al. 1995; Rajesh et al. 2007). Additionally, CB_2 activation stimulates Mitogen activated protein kinase (MAPK), protein kinase C (PKC), phosphoinositide 3-kinase, Raf-1/Erk and the Krox 24 cell signalling pathways (Bouaboula et al. 1995; Sánchez et al. 2003), whereas antagonism of CB_2 causes phospholipase C-mediated release of intracellular Ca^{2+} reservoirs resulting in cellular hyperpolarisation (Zoratti et al. 2003).

In acute inflammatory states, CB_1 expression is up-regulated on epithelial surfaces, and appears submucosally on Th1 cells (Galiègue et al. 1995). Epithelial CB_2 expression is also up-regulated in inflammation, with preference to points of ulceration and within epithelial crypts corresponding with Paneth and M-cells, and on activated innate immune cells (D'Argenio et al. 2007). Expression of these receptors correlates with disease severity (Massa et al. 2004). The reason for these changes in expression is not yet clear, though may act as a mechanism of immune tolerance to luminal contents. Paneth and M cells present antigen to adaptive submucosal immune cells, and act as the interface between the luminal contents and the submucosal immune response (Vaishnava et al. 2008; Kucharzik

et al. 2001). Low or absent levels of CB_2 expression in these zones in health compared with enhanced expression in inflammation suggests either a pro- or anti-inflammatory role for the receptor. As CB_2 activation reduces cytokine secretion and proliferation of T and B cells (Lunn et al. 2006; Cabral & Staab 2005; Klein 2005), we may hypothesize that CB_2 may act as a brake on the immune response, and promote tolerance of the noxious luminal contents.

Transient receptor potential vanilloid receptors

Transient receptor potential vanilloid receptors (TRPVs) are members of the TRP superfamily of receptors. They are six transmembrane-domain cation channels that are non-selective (Jordt & Julius 2002). Other members of this superfamily include transient receptor potential melastatin (TRPM) and transient receptor potential ankyrin (TRPA) (Jordt & Julius 2002).

The TRPVs 1 to 4 are most readily known for TRPV1being the receptor to capsaicin, the "heat" inducing compound found in chilli. TRPV1 specifically is also activated by heat and physical pressure, resulting in the sensation of pain (Caterina et al. 1999). TRPV1 has been widely investigated for its effect on visceral pain perception, and in the gut is located on myenteric neurons and intestinal epithelial surfaces (Sun et al. 2007; Massa et al. 2006; de Jong et al. 2014; Holzer 2011). Phyto- and endocannabinoid ligands activate TRPV1, and also other TRP receptors, though require facilitated transport across the cell membrane prior to activation (De Petrocellis & Di Marzo 2005; L De Petrocellis et al. 2001). Interestingly, stimulation of TRPV1 in the gut has been shown to have both a pro- and anti-inflammatory effect mediated through tachykinin (pro-inflammatory) and somatostatin (anti-inflammatory) release (Holzer 2011). Activation of TRPV1 also causes intracellular calcium release initiating cellular depolarisation leading to cellular signalling through substance P, acetylcholine and calcium gene-related peptide release (CGRP) (Zygmunt et al. 1999). TRPV1 transcription in the gut falls in response to inflammation (De Petrocellis et al. 2012).

Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors are a group of nuclear hormone receptors that act as transcription factors upon activation (O'Sullivan 2007). Three main subtypes have been identified; α, δ(β) and γ. They are found throughout the gastrointestinal tract, within adipose tissue, liver and brain (Lemberger et al. 1996; Moreno et al. 2004) . Despite having little structural resemblance to the classical cannabinoid receptors, PPARs are also activated by phyto- and endocannabinoid ligands (O'Sullivan 2007). During episodes of inflammation PPAR expression within epithelial cells increases. Stimulation of the PAPRα and PPARγ receptors has an

anti-inflammatory effect (O'Sullivan 2007), PPARγ may play a role in shuttling of luminal contents across the gut barrier (Kelly et al. 2004).

GPR55

GPR55 is an "orphan" G protein-coupled receptor, as when initially cloned was not linked conclusively with an endogenous ligand. At first, it was suggested that GPR55 was the "CB_3" receptor, however this has since been found not to be the case as, although endocannabinoid ligands activate this receptor, the concentrations required to do so are much higher than for CB_1 or CB_2 (Lauckner et al. 2008). GPR55 has remained in the family of cannabinoid receptors as it has been shown to be activated by phyto- and synthetic cannabinoids (Krohn et al. 2016). GPR55 is expressed throughout dorsal root ganglia, and within the myenteric plexus of the gastrointestinal tract, as well as on vascular endothelium and the brain (Ryberg et al. 2007; Lauckner et al. 2008). Activation of GPR55 raises intracellular calcium through release from intracellular calcium reservoirs via phospholipase C and RhoA kinase, caused by coupling to $G_{\alpha 13}$ and G_q rather than via $G_{i/o}$ proteins as with CB_1 and CB_2 (Waldeck-Weiermair et al. 2008; Henstridge et al. 2009). GPR55 signalling has been shown to be dampened by concurrent CB_1 activation, and is anti-inflammatory in the gut (Waldeck-Weiermair et al. 2008; Krohn et al. 2016).

GPR119

GPR119 is another orphan G protein-coupled receptor which binds two of the endocannabinoid-like ligands OEA and 2-OG (Overton et al. 2006). GPR119 is exclusively found within the gastrointestinal tract and pancreas in humans and rats (Soga et al. 2005). Activation causes cellular transduction through the cAMP or phosphatidylinositol pathways, and first gained interest because this increases release of insulin (Gilman 1987; Chu et al. 2007). Several synthetic ligands are currently being tested in clinical trials for the treatment of diabetes (Ritter et al. 2016). The effect of GPR119 activation in inflammatory states has not been investigated.

1.2.2 Ligands of the ECS

The endogenous ligands to the CB_1 and CB_2 receptor are derived from arachidonic acid, synthesised locally on demand from membrane precursors rather than being stored in vesicles (Piomelli 2003; Schmid et al. 1990). They have been isolated in all organ systems in the body but their complete functions in physiology are not yet fully defined (Kunos et al. 2002; Fonseca et al. 2005). Molecules found within this group are fatty acids associated around an arachidonic acid-ethanolamine or

arachidonic acid-glycerol bond (Porter et al. 2002). Broad actions of the cannabinoids are summarised in table 1.3.

Anandamide

Anandamide (N-arachidonoyl ethanolamide, AEA) belongs to the N-acylethanolamine (NAE) subclass of fatty acid amides. A two-step process synthesises AEA; firstly N-acyltransferase creates N-arachidonoyl phosphatidylethanolamine (NAPE) by combining the acyl group from abundantly available glycerophospholipid and phosphatidylethanolamine (Cadas et al. 1997; Di Marzo et al. 1994). This molecule then undergoes cleavage of its acylethanolamide group by the enzyme N-acylphosphatidylethanolamine-selective phospholipase D (NAPE-PLD) to create AEA (Deutsch & Chin 1993; Wang & Ueda 2009). AEA, as with all the endocannabinoids, undergoes reuptake into the cell where it is rapidly hydrolysed into the inactive substrates ethanolamine and arachidonic acid by fatty acid amide hydrolase (FAAH) (Wang & Ueda 2009). AEA has highest affinity for the CB_1 receptor, but also activates CB_2, $TRPV_1$, PPARα and PPARγ (Devane et al. 1992; Smart et al. 2000; Maccarrone et al. 2000; O'Sullivan et al. 2009; Bouaboula et al. 2005; Sun et al. 2006).

Within the last decade, AEA has been shown to play physiological roles in the gastrointestinal tract. Administration of AEA induces hyperphagia in partially satiated rats via CB_1 activation (Gómez et al. 2002), suggesting that AEA prepares gut physiology for food or may act as a hunger signal to the brain, a hypothesis supported in both animal and human models (Bellocchio et al. 2008; Cristino et al.; Borrelli & Izzo 2009; Romano et al. 2015).

	THC	CBD	AEA	2-AG	PEA	OEA
Motility/ peristalsis		Decreases motility (J. M. Jamontt et al. 2010)	Decreases intestinal motility (Calignano et al. 1997)	Increases peristalsis (Bashashati et al. 2012)	Decreases motility (Capasso et al. 2014)	
Permeability/ ion transport	Decreases permeability (Alhamoruni et al. 2012)	Decreases permeability (Alhamoruni et al. 2012)	Increases permeability *in vitro* (Alhamoruni et al. 2010)	Increases permeability *in vitro* (Alhamoruni et al. 2010)	Decreases permeability (Borrelli et al. 2015)	Decreases permeability
Secretion			Gastro protective – increases mucus secretion (Sasso et al. 2015)	Gastro protective (Kinsey et al. 2011)	Decreases gut secretion (Borrelli et al. 2015)	
Nociception/ visceral pain	Decreases nociception (Beltramo 2009)	Decreases nociception (Hammell et al. 2015)	Decreases visceral pain (Kaczocha et al. 2015)	Potent analgesic agent (Ghosh et al. 2015)	Centrally acting analgesic (Guida et al. 2015)	Decreases nociception (Thabuis et al. 2008)
Hunger/food intake	Increases food intake (Gorter et al. 1992)	Prevents hyperphagia (Scopinho et al. 2011)	Gut levels rise during periods of starvation Stimulates hyperphagia (Gómez et al. 2002)	Prevents hyperphagia (Dinh et al. 2004)		Decreases hunger and food intake (Astarita et al. 2006)
Gastric emptying			Decreases gastric emptying (Troy-Fioramonti et al. 2015)			
Nausea	Anti-emetic (Tramèr et al. 2001)	Anti-emetic (Parker et al. 2011)	Anti-emetic (O'Brien et al. 2013)	Anti-emetic (Parker et al. 2011)(		
Inflammation	Anti-inflammatory (Do et al. 2004)	Anti-inflammatory (Costa et al. 2004)	Anti-inflammatory (Nicotra et al. 2013; Battista et al. 2012)	Anti-inflammatory (Alhouayek & Muccioli 2012)	Anti-inflammatory (De Filippis et al. 2009)	Anti-inflammatory (Guzmán et al. 2004)
Cell proliferation	Tumour suppressant (Maccarrone 2013)	Tumour suppressant (Chakravarti et al. 2014)	Induces apoptosis in cancer cells (Maccarrone et al. 2000)	Tumour suppressant (Nithipatikom et al. 2004)	Tumour suppressant (Sailler et al. 2014)	

Table 1-3 Summary actions of the endo- and phytocannabinoids

In rodents, exogenous application of AEA inhibits gastric emptying and decreases intestinal motility (Calignano et al. 1997; Troy-Fioramonti et al. 2015), inhibiting excitatory neurons innervating enteral and colonic circular smooth muscle prolonging entero-colonic transit time (Hinds et al. 2006; Wong et al. 2012). AEA has been shown to cause relaxation of the lower oesophageal sphincter via CB_1 receptors (Fioramonti & Bueno 2008). Additionally, oral administration of AEA increases gastric mucus secretion in mice, thereby preventing gastritis caused by capsaicin-induced hyper secretion of gastric acid, which may be a consequence of COX-2 metabolism, increasing prostanoid concentration, rather than direct TRPV1 activation (Sasso et al. 2015; Minowa et al. 2005).

AEA possess anti-nociceptive qualities; prevention of AEA breakdown, by inhibiting FAAH, reduces visceral nociception in mice via CB_1 receptors (Kaczocha et al. 2015; Clapper et al. 2010). This anti-nociceptive effect of increasing AEA concentration at CB_1 and TRPV1 has also been found outside the gut (Starowicz et al. 2013). Prevention of AEA reuptake with the reuptake-inhibitor ARN272, or inhibiting the action of FAAH, reduces vomiting and nausea in rodent models of emesis, though no human clinical evidence currently exists for its use (Van Sickle et al. 2005; Darmani 2002). Lastly, AEA has demonstrated potential as a chemotherapeutic agent. Several centres have reported the apoptosis of colon cancer cells treated *in vitro* with AEA acting via CB_1 and TRPV1 receptors. *In vitro* application of AEA to skin, prostate and breast cancer cells reduces cell proliferation or causes apoptosis via CB_1 receptor activation (Melck et al. 1999; Mimeault et al. 2003; Maccarrone et al. 2000).

The gut inflammatory response is affected by AEA, and may be a component of luminal immune tolerance. Treatment with AEA, or inhibiting its breakdown prevents or reverses inflammation in experimental models of colitis both in human and murine tissue (Nicotra et al. 2013; Di Sabatino et al. 2011; Harvey et al. 2013; Storr et al. 2008). AEA also reduces the concentration of the pro-inflammatory cytokines TNFα and IFNγ from supernatants taken from human IBD samples, though the mechanism by which this occurs is not clear as it does not occur though inhibition of the COX pathway as previously suggested (Di Sabatino et al. 2011; Harvey et al. 2014). In cell culture models, AEA concentration-dependently reduced T-cell proliferation and down-regulated the production of pro-inflammatory cytokines in a CB_2 dominant manner (Cencioni et al. 2010). In addition, CB_2 receptor activation with AEA or synthetic CB_2 agonists reduced cytokine secretion and proliferation of cultured T and B cells (Cabral & Staab 2005; Klein 2005; Lunn et al. 2006). Last, under inflammatory conditions, AEA levels in murine colonic

tissue are increased compared to normal controls, supporting the principle that cannabinoid receptor activation prevents immune hyper-activation (D'Argenio et al. 2006). Despite these effects, AEA has not yet been used in humans because of central side effects including anxiety and depression (Moreira et al. 2009).

2-Arachidonoyl-glycerol (2-AG)

2-AG, the second endocannabinoid to be identified, is an ester of arachidonic acid and glycerol (Sugiura et al. 1995). It is formed on-demand from membrane-derived diglycerides by the action of diacylglycerol lipase (DAGL) by liberation of a mono-acyl group (Hashimotodani et al. 2013). 2-AG is rapidly cleaved by the action of monoacyl-glycerol lipase (MAGL) into arachidonic acid and glycerol (Simpson et al. 1991; Blankman et al. 2007) . 2-AG has a higher affinity for the CB_2 receptor compared to the CB_1, and activates PPARα,PPARγ, and TRPV1 with a similar efficacy to AEA (Steffens et al. 2005; Sugiura et al. 1999; Stella et al. 1997; Mechoulam et al. 1995; Kozak et al. 2002; Rockwell et al. 2006).

Initially considered to be a centrally acting ligand, 2-AG also acts peripherally in a similar pharmacological profile to AEA. Increasing concentrations of 2-AG produces potent anti-nociceptive effects both centrally and peripherally, seen with 2-AG administration and inhibition of MAGL activity with JZL184 and URB602 (Scalvini et al. 2015; Guindon et al. 2011; Ghosh et al. 2015; Long et al. 2008; Long et al. 2009) . 2-AG also modulates inflammation in a similar fashion to AEA. 2-AG prevents gastric haemorrhage caused by diclofenac use in mice, and has been shown to prevent macrophage and neutrophil aggregation in ischaemic brain lesions acting through both CB_1 and CB_2 (Kinsey et al. 2011; Murikinati et al. 2010; Anthony Jalin et al. 2015). Furthermore 2-AG was shown to lessen the impact of ischaemia-reperfusion injury in rat myocardial tissue acting predominantly through CB_2 (Lépicier et al. 2003). During acute neuro-inflammation, 2-AG levels rise sharply, suggesting that, given its anti-inflammatory effects *in vitro*, it may act as an endogenous anti-inflammatory agent. It is likely that any anti-inflammatory effect of 2-AG is mediated by inhibition of COX-2 expression via down-regulation of the MAPK/NF-κB signalling pathway, although this may not be the case as 2-AG is readily metabolised by COX-2, either directly or via prior metabolism into arachidonic acid (Chen et al. 2011). In this study 2-AG was shown to act through CB_2, PPARα and PPARγ receptors. 2-AG has been shown to reduce nausea and vomiting in hyper-emetic rats, acting via both CB_1 and CB_2, though no studies currently exist examining for this effect in humans (Sticht et al. 2015; Parker et al. 2015). 2-AG levels fall upon commencement of a high fat diet, with a concurrent rise in MAGL activity, demonstrating a role in fat assimilation; whilst paradoxical

overexpression of MAGL leads to obesity in mice (Dinh et al. 2004). Furthermore rising 2-AG levels caused by MAGL inhibition produces tumour suppression in prostate cancer cells acting via CB_1 receptors(Nithipatikom et al. 2004).

2-AG concentration is also increased in colonic tissue under inflammatory conditions compared to normal controls, both in human and mouse models (Borrelli et al. 2015; Borrelli & Izzo 2009). Inhibition of 2-AG breakdown though MAGL inhibition decreases macro- and microscopic colitis scores in mice through decreased local production of TNFα and IL-6, suggesting that 2-AG, like AEA, plays a role in immune tolerance and possibly barrier function (Alhouayek et al. 2011).

Palmitoylethanolamide

Palmitoylethanolamide (PEA), also known as *N*-palmitoylethanolamine, is a structural analogue of AEA, and has been detected in multiple organ systems in humans (Long & Martin 1956; Kuehl et al. 1957; Bachur et al. 1965; Schmid et al. 1997; Schuel et al. 2002; Izzo et al. 2010). PEA is an conjugate of a palmitic acid group and ethanolamine, and hence belongs to the NAE subgroup with AEA (Hansen 2010). The final step in the synthesis of PEA is accomplished by NAPE-PLD in a similar fashion to AEA. PEA may be broken down by FAAH, although an additional enzyme, N-acylethanolamine-hydrolyzing acid amidase (NAAA), hydrolyses PEA with 30 times greater efficacy (Tsuboi et al. 2007; Mackie & Stella 2006). PEA is synthesised independently of AEA and 2-AG (Cadas et al. 1997). PEA does not act as a ligand at the CB_1 or CB_2 receptors, and is therefore not classed as a true endocannabinoid (Jesse Lo Verme et al. 2005). It acts as an agonist at TRPV1 PPARα, GPR55 and GPR119 (Costa et al. 2008, Lo Verme et al. 2006, Ryberg et al. 2007)

PEA is noted for its anti-inflammatory, analgesic and neuroprotective effects (De Filippis et al. 2011; Loría et al. 2008) and, as PEA levels rise in the colon in response to inflammation, may also provide a mechanism of luminal-immune tolerance (Borrelli et al. 2015; Darmani et al. 2005; Esposito et al. 2014; Alhouayek et al. 2015). In murine studies using a 2,4,6-trinitrobenzenesulfonic acid-(TNBS) induced colitis model, intraperitoneal administration of PEA prevented severe colitis in two separate experiments. This effect was mediated through PPARα and GPR55 (O'Sullivan 2007; Borrelli et al. 2015; Alhouayek et al. 2015). TNBS and dinitrobenzene sulfonic acid (DNBS) are oxidative stressors that induce an inflammatory reaction in the colon, which on histopathological examination closely resemble ulcerative colitis (Scheiffele & Fuss 2002). In subsequent studies, PEA was shown to reduce cytokine and metalloproteinase production, with associated down regulation of NF-κB activity in a DNBS model of colitis (Cuzzocrea et al. 2014),

and reduced neutrophil chemotaxis and myeloperoxidase (MPO) activity (Esposito et al. 2014; Borrelli et al. 2015). Several other animal studies have demonstrated that PEA is anti-inflammatory peripherally by decreasing mast cell activity and neutrophil proliferation (Cerrato et al. 2010; De Filippis et al. 2009).

The mechanism by which PEA brings about these effects has been debated. It has been suggested that through PPARα and GPR55 activation, PEA indirectly activates the CB_1 and CB_2 receptors by increasing local AEA concentrations via substrate competition for FAAH, and increases 2-AG concentrations through TRPV1 agonism, however none of these assertions have yet been proven definitively (Lambert & Di Marzo 1999; Capasso et al. 2014; Petrosino et al. 2015). This has been called the entourage effect (Jonsson et al. 2001). Furthermore, PEA administration prevents falls in TRPV1 and GPR55 expression caused by inflammation, and up regulates CB_1 receptor expression (De Petrocellis et al. 2002). This suggests that, in addition to competing for FAAH, PEA also up-regulates the transcription of CB_1 receptor which may then be activated by AEA, bringing about anti-inflammatory actions. Could be more detailed in terms of molecular detail

PEA, which has no observed side effects (Keppel Hesselink & Hekker 2012), is available throughout Europe as a herbal remedy. At least six trials in the last 30 years have suggested that administration of PEA for influenza and hypersensitivity reactions may relieve symptoms. However these trials were not of a high-enough standard to draw firm conclusions (Keppel Hesselink et al. 2013). Significantly, no adverse events in experimental groups were reported. In two clinical trials and one case series examining the effects of PEA on ocular hypertension, improved symptomatology and no side effects were found at 600 mg oral daily dosage (Keppel Hesselink & Hekker 2012; Strobbe et al. 2013; Costagliola et al. 2014). PEA is currently being used in a phase two clinical trial measuring the effect of the drug on mast cell activity and pain in irritable bowel syndrome patients (Comelli 2012).

Oleoylethanolamide

Oleoylethanolamide (OEA) is an amide of ethanolamine and oleic acid; like AEA, the critical enzyme responsible for its production is NAPE-PLD (Martínez de Ubago et al. 2009; Borrelli & Izzo 2009; Thabuis et al. 2008). NAAA and FAAH hydrolyse OEA into ethanolamine and oleic acid. OEA is an agonist of the PPARα, TPRV1, GPR55 and GPR119 receptors (Fu et al. 2003; Borrelli & Izzo 2009).

OEA plays a role in the regulation of feeding and energy balance. OEA levels within the gut are low during periods of starvation, and rise steeply after feeding (Astarita et al. 2006). Administration of OEA to partially starved mice prevented feeding;

this effect was also seen in mice who had undergone prolonged starvation (Romano et al. 2014). However, these effects were not reproducible in knock-out (KO) mice lacking the PPARα receptor (Fu et al. 2003). OEA rises may therefore act as a mechanism to provide sensations of satiety and prevent overfeeding. OEA administration also prevented the overfeeding caused by AEA administration, demonstrating an inverse balance mechanism between the two compounds, although not acting at the same receptor (Gómez et al. 2002). OEA must act peripherally in controlling hunger, as intracerebral administration of OEA to mice does not prevent feeding, unlike intraperitoneal administration (J Lo Verme et al. 2005; Capasso & Izzo 2008). However, its release may be centrally controlled as division of the coeliac and superior mesenteric ganglions of mice prevented feeding-induced rises in OEA production (Fu et al. 2011). This has not yet been tested in humans, though might provide an alternative to contemporary anti-obesity therapy as OEA administration to obese rats has been shown to reduce body weight and serum cholesterol levels (Decara et al. 2012).

In addition to its effects on hunger and feeding, OEA has demonstrated, in a peripheral model of chemical inflammation, that it may reduce oedema in soft tissues acting via PPARα (Guzmán et al. 2004). It has also been shown to prevent neurological damage induced by inflammation secondary to ischaemia and reperfusion injury in the mouse brain, acting through PPARα (Zhou et al. 2012). OEA also possesses anti-nociceptive properties, though this is achieved through an unknown receptor including PPARα (Thabuis et al. 2008).

1.2.3 Phytocannabinoid ligands

Δ^9-Tetrahydrocannabinol

THC activates CB_1, CB_2, TPRV1 and PPARγ receptors (O'Sullivan et al. 2005; Jordt et al. 2004; Izzo et al. 2000). It undergoes hepatic metabolism by the cytochrome P450 enzymatic system into 11-hydroxytetrahydrocannabinol, which is also able to activate CB_1 and CB_2 receptors (Maykut 1985; Ashton 2001). In humans, complete excretion of THC may take up to 30 days, as gut excretion allows reuptake and then further pharmacological effects (Ashton 2001). THC has been used colloquially as a treatment for IBD, epilepsy and pain for many centuries despite inconsistent side effects such as anxiety, paranoia, hypotension and depression (Moreira et al. 2009). THC is currently licenced in the United Kingdom for the treatment of spasticity in multiple sclerosis as Sativex (which includes cannabidiol). THC is also available as dronabinol (p.o.) or the synthetic drug levonantradol (i.m.), which have been used to alleviate anorexia in patients with human immunodeficiency virus or prevent

anorexia during chemotherapy (Struwe et al.; Gorter et al. 1992). A large systematic review of THC found it to be at least as effective as centrally acting anti-emetics such as ondansetron, though was limited in its effectiveness by psychotropic side effects and reduction in alertness (Tramèr et al. 2001). The anti-nociceptive properties of THC act via CB_1 and CB_2, and is likely to act both on neurons peripherally and centrally (Beltramo 2009). Central activation of these receptors with THC, predominantly CB_1, leads to region-dependent dopamine release and, in addition to anti-nociception, is likely to be the basis of its recreational use (Tanda et al. 1997).

THC is an anti-inflammatory agent. Treatment of dendritic cells with THC, acting via CB_1 and CB_2, promotes apoptosis; caused by induction of caspase -2, -8 and -9 acting through NF-κB (Do et al. 2004). Furthermore, treatment of neutrophils with THC caused down regulation of cytokine production acting via the same mechanism (Nagarkatti et al. 2009). THC has also been shown to induce apoptosis in T-lymphocytes, macrophages and murine thymocytes, achieved primarily through CB_2 agonism (Guzmán 2005; Jia et al. 2006; McKallip et al. 2002). Lastly, it has been proposed that activation of the CB_1 and CB_2 receptors with THC is potentially tumoricidal (Maccarrone 2013). THC was shown to prevent progression of prostate cancer cells via CB_1 (Ruiz et al. 1999; Carrier et al. 2006).

Cannabidiol

CBD is a non-psychotropic compound also present in *Cannabis sativa* (Adams & Hunt 1940). It is a weak agonist of CB_1 and CB_2 compared to THC (Pertwee 1999), though acts as a ligand at TRPV1 (Bisogno et al. 2001), GPR55 (Ryberg et al. 2007), the 5HT-1a serotonergic receptor (Mishima et al. 2005) and the adenosine A_2 receptor on microglial cells. CBD is also metabolised by cytochrome P450, and excreted by the kidneys and into the gut where further reabsorption is possible, leading to a long half-life (Huestis 2007).

CBD has been shown to act as an anti-convulsant, anxiolytic, antioxidant, anti-emetic and potential anti-psychotic agent (Jones et al. 2012; Crippa et al. 2004; Hampson et al. 1998; Parker et al. 2011). Currently CBD is available clinically in the US from 2mg/kg to 25mg/kg orally as Epidiolex, used in the prevention of infantile epilepsy (O'Connell et al. 2017). Although the use of this drug is possible in the UK, it has not yet been recommended for routine use in clinical commissioning groups in the UK due to the cost. CBD has demonstrated anti-inflammatory effects through four mechanisms (Costa et al. 2004): apoptosis of activated innate immune cells, suppression of immune cell proliferation, reduction of cytokine and chemokine transcription and proliferation of regulatory T cells (Rieder et al. 2010). This was

evidenced by demonstrating that CBD induced formation of caspase-3 and -8 in activated splenocytes. Furthermore, CBD supressed activated peritoneal macrophage IFNγ and interleukin-8 (IL-8) production and down-regulated IL-2 cell surface receptor expression in human B cell lines (Kaplan et al. 2008). This experiment was repeated using CB_1-/- and CB_2 -/- KO splenocytes and found the effects of CBD not to act through these receptors, but is regulated by several transcription factors, including Activator Protein-1 and the Nuclear Factor of Activated T Cells.

In the gut, CBD has been shown to reduce colonic inflammation. In several murine models of caustic agent-induced colitis, CBD prevented both macroscopic and microscopic inflammatory changes in the colon, prevented increased production of inducible nitric oxide synthase and IL-1β, decreased activity of MPO and increased the production of the anti-inflammatory cytokine IL-10 (Schicho & Storr 2012; de Filippis et al. 2011; Borrelli et al. 2009; J. Jamontt et al. 2010). This was also shown to translate into human colonic inflammation, as CBD decreased inflammation-induced histological changes in human colon mucosal biopsies treated with pro-inflammatory cytokines (Harvey et al. 2014). There is further evidence that CBD may prevent the breakdown of the epithelial barrier, as one study using caecal ligation and puncture to induce inflammation found that mice treated with CBD showed decreased oxidation of peripheral organs (such as lung tissue) compared to controls, suggesting a decreased systemic inflammatory load. It is possible in this experiment that CBD prevented increased transfer of cytokines into the systemic circulation, preventing these peripheral changes. However, this hypothesis has not been proven.

CBD has few side effects, and up to 600 mg p.o. daily has been reported as a safe dose in humans (Zhornitsky & Potvin 2012). Potential side effects may be inhibition of hepatic drug metabolism and decreased activity of P-glycoprotein drug transport (Bergamaschi et al. 2011).

Other phytocannabinoid ligands

In addition to THC and CBD, some of the other compounds found within *Cannabis sativa* have been used to treat experimental colitis. For example, cannabigerol (CBG), cannabichromene (CBC) and cannabinol (CBN) have been used to treat murine colitis induced by CO or DNBS administration, and reduced inflammation-induced hypermotility, MPO activity, histological disease activity index (DAI), inducible nitric oxide synthase and IL-1β production (Izzo, Fezza, et al. 2001; Izzo et al. 2012; Borrelli et al. 2013). These compounds have not yet individually been investigated in humans, although they are present in cannabis sativa.

1.2.4 Cannabinoids as potential therapies for IBD

Understanding the mechanisms underlying the function of the ECS may shed light on new therapies to prevent the barrier breakdown seen in Crohn's disease and ulcerative colitis. There is already a body of cell-based evidence which demonstrates that cannabinoids play a role in epithelial permeability both in physiological conditions and during episodes of inflammation.

In vitro studies

Several studies have examined the effect of the cannabinoids on cultured monolayers. In a previous series from this centre using Caco-2 cells, monolayers were treated with inflammatory cytokines (TNFα and IFNγ 10ng/ml) causing a fall in TEER (Alhamoruni et al. 2012). The cannabinoids THC, CBD, AEA and 2-AG were applied to the membranes either apically (to simulate luminal drug application), or basolaterally (to simulate systemic drug application). The authors found that the increased permeability caused by cytokine application was returned to baseline with apical application of THC or CBD (10 μM) in a concentration-dependent fashion. This was abolished by administration of the CB_1 receptor antagonist AM251. Conversely, apical application of AEA and 2-AG (10 μM) worsened the increase in permeability, again acting through CB_1. These changes were potentiated further by the additional administration of the FAAH inhibitor URB597 and MAGL inhibitor JZL184. In a further experiment, basolateral PEA and OEA application prevented falls in TEER acting via TRPV1 receptors (Karwad et al. 2016). In the same experiments, basolateral application of OEA, acting via PPARα, increased falls in TEER. This set of experiments demonstrated that cannabinoids modulate permeability of epithelial monolayers, though did not investigate how this occurs.

In a parallel study from another centre, Caco-2 monolayers were treated with a different inflammatory protocol (TNFα and IL-1β 100ng/ml) (Harvey et al. 2013). The falls in TEER observed with the inflammatory protocol were not affected by basolateral application of CBD (10^{-5} M), AEA (10^{-6} M), the CB_2 selective inverse agonist JTE-907 (10^{-7} M) or the CB_1 selective agonist arachidonoyl chloroethylamide (ACEA, 10^{-7} M). Apical administration was not tested. The difference in results in these studies may be due to a differing inflammatory protocol, differing concentrations of ligands used in the studies or may be explained by a tendency towards a higher cell-line passage in the study from Alhamoruni (Alhamoruni et al. 2012).

Studies from cell lines outside of a gut origin also support the ability of the ECS to modulate permeability. In one study, endothelial cells from murine brain microvasculature were co-cultured with astrocytes (Hind, Tufarelli, et al. 2015). An

ischaemia model using oxygen-glucose deprivation achieved decreases in TEER. Luminal application of AEA (10 μM) and OEA (10 μM) prevented falls in TEER under normal conditions. AEA was found to act through CB_2, TRPV1 and CGRP receptors, while OEA acted via CB_2, TRPV1 and PPARα receptors. Application of PEA, OEA and virodhamine reduced the increased permeability caused by reperfusion mediated by PPARα receptors. Further work from this centre demonstrated that, in cultured human brain microvascular endothelium, CBD also prevented increases in endothelial permeability caused by oxygen-glucose deprivation (Hind, England, et al. 2015). From another centre co-culturing astrocytes and brain endothelial cells, the non-selective synthetic CB_1 agonists CP55940 and ACEA prevented cytokine-induced falls in permeability (Lu et al. 2008).

Although there is some heterogeneity across these studies, there is strong evidence that the endocannabinoids are able to influence permeability, however the mechanism is unclear. Given that all the cell culture models above used an inflammatory protocol to initiate changes in baseline permeability, we are able to determine if TEER was protected by reducing the inflammatory response from the cultured cells or by protecting the paracellular apparatus from the inflammatory cascade.

***In vivo* studies**

Few studies examining the effect of the cannabinoids *in vivo* focus on permeability. In a murine study of TNBS-induced colitis, circulating levels of lipopolysaccharide (LPS) were used as a surrogate marker of increased permeability (Alhouayek et al. 2011). Mice which were administered the MAGL inhibitor JZL184 (16 mg/kg i.p.) demonstrated lower circulating serum levels of LPS compared to control mice. Furthermore, mice in the treatment group exhibited lower mRNA expression for pro-inflammatory cytokines in the liver and brain, with lower circulating concentrations of IL-1β, TNFα, Macrophage Inflammatory Protein-1, IL-6, Monocyte Chemotactic Protein-1 (MCP-1) and IFNγ.

In a murine experiment, Borelli administered oral fluorescein isothiocyanate-conjugated dextran (600 mg/kg body weight p.o.), a polysaccharide which is not normally absorbed by the gut, two days after causing TNBS-induced colitis. Serum levels of the dextran were then measured. The authors found that control mice exhibited high levels of serum dextran, whereas mice treated with CBG (1-30 mg/kg i.p.) demonstrated no serum dextrans. Further experimentation found that CBG also had this effect when administered in a prophylactic manner, mediated by CB_2 receptors (Borrelli et al. 2013). An identical experiment from the same centre using PEA (1 mg/kg p.o.) found similarly low serum dextran concentrations in treatment

groups (Borrelli et al. 2015). These effects were abolished by CB_2, PPARα and GPR55 receptor antagonism, but further potentiated by TRPV1 antagonism. These promising results suggest that cannabinoid or related ligands, CBG and PEA in these cases, may prevent the increased gut permeability seen in sepsis. Mechanisms of change in permeability in terms of anti-inflammatory actions or the paracellular apparatus were not investigated.

1.3 Project aims

An underlying defect in Crohn's disease and ulcerative colitis is a hyper-inflammatory state that may be caused by the breakdown of the epithelial barrier. Furthermore, several cannabinoids are able to prevent hyperpermeability of cultured epithelial cells to ions during episodes of inflammation *in vitro*, which may also be the case *in vivo*. Although stimulation of cannabinoid and related receptors has a potent anti-inflammatory action, it is not clear if this anti-inflammatory effect is the mechanism by which changes in permeability are prevented, or if this achieved by direct actions on membrane-bound and paracellular proteins discussed above.

The specific aims of this is PhD project are as follows:

1) To determine, by means of systematic review and meta-analysis, the utility of different cannabinoid drugs in treating inflammatory bowel disorders.

2) To investigate potential cannabinoid agents for their effect on inflammation-induced hyperpermeability using cultured cell lines and human tissue in vitro, identifying a mechanism of action.

3) To investigate the effects of these agents on colonic hyperpermeability in humans *in vivo*.

2 The use of cannabinoids in colitis: a systematic review and meta-analysis

2.1 Introduction

IBD affects 200 per 100,000 adults in the United States and 400 per 100,000 in the UK (Kappelman et al. 2007; Rubin et al. 2000). Major subtypes consist of Crohn's disease and ulcerative colitis. A definitive clinical treatment for these chronic relapsing diseases remains elusive, as currently no therapy exists to reverse the clinical pathology without a risk of significant side effects. 5-ASA agents, corticosteroids, TNFα antagonists and other immunomodulatory drugs have all been shown to induce significant remission in IBD, but are associated with bone marrow suppression, opportunistic infection, infusion reactions and malignancy secondary to immunosuppression (Sandborn et al. 2009; Sutherland et al. 1997; Herrinton et al. 2011).

There is now significant promising preclinical evidence for the use of cannabinoid agents in the treatment of colitis. Early experimentation in murine models has demonstrated that endogenous, plant-derived or synthetic cannabinoids prevent the onset of experimental colitis or reduce its severity (Massa et al. 2004). Since these initial findings, many reports, including clinical trials, have now investigated the effect of cannabinoid ligands, or the effect of blockade of their metabolising enzymes, on inflammation of the gut.

The primary aims of this study were:

- To review systematically existing preclinical and clinical evidence on the use of cannabinoid compounds in colitis.
- To perform meta-analyses across studies using clinically relevant endpoints where possible.

A secondary aim of this systematic review was :

- To identify underlying mechanisms of action of cannabinoid compounds in the gut from existing literature.

2.2 Methods

2.2.1 Search strategy

All studies evaluating the effect of cannabinoid drugs on inflammation of the colon were searched from March 1980 until March 2017 by two independent researchers in Medline, EMBASE and Pubmed. The second independent researcher was Henry Maudslay at the School of Medicine, University of Nottingham. Keywords included cannabidiol, tetrahydrocannabinol, anandamide, 2-AG, cannibichromene, cannabigerol, cannabinoid, Cannabis sativa, colon, intestine, gut, inflammation, Crohn's, ulcerative and colitis. Names of all synthetic cannabinoid agents included in the glossary on page 24 were also included. References from included studies were searched by hand. Pre-specified inclusion and exclusion criteria were used to prevent bias; original research must have been performed in the context of administration of cannabinoid drugs to inflammatory states of the colon in humans or animals, either experimental or due to Crohn's disease or ulcerative colitis. *In vitro* studies or studies not examining the effect of cannabinoids in intestinal inflammation specifically, or studies using cannabinoid antagonists as a primary agent were excluded. A PRISMA checklist is included in appendix 1.

2.2.2 Data acquisition

The mode of colitis induction in preclinical studies was recorded in addition to the timing of cannabinoid application. For the purposes of meta-analysis, data on the macroscopic or histological DAI, and MPO activity were collected. If the exact number of animals was not available, the lowest number of animals within the given range were used for the experimental group, and the highest number used for the control/vehicle group. Where studies reported the effects of more than one cannabinoid sharing a single control group for comparison, control group numbers were equally distributed between comparisons to avoid unit of analysis issues. WebPlotDigitiser (version 3.11 http://arohatgi.info/WebPlotDigitizer/) was used to extract values from figures in published articles where no data values were given in the text.

2.2.3 Quality and risk of bias

In order to assess the quality and risk of bias of preclinical studies, the STAIR and Arrive preclinical assessment tools were adapted (Fisher et al. 2009; Kilkenny et al. 2010). Each of the below were awarded one point: randomisation, assessor blinding, results replicated in a second species, dose-response experiments, results replicated in a second model of colitis, n=5 or greater in each group, the use of

clinically relevant endpoint to assess response of colitis, definitive statement of animal numbers in each group, a statement regarding the housing of animals and a statement describing the location and timing of animal experimentation (i.e. in animal housing or a separate cage, time of day of administration), giving a highest possible score of 10.

As this modified STAIR score (mSTAIR) had not been previously validated, we also assessed risk of bias in animal studies using the SYRCLE (SYstematic Review Centre for Laboratory animal Experimentation) risk of bias score with studies graded out of 10 (Hooijmans et al. 2014).

2.2.4 Data analysis

Where possible, data were grouped into DAI and MPO activity, and subdivided by species and compound. Data from each group were analysed as forest plots using Cochrane Review Manager Software (Review Manager 5.3, Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014), and as funnel plots using STATA (Stat Corp. 2009 Stat Statistical Software: Release 11. College Station, TX, USA). Funnel plot asymmetry was tested using Egger's linear regression test. Correlation between mSTAIR and SYRCLE scores were assessed using Pearson's correlation coefficient. As different studies measured MPO activity and DAI using various scales, effect estimates are presented as standardized mean differences (SMD) with 95% confidence intervals (CI). We used the following SMD values to assess results for clinical significance: < -0.5 small clinical significance, -0.5 to -0.8 moderate clinical significance and >-0.8 high clinical significance based on the observations from Cohen et al (Cohen 1988). Due to clinical heterogeneity between the various studies, a random-effects model was used. We assessed statistical heterogeneity using the I^2 statistic, with >50% regarded as evidence of statistical heterogeneity. Studies were weighted by sample size and statistical significance was set at a minimum of $p<0.05$.

2.3 Results

2.3.1 Search results

The search strategy returned 2008 results from which 199 relevant publications were identified. From these, 53 publications comprising 106 experiments met the inclusion criteria (figure 2.1, table 2.1). Thirty-four studies were included in the meta-analysis.

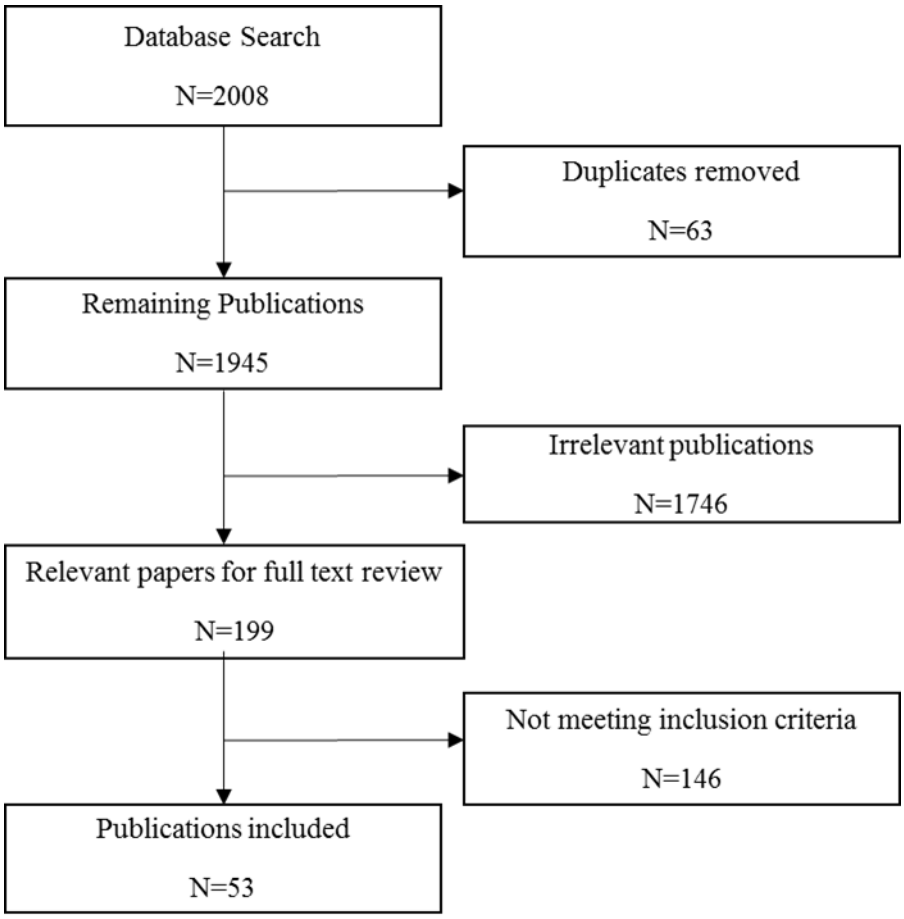


Figure 2.1 PRISMA flow chart of the record identification process.

2.3.2 Study characteristics

Forty-three publications studied the effects of cannabinoids on experimental colitis in mice, 5 in rats, and 3 in both mice and rats. Two clinical trials examined the effect of a cannabinoid (THC and CBD) in Crohn's disease in humans. Within animal publications, 43 used caustic agents (DNBS,TNBS, oil of mustard (OM), dextran sulphate sodium (DSS) and croton oil (CO)) to induce colitis, 6 used intravenous or topical lipopolysaccharide, 2 induced colonic inflammation using surgical arterial ligation or puncture of the colon and 1 induced colitis with IL-10 knock-down and DSS (figure 2.2 A). Across all publications, including clinical trials, 71 endpoints were examined to evaluate the effect of cannabinoid drugs on colitis. Forty-nine publications (89 experiments) examined more than one endpoint. Of these endpoints, DAI and MPO activities were the most consistently used (34 and 26 experiments respectively), and were therefore selected for meta-analysis. Incidence of endpoints is given in figure 2.2 B. what about acute vs repeated treatments?

The effect of 6 phytocannabinoids were studied across 18 publications; CBN, CBD, THC, CBC, CBG and medicinal Cannabis (MMJ). 3 endocannabinoids were studied across 11 publications (PEA, ultramicronized PEA (uPEA), and AEA), 17 synthetic cannabinoid agonists were studied across 22 publications (AM841, Adelmidrol, HU210, CP55,940, WIN55,212-2, AM1241, JHW015, JWH133, β-caryophyllene, O-1602, ACEA, HU308, αβ-amyrin, CID 16020046, compound 26, SAB378 and abnormal CBD (Ab-CBD)), and 9 compounds targeting the catabolism or transport of endogenous cannabinoids were studied across 13 publications (ARN2508, PF-3845, compound 39, JZL184, AA-5HT, VDM11, URB597, AM9053, AM3506). The degree of positivity or negativity of the outcomes of these studies are displayed in figure 2.2 C. Twenty-three studies investigated underlying receptor mechanisms using KO animals or receptor antagonists.

Study	Species	Model	Compound	Route/dosage	Time of administration verses inflammation	Time of assessment post inflammation	Modified STAIR score	SRCYCLE Score
Capasso 2001 (Capasso et al. 2001)	ICR mice	CO	PEA	i.p 2.5-30 mg/kg	20 minutes pre	4 days	4	1
Izzo 2001 (Izzo, *et al.*, 2001)	ICR mice	CO	CP 55,940 Cannabinol	i.p. 0.03–10 nmol/m i.p. 10-3000nmol/m	4 days post	20 minutes	3	0
Massa 2004 (Massa et al. 2004)	C57BL/6 N mice	DNBS	Rimonabant HU210	i.p. 3mg/kg i.p. 0.05 mg/kg	Pre, 24 and 48 hours post	3 & 7 days	4	2
Mathison 2004 (Mathison et al. 2004)	Spr-Dawley rats	LPS	ACEA JWH133	i.p. 1mg/kg i.p. 1mg/kg	70 minutes post	120 minutes	5	0
D'Argenio 2006 (D'Argenio et al. 2006)	C57/BJ mice Wistar rats	DNBS TNBS	VDM11 AA-5-HT	s.c. 5mg/kg s.c. 10mg/kg	Post	3 & 7 days	6	0
Kimball 2006 (Kimball et al. 2006)	CD-1 mice	OM	ACEA JWH133	i.p. 10mg/kg i.p. 2.5mg/kg	24 hours pre	3 days	3	1
Capasso 2008 (Capasso et al. 2008)	ICR mice	CO	CBD JWH015	i.p. 5mg/kg i.p. 10mg/kg	20 minutes pre Ach	4 days	5	0
Engel 2008 (Engel et al. 2008)	AKR mice	TNBS	AEA	i.p. 5mg/kg	30 minutes pre	3 days	3	1
Storr 2008 (Storr et al. 2008)	C57/BL mice	TNBS	URB597 VDM11	i.p. 5mg/kg i.p.5mg/kg	30 minutes pre or 24 hours post	3 days	4	1
Borelli 2009 (Borrelli et al. 2009)	ICR mice	DNBS	CBD	i.p. 1, 2, 5, 10mg/kg	24 hours post	3 days	3	0
Li 2009 (Li et al. 2010)	Rats Mice	LPS	HU210 JWH133 AM630 AM251	100 µg/kg 100 µg/kg 3 mg/kg	5 minutes	30 minutes	8	1
Storr 2009 (Storr et al. 2009)	C57/BL mice	TNBS DSS	JWH133 AM1241 AM630	i.p. 20mg/kg i.p. 10-20 mg/kg i.p. 10mg/kg	30 minutes pre or 24 hours post	1, 3, 5, 7 days	7	1
Cassol Jr 2010 (Cassol-Jr et al. 2010)	Wistar rats	CLP	CBD	i.p. 2.5, 4, 10mg/kg	Simultaneous	9 days	8	2
Cluny 2010 (Cluny et al. 2010)	C57/BL mice	DSS TNBS	SAB378 AM251 AM630 WIN55,212-2	i.p 0.1 or 1.0mg/kg i.p 1.0mg/kg i.p 1.0mg/kg i.p 1, 2mg/kg	4 days post	8 days	5	1

Study	Species	Model	Compound	Route/dosage	Time of administration verses inflammation	Time of assessment post inflammation	Modified STAIR score	SRCYCLE Score
Kimball 2010 (Kimball et al. 2010)	CD1 mice	OM	ACEA JWH133	i.p. 1mg/kg i.p. 1mg/kg	30 minutes pre	28 days	4	3
Jamontt 2010 (J. M. Jamontt et al. 2010)	Wistar rats	TNBS	THC CBD	i.p. 5-20mg/kg i.p. 5-20mg/kg	30 minutes pre	3 days	5	1
Alhouayek 2011 (Alhouayek et al. 2011)	C57BL/6 mice	TNBS	JZL184	i.p. 16mg/kg	Pre onset	3 days	2	1
Andrejak 2011 (Andrzejak et al. 2011)	C57/BL mice	TNBS	Compound 39	i.p. 5mg/kg	3 days pre	3 days	6	1
Bento 2011 (Bento et al. 2011)	CD1 mice	DSS	βCaryophyllene	i.p. 12.5, 25, 50mg/kg	3 -7 days post	7 days	4	1
Defilipis 2011 (de Filippis et al. 2011)	OF1 mice	LPS	CBD	i.p. 10mg/kg	6 hours post	120 minutes	6	1
Lin 2011 (Lin et al. 2011)	C57/BL mice Spr-Dawley rats	LPS	CBD O-1602	i.p. 10mg/kg I.p. 1mg/kg	30 minutes pre	20 minutes	5	1
Schicho 2011 (Schicho et al. 2011)	C57/BL mice	DSS TNBS	O-1602	i.p. 5mg/kg	30 minutes pre	7 days	3	3
Bashashati 2012 (Bashashati et al. 2012)	CD1 mice	LPS	AM3506	i.p. 100ug.kg	20 minutes pre	120 minutes	3	0
Izzo 2012 (Izzo et al. 2012)	ICR mice	CO	CBC	i.p. 15mg/kg	20 minutes pre exam	4 days	5	2
Lehmann 2012 (Lehmann et al. 2012)	Lewis rats	LPS CASP	HU308	2.5mg/kg	15 minutes post	2 – 16 hours	4	0
Schicho 2012 (Schicho & Storr 2012)	C57/BL mice	TNBS	CBD	i.p. 10mg/kg p.o. 20mg/kg p.r. 20mg/kg	30 minutes pre onset	7 days	4	0
Singh 2012 (Singh et al. 2012)	C57/BL mice	IL-10 -/- DSS	JWH133	i.p. 2.5mg/kg	Simultaneous	7 – 14 days	5	1

Study	Species	Model	Compound	Route/dosage	Time of administration verses inflammation	Time of assessment post inflammation	Modified STAIR score	SRCYCLE Score
Borrelli 2013 (Borrelli et al. 2013)	ICR mice	DNBS	CBG	i.p. 30mg/kg	3 days pre	3 days	5	1
Esposito 2014 (Esposito et al. 2014)	CD-1 mice	DSS	PEA	i.p. 10mg/kg	2 days post	7 days	5	2
Li 2013 (Li et al. 2013)	C57/BL mice	DSS	WIN55,212-2	i.p. 5mg/kg	Simultaneous	7 days	4	1
Matos 2013 (Matos et al. 2013)	CD1 mice	DSS	αβ Amyrin	p.o. 1, 3, 10mg/kg	Pre and 3 days post	7 days	6	1
Naftali 2013 (Naftali et al. 2013)	Clinical trial	Crohn's	Cannabis sativa extract (THC)	115 mg inhaled	N/A	8 weeks	NA	NA
Romano 2013 (Romano et al. 2013)	ICR mice	DNBS	CBC	i.p 0.1-1.0mg/kg	24 hours post	3 days	6	0
Wallace 2013 (Wallace et al. 2013)	Wistar rats	DNBS	C. sativa extract AM630	i.c. 6 mg/kg p.o. 10mg/kg	30 minutes pre and 24 hours post	7 days	4	1
Borelli 2015 (Borrelli et al. 2015)	ICR mice	DNBS	PEA	i.p 1mg/kg p.o. 1mg/kg	3 days pre	3 days	5	1
Capasso 2014 (Capasso et al. 2014)	ICR mice	OM	PEA	i.p. 10mg/kg	30 minutes	3 and 7 days	6	2
Fichna 2014 (Fichna et al. 2014)	CD1 mice	DSS DNBS	AM841 CB13	i.p. 0.01, 0.1,1 mg/kg i.p. 0.1 mg/kg	15 minutes pre	3 and 7 days	4	0
Salaga 2014 (Sałaga et al. 2014)	C57/BL mice	TNBS DSS	PF3845	i.p. 10mg/kg p.o. 5mg/kg i.c. 5mg/kg	30 minutes	3 and 7 days	2	0
Sardinha 2014 (Sardinha et al. 2014)	C57/BL mice	LPS	HU308 AM630 URB597 JZL184	i.v. 2.5mg/kg i.v.2.5mg/kg i.p. 0.6mg/kg i.p. 16mg/kg	15 minutes pre	Simultaneous	6	0
Alhouayek 2015 (Alhouayek et al. 2015)	CD57/BL mice	TNBS DSS	PEA PF-3845 AM9503	i.p. 10mg/kg i.p. 10mg/kg i.p. 10mg/kg	Simultaneous and 5 days post	7 days	4	1
El bakali 2015 (El Bakali et al. 2015)	C57/BL mice	TNBS	Compound 26	p.o. 10mg/kg	2 days pre	7 days	6	0
Impellizzeri 2015 (Impellizzeri et al. 2016)	CD1 mice	DNBS	uPEA	i.p. 10mg/kg	1 hour post	4 days	9	2

Study	Species	Model	Compound	Route/dosage	Time of administration verses inflammation	Time of assessment post inflammation	Modified STAIR score	SRCYCLE Score
Sasso 2015 (Sasso et al. 2015)	CD1 mice	TNBS DSS	ARN2508	p.o. 5mg/kg	Simultaneous	7 days	8	3
Stančić 2015 (Stančić et al. 2015)	C57/BL mice	DSS TNBS	CID16020046	s.c. 20mg/kg	30 minutes	7 days	6	1
Cordaro 2016 (Cordaro, Impellizzeri, Gugliandolo, et al. 2016)	CD1 mice	DNBS	Adelmidrol	p.o. 10mg/kg	60 minutes post	4 days	4	1
Feng 2016 (Feng et al. 2016)	C57/BL mice	DSS	WIN55,212-2	i.p. 5mg/kg	Simultaneous and 60 hours post	7 days	5	1
Ke 2016 (Ke et al. 2016)	C57/BL mice	DSS	HU308	i.p. 1mg/kg	Simultaneous and daily	8 days	4	2
Krohn 2016 (Krohn et al. 2016)	CD1 mice	TNBS	Ab-CBD O-1918 AM251 AM630	i.p. 5mg/kg i.p. 5mg/kg i.p. 5mg/kg i.p. 5mg/kg	45 minutes pre	4 days	6	1
Pagano 2016 (Pagano et al. 2016)	ICR mice	DNBS CO	CBD Pure CBD	i.p. 30mg/kg p.o. 60mg/kg	24 hours post	3 days	3	0
Sarnelli 2016 (Sarnelli et al. 2016)	CD1 mice	DSS	PEA	i.p. 2, 10mg/kg	2 days post	7 days	6	1
Lin 2017 (Lin et al. 2017)	C57/BL mice	DSS	HU210	i.p. 0.05mg/kg	30 minutes pre	7 days	5	1
Shamran 2017 (Shamran et al. 2017)	C57/BL mice	DSS	FAAH-II	i.p. 5 – 40mg/kg	24 hours post	7 days	6	1
Naftali 2017 (Naftali et al. 2017)	Clinical trial	Crohn's	CBD	10mg p.o. BD	N/A	8 weeks	NA	NA

CO, croton oil; DNBS, dinitrobenzosulphonic acid; LPS, lipopolysaccharide; TNBS, trinitrobenzosulphonic acid; DSS, dextran sulphate sodium; OM, oil of mustard; CASP, colon ascendens stent peritonitis; IL-10, interleukin 10; PEA, palmitoylethanolamide; AEA, anandamide; CBD, cannabidiol; THC; tetrahydrocannabinol; CBC, cannabichromene; CBG, cannabigerol; uPEA, C. Sativa; *Cannabis Sativa* ultramicronised PEA, AB-CBD, abnormal CBD; FAAH-II, fatty acid aminohydrolase II; i.p. intraperitoneal, i.c. intracolonic, p.o. oral administration; s.c. sub

Table 2-1 Characteristics of studies included for systematic review.

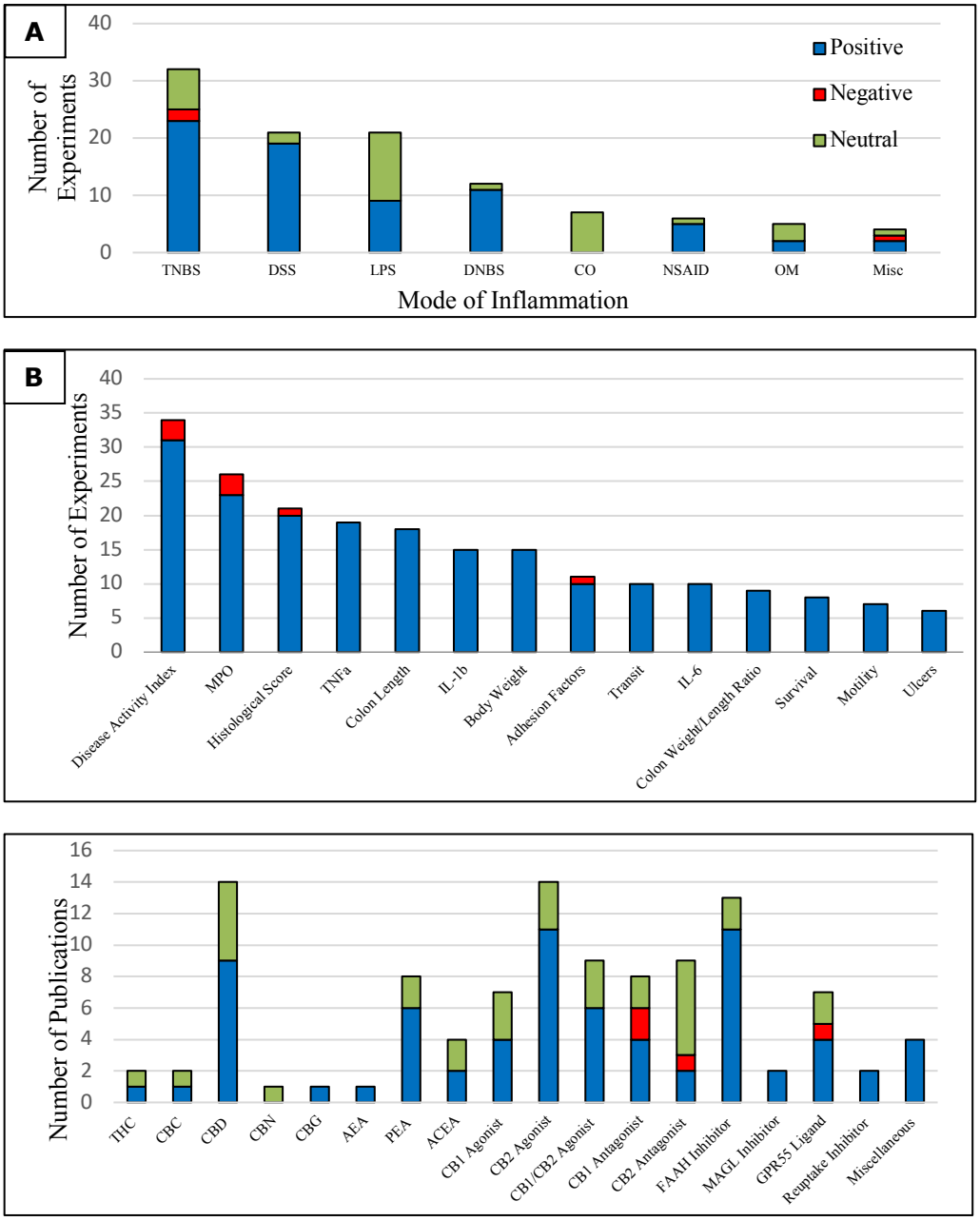


Figure 2.2 Positive, negative and neutral outcomes of cannabinoid treatment across modes of inflammation (A). Incidence of endpoints across all experiments comparing cannabinoid treatment with control (B). The effect of cannabinoid drugs compared to control across all endpoints expressed as primary drug investigated (C).

Preclinical studies

Of the 105 experiments comparing cannabinoids with vehicle or placebo, 67 (63.8%) favoured cannabinoids, 34 (32.3%) reported no difference, and 4 (3.8%) favoured vehicle. Mice were used in 89 experiments (68.5% of which favoured cannabinoids), rats in 14 (71.4% favoured cannabinoids), in 4 experiments both mice and rats were used showing no difference between cannabinoids and vehicle. Across preclinical studies, the two compound classes with the highest rate of positive outcome were synthetic CB_2 agonists and FAAH inhibitors. 11 of 14 publications (78.6%) using synthetic CB_2 receptor agonists favoured cannabinoid use over vehicle, and a further 11 of 13 (84.6%) favoured using FAAH inhibitors over vehicle. The outcome of all cannabinoids across publications is given in figure 2.2 C.

Of the 104 preclinical experiments where timing of drug administration was stated, 37 administered cannabinoids therapeutically, of which 62.2% favoured cannabinoid treatment. 19 experiments administered cannabinoids prophylactically, of which 52.6% favoured cannabinoid treatment. 48 experiments administered cannabinoids both prophylactically and therapeutically, of which 75% favoured cannabinoid treatment versus vehicle.

Clinical studies

Two clinical trials examining the effect of CBD and THC in Crohn's disease were found. Naftali et al. (Naftali et al. 2013) conducted a placebo controlled study in Crohn's disease patients, comparing THC 115 mg inhaled alone with placebo. Disease activity was compared between groups by means of validated questionnaire (Crohn's disease activity index – CDAI) after 8 weeks of treatment. A non-significant increase disease remission rate, as defined by the authors, was found at the end of the study period, however a secondary endpoint of reduction in overall CDAI scores was found between groups ($p=0.028$). In a second study, Naftali et al. (2017) compared oral CBD 10 mg p.o. twice daily with placebo in Crohn's disease, using CDAI in an identical fashion. No reduction in disease activity was detected between groups. In both studies, the authors measured changes in serum C-reactive protein (CRP). In both experimental and placebo groups, CRP levels were below 5 mg per litre at the beginning and end of the study periods, which are regarded as clinically normal levels.

2.3.3 Meta-analyses

Two human studies examined CDAI as an endpoint, 34 and 26 preclinical publications reported DAI or MPO activity as endpoints, allowing for meta-analysis. Of the remaining studies, significant heterogeneity of endpoints prevented further meta-analysis.

Crohn's Disease Activity Index

The use of two phytocannabinoids, THC or CBD, in two human studies were meta-analysed. Phytocannabinoid use decreased severity scores in comparison with placebo (mean difference (MD) -74.97, 95% CI –229, 0.79, I^2=75%. Figure 2.3). THC alone had a significant effect on reducing CDAI (MD -154.00, 95% CI -2.68.57, -44.43), whereas CBD alone did not (MD +4.00 95% CI -1.5.39, +113.39). Heterogeneity was found between these two groups.

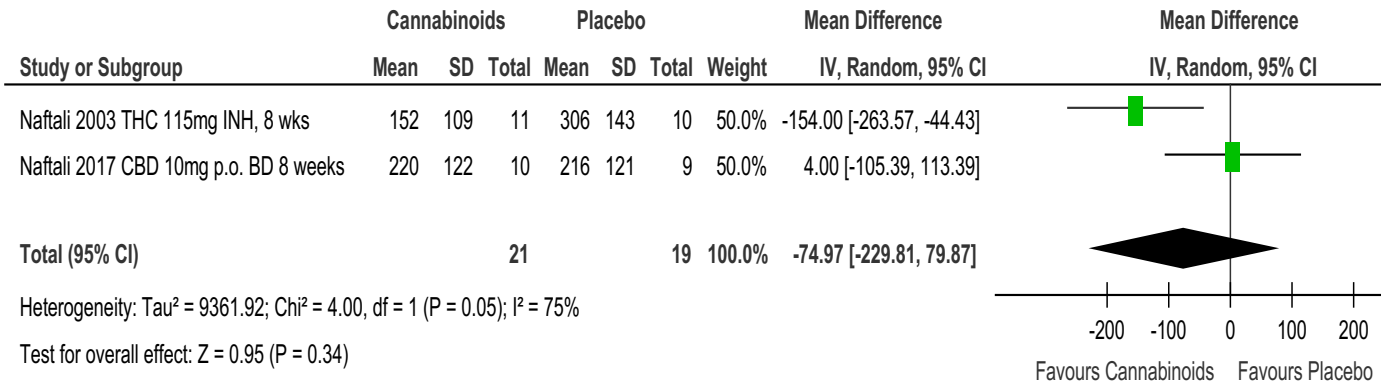


Figure 2.3 Forest plot of the effects of cannabinoid treatment on Crohn's Disease, assessed by reduction in CDAI in human studies.

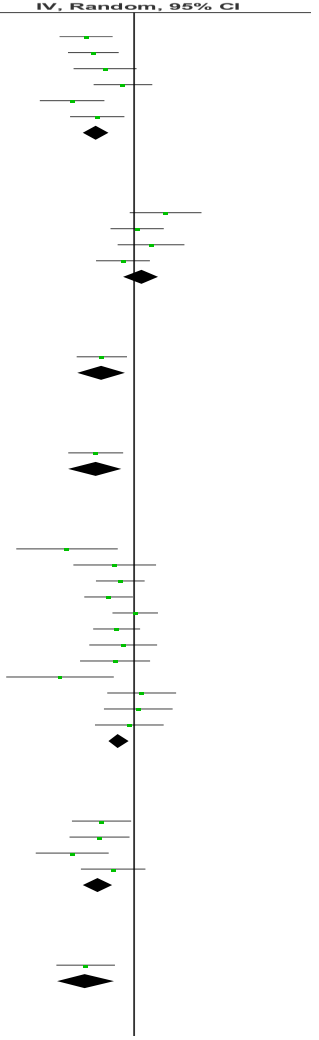
Disease Activity Index

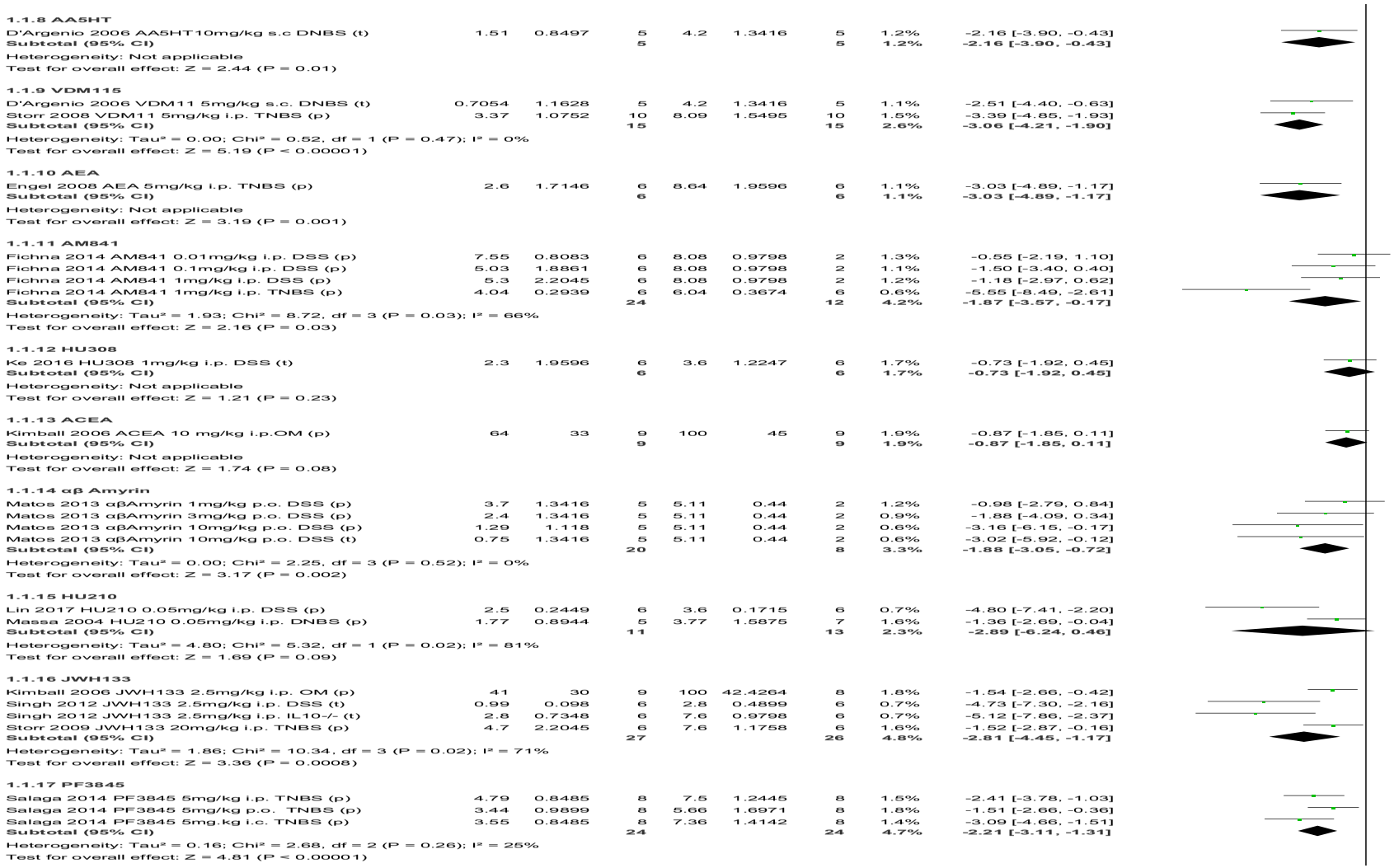
34 publications examined the effects of 25 cannabinoid drugs across 68 experiments, within mouse and rat models (n = 948; n = 519 experimental vs 429 in control groups). Overall, the use of cannabinoid drugs reduced DAI in comparison with vehicle; SMD -1.36, 95% CI -1.62 to -1.09, I^2=61% (figure 2.4, table 2.2). On subgroup analysis there was a significant difference between drug subtypes (P<0.001).

DAI was significantly reduced in mice (SMD -1.49, 95% CI -1.77 to -1.22; I^2=61%; low quality evidence). Seven experiments within one publication examined the effects of cannabinoids on rat colitis (THC and CBD, both conducted in a dose response manner), but did not reach significance at any dose; SMD -0.29, 95% CI -0.77 to 0.20, I^2=0%. SMD and confidence intervals for individual drugs on DAI are given in table 2.2.

The largest effect size on DAI reduction was caused by the FAAH inhibitor URB597 (SMD-4.43, 95% CI-6.32,-2.55). The largest effect on DAI reduction by an endocannabinoid was AEA (SMD-3.03, 95% CI -4.89,-1.17), the largest reduction by a phytocannabinoid was CBD (SMD -0.56, 95% CI-0.97, -0.16, I^2= 29%). The largest effect size by a synthetic cannabinoid was AM1241 (SMD –3.11, 95% CI -5.01, -1.22). 18 of 25 drugs had a large effect size, one had a moderate effect size, and six had no significant effect on DAI.

Study or Subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
1.1.1 PEA								
Borrelli 2014 PEA 1mg/kg p.o. DNBS (t)	50.1	4.5033	12	77.3	19.7454	12	1.9%	-1.83 [-2.81, -0.85]
Borrelli 2014 PEA 1mg/kg i.p. DNBS (t)	48.5	24.2487	12	84.94	20.7846	12	2.0%	-1.56 [-2.49, -0.62]
Esposito 2014 PEA 2mg/kg i.p. DSS (t)	1.12	0.6325	10	1.8	0.4249	5	1.7%	-1.11 [-2.28, 0.06]
Esposito 2014 PEA 10mg/kg i.p. DSS (t)	0.72	2.846	10	1.8	0.4249	5	1.8%	-0.43 [-1.52, 0.66]
Impellizzeri 2015 PEA 10mg/kg i.p. DNBS (t)	40.66	31.3065	10	100	13.0286	10	1.7%	-2.37 [-3.57, -1.17]
Alhouayek 2015 PEA 10mg/kg i.p. DSS (p)	78.3	15.8114	10	100	13.5978	10	1.9%	-1.41 [-2.41, -0.41]
Subtotal (95% CI)			**64**			**54**	**11.1%**	**-1.45 [-1.94, -0.96]**
Heterogeneity: Tau² = 0.09; Chi² = 6.62, df = 5 (P = 0.25); I² = 25%								
Test for overall effect: Z = 5.74 (P < 0.00001)								
1.1.2 SAB378								
Cluny 2010 SAB378 0.1mg/kg i.p. DSS (t)	9.53	1.3294	8	7.2	2.5456	4	1.6%	1.21 [-0.13, 2.54]
Cluny 2010 SAB378 1mg/kg i.p. DSS (t)	7.5	2.2627	8	7.2	2.5456	8	1.9%	0.12 [-0.86, 1.10]
Cluny 2010 SAB378 0.1mg/kg i.p. TNBS (p)	7.45	1.5274	8	6.2	2.2627	4	1.7%	0.65 [-0.59, 1.89]
Cluny 2010 SAB378 1mg/kg i.p.TNBS (p)	5.12	2.5456	8	6.2	2.2627	8	1.9%	-0.42 [-1.42, 0.57]
Subtotal (95% CI)			**32**			**24**	**7.1%**	**0.28 [-0.38, 0.94]**
Heterogeneity: Tau² = 0.13; Chi² = 4.19, df = 3 (P = 0.24); I² = 28%								
Test for overall effect: Z = 0.83 (P = 0.41)								
1.1.3 JZL184								
Alhouayek 2011 JZL184 16mg/kg i.p. TNBS (p)	2.7	2.3216	11	5.13	1.3266	11	2.0%	-1.24 [-2.16, -0.31]
Subtotal (95% CI)			**11**			**11**	**2.0%**	**-1.24 [-2.16, -0.31]**
Heterogeneity: Not applicable								
Test for overall effect: Z = 2.61 (P = 0.009)								
1.1.4 Compound 39								
Andrzejak 2011 Compound 39 5mg/kg i.p. TNBS (p)	2.2	0.9487	10	4.2	1.5811	10	1.9%	-1.47 [-2.48, -0.46]
Subtotal (95% CI)			**10**			**10**	**1.9%**	**-1.47 [-2.48, -0.46]**
Heterogeneity: Not applicable								
Test for overall effect: Z = 2.84 (P = 0.004)								
1.1.5 CBD								
Borrelli 2009 CBD 10mg/kg i.p. DNBS (p)	2.26	0.7155	5	4.2	0.6485	5	1.1%	-2.57 [-4.47, -0.66]
Jamontt 2010 CBD 10mg/kg i.p. TNBS (p)	2.9	3.3166	11	5.51	2.8523	2	1.4%	-0.74 [-2.29, 0.80]
Krohn 2016 abCBD 5mg/kg i.p. TNBS (t)	4.05	2.3085	10	5.38	2.5298	10	2.0%	-0.53 [-1.42, 0.37]
Schicho 2012 CBD 10mg.kg i.p. TNBS (p)	4.3	0.995	11	5.9	1.99	11	2.0%	-0.98 [-1.87, -0.08]
Schicho 2012 CBD 20mg/kg p.o. TNBS (p)	5.57	0.995	11	5.5	1.8905	11	2.1%	0.04 [-0.79, 0.88]
Schicho 2012 CBD 20mg/kg p.r. TNBS (p)	6.15	1.3266	11	7.07	1.3266	11	2.1%	-0.67 [-1.53, 0.20]
Borrelli 2009 CBD 1mg/kg i.p. DNBS (p)	3.9	0.6485	5	4.2	0.6485	5	1.6%	-0.42 [-1.68, 0.84]
Borrelli 2009 CBD 2.5mg/kg i.p. DNBS (p)	3.55	0.9391	5	4.2	0.6485	5	1.6%	-0.73 [-2.03, 0.58]
Borrelli 2009 CBD 5mg/kg i.p. DNBS (p)	2.13	0.6708	5	4.2	0.6485	5	1.0%	-2.83 [-4.85, -0.81]
Jamontt 2010 CBD 5mg/kg i.p. TNBS (p)	6.7	5.3066	11	5.15	2.8523	3	1.6%	0.29 [-0.99, 1.57]
Jamontt 2010 CBD 15mg/kg i.p. TNBS (p)	5.52	1.99	11	5.15	2.8523	3	1.6%	0.16 [-1.12, 1.44]
Jamontt 2010 CBD 20mg/kg i.p. TNBS (p)	4.65	4.6433	11	5.51	2.8523	3	1.6%	-0.18 [-1.46, 1.10]
Subtotal (95% CI)			**107**			**74**	**19.9%**	**-0.56 [-0.97, -0.16]**
Heterogeneity: Tau² = 0.14; Chi² = 15.43, df = 11 (P = 0.16); I² = 29%								
Test for overall effect: Z = 2.75 (P = 0.006)								
1.1.6 WIN 522,212								
Cluny 2010 WIN 52212-2 2mg/kg i.p. DSS (t)	4.2	1.9799	8	7.2	2.5456	8	1.8%	-1.24 [-2.34, -0.15]
Cluny 2010 WIN 52212-2 2mg/kg i.p. TNBS (t)	3.7	1.1314	8	6.2	2.2627	8	1.8%	-1.32 [-2.43, -0.21]
Feng 2016 WIN55,212 5mg/kg i.p. DSS (t)	5.05	2.2627	8	9.77	1.4142	8	1.6%	-2.37 [-3.73, -1.00]
Li 2013 WIN55212-2 5mg/kg i.p. DSS (t)	2.29	0.9798	6	3.5	1.7146	6	1.7%	-0.80 [-2.00, 0.40]
Subtotal (95% CI)			**30**			**30**	**6.9%**	**-1.37 [-1.96, -0.78]**
Heterogeneity: Tau² = 0.00; Chi² = 2.98, df = 3 (P = 0.39); I² = 0%								
Test for overall effect: Z = 4.55 (P < 0.00001)								
1.1.7 Adelmidrol								
Cordaro 2016 Ademidrol 10mg/kg p.o DNBS (t)	4.3	1.8974	10	7.2	0.9487	10	1.8%	-1.85 [-2.94, -0.77]
Subtotal (95% CI)			**10**			**10**	**1.8%**	**-1.85 [-2.94, -0.77]**
Heterogeneity: Not applicable								
Test for overall effect: Z = 3.34 (P = 0.0008)								




1.1.8 AA5HT
D'Argenio 2006 AA5HT10mg/kg s.c DNBS (t) 1.51 0.8497 5 4.2 1.3416 5 1.2% -2.16 [-3.90, -0.43]
Subtotal (95% CI) 5 5 1.2% -2.16 [-3.90, -0.43]
Heterogeneity: Not applicable
Test for overall effect: Z = 2.44 (P = 0.01)
1.1.9 VDM115
D'Argenio 2006 VDM11 5mg/kg s.c. DNBS (t) 0.7054 1.1628 5 4.2 1.3416 5 1.1% -2.51 [-4.40, -0.63]
Storr 2008 VDM11 5mg/kg i.p. TNBS (p) 3.37 1.0752 10 8.09 1.5495 10 1.5% -3.39 [-4.85, -1.93]
Subtotal (95% CI) 15 15 2.6% -3.06 [-4.21, -1.90]
Heterogeneity: Tau² = 0.00; Chi² = 0.52, df = 1 (P = 0.47); I² = 0%
Test for overall effect: Z = 5.19 (P < 0.00001)
1.1.10 AEA
Engel 2008 AEA 5mg/kg i.p. TNBS (p) 2.6 1.7146 6 8.64 1.9596 6 1.1% -3.03 [-4.89, -1.17]
Subtotal (95% CI) 6 6 1.1% -3.03 [-4.89, -1.17]
Heterogeneity: Not applicable
Test for overall effect: Z = 3.19 (P = 0.001)
1.1.11 AM841
Fichna 2014 AM841 0.01mg/kg i.p. DSS (p) 7.55 0.8083 6 8.08 0.9798 2 1.3% -0.55 [-2.19, 1.10]
Fichna 2014 AM841 0.1mg/kg i.p. DSS (p) 5.03 1.8861 6 8.08 0.9798 2 1.1% -1.50 [-3.40, 0.40]
Fichna 2014 AM841 1mg/kg i.p. DSS (p) 5.3 2.2045 6 8.08 0.9798 2 1.2% -1.18 [-2.97, 0.62]
Fichna 2014 AM841 1mg/kg i.p. TNBS (p) 4.04 0.2939 6 6.04 0.3674 6 0.6% -5.55 [-8.49, -2.61]
Subtotal (95% CI) 24 12 4.2% -1.87 [-3.57, -0.17]
Heterogeneity: Tau² = 1.93; Chi² = 8.72, df = 3 (P = 0.03); I² = 66%
Test for overall effect: Z = 2.16 (P = 0.03)
1.1.12 HU308
Ke 2016 HU308 1mg/kg i.p. DSS (t) 2.3 1.9596 6 3.6 1.2247 6 1.7% -0.73 [-1.92, 0.45]
Subtotal (95% CI) 6 6 1.7% -0.73 [-1.92, 0.45]
Heterogeneity: Not applicable
Test for overall effect: Z = 1.21 (P = 0.23)
1.1.13 ACEA
Kimball 2006 ACEA 10 mg/kg i.p.OM (p) 64 33 9 100 45 9 1.9% -0.87 [-1.85, 0.11]
Subtotal (95% CI) 9 9 1.9% -0.87 [-1.85, 0.11]
Heterogeneity: Not applicable
Test for overall effect: Z = 1.74 (P = 0.08)
1.1.14 αβ Amyrin
Matos 2013 αβAmyrin 1mg/kg p.o. DSS (p) 3.7 1.3416 5 5.11 0.44 2 1.2% -0.98 [-2.79, 0.84]
Matos 2013 αβAmyrin 3mg/kg p.o. DSS (p) 2.4 1.3416 5 5.11 0.44 2 0.9% -1.88 [-4.09, 0.34]
Matos 2013 αβAmyrin 10mg/kg p.o. DSS (p) 1.29 1.118 5 5.11 0.44 2 0.6% -3.16 [-6.15, -0.17]
Matos 2013 αβAmyrin 10mg/kg p.o. DSS (t) 0.75 1.3416 5 5.11 0.44 2 0.6% -3.02 [-5.92, -0.12]
Subtotal (95% CI) 20 8 3.3% -1.88 [-3.05, -0.72]
Heterogeneity: Tau² = 0.00; Chi² = 2.25, df = 3 (P = 0.52); I² = 0%
Test for overall effect: Z = 3.17 (P = 0.002)
1.1.15 HU210
Lin 2017 HU210 0.05mg/kg i.p. DSS (p) 2.5 0.2449 6 3.6 0.1715 6 0.7% -4.80 [-7.41, -2.20]
Massa 2004 HU210 0.05mg/kg i.p. DNBS (p) 1.77 0.8944 5 3.77 1.5875 7 1.6% -1.36 [-2.69, -0.04]
Subtotal (95% CI) 11 13 2.3% -2.89 [-6.24, 0.46]
Heterogeneity: Tau² = 4.80; Chi² = 5.32, df = 1 (P = 0.02); I² = 81%
Test for overall effect: Z = 1.69 (P = 0.09)
1.1.16 JWH133
Kimball 2006 JWH133 2.5mg/kg i.p. OM (p) 41 30 9 100 42.4264 8 1.8% -1.54 [-2.66, -0.42]
Singh 2012 JWH133 2.5mg/kg i.p. DSS (t) 0.99 0.098 6 2.8 0.4899 6 0.7% -4.73 [-7.30, -2.16]
Singh 2012 JWH133 2.5mg/kg i.p. IL10-/- (t) 2.8 0.7348 6 7.6 0.9798 6 0.7% -5.12 [-7.86, -2.37]
Storr 2009 JWH133 20mg/kg i.p. TNBS (p) 4.7 2.2045 6 7.6 1.1758 6 1.6% -1.52 [-2.87, -0.16]
Subtotal (95% CI) 27 26 4.8% -2.81 [-4.45, -1.17]
Heterogeneity: Tau² = 1.86; Chi² = 10.34, df = 3 (P = 0.02); I² = 71%
Test for overall effect: Z = 3.36 (P = 0.0008)
1.1.17 PF3845
Salaga 2014 PF3845 5mg/kg i.p. TNBS (p) 4.79 0.8485 8 7.5 1.2445 8 1.5% -2.41 [-3.78, -1.03]
Salaga 2014 PF3845 5mg/kg p.o. TNBS (p) 3.44 0.9899 8 5.66 1.6971 8 1.8% -1.51 [-2.66, -0.36]
Salaga 2014 PF3845 5mg.kg i.c. TNBS (p) 3.55 0.8485 8 7.36 1.4142 8 1.4% -3.09 [-4.66, -1.51]
Subtotal (95% CI) 24 24 4.7% -2.21 [-3.11, -1.31]
Heterogeneity: Tau² = 0.16; Chi² = 2.68, df = 2 (P = 0.26); I² = 25%
Test for overall effect: Z = 4.81 (P < 0.00001)

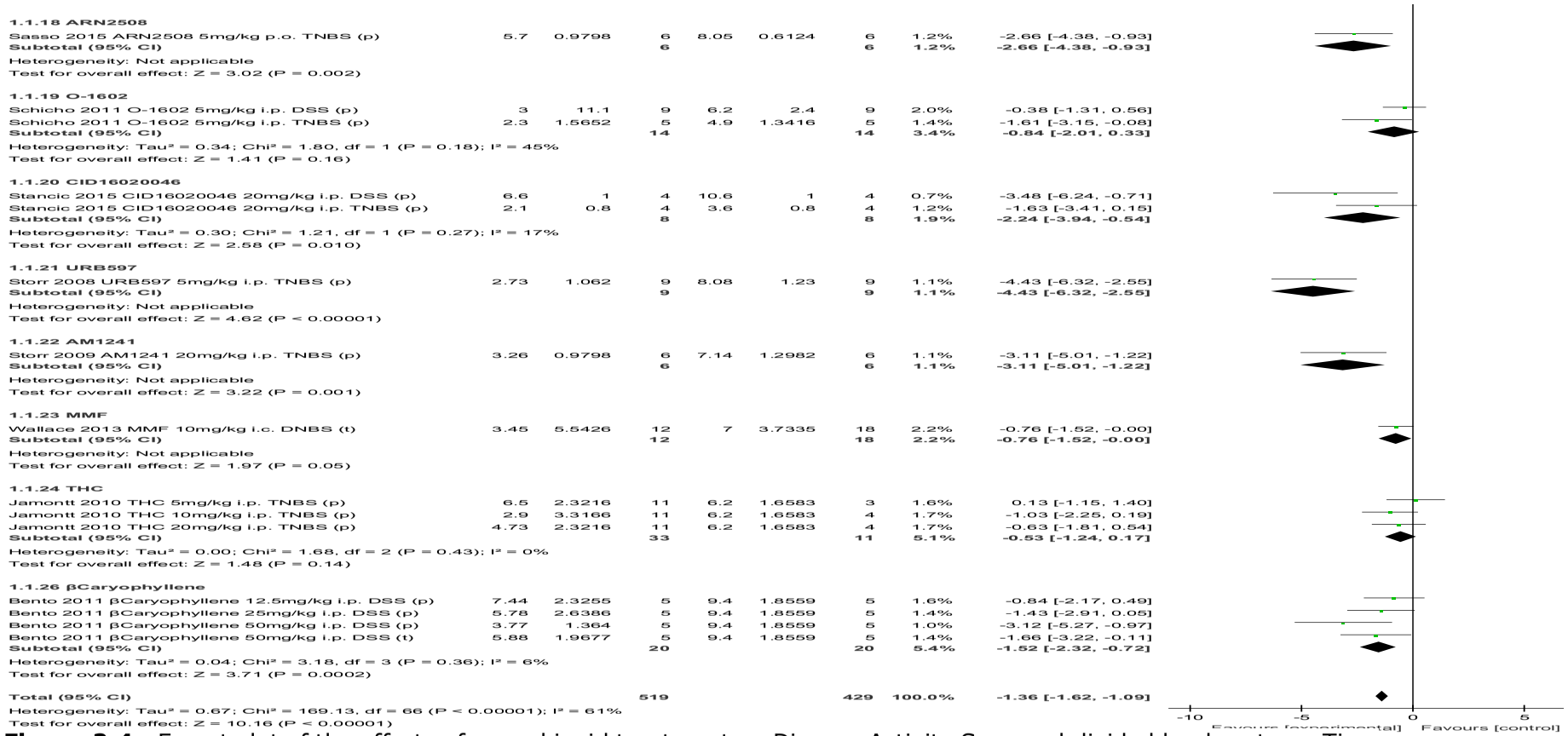


Figure 2.4 - Forest plot of the effects of cannabinoid treatment on Disease Activity Score subdivided by drug type. Time of administration in relation to onset of colitis is given where 'p' represents prophylactic administration, and 't' represents therapeutic administration.

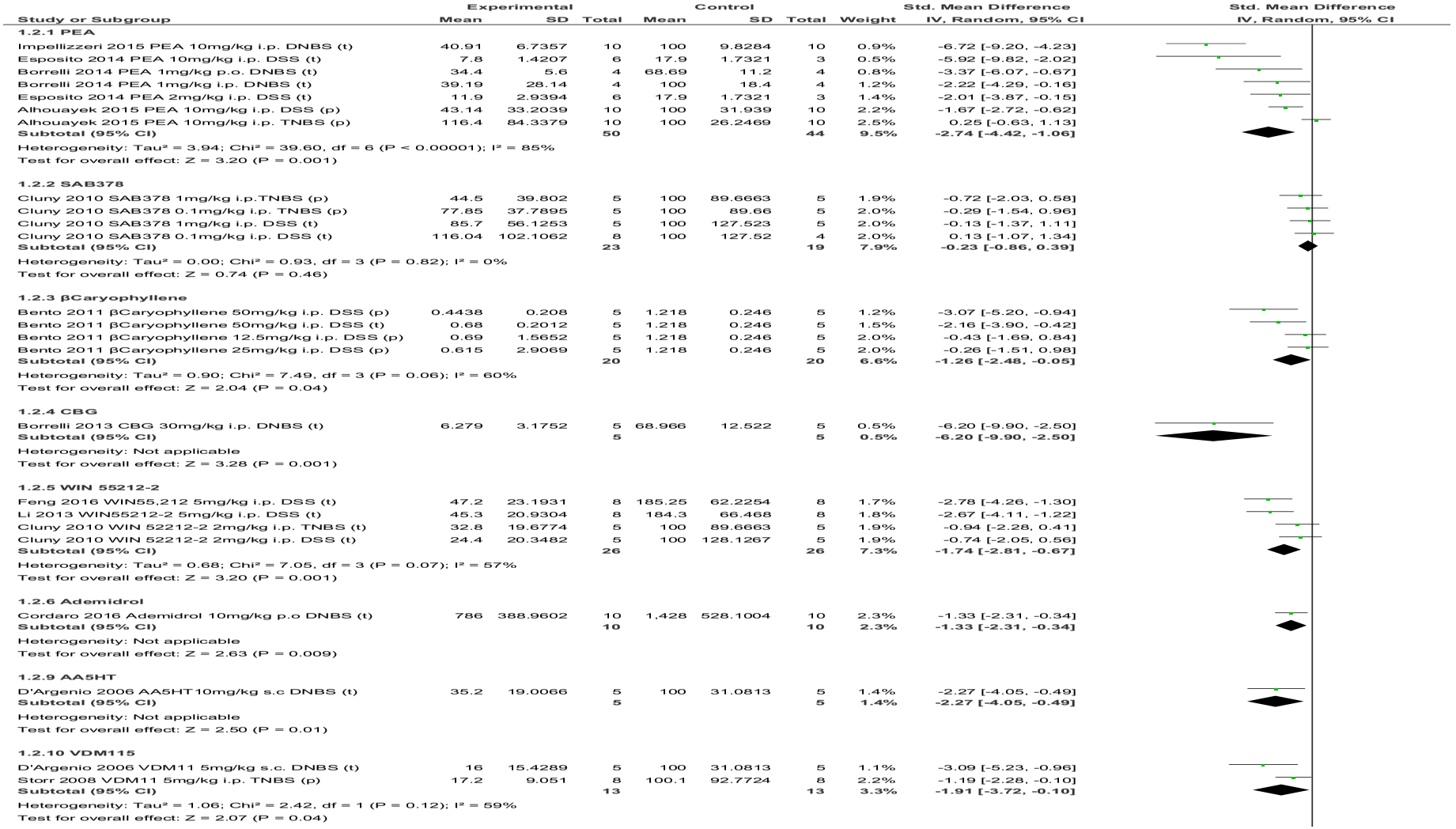
	No. of Studies	No. of animals	SMD [95% CI]	p value	I^2 (%)	Clinical significance
Endocannabinoids						
PEA	6	118	-1.45 [-1.94, -0.96]	<0.00001	25	High
AEA	1	12	-3.03 [-4.89, -1.17]	0.001	N/A	High
Phytocannabinoids						
CBD	12	181	-0.56 [-0.97, -0.16]	0.006	29	NS
THC	3	44	-0.53 [-1.24, 0.17]	0.14	0	NS
MMJ	1	30	-0.76 [-1.52, -0.00]	0.05	N/A	Moderate
Cannabinomimetics						
αβ Amyrin	4	28	-1.88 [-3.05, -0.72]	0.002	0	High
AM841	4	36	-1.87 [-3.57, -0.17]	0.03	66	High
βCaryophyllene	4	40	-1.52 [-2.32, -0.72]	0.0002	6	High
SAB378	4	56	0.28 [-0.38, 0.94]	0.41	28	NS
WIN55,212-2	4	60	-1.37 [-1.96, -0.78]	<0.00001	0	High
CID16020046	2	16	-2.24 [-3.94, -0.54]	0.01	17	High
HU210	2	24	-2.89 [-6.24, 0.46]	0.09	81	NS
O-1602	2	28	-0.84 [-2.01, 0.33]	0.16	45	NS
ACEA	1	18	-0.87 [-1.85, 0.11]	0.08	N/A	High
Adelmidrol	1	20	-1.85 [-2.94, -0.77]	0.0008	N/A	High
AM1241	1	12	-3.11 [-5.01, -1.22]	0.001	N/A	High
HU308	1	12	-0.73 [-1.92, 0.45]	0.23	N/A	NS
Enzyme inhibitors						
JWH133	4	53	-2.81 [-4.45, -1.17]	0.0008	71	High
PF3845	3	48	-2.21 [-3.11, -1.31]	<0.00001	25	High
AA5HT	1	10	-2.16 [-3.90, -0.43]	0.01	N/A	High
ARN2508	1	12	-2.66 [-4.38, -0.93]	0.002	N/A	High
Compound 39	1	20	-1.47 [-2.48, -0.46]	0.004	N/A	High
JZL184	1	22	-1.24 [-2.16, -0.31]	0.009	N/A	High
URB597	1	18	-4.43 [-6.32, -2.55]	<0.00001	N/A	High
Transport inhibitors						
VDM11	2	30	-3.06 [-4.21, -1.90]	<0.00001	0	High
Total	**68**	**948**	**-1.36 [-1.62, -1.09]**	**<0.00001**	**61**	**High**

Table 2-2 The effects of cannabinoids on disease activity score caused by experimental colitis, grouped by compound (NS – non significant).

Myeloperoxidase activity

26 publications investigated the effects of 21 cannabinoid drugs on MPO activity throughout 57 individual experiments (total n = 757, n = 419 in experimental vs 338 in control groups). Overall, cannabinoid drugs reduced MPO activity in comparison with vehicle; SMD -1.26, 95% CI-1.54 to -0.97, I^2=48.1% (figure 2.5, table 2.3). There was significant heterogeneity between studies examining the same compound, and there were significant subgroup differences (I^2=48.1%, P<0.008). MPO activity was significantly reduced in mice and rats (SMD -1.28, 95% CI -1.59 to -0.98 I^2=61% and -1.06, 95% CI -1.99 to -0.13, I^2=56%, respectively). SMD and confidence intervals of individual drugs on MPO activity are given in table 2.3.

The largest effect size on MPO activity reduction was caused by the phytocannabinoid CBG (SMD -6.20, 95% CI -9.90 to -2.50, I^2 not assessed). The largest effect on DAI reduction by an endocannabinoid was PEA (SMD -2.74, 95% CI -4.42, -1.06, I^2=85%). The largest effect size by a synthetic cannabinoid was ACEA (SMD -3.15, 95% CI -4.75, -1.55, I^2 not assessed), and the largest effect size of any enzyme or transport inhibitor was AA5HT (SMD -2.27, 95% CI -4.05, -0.49, I^2 not assessed). Thirteen of 21 cannabinoid drugs had a large clinical effect, the remaining of which had no significant effect on MPO activity.


Experimental
Control
Std. Mean Difference
Std. Mean Difference
Study or Subgroup Mean SD Total Mean SD Total Weight IV, Random, 95% CI
IV, Random, 95% CI
1.2.1 PEA
Impellizzeri 2015 PEA 10mg/kg i.p. DNBS (t) 40.91 6.7357 10 100 9.8284 10 0.9% -6.72 [-9.20, -4.23]
Esposito 2014 PEA 10mg/kg i.p. DSS (t) 7.8 1.4207 6 17.9 1.7321 3 0.5% -5.92 [-9.82, -2.02]
Borrelli 2014 PEA 1mg/kg p.o. DNBS (t) 34.4 5.6 4 68.69 11.2 4 0.8% -3.37 [-6.07, -0.67]
Borrelli 2014 PEA 1mg/kg i.p. DNBS (t) 39.19 28.14 4 100 18.4 4 1.2% -2.22 [-4.29, -0.16]
Esposito 2014 PEA 2mg/kg i.p. DSS (t) 11.9 2.9394 6 17.9 1.7321 3 1.4% -2.01 [-3.87, -0.15]
Alhouayek 2015 PEA 10mg/kg i.p. DSS (p) 43.14 33.2039 10 100 31.939 10 2.2% -1.67 [-2.72, -0.62]
Alhouayek 2015 PEA 10mg/kg i.p. TNBS (p) 116.4 84.3379 10 100 26.2469 10 2.5% 0.25 [-0.63, 1.13]
Subtotal (95% CI) 50 44 9.5% -2.74 [-4.42, -1.06]
Heterogeneity: Tau² = 3.94; Chi² = 39.60, df = 6 (P < 0.00001); I² = 85%
Test for overall effect: Z = 3.20 (P = 0.001)
1.2.2 SAB378
Cluny 2010 SAB378 1mg/kg i.p.TNBS (p) 44.5 39.802 5 100 89.6663 5 1.9% -0.72 [-2.03, 0.58]
Cluny 2010 SAB378 0.1mg/kg i.p. TNBS (p) 77.85 37.7895 5 100 89.66 5 2.0% -0.29 [-1.54, 0.96]
Cluny 2010 SAB378 1mg/kg i.p. DSS (t) 85.7 56.1253 5 100 127.523 5 2.0% -0.13 [-1.37, 1.11]
Cluny 2010 SAB378 0.1mg/kg i.p. DSS (t) 116.04 102.1062 8 100 127.52 4 2.0% 0.13 [-1.07, 1.34]
Subtotal (95% CI) 23 19 7.9% -0.23 [-0.86, 0.39]
Heterogeneity: Tau² = 0.00; Chi² = 0.93, df = 3 (P = 0.82); I² = 0%
Test for overall effect: Z = 0.74 (P = 0.46)
1.2.3 βCaryophyllene
Bento 2011 βCaryophyllene 50mg/kg i.p. DSS (p) 0.4438 0.208 5 1.218 0.246 5 1.2% -3.07 [-5.20, -0.94]
Bento 2011 βCaryophyllene 50mg/kg i.p. DSS (t) 0.68 0.2012 5 1.218 0.246 5 1.5% -2.16 [-3.90, -0.42]
Bento 2011 βCaryophyllene 12.5mg/kg i.p. DSS (p) 0.69 1.5652 5 1.218 0.246 5 2.0% -0.43 [-1.69, 0.84]
Bento 2011 βCaryophyllene 25mg/kg i.p. DSS (p) 0.615 2.9069 5 1.218 0.246 5 2.0% -0.26 [-1.51, 0.98]
Subtotal (95% CI) 20 20 6.6% -1.26 [-2.48, -0.05]
Heterogeneity: Tau² = 0.90; Chi² = 7.49, df = 3 (P = 0.06); I² = 60%
Test for overall effect: Z = 2.04 (P = 0.04)
1.2.4 CBG
Borrelli 2013 CBG 30mg/kg i.p. DNBS (t) 6.279 3.1752 5 68.966 12.522 5 0.5% -6.20 [-9.90, -2.50]
Subtotal (95% CI) 5 5 0.5% -6.20 [-9.90, -2.50]
Heterogeneity: Not applicable
Test for overall effect: Z = 3.28 (P = 0.001)
1.2.5 WIN 55212-2
Feng 2016 WIN55,212 5mg/kg i.p. DSS (t) 47.2 23.1931 8 185.25 62.2254 8 1.7% -2.78 [-4.26, -1.30]
Li 2013 WIN55212-2 5mg/kg i.p. DSS (t) 45.3 20.9304 8 184.3 66.468 8 1.8% -2.67 [-4.11, -1.22]
Cluny 2010 WIN 52212-2 2mg/kg i.p. TNBS (t) 32.8 19.6774 5 100 89.6663 5 1.9% -0.94 [-2.28, 0.41]
Cluny 2010 WIN 52212-2 2mg/kg i.p. DSS (t) 24.4 20.3482 5 100 128.1267 5 1.9% -0.74 [-2.05, 0.56]
Subtotal (95% CI) 26 26 7.3% -1.74 [-2.81, -0.67]
Heterogeneity: Tau² = 0.68; Chi² = 7.05, df = 3 (P = 0.07); I² = 57%
Test for overall effect: Z = 3.20 (P = 0.001)
1.2.6 Ademidrol
Cordaro 2016 Ademidrol 10mg/kg p.o DNBS (t) 786 388.9602 10 1,428 528.1004 10 2.3% -1.33 [-2.31, -0.34]
Subtotal (95% CI) 10 10 2.3% -1.33 [-2.31, -0.34]
Heterogeneity: Not applicable
Test for overall effect: Z = 2.63 (P = 0.009)
1.2.9 AA5HT
D'Argenio 2006 AA5HT 10mg/kg s.c DNBS (t) 35.2 19.0066 5 100 31.0813 5 1.4% -2.27 [-4.05, -0.49]
Subtotal (95% CI) 5 5 1.4% -2.27 [-4.05, -0.49]
Heterogeneity: Not applicable
Test for overall effect: Z = 2.50 (P = 0.01)
1.2.10 VDM115
D'Argenio 2006 VDM11 5mg/kg s.c. DNBS (t) 16 15.4289 5 100 31.0813 5 1.1% -3.09 [-5.23, -0.96]
Storr 2008 VDM11 5mg/kg i.p. TNBS (p) 17.2 9.051 8 100.1 92.7724 8 2.2% -1.19 [-2.28, -0.10]
Subtotal (95% CI) 13 13 3.3% -1.91 [-3.72, -0.10]
Heterogeneity: Tau² = 1.06; Chi² = 2.42, df = 1 (P = 0.12); I² = 59%
Test for overall effect: Z = 2.07 (P = 0.04)

1.2.11 AM841								
Fichna 2014 AM841 1mg/kg i.p. DSS (p)	16.15	10.748	8	47.45	14.99	2	1.2%	-2.49 [-4.58, -0.40]
Fichna 2014 AM841 1mg/kg i.p. TNBS (p)	18.43	7.3539	8	39.43	9.8995	8	1.9%	-2.28 [-3.61, -0.94]
Fichna 2014 AM841 0.1mg/kg i.p. DSS (p)	27.14	8.8813	8	47.45	14.99	3	1.6%	-1.76 [-3.37, -0.15]
Fichna 2014 AM841 0.01mg/kg i.p. DSS (p)	44.9	27.4357	8	47.45	14.99	3	1.9%	-0.09 [-1.42, 1.24]
Subtotal (95% CI)			**32**			**16**	**6.5%**	**-1.56 [-2.71, -0.41]**
Heterogeneity: Tau² = 0.74; Chi² = 6.57, df = 3 (P = 0.09); I² = 54%								
Test for overall effect: Z = 2.66 (P = 0.008)								
1.2.12 ACEA								
Kimball 2006 ACEA 10 mg/kg i.p.OM (p)	5.7	13.2	9	199	87.3098	7	1.6%	-3.15 [-4.75, -1.55]
Subtotal (95% CI)			**9**			**7**	**1.6%**	**-3.15 [-4.75, -1.55]**
Heterogeneity: Not applicable								
Test for overall effect: Z = 3.86 (P = 0.0001)								
1.2.13 CBD								
Pagano 2016 CBD 30mg/kg i.p. DNBS (t)	4.62	0.5814	5	6.06	0.5143	5	1.4%	-2.37 [-4.19, -0.55]
Pagano 2016 CBD 60mg/kg p.o. DNBS (t)	5.7	1.7889	5	9.03	0.2236	5	1.4%	-2.36 [-4.18, -0.54]
Jamontt 2010 CBD 20mg/kg i.p. TNBS (p)	2.87	1.3266	11	6.05	2.3548	3	1.7%	-1.93 [-3.45, -0.40]
Jamontt 2010 CBD 10mg/kg i.p. TNBS (p)	4.6	0.3317	11	6.05	2.3548	3	1.8%	-1.35 [-2.75, 0.06]
Schicho 2012 CBD 20mg/kg p.r. TNBS (p)	69.4	21.8197	10	105.55	43.9557	10	2.4%	-1.00 [-1.94, -0.06]
Krohn 2016 abCBD 5mg/kg i.p. TNBS (t)	60.03	32.5715	10	100	43.6394	10	2.4%	-0.99 [-1.94, -0.05]
Schicho 2012 CBD 10mg/kg i.p. TNBS (p)	80.5	17.7088	10	106.9	35.1013	10	2.4%	-0.91 [-1.84, 0.02]
Schicho 2012 CBD 20mg/kg p.o. TNBS (p)	70.83	50.7444	11	101.38	18.5731	11	2.5%	-0.77 [-1.64, 0.10]
Jamontt 2010 CBD 15mg/kg i.p. TNBS (p)	4.1	4.9749	11	6.05	2.3548	3	1.9%	-0.39 [-1.68, 0.89]
Jamontt 2010 CBD 5mg/kg i.p. TNBS (p)	5.5	3.6483	11	6.05	2.3548	2	1.7%	-0.14 [-1.65, 1.36]
Subtotal (95% CI)			**95**			**62**	**19.5%**	**-1.03 [-1.40, -0.66]**
Heterogeneity: Tau² = 0.00; Chi² = 8.33, df = 9 (P = 0.50); I² = 0%								
Test for overall effect: Z = 5.47 (P < 0.00001)								
1.2.14 HU210								
Massa 2004 HU210 0.05mg/kg i.p. DNBS (p)	2.9	9.1679	5	100	103.4489	7	2.0%	-1.12 [-2.39, 0.15]
Lin 2017 HU210 0.05mg/kg i.p. DSS (p)	1.74	0.4164	6	3.21	8.0833	6	2.1%	-0.24 [-1.37, 0.90]
Subtotal (95% CI)			**11**			**13**	**4.1%**	**-0.63 [-1.48, 0.23]**
Heterogeneity: Tau² = 0.01; Chi² = 1.02, df = 1 (P = 0.31); I² = 2%								
Test for overall effect: Z = 1.44 (P = 0.15)								
1.2.15 CBC								
Romano 2013 CBC 1mg.kg i.p. DNBS (t)	33.14	7.2001	5	67.64	12.9692	5	1.2%	-2.97 [-5.05, -0.89]
Subtotal (95% CI)			**5**			**5**	**1.2%**	**-2.97 [-5.05, -0.89]**
Heterogeneity: Not applicable								
Test for overall effect: Z = 2.80 (P = 0.005)								
1.2.16 PF3745								
Salaga 2014 PF3845 5mg/kg p.o. TNBS (p)	61.01	29.1297	7	100	24.8701	7	2.0%	-1.35 [-2.55, -0.15]
Salaga 2014 PF3845 5mg.kg i.c. TNBS (p)	86.36	48.1964	8	100	25.7104	8	2.3%	-0.33 [-1.32, 0.66]
Salaga 2014 PF3845 5mg/kg i.p. TNBS (p)	142	35.3553	8	100	25.4558	8	2.2%	1.29 [0.18, 2.40]
Subtotal (95% CI)			**23**			**23**	**6.5%**	**-0.12 [-1.56, 1.32]**
Heterogeneity: Tau² = 1.31; Chi² = 10.40, df = 2 (P = 0.006); I² = 81%								
Test for overall effect: Z = 0.16 (P = 0.87)								
1.2.17 O-1602								
Schicho 2011 O-1602 5mg/kg i.p. TNBS (p)	21.98	33.541	5	100	46.7338	5	1.6%	-1.73 [-3.31, -0.16]
Schicho 2011 O-1602 5mg/kg i.p. DSS (p)	67.06	16.7705	5	100.31	19.0066	5	1.6%	-1.68 [-3.23, -0.12]
Subtotal (95% CI)			**10**			**10**	**3.3%**	**-1.70 [-2.81, -0.60]**
Heterogeneity: Tau² = 0.00; Chi² = 0.00, df = 1 (P = 0.96); I² = 0%								
Test for overall effect: Z = 3.01 (P = 0.003)								
1.2.18 CID16020046								
Stancic 2015 CID16020046 20mg/kg i.p. TNBS (p)	313.5	69.2207	14	435	138.4413	14	2.6%	-1.08 [-1.88, -0.28]
Stancic 2015 CID16020046 20mg/kg i.p. DSS (p)	172	53.1315	14	428.37	344.2325	14	2.6%	-1.01 [-1.80, -0.22]
Subtotal (95% CI)			**28**			**28**	**5.1%**	**-1.04 [-1.61, -0.48]**
Heterogeneity: Tau² = 0.00; Chi² = 0.01, df = 1 (P = 0.91); I² = 0%								
Test for overall effect: Z = 3.63 (P = 0.0003)								

Figure 2.5 Forest plot of the effects of cannabinoid treatment on MPO activity subdivided by drug type. Time of administration in relation to onset of colitis is given where 'p' represents prophylactic administration, and 't' represents therapeutic administration.

	No. of Studies	No. of animals	SMD [95% CI]	p value	I^2 (%)	Clinical significance
Endocannabinoids						
PEA	7	94	-2.74 [-4.42, -1.06]	0.001	85	High
Phytocannabinoids						
CBD	10	157	-1.03 [-1.40, -0.66]	<0.00001	0	High
THC	3	29	-1.40 [-3.97, 1.17]	0.28	80	NS
CBC	1	10	-2.97 [-5.05, -0.89]	0.005	N/A	High
CBG	1	10	-6.20 [-9.90, -2.50]	0.01	N/A	High
Cannabinomimetics						
βCaryophyllene	4	40	-1.26 [-2.48, -0.05]	0.04	60	High
AM841	4	48	-1.56 [-2.71, -0.41]	0.008	54	High
SAB378	4	42	-0.23 [-0.86, 0.39]	0.46	0	NS
WIN55,212-2	4	52	-1.74 [-2.81, -0.67]	0.001	57	High
αβ Amyrin	2	15	-0.38 [-1.48, 0.71]	0.5	0	NS
CID16020046	2	56	-1.04 [-1.61, -0.48]	0.0003	0	High
HU210	2	24	-0.63 [-1.48, 0.23]	0.15	2	NS
O-1602	2	20	-1.70 [-2.81, -0.60]	0.003	0	High
ACEA	1	16	-3.15 [-4.75, -1.55]	0.0001	N/A	High
AM1241	1	10	-0.96 [-2.31, 0.39]	0.16	N/A	NS
JWH133	1	16	-0.98 [-2.04, 0.07]	0.09	N/A	NS
Ademidrol	1	20	-1.33 [-2.31, -0.34]	0.009	N/A	High
Enzyme inhibitors						
PF3845	3	46	-0.12 [-1.56, 1.32]	0.81	81	NS
AA5HT	1	10	-2.27 [-4.05, -0.49]	0.01	N/A	High
URB597	1	16	-1.00 [-2.06, 0.06]	0.06	N/A	NS
Transport inhibitors						
VDM11	2	26	-1.91 [-3.72, -0.10]	0.04	59	High
Total	**57**	**757**	**-1.26 [-1.54, -0.97]**	**<0.00001**	**48.1**	**High**

Table 2-3 The effects of cannabinoids on MPO activity caused by experimental colitis, grouped by drug (NS – non significant).

Timing of administration

From the 51 publications examining the effect of cannabinoids on an animal model of colitis, 28 studies administered cannabinoid agents either simultaneously with colitis onset or prophylactically. 16 studies administered drugs between 15 minutes and 7 days after the onset of colitis. Additionally, 7 studies compared the benefit of prophylactic cannabinoid use to therapeutic, but did not find any difference in efficacy. To investigate if timing of drug treatment affected DAI or MPO activity, we compared study size-weighted effect sizes (dependent variable) with time of administration (covariate) using meta-regression. We found that earlier drug administration weakly predicted a larger effect size in reducing DAI and MPO activity, although this was not statistically significant (P=0.09 R^2=11% and P=0.055 R^2=41% respectively, figure 2.6 A and B).

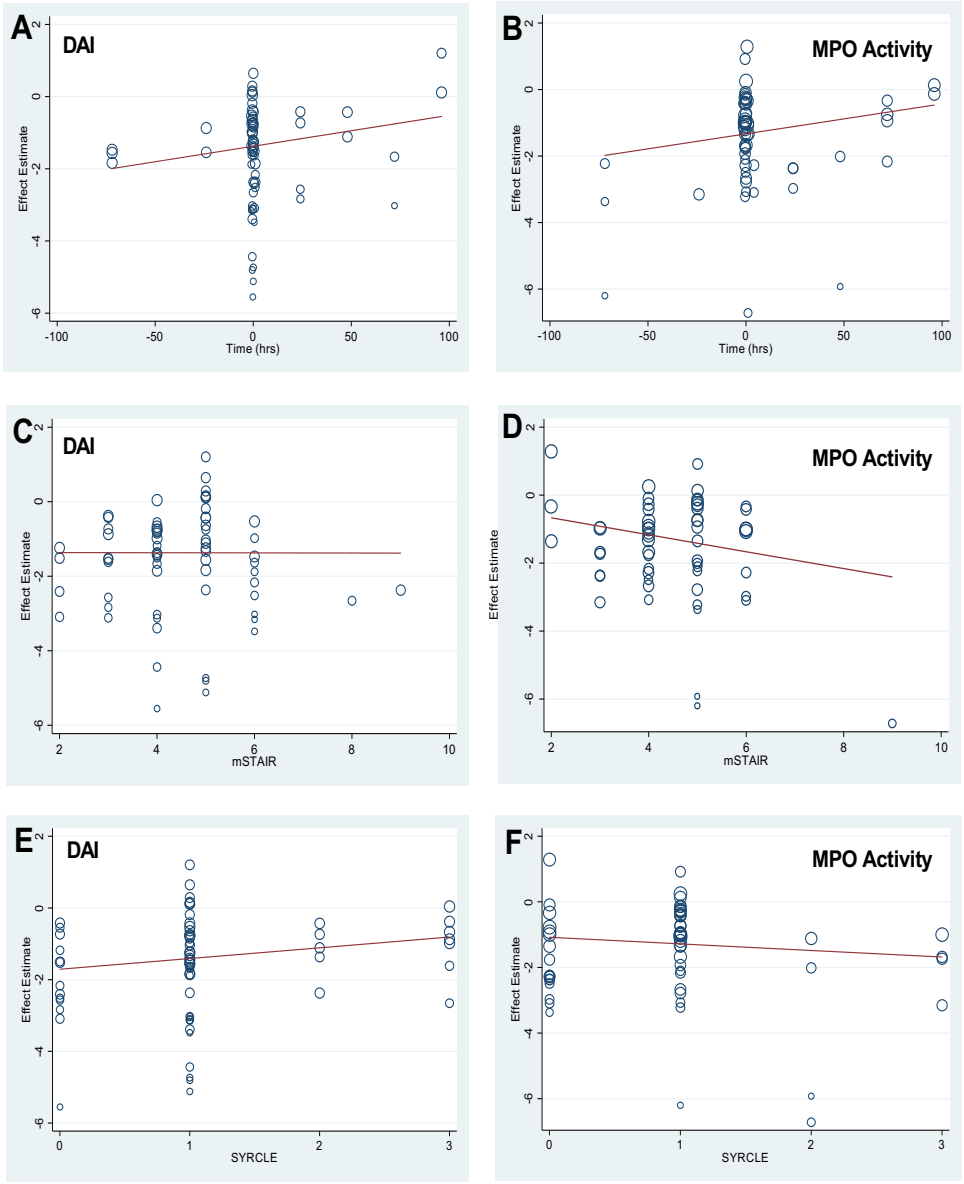


Figure 2.6 The effect of cannabinoid treatment on experimentally induced colitis in animals, determined by DAI (A) and MPO activity (B), predicted by timing of drug administration in relation to colitis onset. The effect of study quality, determined by mSTAIR score and SYRCLE score, on effect size in DAI (C, E) and MPO activity (D, F). Study weights are represented by diameter of circles, with larger circles representing studies with largest weight in the analysis.

Quality and risk of bias

Of the 53 papers, 21 used randomisation in their design, 7 reported blinding of assessment, 5 replicated their results in a second species, and 14 replicated their findings in a second model of colitis. 50 reported n≥5 in control and experimental groups. 15 publications reported specific numbers within groups. All papers reported a clinically relevant endpoint. Median study mSTAIR score was 5 out of 10 (mean 4.9, SD 2.29). Using meta-regression, higher mSTAIR scores predicted greater reductions in MPO activity (P=0.043 R^2=65%, figure 2.6 D), but not in reduction of DAI (P=0.98 R^2= 35%, Figure 2.6 C).

The SYRCLE risk of bias score for each endpoint showed a trend to larger reduction in DAI in studies with a larger risk of bias (P=0.084 R^2=69%, figure 2.6 E), but not MPO activity (P=0.345 R^2=8%, figure 2.6 F).

There were no correlation between mSTAIR score and SYRCLE scores (p=0.0705 R^2= 0.6521, Pearson's correlation coefficient).

Publication bias

Funnel plots comparing MPO activity and DAI were constructed and analysed statistically for bias. The presence of publication bias was not found in either group (MPO; Egger's statistic P=0.570, figure 2.7 A; DAI; Egger's statistic P=0.274, figure 2.7 B).

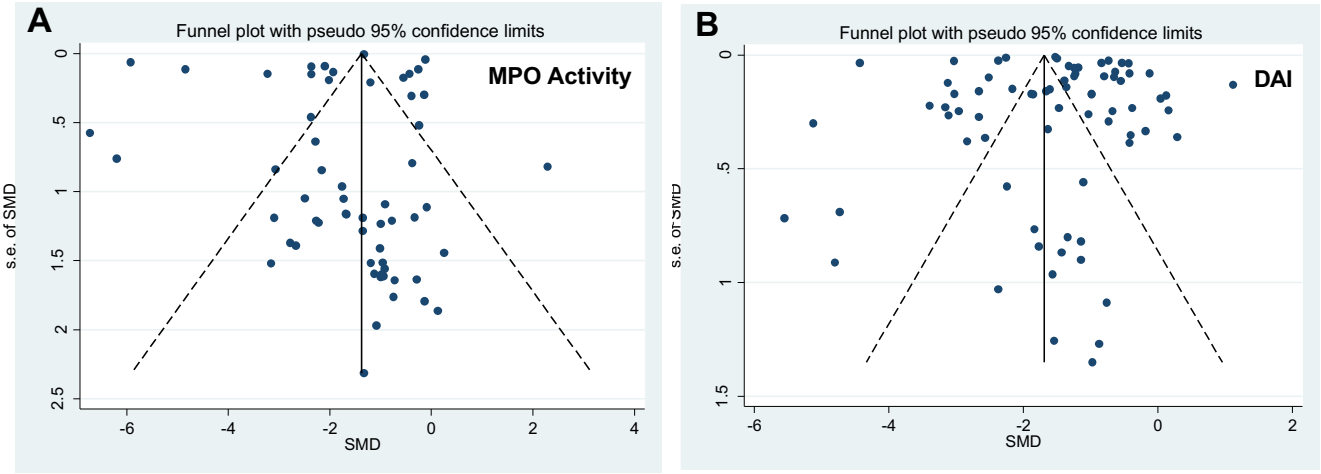


Figure 2.7 Funnel plots evaluating for publication bias in (A) MPO activity and (B) DAI. Standard error of the standardized mean difference (SE (SMD), y axes) for each study is plotted against its effect size (SMD, x axes).

2.4 Discussion

The aim of this study was to review available data on cannabinoid compound effects on colonic inflammation, assessing the efficacy of those agents, and to suggest compounds showing potential for clinical use to take forward for further investigation. We found 53 studies that examined this effect using endocannabinoids, phytocannabinoids, synthetic cannabinoids and enzyme inhibitors across multiple models of animal and human colitis. In both qualitative assessment and meta-analysis, controlled studies demonstrate that the use of cannabinoid drugs were beneficial in reducing colonic inflammation in rats and mice, with unclear effects in human subjects.

2.4.1 Preclinical studies

In animal studies, cannabinoids were shown to reduce inflammation both qualitatively and at meta-analysis. Across experiments included in this review, CB_2 agonists, FAAH inhibitors and CBD were the most widely studied and showed the greatest therapeutic benefit across all endpoints. Subgroup analyses suggested that CBG caused the greatest reduction in MPO activity scores followed by the synthetic CB_1 agonist ACEA. However, both agents were only studied within a single publication. In the MPO activity analysis, the most studied drug was CBD, with 157 individual animals examined across 7 publications. Similarly, within DAI analysis, CBD was again the most studied single drug including 181 animal studies? across 6 publications, though the largest reduction in DAI was caused by the FAAH antagonist URB597, studied in one publication. There was statistical heterogeneity in both MPO activity and DAI analyses, which was partly accounted for by subgroup differences. At meta-regression, factors leading to subgroup differences were quality, timing and risk of bias.

We found that most of the existing cannabinoid-gut research focusses on the therapeutic potential of CBD. This is unsurprising as CBD is clinically available in the US, and a legal substance in many western countries including the UK, Israel, South Africa and throughout Western Europe, well tolerated and has shown consistent, positive results. Nine studies found a positive, dose dependent effect of CBD on local inflammatory cytokine expression, COX2 activation, MPO activity, enteric glial cell activation and caspase-3 production, with associated improvements in macroscopic and histologic grades of inflammation (Pagano et al. 2016; Krohn et al. 2016; Harvey et al. 2013; Schicho & Storr 2012; Lin et al. 2011; de Filippis et al. 2008; J. M. Jamontt et al. 2010; Borrelli et al. 2009). One study also showed that intraperitoneal CBD administration decreased oxidative-stress scores of

peripheral lung and brain tissue following intestinal inflammation (Cassol-Jr et al. 2010), adding to the existing evidence that CBD maintains the gut barrier during inflammation (Alhamoruni et al. 2010). We also found four publications demonstrating the beneficial effect of PEA on DAI and MPO activity (Impellizzeri et al. 2015; Alhouayek et al. 2015; Borrelli et al. 2015; Esposito et al. 2014), and an additional 3 publications demonstrating benefit on other inflammatory endpoints (Sarnelli et al. 2016; Capasso et al. 2014; Capasso et al. 2001). Multiple other compounds were shown at meta-analysis to be beneficial in colitis. From these, synthetic compounds showed the greatest potential reduction in inflammation, however limited data available regarding their side-effect profiles suggests that they are not well tolerated (Cooper et al. 2016). Additionally, THC was shown to be beneficial in this review, even though it has the potential to induce paranoia, anxiety and depression when given alone.

2.4.2 Clinical studies

From the clinical literature, we found two randomised placebo-controlled studies examining the effect of phytocannabinoids in humans. Our analysis found no effect of THC or CBD on disease scores, however there was large statistical and clinical heterogeneity between these studies. We found from meta-analysis that inhaled THC did have a beneficial effect on CDAI at 8 weeks, although CBD did not.

There may be several reasons for this heterogeneity; first in all groups, small cohort sizes were used, making meaningful conclusions difficult regarding the use of CBD or THC in IBD. Second, within the CBD study, very low doses of CBD were utilized compared to conventional use of CBD in other clinical trials, which commonly used 600 mg twice daily (Naftali et al. 2017). A recent trial in drug-resistant epilepsy found benefit with 20 $mg.kg^{-1}$ daily for 4 weeks (Devinsky et al. 2017), with a small number of participants experiencing side effects such as vomiting and diarrhoea. It is possible that such 10 mg doses had no clinical effect on Crohn's disease as insufficient plasma concentrations may have been reached due to the poor bioavailability of oral CBD.

A major flaw within the THC trial is sham cigarettes administered to participants contained *Cannabis sativa* flowers, from which active cannabinoids had been removed. It is unlikely that other compounds present in *Cannabis*, such as terpenes (the compounds which provide the characteristic smell of smoked cannabis), which also have an anti-inflammatory effect, had been removed (de las Heras & Hortelano 2009). This factor may have introduced positive bias into the study. Despite these drawbacks, inhaled THC did demonstrate, as subgroup analysis, reduction on

steroid dependence, decreased pain and improved sleep scores compared to placebo. This does allow a basis for further studies examining the effects of phytocannabinoids in Crohn's disease. Although not included in this review a paper from Storr et al (Storr et al. 2014) demonstrated in a retrospective, non-placebo controlled study that users of MMJ, although reporting improved symptomatic control in terms of diarrhoea and pain, had a significantly higher risk of undergoing surgery and bowel resection within 6 months of use (odds ratio = 5.03, 95% CI = 1.45-17.46). This finding, which apparently lies in opposition to preclinical findings, should be examined further with placebo controlled trials.

2.4.3 Suggested compounds for further study

Two compounds, CBD and PEA, were investigated most commonly within our meta-analysis, both of which have been shown previously to have a favourable side effect profile in humans. Although CBD did not show any benefit within a clinical trial, this may have been due to inadequate dosage or small study numbers. In addition to the studies identified in this review, both PEA and CBD have previously been investigated for their effects on permeability in human cell lines, demonstrating the ability to reduce inflammation-induced increases in permeability (Alhamoruni et al. 2012; Karwad et al. 2016). As CBD is already used in the treatment of epilepsy (Bergamaschi et al. 2011), and PEA is available over the counter, these two agents independently should be further investigated for their effect in humans. THC was shown to be beneficial at meta-analysis, and may be of benefit in humans. However due to its psychotropic effects and current status as a class B drug in the UK, progression to clinical study in the United Kingdom may not be timely, and therefore at this stage not investigated further.

2.4.4 Secondary endpoints

Mechanisms of action

Receptor targets were explored in 23 publications using receptor-specific agonists or antagonists, and receptor knock-out. In murine models of colitis, agonism of the CB_1 or CB_2 receptor lead to a reduction in inflammation, and at subgroup analysis use of the synthetic CB_1/CB_2 agonists acting demonstrated the greatest reduction in disease scores and MPO activity. In addition, studies also reported that activation of PPARα, GPR55 and GPR18 receptors reduced inflammation of the colon. The wide variation in the measured inflammatory endpoints across these studies prevented further meta-analysis. Interestingly the use of the peripherally restricted synthetic agonist SAB378, which activates both CB_1 and CB_2 receptors, had no significant effect on either MPO activity or DAI. This is in contrast to *ex vivo* human colonic

explant data, which demonstrated that cannabinoid activity with AEA or CBD was beneficial in colonic mucosal inflammation, which were peripherally restricted by definition of the explant model (Harvey et al. 2014; Nicotra et al. 2013). Izzo (Izzo *et al.*, 2001) found through receptor antagonism that the effect of CBN in preventing hypermobility caused by croton oil was mediated by CB_1, but not $_{CB2}$ receptors.

PEA was investigated by Capasso (Capasso et al. 2014; Capasso et al. 2001) using two models of inflammation-induced hypermotility. Using receptor antagonists in both experiments, Capasso found that PEA in an OM model acted through CB_1 but not CB_2 or PPARα, but in a CO model PEA was still effective but did not act through CB_1 or CB_2, suggesting that the mechanism by which PEA acts as an anti-inflammatory agent was not mediated by a single receptor, but by receptor co-dependence. ACEA was investigated for receptor mechanism in two publications, both of which found ACEA action is dependent on CB_1 receptors. None of the reviewed studies investigated a mechanism of action for AEA in gut inflammation; however, one *ex vivo* human study from Harvey found that AEA prevented increased cytokine production in experimentally inflamed human mucosa was dependent on CB_2 receptors, although the authors did not report antagonism of any other receptor (Nicotra et al. 2013).

Despite being the most-studied drug, the mechanism by which CBD acts was not made clear by this review. One study by De Fillipis et al (de Filippis et al. 2008) found that hyper-motility caused by LPS administration in mice was reduced by CBD through a CB_1 receptor-dependent mechanism. Similarly, Capasso in 2008 found that CBD prevented croton oil-induced hypermotility via CB_1. *In vitro*, de Fillipis in 2011 demonstrated that in human explant tissue S100B levels, as a marker of glial cell activation was decreased by CBD in a PPARγ dependent mechanism (although other antagonists were not investigated) (de Filippis et al. 2011).

Timing of administration

The timing of cannabinoid administration was weakly correlated with reduction in effect on colitis activity. There was also a correlation between time of drug administration and effect size in both DAI and MPO activity reduction, with earlier administration of cannabinoids drugs producing a greater effect size, suggesting that, for the purposes of clinical investigation, cannabinoids may be used prophylactically and therapeutically. As no clinical trials have investigated the prophylactic use of either MMJ or a specific cannabinoid compound compared to the therapeutic use, the hypothesis that cannabinoid compounds may be used in either setting remains unanswered and remains an area of potential study.

Combination therapy

One potentially important area for research is the combination of cannabinoid drugs with existing treatments for inflammatory bowel disease. In clinical practice, it is common to treat patients with acute severe Crohn's and ulcerative colitis with combination of agents, such as antibiotic, anti-TNFα, and corticosteroid therapy. One study compared the efficacy of CBD and THC with that of sulphasalazine, a 5-ASA, a drug commonly used in clinical practice (J. M. Jamontt et al. 2010). Although in this study CBD and THC efficacy were comparable to that of sulphasalazine, the authors did not examine the potential additive or subtractive effect of these agents in the context of colitis.

2.4.5 Limiting factors

The findings of this study are limited by several factors typically seen in meta-analyses and systematic reviews. We found significant heterogeneity between sub-groups in both DAI and MPO analyses, and suggested that 11% and 41% of this was due to the difference in time of administration in terms of changes in DAI and MPO respectively. Additionally, we found a high risk of bias in retrieved studies, and median study quality to be relatively low. Meta-regression demonstrated these factors significantly correlated with study outcomes. Although we did not analyse for differences between scoring systems and mode of colitis, these factors may have also contributed to heterogeneity and influenced outcome. We sought to overcome this variability between scoring systems with random effects analysis. Additionally, within this review we have examined the effect of cannabinoid drugs *en masse*, which may have affected the overall outcome of meta-analyses. It is possible that some articles may have not been identified in initial searches, or conference abstracts missed from the search period. Lastly, where control groups were compared to multiple experimental groups within the same set of experiments variance and SMD may be exaggerated, leading to further bias.

2.4.6 Summary

In conclusion, we have shown in this systematic review and meta-analysis that cannabinoid drugs are beneficial in treating experimentally-induced colitis in animal models. CBD and PEA have shown positive outcomes, showing potential for clinical use, and should therefore be investigated further.

3 Investigating the effect of CBD and PEA on the inflammatory response of the human colon

3.1 Introduction

In health, the gut absorbs nutrients from the luminal environment into the sterile submucosa without absorbing noxious material, such as bacteria. The barrier between lumen and submucosa is formed by epithelial cells, which allow selective absorption of particles into the enteric circulation via paracellular and transcellular pathways, whilst preventing bacterial translocation (van der Flier & Clevers 2009; Kim & Ho 2010; Coskun 2014; Peterson & Artis 2014; Lee 2015). Inflammation causes this barrier to become compromised (de Punder & Pruimboom 2015). Inflammation is present in conditions such as diverticulitis, infective colitis, appendicitis, and IBD (Okamoto & Watanabe 2016). When inflammation occurs, luminal bacteria and lipopolysaccharide are able to translocate into the submucosal space and beyond, resulting in secondary complications such as endotoxaemia, sepsis and death (Harrison et al. 2006; Vaishnavi 2013). At the cellular level, the increased presence of locally produced pro-inflammatory cytokines contributes to this increase in permeability by modification of the cellular apparatus governing paracellular and transcellular flow (section 1.1.3). Currently, there are no clinical treatments to counter the permeability changes seen in systemic inflammation and sepsis. Development of an agent to prevent bacterial and lipopolysaccharide translocation across the gut wall with the intention of reducing or preventing the triggering of sepsis is therefore of high clinical importance.

3.1.1 PEA and CBD

The effects of PEA and CBD on inflammation-induced membrane permeability have previously been investigated. In Caco-2 culture models, increased permeability was induced using TNFα and IFNγ, demonstrated by falls in TEER (Alhamoruni et al. 2012). PEA and CBD were shown to prevent these permeability changes when used prophylactically, and restored membrane resistance when given therapeutically, acting via PPARα and CB_1 receptors, respectively (Alhamoruni et al. 2010; Alhamoruni et al. 2012; Karwad et al. 2016). Previous studies have also shown that PEA and CBD can prevent increases in permeability in experimentally inflamed explant colonic tissue (Harvey et al. 2014; Esposito et al. 2014; de Filippis et al. 2011). Others have also observed a protective effect of PEA and CBD on the gut barrier during inflammation *in vivo* (Borrelli et al. 2015; Cassol-Jr et al. 2010).

However, the mechanisms by which PEA and CBD exert these effects on permeability is not clear. Existing literature provides two hypotheses. First, both PEA and CBD have been shown to exert an anti-inflammatory effect on enteric glial cells, and can prevent the immune response *in vivo* (Parrella et al. 2016; Skaper et al. 2013; de Filippis et al. 2011; Esposito et al. 2014). Therefore, any permeability effects caused by these compounds may be secondary to an anti-inflammatory effect. Secondly, Karwad et al. (2016) showed that PEA may affect permeability through modification of the actin cytoskeleton by inducing Focal Adhesion Kinase phosphorylation (Karwad et al. 2016). Within this study it was not clear if this effect was secondary to an anti-inflammatory effect or due to the direct action of PEA on permeability-governing structures.

3.1.2 Study aims

In this study, we sought to investigate further the effect of PEA and CBD on the local inflammatory response in cultured Caco-2 cells and normal explant human colonic tissue. We hypothesised that CBD and PEA would cause changes in intestinal permeability through suppression of the local immune response. We therefore examined the effect of CBD and PEA on intracellular signalling phosphoproteins in response to inflammation, and on downstream production of inflammatory cytokines. By examining these effects in Caco-2 cultures and colonic explant tissue, we were able to compare the effects of PEA and CBD on epithelial Caco-2 cells alone to their effect on whole tissue. We then aimed to, for the first time in humans, examine the effects of these compounds on colon explants from patients with established inflammation caused by acute appendicitis and inflammatory bowel disease.

3.2 Methods

3.2.1 Ethical considerations

All experiments and procedures received prior approval of the University of Nottingham Ethics Committee and local NHS Research Ethics Committee (ethics number DHRD/2008/060).

3.2.2 Caco-2 cell culture

Caco-2 cells were purchased from European Collection of Cell Culture (Wiltshire, UK; passages 21-42). Cells were cultured in Eagle's minimum essential medium supplemented with L-glutamine, 10% foetal bovine serum (FBS), 1% penicillin/streptomycin and 1% non-essential amino acids mixture (all Sigma-Aldrich). Cells were kept at 37 °C in 5% CO_2 and 95% humidity. Cells were seeded at $4x10^5$ cells per well in polystyrene 12 well plates (Corning Incorporated, USA), and grown for 2 weeks until fully differentiated. Cells were used for experimentation at day 14-16. The medium was changed on alternate days as this was found to prevent shedding of cells from the basement of the well secondary to glucose deprivation.

3.2.3 Caco-2 experimentation

Randomly assigned wells (n=8) were treated with the following drug treatments: vehicle, an inflammatory protocol of IFNγ (10 $ng.ml^{-1}$, Sigma-Aldrich) for 8 hours, followed by TNFα (10 $ng.ml^{-1}$, Sigma-Aldrich) for 16 hours, inflammation and PEA (10 μM, PEA was added simultaneously with IFNγ at the start of the 24-hour inflammatory period), inflammation and CBD (10 μM, CBD was added simultaneously with IFNγ at the start of the 24-hour inflammatory period), PEA (10 μM) alone, or CBD (10 μM) alone. PEA and CBD were purchased from Tocris Bioscience (Bristol, UK). At the end of the 24-hour experimental period, media were collected and stored at -80 °C until analysis. Cells were washed twice with ice-cold phosphate-buffered saline (PBS), and treated with radioimmunoprecipitation assay (RIPA) buffer supplemented with phosphatase and protease inhibitors (Sigma-Aldrich) at 4 °C for one hour on a rocking platform to cause cell lysis. Cell lysates were then collected and stored at -80 °C until analysis.

3.2.4 Human colon culture

Experiments on *ex vivo* human tissue were performed by obtaining colonic samples from the appendix, caecum or ascending colon from patients having undergone elective bowel resections for bowel cancer (n=13), planned resections for quiescent

inflammatory bowel disease (n=6) or emergency appendectomies for acute appendicitis (n=6) at Derby Teaching Hospitals NHS Trust, Derbyshire, UK. Samples of normal colon at least 10 cm proximal to right sided bowel tumours (in the case of bowel cancer resections), sections of inflamed colon (in inflammatory bowel disease resection), or sections of inflamed appendix (in the case of emergency appendicectomy) were obtained immediately after resection in the operating theatre. Sections of tissue 2 x 2 cm were removed from the resected specimen and transferred on ice to the laboratory within ten minutes, in pre-chilled Eagle's minimum essential medium supplemented with 1% FBS, 1% penicillin/streptomycin and 1% non-essential amino acids mixture (Sigma-Aldrich). The remaining operative specimen was sent to pathology for routine analysis.

Once in the laboratory, samples were pinned on Stylgard plates. Mucosa with submucosa was dissected free from the underlying muscularis layer. Mucosal samples were then further dissected into 2 x 2 mm sections and placed in individual wells of 24-well polystyrene plates (Corning Incorporated, USA), each containing 1 ml of media.

3.2.5 Human colon experimentation

Samples of colonic tissue were treated with TNFα and IFNγ, in the absence or presence of PEA and CBD as described with Caco-2 experiments in section 3.2.2. Experiments were carried out in triplicate, with final values derived from the mean result of three measurements. Samples were incubated for 24 h at 37 °C in 5% CO_2 and 95% humidity. At the end of the 24-h experimental period media was collected and stored at -80 °C until analysis. Explant tissue was washed with ice cold PBS and stored frozen at -80 °C until homogenisation and analysis. Prior to analysis, colonic samples were thawed on ice and cryohomogenised using the method described by von Ziegler (von Ziegler et al. 2013). Collected homogenates were then dissolved in 215 µl of RIPA buffer, incubated on an oscillating thermomixer for 30 min at 60 °C, then centrifuged at 10,000 g for 15 min. Supernatant layers were collected, vortexed for 20 seconds and then analysed.

3.2.6 Intracellular signalling pathways

To determine the effect of CBD and PEA on signalling proteins, Luminex xMAP technology (Austin, TX, USA) was used to detect changes in phosphorylated cAMP response element-binding protein (CREB; pS133), extra-cellular signal regulated kinase (Erk) (pT185/pY187), NF-κB (pS536), c-Jun N-terminal kinase (JNK) (pT183/pY185), p38 (pT180/pY182), p70-S6 Kinase 1 (P70 S6K) (pT412), Signal transducer and activator of transcription-3 (STAT3) (pS727),

Signal transducer and activator of transcription-5 (STAT5) (pY694/699), and alpha serine/threonine-protein kinase (Akt) (pS473) (Milliplex 48-680MAG; Merck Millipore, Darmstadt, Germany) from undiluted cell lysates or homogenised colonic lysates according to the manufacturer's instructions using a Magpix plate reader (MAGPX 11326002, Luminex, Texas, US). Signalling phosphoprotein concentrations were corrected for total protein content using the bicinchoninic acid (BCA) assay determination of lysate protein concentration against a standard curve.

3.2.7 Cytokine production

To quantify the effects of PEA and CBD on the secreted inflammatory response, we measured media concentrations from cell or colonic cultures of seven cytokines at the end of the 24-h experimental period using enzyme-linked immunosorbent assay (ELISA). Cytokines measured were: IL-8, MCP-1, intercellular adhesion molecule-1 (ICAM-1), matrix metallopeptidase-3 (MMP-3); DUOSET ELISA kits R&D Systems Minneapolis, US, IL-17, granulocyte-macrophage-colony stimulating factor (GM-CSF) and IL-6; ready-set-go ELISA kits, Affymetrix eBioscience, San Diego, CA. Cytokine concentrations were normalised for protein content as previously using BCA assay determination of cell lysate protein concentration against a standard curve. Experimental conditions were averaged from triplicate readings, as above.

3.2.8 Target sites of action of PEA & CBD

To identify target sites of action of PEA and CBD, the following antagonists were co-applied to healthy colonic explant tissue with PEA or CBD under inflammatory conditions: AM251 100 nM (CB_1 receptor-selective antagonist, Tocris Bioscience, Bristol, UK), AM630 100 nM (CB_2 receptor-selective antagonist, Tocris Bioscience, Bristol, UK), GW6471 500 nM (PPARα receptor-selective antagonist, Tocris Bioscience, Bristol, UK), GW9662 100 nM (PPARγ receptor-selective antagonist, Tocris Bioscience, Bristol, UK), SB366791 500 nM (TRPV1 receptor-selective antagonist, Tocris Bioscience, Bristol, UK), and CID16020046 500 nM (GPR55 antagonist, Tocris Bioscience, Bristol, UK). Concentrations of antagonists were based on previously published experimentation (Alhamoruni et al. 2012). Receptor occupancies were not calculated during this study. Following co-incubation for 24 h, media concentrations of IL-8, IL-6 and MCP-1 were measured using ELISA.

3.2.9 Statistical analysis

Data are presented as mean (or mean percentage change from baseline where indicated) ± standard error of the mean (SEM). For each well containing Caco-2 cultures or human explant tissue, three readings were taken, calculating the mean

result. Caco-2 cytokine and signalling protein concentration results were compared using one-way analysis of variance (ANOVA). Human tissue cytokine secretion and phosphorylated protein levels are expressed as percentage change compared to vehicle per patient or [pg/ml]/[mg/ml] where indicated, and analysed using one-way ANOVA. For human tissue, each value represents the mean of three independent values. A p-value of <0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 7.01 for Windows (GraphPad Software, San Diego, USA).

3.3 Results

3.3.1 Caco-2 intracellular response to inflammation

Stimulation of Caco-2 cells with IFNγ and TNFα increased the levels of phosphorylated CREB ($p<0.001$), JNK ($p<0.001$), NF-κB ($p<0.001$), p38 ($p<0.01$), ERK1/2 ($p<0.05$), Akt ($p<0.01$), p70S6k ($p<0.001$), STAT3 ($p<0.01$), and STAT5 ($p<0.01$) (figure 3.1).

PEA in the presence of inflammation, compared to inflammation alone, prevented the increases of phosphorylated CREB ($p<0.01$) and JNK ($p<0.01$).

CBD, in the presence of inflammation compared to inflammation alone, prevented the production of CREB ($p<0.05$), p38 ($p<0.05$), NF-κB ($p<0.01$), JNK ($p<0.05$) and STAT5 ($p<0.05$).

CBD or PEA treatment alone did not affect the level of any of the phosphorylated proteins compared to control tissue.

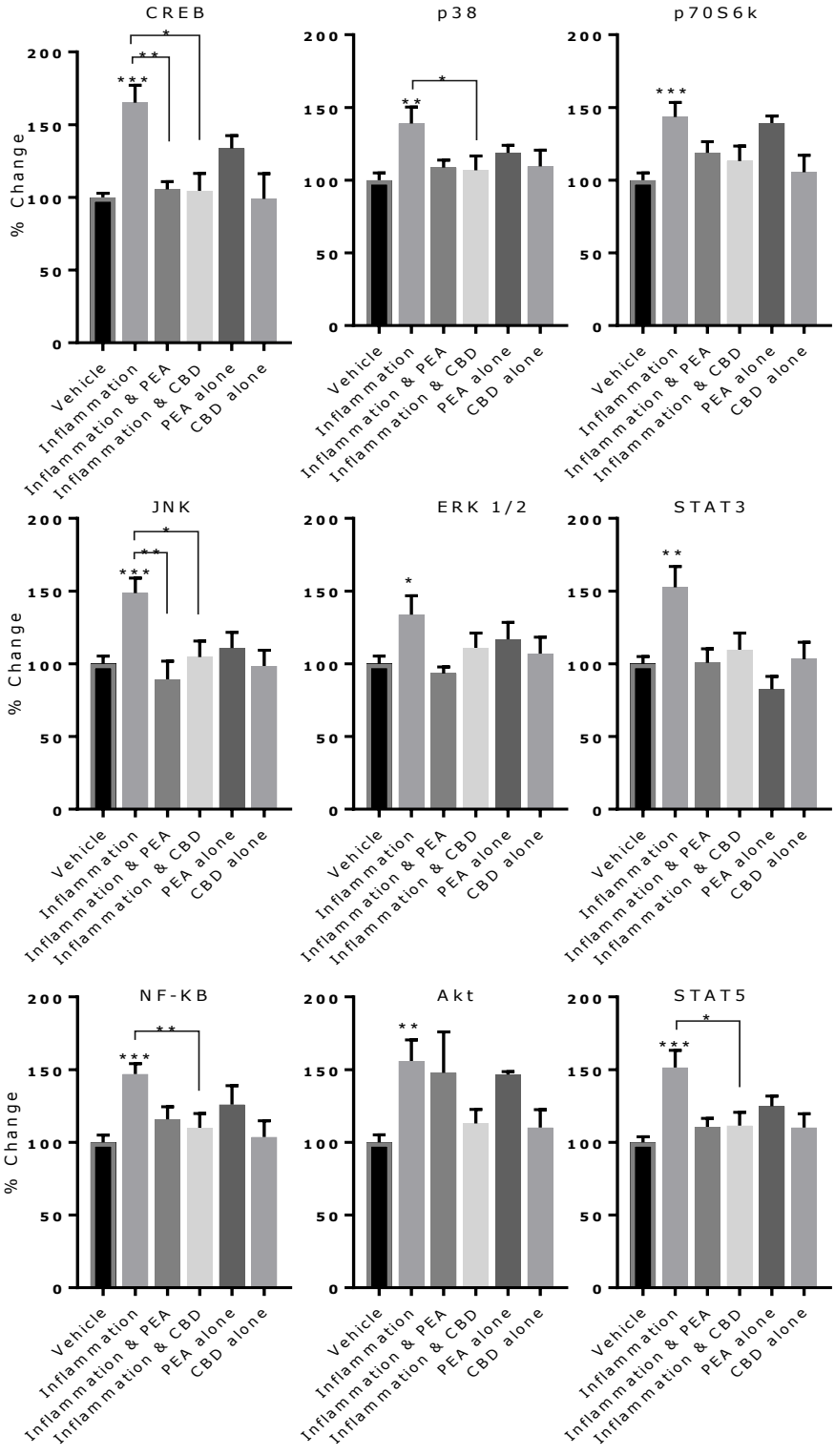


Figure 3.1 The effects of PEA and CBD on the phosphorylation of signalling proteins in response to an inflammatory protocol in cultured Caco-2 monolayers, measured by multiplex. Data are presented as percentage change from vehicle per plate +/- SEM, n=8 per condition. Data were analysed by one-way ANOVA comparing against the vehicle control or inflammation where indicated (*P<0.05, **P<0.01 and ***P<0.001).

3.3.2 Human colon intracellular response to inflammation

Stimulation of explant colonic tissue with IFNγ and TNFα resulted in an increase in phosphorylated CREB ($p<0.05$), NF-κB ($p<0.05$), Akt ($p<0.01$), p70S6k ($p<0.001$), STAT3 ($p<0.001$) and STAT5 ($p<0.001$) (figure 3.2).

PEA in the presence of inflammation, compared to inflammation alone, prevented increases in phosphorylated CREB ($p<0.05$), NF-κβ ($p<0.01$), Akt ($p<0.01$), p70S6k ($p<0.001$), STAT3 ($p<0.01$) and STAT5 ($p<0.01$).

CBD in the presence of inflammation, compared to inflammation alone, did not significantly reduce any measured phosphoprotein.

PEA or CBD alone did not affect any phosphoprotein compared to vehicle.

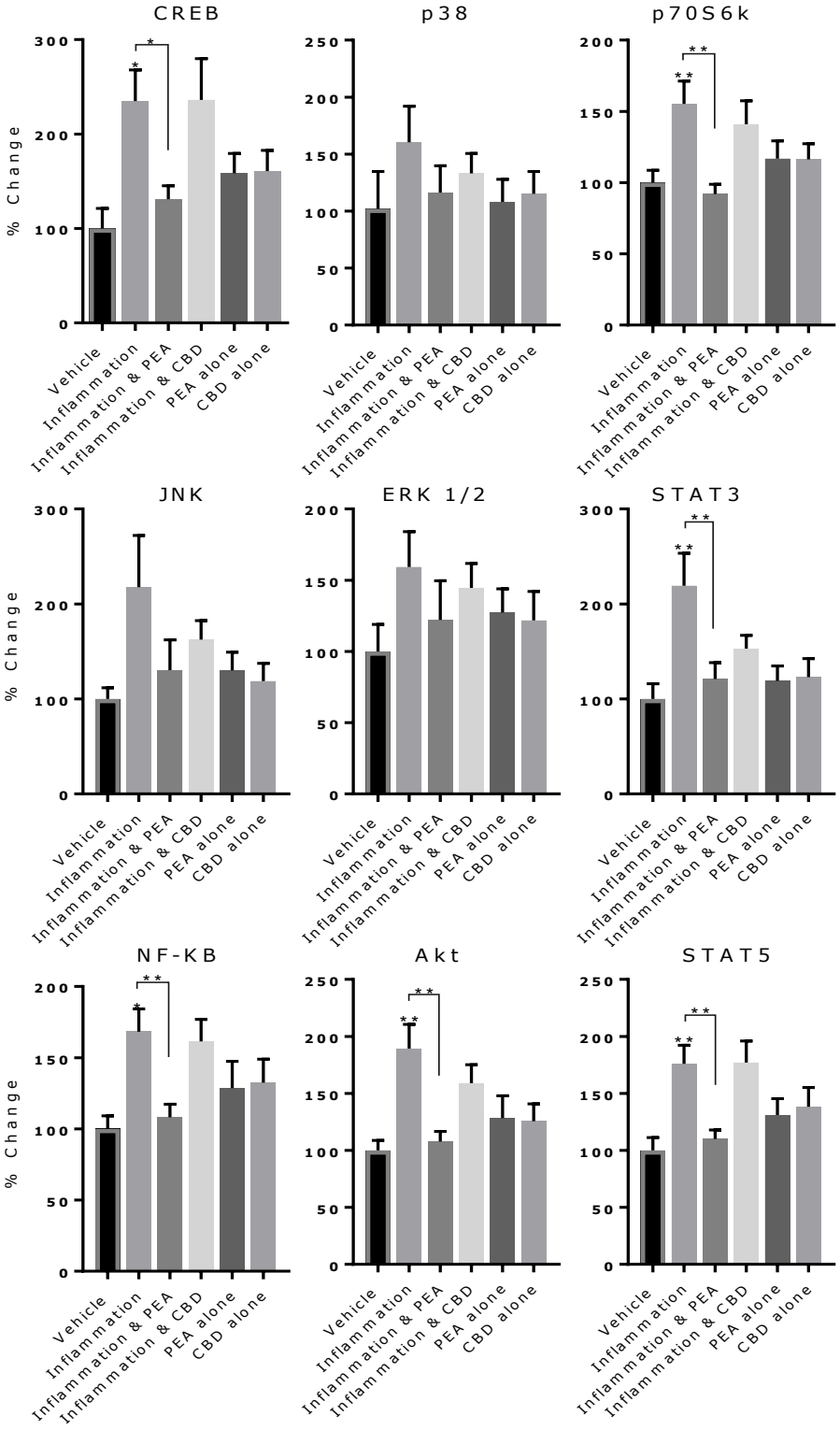


Figure 3.2 The effects of PEA and CBD on levels of phosphorylated signalling proteins in response to 24 h exposure to TNFα and IFNγ in cultured human colonic explants, measured by multiplex. Data are presented as percentage change from vehicle +/- SEM, n=13 per condition. Data were analysed by one-way ANOVA comparing against the vehicle control or inflammation where indicated (*P<0.05, **P<0.01).

3.3.3 Effects of PEA and CBD on cytokine production in response to inflammation

IL-6, IL-8 and IL-17

Stimulation of Caco-2 cells with IFNγ and TNFα caused an increase in the secretion of IL-8 ($p<0.001$) and IL-6 ($p<0.05$), although did not increase in the secretion of IL-17 (figure 3.3). PEA and CBD did not affect the production of these cytokines in the presence of inflammation.

In Caco-2 cells, PEA and CBD did not change the production of IL-8, IL-6 of IL-17 compared to vehicle.

In human colonic tissue IFNγ and TNFα caused and increase in the production of IL-8 ($p<0.01$), IL-6 ($p<0.05$) and IL-17 ($p<0.05$) compared to vehicle (figure 3.3). PEA prevented the increased production of IL-8 ($p<0.05$), IL-6 ($p<0.05$) and IL-17 ($p<0.05$). CBD also prevented the increased production of IL-8 ($p<0.01$), IL-6 ($p<0.05$) and IL-17 ($p<0.05$).

In human colonic tissue, PEA and CBD did not change the production of IL-8, IL-6 or IL-17 compared to vehicle.

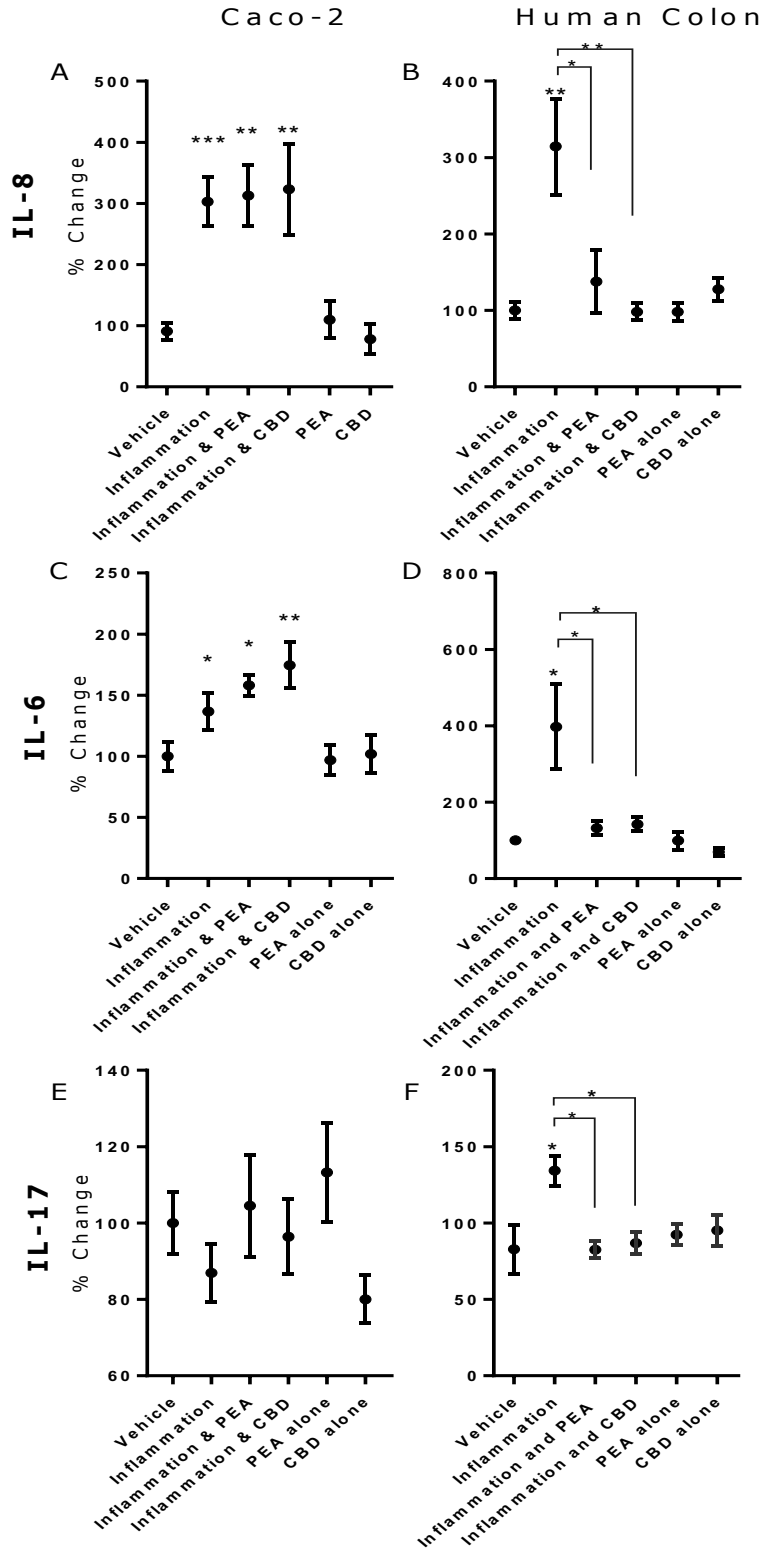


Figure 3.3 The effects of PEA and CBD on the secreted cytokine response to an inflammatory protocol in cultured Caco-2 monolayers (A, C and E, column 1, n=8) and human colonic tissue (B, D and F, column 2, n=7), measured by ELISA. Error bars represent +/- SEM per condition. Data were analysed by one-way ANOVA comparing against the vehicle control or inflammation where indicated (*P<0.05, **P<0.01 and ***P<0.001).

GM-CSF

Stimulation of Caco-2 cells with IFNγ and TNFα did not increase the production of GM-CSF, but both PEA alone and CBD alone increased GM-CSF production compared to vehicle ($p<0.01$ and <0.0001 respectively) (figure 3.4 A).

In human tissue, IFNγ and TNFα increased the production of GM-CSF compared to vehicle ($p<0.05$) (figure 3.4 C). CBD, but not PEA, prevented this increase in GM-CSF production ($p<0.05$).

MCP-1

IFNγ and TNFα increased MCP-1 production in both Caco-2 cells ($p<0.0001$ and human colonic tissue ($p<0.001$) (figure 3.4 B D).

In Caco-2 cells, in the presence of inflammation, CBD and PEA had no effect on the production of MCP-1 compared to inflammation alone.

In human colonic tissue, in the presence of inflammation, CBD and PEA reduced the production of MCP-1 compared to inflammation alone (both $p<0.05$).

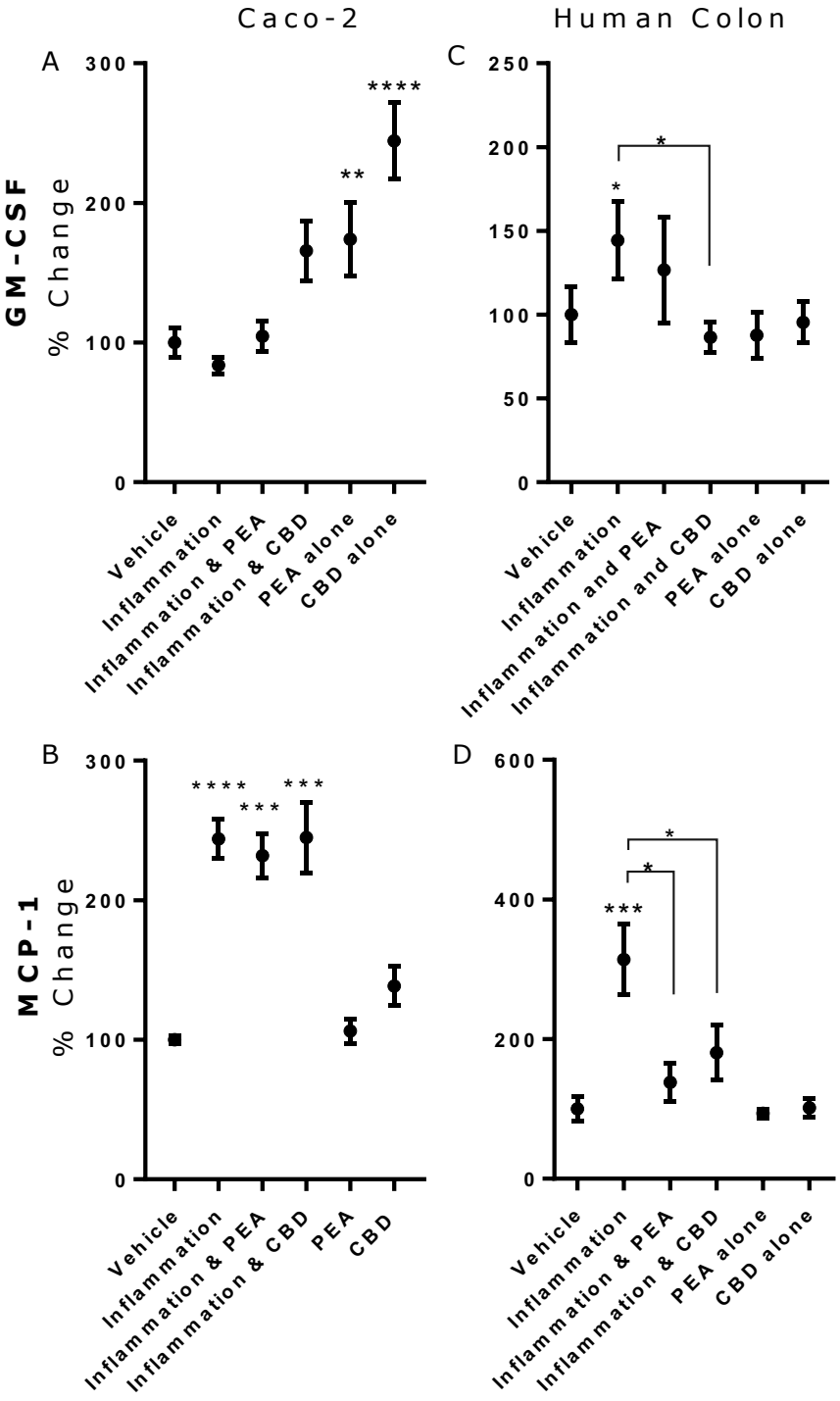


Figure 3.4 The effects of PEA and CBD on granulocyte macrophage colony stimulation (A, C) and monocyte chemotaxis (B, D) in response to an inflammatory protocol in cultured Caco-2 monolayers (column 1, n=8) and human colonic tissue (column 2, n=7), measured by ELISA. Error bars represent +/- SEM per condition. Data were analysed by one-way ANOVA comparing against the vehicle control or inflammation where indicated (*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001).

ICAM-1

Stimulation of Caco-2 cells with IFNγ and TNFα increased the production of ICAM-1 ($p<0.05$) (figure 3.5 A).

CBD and PEA did not prevent this increase in production, but PEA alone caused an increase in ICAM-1 production compared to vehicle ($p<0.001$).

In human colonic tissue, IFNγ and TNFα caused an increase in ICAM-1 production compared to vehicle ($p<0.05$). This was prevented by PEA and CBD (both $p<0.05$) (figure 3.5 C).

PEA alone, and CBD alone had no effect on ICAM-1 production in colonic tissue compared to vehicle.

MMP-3

Treatment of Caco-2 cells with IFNγ and TNFα or PEA or CBD had no effect on the production of MMP-3 (figure 3.5 B).

In human colonic tissue, IFNγ and TNFα increased the production of MMP-3 ($p<0.05$). PEA and CBD non-significantly reduced the production of MMP-3 in the presence of IFNγ and TNFα (figure 3.5 D).

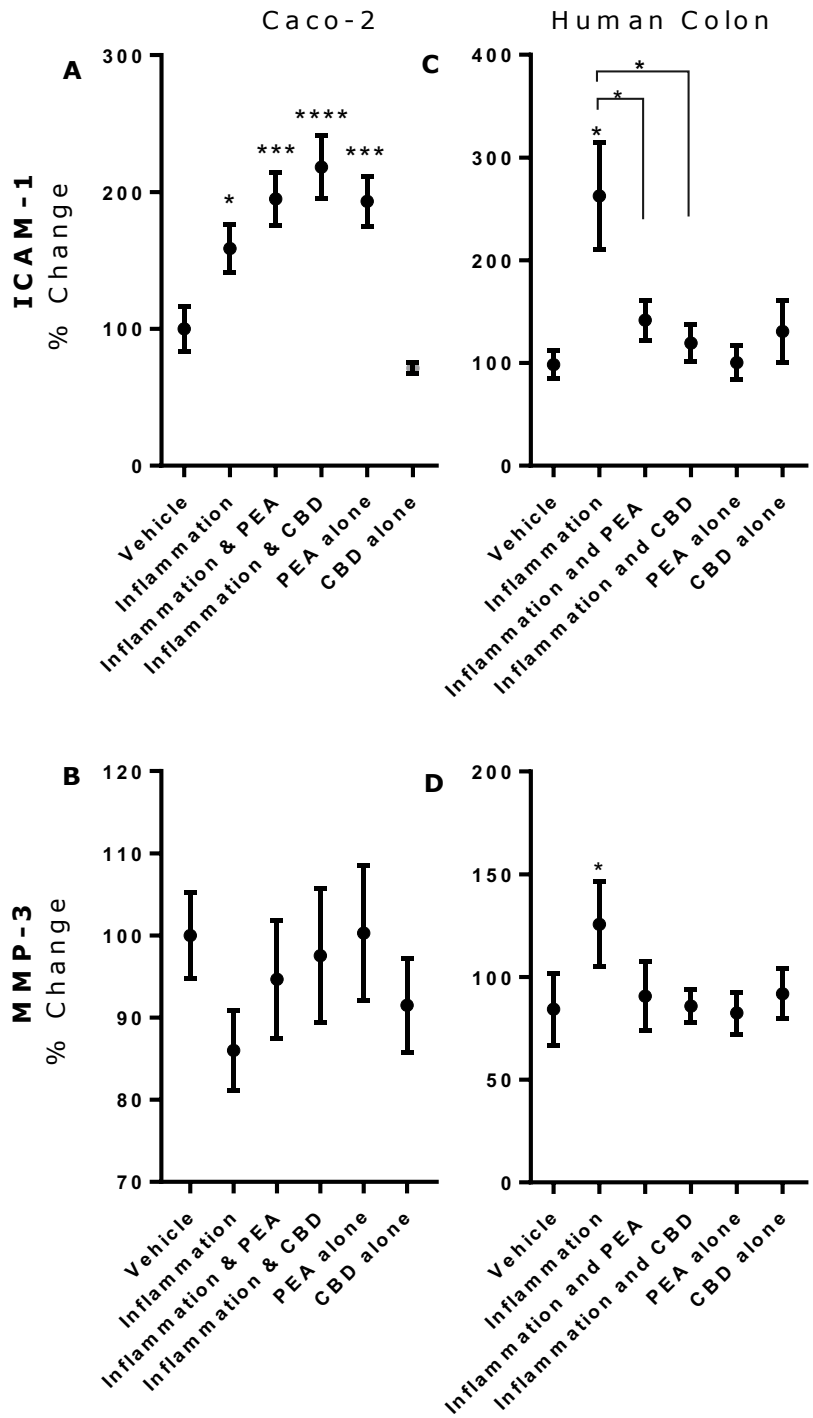


Figure 3.5 The effects of PEA and CBD on intracellular adhesion molecule-1 (A,D) and matrix metalloproteinase secretion (B,D) response to an inflammatory protocol in cultured Caco-2 monolayers (column 1, n=7) and human colonic tissue (column 2, n=7), measured by ELISA. Error bars represent +/- SEM per condition. Data were analysed by one-way ANOVA comparing against the vehicle control (*P<0.05, ***P<0.001 and ****P<0.0001).

3.3.4 Antagonist studies

In antagonist studies, stimulation of human colonic tissue with IFNγ and TNFα caused a significant rise in IL-8, IL-6 and MCP-1 production compared to vehicle, whilst treatment of inflamed colon with simultaneous PEA or CBD prevented these increases in cytokine production (figure 3.6 A to F).

The anti-inflammatory effects of PEA on IL-8, IL-6 and MCP-1 production were prevented by the addition of the PPARα antagonist GW6471 ($p<0.05$, for all). The antagonists AM251, AM630, GW9662, SB366791 and CID16020046 did not affect the action of PEA on cytokine production.

The anti-inflammatory effects of CBD on IL-8, IL-6 and MCP-1 production were prevented by the CB_2 receptor antagonist AM630 ($p<0.01$, $p<0.05$ and $p<0.05$ for all). The anti-inflammatory effects of CBD on MCP-1 production were also inhibited by the addition of the TRPV1 receptor antagonist SB366791 ($p<0.05$).

SB366791 appeared to (non-significantly) prevent the anti-inflammatory effect of CBD on IL-8 production ($p=0.31$), but had no effect on the anti-inflammatory effect of CBD on IL-6 production. The antagonists AM251, GW6471, GW9662 and CID16020046 did not affect the action of CBD on cytokine production.

GW6471, AM630 and SB366791 had no effect on cytokine production when administered alone (figure 3.7).

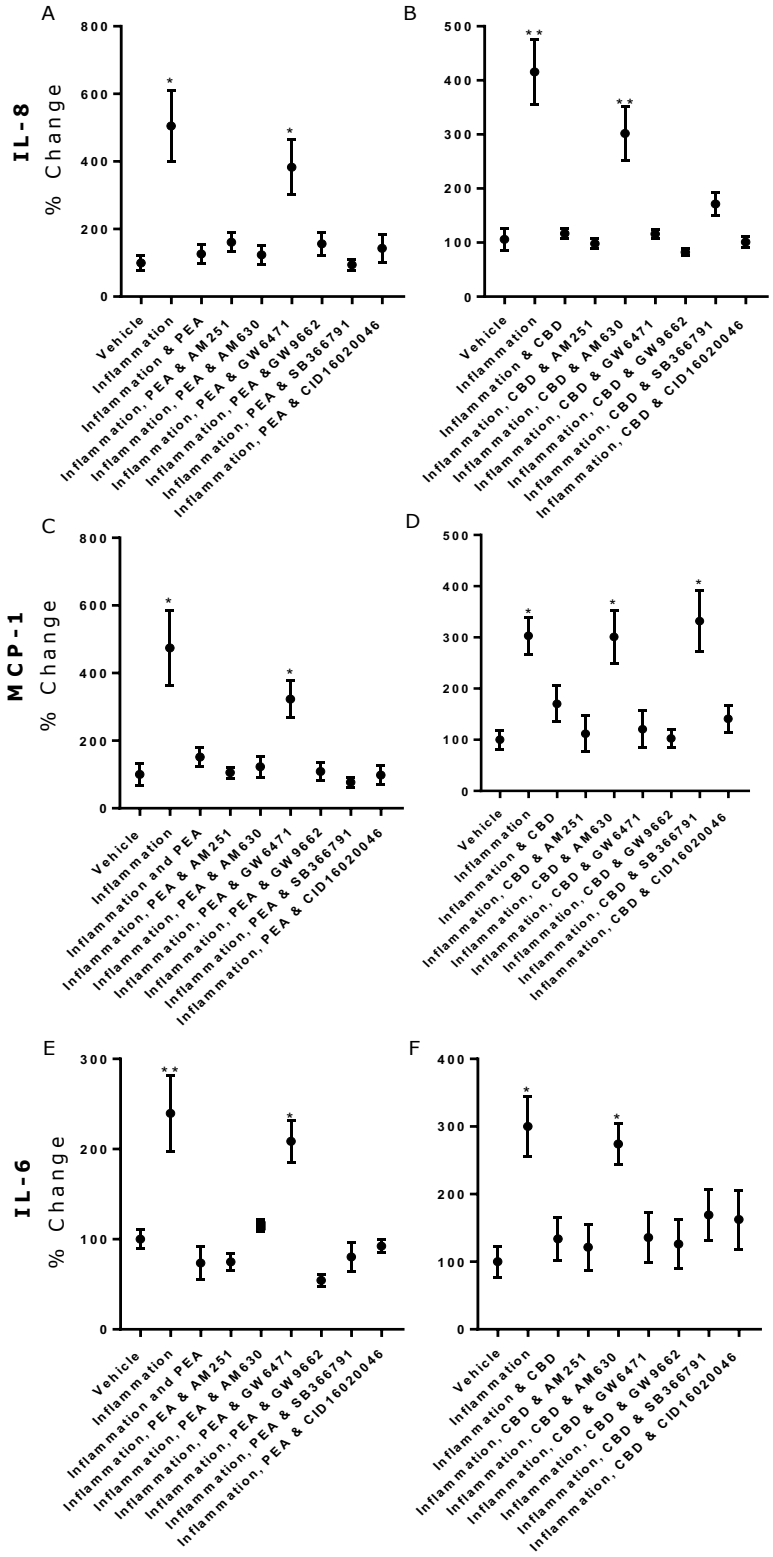


Figure 3.6 The effects of PEA (A,C,E) and CBD (B,D,F) on the secretion of IL-8, MCP-1 and IL-6 in response to an inflammatory protocol in explant human colonic tissue in the presence of receptor antagonists, measured by ELISA (n=10). Error bars represent +/- SEM per condition. Data were analysed by one-way ANOVA comparing against the vehicle control (*P<0.05, **P<0.01).

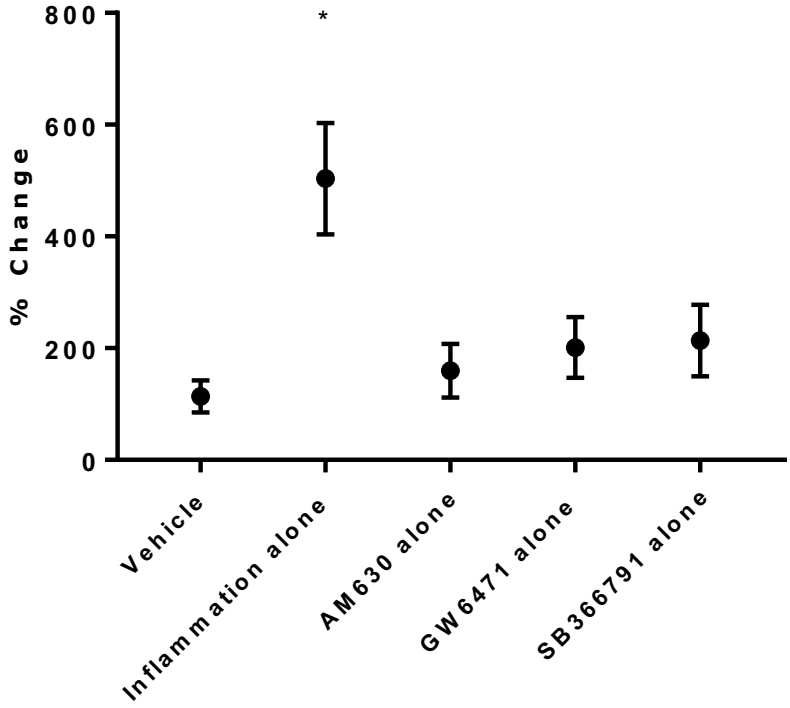


Figure 3.7 The effects of the receptor antagonists AM630, GW6471 & SB366791 on the secretion of MCP-1 of explant human colonic tissue, contrasted with the effect of an inflammatory protocol, measured by ELISA. Error bars represent +/- SEM per condition (n=7). Data were analysed by one-way ANOVA comparing against the vehicle control (*P<0.05).

3.3.5 Effects of PEA and CBD on cytokine production in response to inflammation in IBD and appendicitis explants

Because we found PEA and CBD had an anti-inflammatory effect on experimentally inflamed colonic tissue, we sought to find if CBD and PEA would exert the same anti-inflammatory effects in clinically inflamed tissue. We tested this by collecting samples of inflamed colon from patients with IBD and acute appendicitis.

IBD explants

Explants of six patients with quiescent IBD were collected. All participants included had elective resections for ulcerative colitis. Baseline measurements of untreated IBD tissue demonstrated an increase of between approximately 40% and 930% of cytokine production comparison to vehicle-treated sections of healthy tissue (figure 3.8). This reached significance in IL-8 ($P<0.01$), IL-6 ($p<0.01$), IL-17 ($p<0.05$), GM-CSF ($p<0.05$), and ICAM-1 ($p<0.01$).

As before, explants were treated with IFNγ and TNFα to simulate acutely active IBD. Treatment of IBD explants with IFNγ and TNFα further increased the secretion of IL-6, IL-17, GM-CSF, MCP-1 and ICAM-1 (all $p<0.05$) (figure 3.9).

PEA prevented the cytokine-stimulated increased production of IL-6, IL-17, and GM-CSF (all $p<0.05$).

CBD prevented the cytokine-stimulated increased production of IL-8 and IL-17 (both $p<0.05$).

Treatment of unstimulated IBD tissue with either PEA or CBD alone produced a trend of decreased inflammatory cytokine secretion, however this only reached significance in the case of IL-6 in CBD-treated tissue ($p<0.05$).

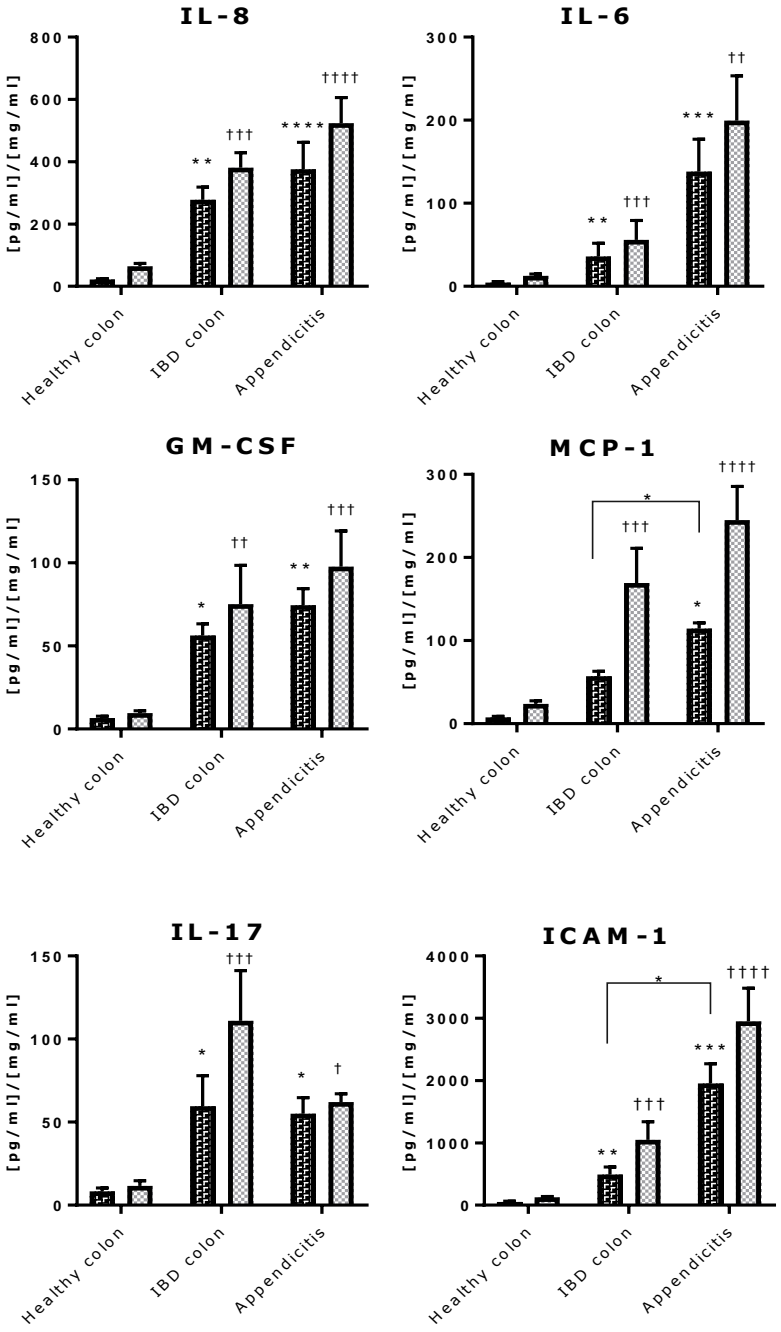


Figure 3.8 The effect of an inflammatory protocol on the secretion of inflammatory cytokines on normal colon, sections of IBD tissue and samples of appendicitis, measured by ELISA. Darkly shaded columns represent vehicle treated sections of tissue, lightly shaded bars represent tissue sections treated with an inflammatory protocol. Vehicle-treated normal colonic sections are compared with vehicle-treated IBD and appendicitis samples with two-way ANOVA (* represent significant difference from vehicle-treated healthy colon), inflammatory-treated normal colonic sections are also compared with inflammatory-treated IBD and appendicitis samples (significant differences represented by †). Error bars represent +/- SEM per condition. * or † $p<0.05$, ** or †† $p<0.01$, *** or ††† $p<0.0001$, **** or †††† $p<0.00001$.

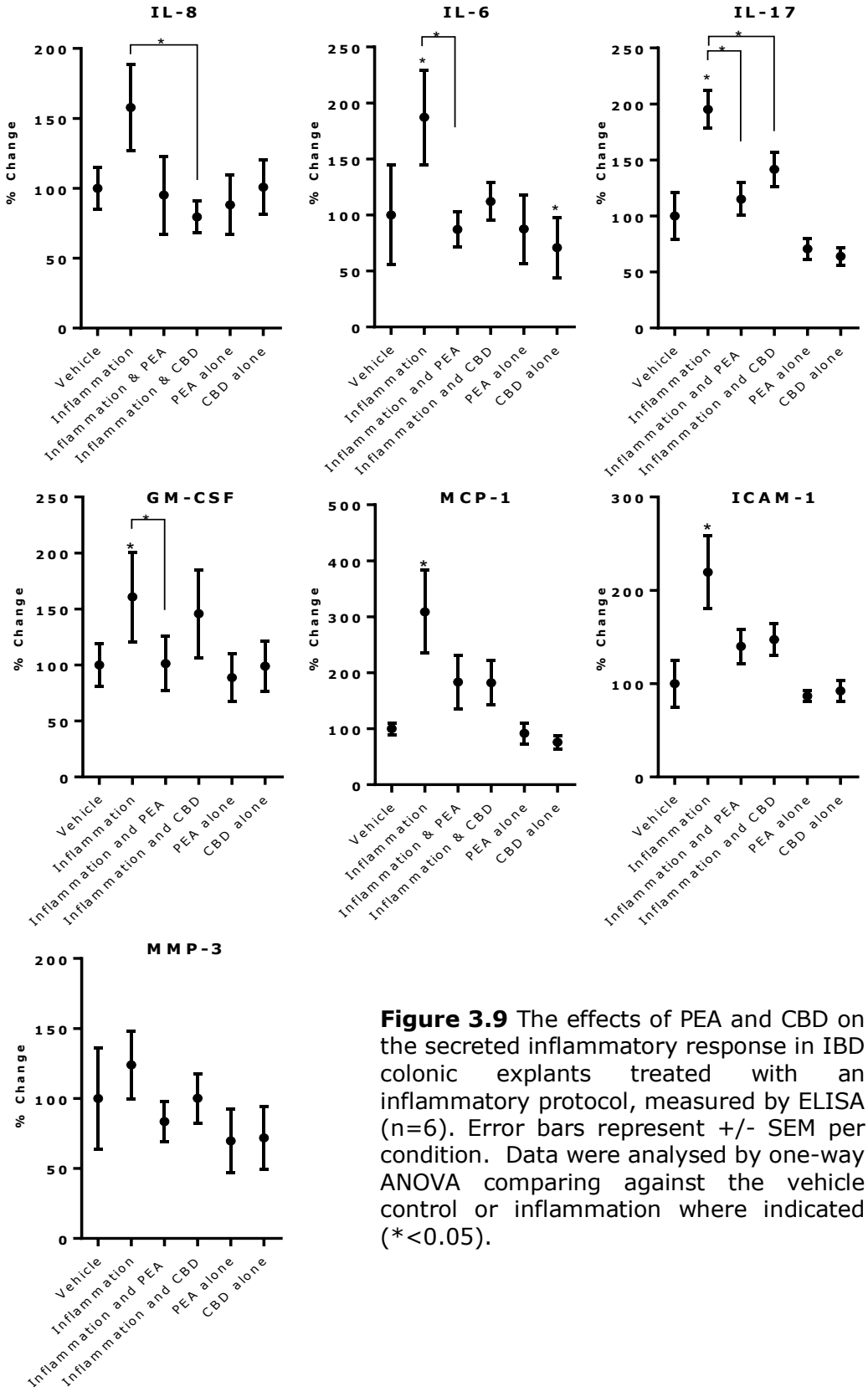


Figure 3.9 The effects of PEA and CBD on the secreted inflammatory response in IBD colonic explants treated with an inflammatory protocol, measured by ELISA (n=6). Error bars represent +/- SEM per condition. Data were analysed by one-way ANOVA comparing against the vehicle control or inflammation where indicated (*<0.05).

Appendicitis explants

Explants of six patients with acute appendicitis were collected. No samples included contained areas of macroscopic gangrene.

Baseline measurements of untreated appendicitis tissue demonstrated a significant increase in cytokine production compared to untreated normal tissue (figure 3.8). This reached significance in IL-8 ($P<0.0001$), IL-6 ($p<0.001$), IL-17 ($p<0.05$), GM-CSF ($p<0.01$), MCP-1 ($p<0.05$) and ICAM-1 ($p<0.001$). Compared to untreated IBD tissue, appendicitis tissue trended to produce increased levels of cytokines. This reached significance in MCP-1 and ICAM-1 (both $p<0.05$).

Compared to vehicle-treated appendicitis tissue, PEA alone supressed the secretion of IL-6 ($p<0.05$), MCP-1 ($p<0.01$) and ICAM-1 ($p<0.01$) (figure 3.10).

Compared to vehicle-treated appendicitis tissue, CBD alone supressed the secretion of GM-CSF, MCP-1 and ICAM-1 (all $p<0.05$).

Treatment of appendicitis explants with IFNγ and TNFα caused an increase in the secretion of inflammatory cytokines, though this only reached significance in MCP-1 ($p<0.05$).

Treatment of IFNγ and TNFα-stimulated appendicitis tissue with PEA decreased the secretion of MCP-1 compared to appendicitis tissue stimulated with cytokines alone ($p<0.05$).

Treatment of IFNγ and TNFα-stimulated appendicitis tissue with CBD decreased the secretion of MCP-1 compared to appendicitis tissue stimulated with IFNγ and TNFα alone ($p<0.05$).

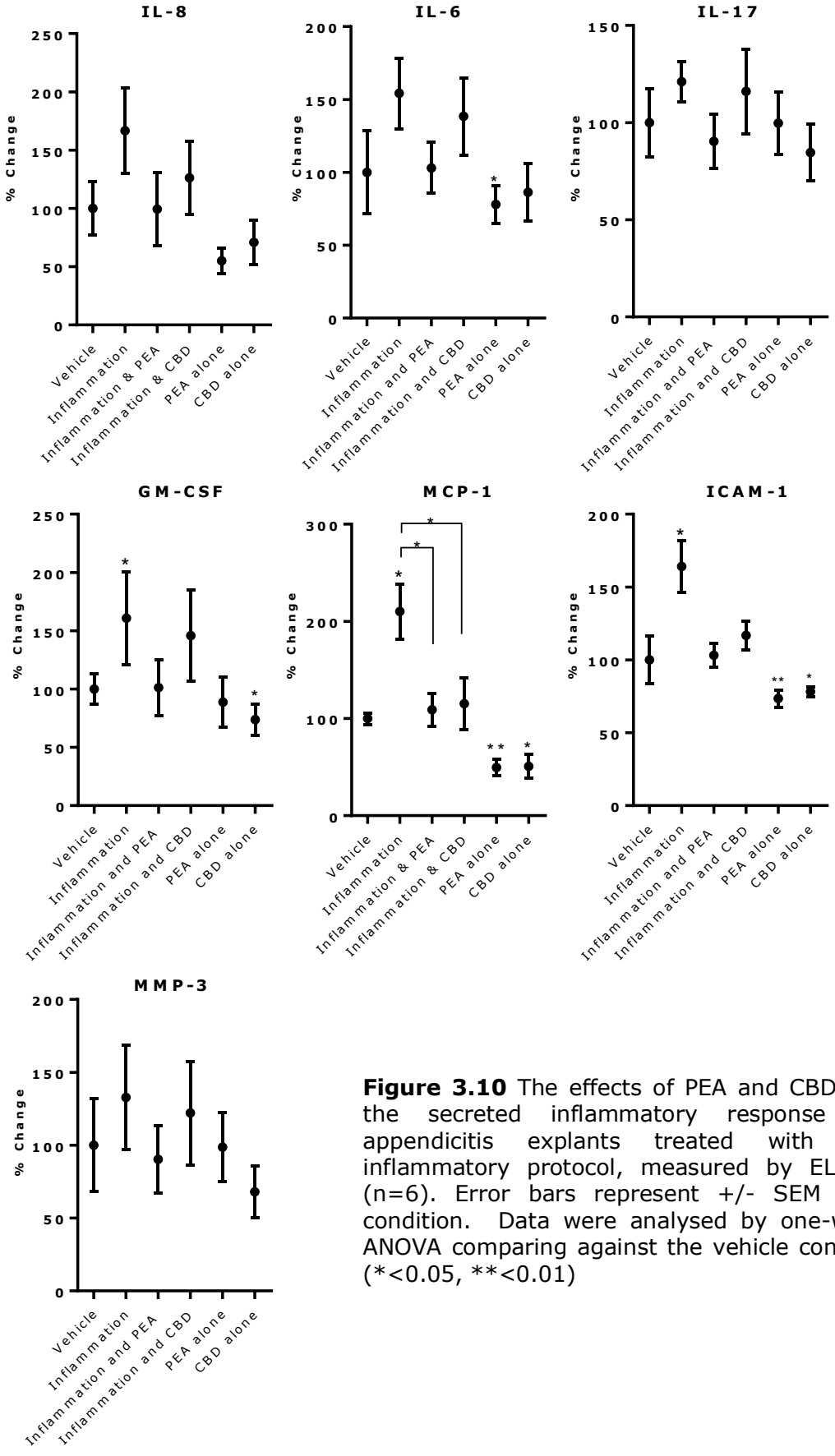


Figure 3.10 The effects of PEA and CBD on the secreted inflammatory response in appendicitis explants treated with an inflammatory protocol, measured by ELISA (n=6). Error bars represent +/- SEM per condition. Data were analysed by one-way ANOVA comparing against the vehicle control (*<0.05, **<0.01)

3.4 Discussion

The aim of this study was to examine the anti-inflammatory properties of PEA and CBD, comparing Caco-2 cell cultures (representing epithelial cells in the absence of innate immune cells) and mucosal explants from human colon. We demonstrate that under inflammatory conditions, PEA and CBD supress the phosphorylation of several intracellular proteins in Caco-2 cells, however this does not supress the secretion of pro-inflammatory cytokines. Conversely, in explant human colonic tissue stimulated with IFNγ and TNFα, PEA and CBD both supress the phosphorylation of intracellular proteins which were up-regulated by inflammation, and also prevented the increased secretion of pro-inflammatory cytokines. Additionally, we have shown that PEA and CBD have an anti-inflammatory effect in explant IBD and appendicitis tissue.

3.4.1 Caco-2 cell cultures

It has been previously demonstrated that PEA and CBD prevent changes in the permeability of Caco-2 monolayers under inflammatory conditions (Alhamoruni et al. 2012; Karwad et al. 2016). Prior to conducting this study we had hypothesized that this effect on permeability was secondary to a local anti-inflammatory action, as PEA and CBD have been previously shown to supress the inflammatory response in the inflamed colon of mice and humans (Borrelli & Izzo 2009; J. M. Jamontt et al. 2010; de Filippis et al. 2011; Cuzzocrea et al. 2014; Esposito et al. 2014). In order for this hypothesis to be correct, we would expect PEA and CBD to have an anti-inflammatory action in Caco-2 monolayers.

IFNγ and TNFα caused an increase in all measured intracellular signalling phosphoproteins, in line with similar experiments using TNFα stimulation on Caco-2 cell lines (Erlejman et al. 2008). We found that increases were suppressed by PEA and CBD. We then quantified the effect of PEA and CBD on the extracellular inflammatory response, measuring seven pro-inflammatory cytokines representing five aspects of immune activation. As an indicator of pro-inflammatory cytokine production we assayed IL-8, IL-6, and IL-17 (Harada et al. 1994; Xing et al. 1998; Zhang et al. 2006). As a marker of leucocyte recruitment and activation we assayed MCP-1 and GM-CSF (Deshmane et al. 2009; Wicks & Roberts 2015). As a marker of extracellular matrix remodelling, we measured MMP-3 (Nagase & Woessner 1999), and as a marker of cell-to-cell adhesion we measured concentrations of ICAM-1. We found that stimulation of Caco-2 cultures with IFNγ and TNFα caused an increase in all measured cytokines, except MMP-3. Surprisingly, neither PEA nor CBD prevented the increased secretion of these cytokines. We may suggest

therefore that the effects of PEA and CBD on signal phosphorylation and permeability are distinct from their effects on inflammation, and not due to suppression of a local extracellular immune response, or are not generalised anti-inflammatory agents, but in the context of Caco-2 cells prevent signal phosphorylation with secondary effects other than production of pro-inflammatory cytokines. Of interest, we found that treatment of unstimulated Caco-2 cells with both CBD and PEA increased the secretion of GM-CSF. This result in particular was surprising. Caco-2 cells are cultured because of their rapidly dividing nature, and have previously been found to contain frequent significant genotype mutations subject to environmental conditions, occasionally within the same culture well (Gayet et al. 2001). Our GM-CSF results may be secondary to such mutations, however it is less likely as it is out of context with our other cytokine findings. This could, however be a less obvious mechanism by which PEA and CBD have been shown to exhibit prophylactic effects, but again is less likely because we did not seen these same effects in ex plant human tissue. These two hypotheses could be explored with further experimentation, specifically with co-culture models. For example, it would be possible to examine the hypothesis that PEA and CBD possess anti-inflammatory effects because of their action on the secondary immune response by administration of CBD and PEA to cultured macrophages and dendritic cells, with or without the presence of Caco-2 cells. Should we find that these agents become anti-inflammatory only in the presence of such complementary cultures we could ascertain that their effect is not epithelial, but rather systemic. Indeed, we may also hypothesize that the effect of PEA and CBD on GM-CSF has a wider secondary immune significance and may be the mechanism by which the severity of inflammation is decreased as GM-CSF-induced dendritic cell activation has been shown to bring about both pro- and anti-inflammatory effects (Bhattacharya et al. 2015).

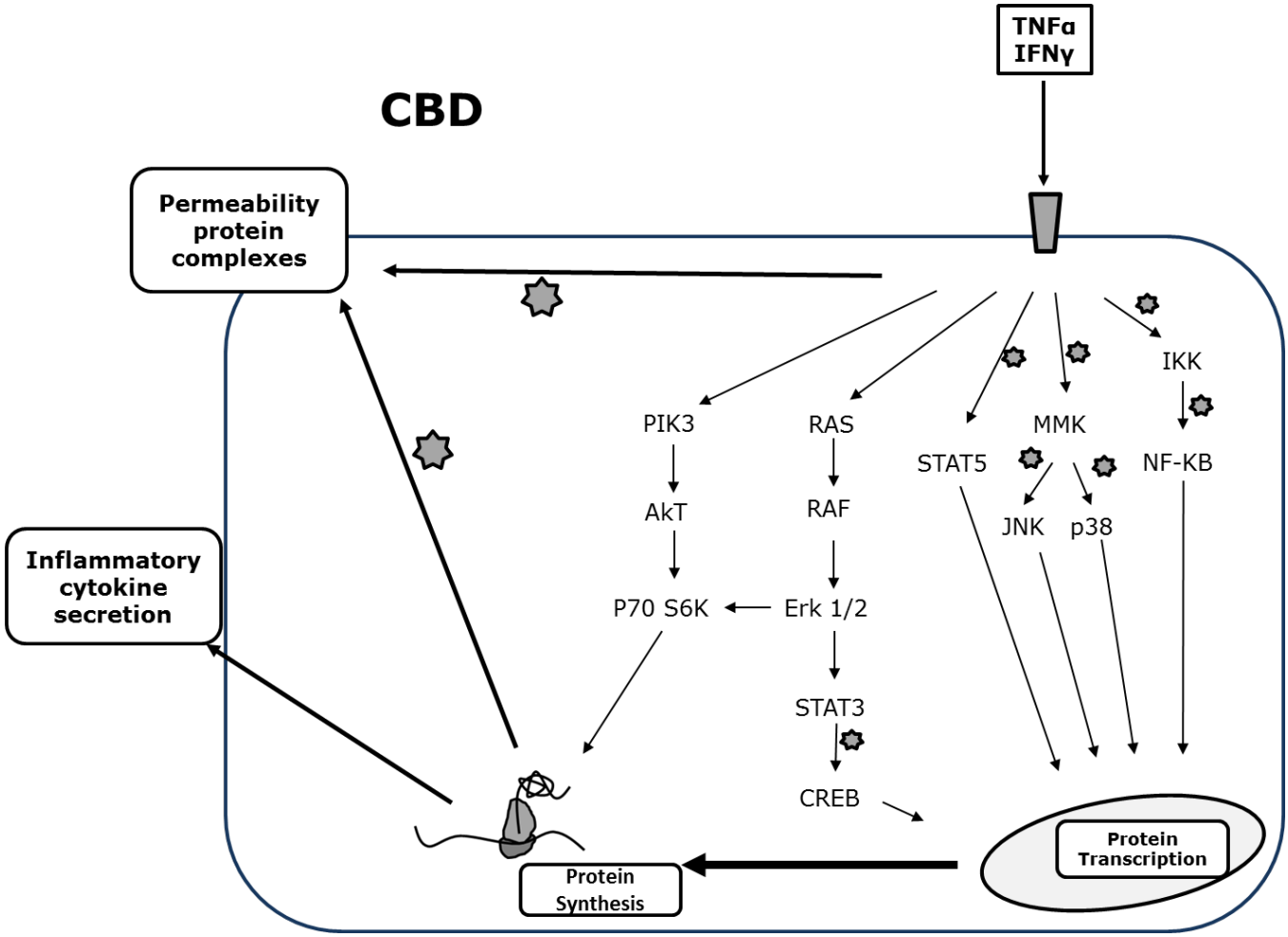
3.4.2 Explant colonic Tissue

In explant human tissue, we found that IFNγ and TNFα stimulated pro-inflammatory cytokine production, and that this was inhibited by treatment with PEA and CBD along the majority of the measured cytokines. This finding, in contrast to results from cultured Caco-2 cells, could be explained by the presence of submucosal immunocytes such as dendritic cells and macrophages. Explant colonic tissue contains lymphoid aggregates, innate immune cells and enteric glia, as opposed to cultured Caco-2 cells (Jarry et al. 2015). Within the gut, the CB_2 receptor, AEA and 2-AG are found in highest abundance within submucosal immune and nervous tissues and this may be the primary site of cannabinoid action on the inflammatory

response (Nagarkatti et al. 2009; Borrelli et al. 2015). Within our colonic explant tissue, PEA and CBD may be acting on such innate macrophage and dendritic cell colonies, therefore supressing the production of pro-inflammatory cytokines (Kozela et al. 2010; Jarry et al. 2015). This would explain why, in explant tissue in the presence of IFNγ and TNFα, PEA and CBD acted as anti-inflammatory agents, but no anti-inflammatory action was found in Caco-2 cells or tissue treated with PEA or CBD alone. Alternatively, it is possible that CBD and PEA are unable to act in Caco-2 cells because the receptors for these agonists are absent or do not function. This hypothesis could be explored further by examining the effect of CBD and PEA on Caco-2 cells in a co-culture model with macrophages.

3.4.3 PEA and CBD on permeability

These data suggest that PEA and CBD are anti-inflammatory agents in the inflamed human colon, and support clinical data showing that use of *Cannabis sativa* in Crohn's disease patients improves symptoms during disease flares (Storr et al. 2014). However, comparing Caco-2 cells with explant colonic findings, these data also suggest that the effect of PEA and CBD on permeability is not due to a local anti-inflammatory action, and may be due to an additional effect on the structures



governing permeability such as tight junctions or membrane-bound transcellular transport proteins. If this is the case, prevention of signal phosphorylation may be a common pathway for these effects as shown in figures 3.11 and 3.12. For example, when treating Caco-2 cultures with CBD we found that several parallel and overlapping pathways for induction of protein transcription were prevented, namely NF-κβ, JNK, p38, STAT5 and CREB. In opposition we found that PEA only reduced the phosphorylation of JNK and CREB. As both PEA and CBD have been shown previously to prevent in inflammation-induced falls in TEER, we may suggest that prevention of CREB phosphorylation, resulting in the downstream production or activation of paracellular proteins may be the mechanism by which these effects are brought about. Therefore, further study therefore may examine the effect of these compounds on the function or presence of these proteins, and the pathways along which they act. These effects could also be further confirmed with the use of specific pathway inhibitors to confirm or disprove these hypotheses. Logically the next step would be to examine the effect of PEA and CBD on paracellular protein production at the epithelial level.

Figure 3.11 Cell model suggesting putative sites of action of CBD indicated by asterisks.

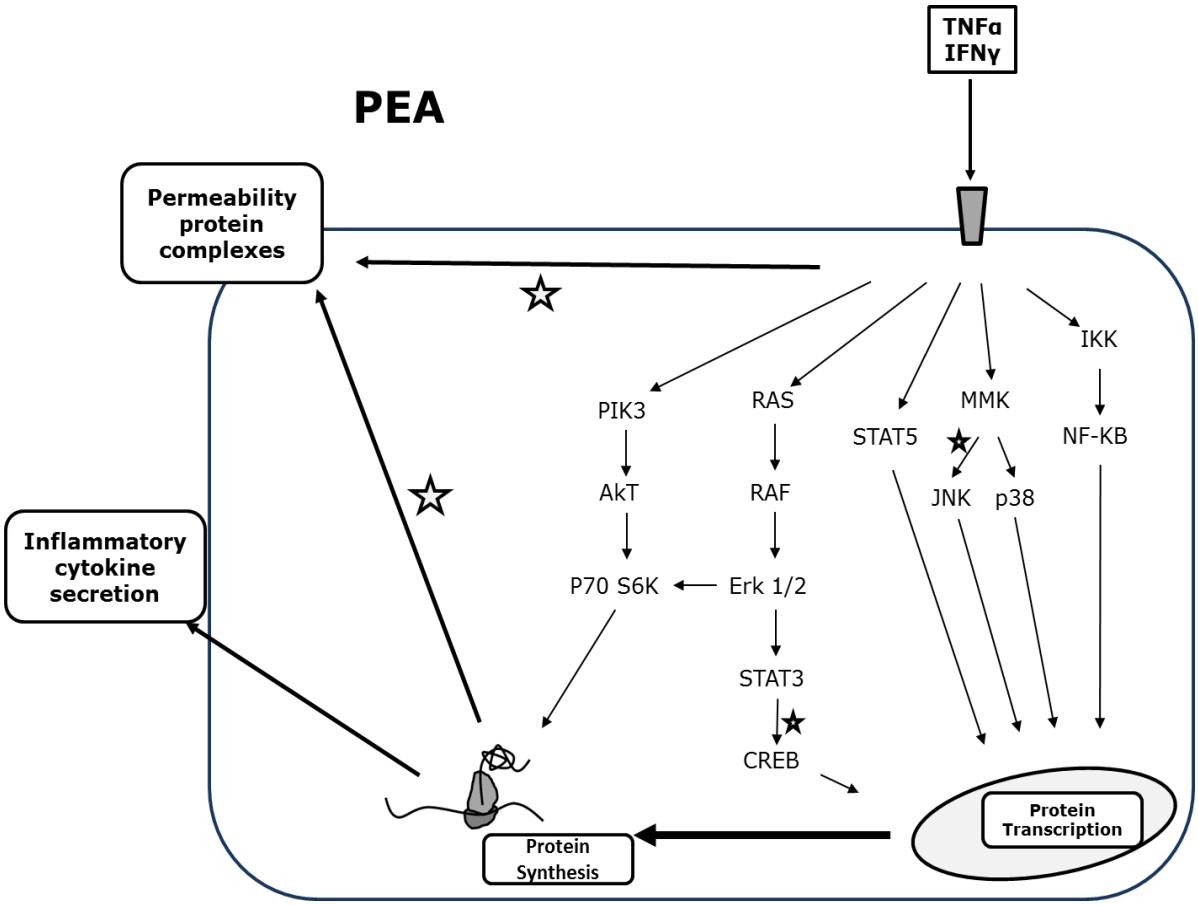


Figure 3.12 Cell model suggesting putative sites of action of PEA, indicated by starred sites.

3.4.4 Receptor sites

Receptor sites of PEA and CBD are not clear. Capasso et al. (2001) first demonstrated that the action of PEA on gut hypermotility in mice caused by caustic inflammation (croton oil), was prevented by blockade of the CB_2, but not CB_1 receptors (Capasso, *et al.*, 2001). The same group also reported that, in a different model of hypermotility (oil of mustard), PEA had an anti-inflammatory effect that was inhibited by blockade of the CB_1 and PPARα receptors, but not by blockade of CB_2 receptors (Capasso et al. 2014). Esposito et al. demonstrated that the anti-inflammatory effects of PEA in both dextran-sulphate sodium treated mice and colonic biopsies from patients with ulcerative colitis were inhibited by blockade of the PPARα receptor (Esposito et al. 2014). It has been suggested that PEA may exert its effects through increasing the local concentration or potency of a second agent, such as AEA, and therefore antagonising receptor targets for PEA or the second agent would inhibit its biologic effects (Ho et al. 2008). In view of this, we examined the anti-inflammatory effect of PEA in the presence of six receptor antagonists at which cannabinoid agonists have been postulated to act: CB_1, CB_2, PPARα, PPARγ, TRPV1 and GPR55. We found that across three measured cytokines the effect of PEA was prevented by antagonism of PPARα. This is the second human colonic study to demonstrate PEA action at PPARα, suggesting that this is the primary site of action for PEA in colonic mucosa (Karwad et al. 2016).

As PEA and CBD appeared to have similar effects we hypothesized that they may share a common mechanism of action, such as causing the increased presence of a second compound. PEA has been shown to increase the action of local AEA either by preventing hydrolysis of AEA through substrate competition or FAAH inhibition (Di Marzo et al. 2001) or by enhancing AEA potency at vanilloid receptors (Luciano De Petrocellis et al. 2001). Secondly, CBD has been shown to prevent AEA uptake and catabolism (Bisogno et al. 2001). AEA itself has been shown to down regulate NF-κB and exert anti-inflammatory properties through IL-10, and may therefore been at least partly responsible for the effects on phosphorylated signalling proteins (Correa et al. 2010). If this hypothesis were to be true, we would expect the effects of CBD and PEA to be prevented by the same antagonists. We did not find this to be the case, suggesting CB_2 and TRPV1 receptors to be the sites of action of CBD in human colonic mucosa.

There is conflicting literature regarding the site of action of CBD in the mammalian gut. Two murine colitis studies demonstrating the beneficial effect of CBD on

inflammation-induced hypermotility showed that CB_1 rather than CB_2 was the target receptor of CBD (Capasso et al. 2008; de Filippis et al. 2008). However, two independent studies examining the effect of CBD on the immune response in human and murine colonic tissue suggested that PPARα was the dominant receptor target of CBD, with a possible role for CB_1, but again suggesting that CB_2 was not a receptor target (de Filippis et al. 2011; Krohn et al. 2016). The data displayed in this study may differ from pre-existing literature because of the anatomical site of samples, and the mode of inflammation employed. It has been demonstrated that the distribution of cannabinoid receptors differs across the colon, and that these receptors can be up regulated by inflammation (D'Argenio et al. 2007). In our study, we collected colonic samples from right-sided colonic resections, whereas previously cited murine studies have used whole colonic homogenates, and the pre-existing human colon studies have used left-sided (sigmoid) colonic biopsies (Harvey et al. 2013). It is possible that the sites of CBD activation differ throughout the gut, hence in the proximal gastrointestinal tract PPARα is the main site of activation, whereas distally CB_2, TRPV1 and then CB_1 become more dominant. This may be supported by evidence showing that in established inflammation of the gut and other organ systems, CBD has epithelial protective effects mediated by CB_2 and TRPV1 (De Petrocellis et al. 2012; Pazos et al. 2013). Further work could compare the receptor targets of CBD across the length of the gut during inflammation.

3.4.5 IBD and appendicitis tissue

We then compared the anti-inflammatory effect of PEA and CBD in experimentally inflamed normal colonic tissue, explant IBD tissue, and explant appendicitis tissue. We found that appendicitis tissue, followed by IBD tissue when compared to healthy control tissue had higher baseline levels of cytokine production, demonstrated in figure 3.8. These differences in baseline cytokine production are likely to represent the acute inflammatory nature of acute appendicitis, versus the chronic low-levels of inflammation in long-standing IBD, compared with healthy tissue. CBD and PEA were anti-inflammatory in acute appendicitis tissue across several endpoints, whereas only CBD reduced inflammation in IBD tissue at one of the measured endpoints. Also, PEA and CBD were effective in preventing increased cytokine production in cytokine-treated IBD and appendicitis tissue. It is important to add that these data do not suggest that PEA or CBD may be an alternative treatment for appendicitis. In this study, appendicitis tissue was harvested because this was a readily available source of acutely inflamed colonic tissue. These data, however, do support the further investigation of PEA and CBD for treating inflammation on the human colon.

3.4.6 Limitations

Our data are limited by using explant tissue, rather than any clinical *in vivo* data, and the generic nature of the explant models used. Tissue viability studies were not carried out on explant tissue to ensure the mucosa had not been damaged by dissection, or had become necrotic. Lastly, within this study we did not examine for any additive effects of CBD together with PEA on the immune response, which may have been positive.

3.4.7 Summary and reviewed hypotheses

We have demonstrated that CBD and PEA prevent cytokine production in human colonic explant tissue via PPARα, CB_2 and TRPV1, but are not anti-inflammatory in cultured epithelial cells. These effects extend into chronic inflammatory states such as IBD, but also acute inflammatory conditions such as appendicitis. We can conclude that the effects of PEA and CBD on inflammation-induced hyperpermeability in the colon are not due to an anti-inflammatory effect alone, and may be due to direct effects on cellular structures governing permeability such as tight junctions or AQPs. Further studies should examine the effect of PEA and CBD on the presence or function of these structures, or receptors which govern their function.

4 Investigating the effects of PEA and CBD on colonic permeability

4.1 Introduction

In Chapter 3, we demonstrated that the effect of PEA and CBD on lowering inflammation-induced permeability was not due to immunosuppression alone, and suggested that alternative mechanisms of action may be by direct action on the cellular structures which govern permeability, such as the tight junction complex or AQPs. This experiment was based on observations that Caco-2 cultures treated with IFNγ and TNFα demonstrated increased permeability as measured by TEER, which was prevented by PEA and CBD (Alhamoruni et al. 2012; Alhamoruni et al. 2010). One potential flaw with these previous findings is that TEER is a measure of ion flow across membranes, and may not translate into permeability shifts to larger macromolecules such as LPS and bacteria. IF PEA and CBD only prevented changes in ion flow, they would be unlikely to be of significant clinical use in treating or preventing the consequences of colonic inflammation.

The aims of this study were:

- To verify that changes in TEER observed in previous experiments corresponded to changes in permeability to macromolecules using fluorescein-labelled dextrans.
- To identify underlying mechanisms of action by examining receptor pathways.
- To examine the effect of CBD and PEA on the expression of tight junction proteins, aquaporins and key cannabinoid receptors in the presence of inflammation.

4.2 Methods

4.2.1 Caco-2 cell culture

For permeability experiments, Caco-2 cells (purchased as described in section 3.2.2) were seeded at $5x10^4$ /ml per well in the apical compartment of 12 mm diameter 3.0 µm pore polyester membrane inserts within 24-well polyester plates (Corning Incorporated, ME, USA), and cultured for 14-16 days until fully confluent (figure 4.1). The medium was changed on alternate days. Cells were used for experimentation at day 14-16.

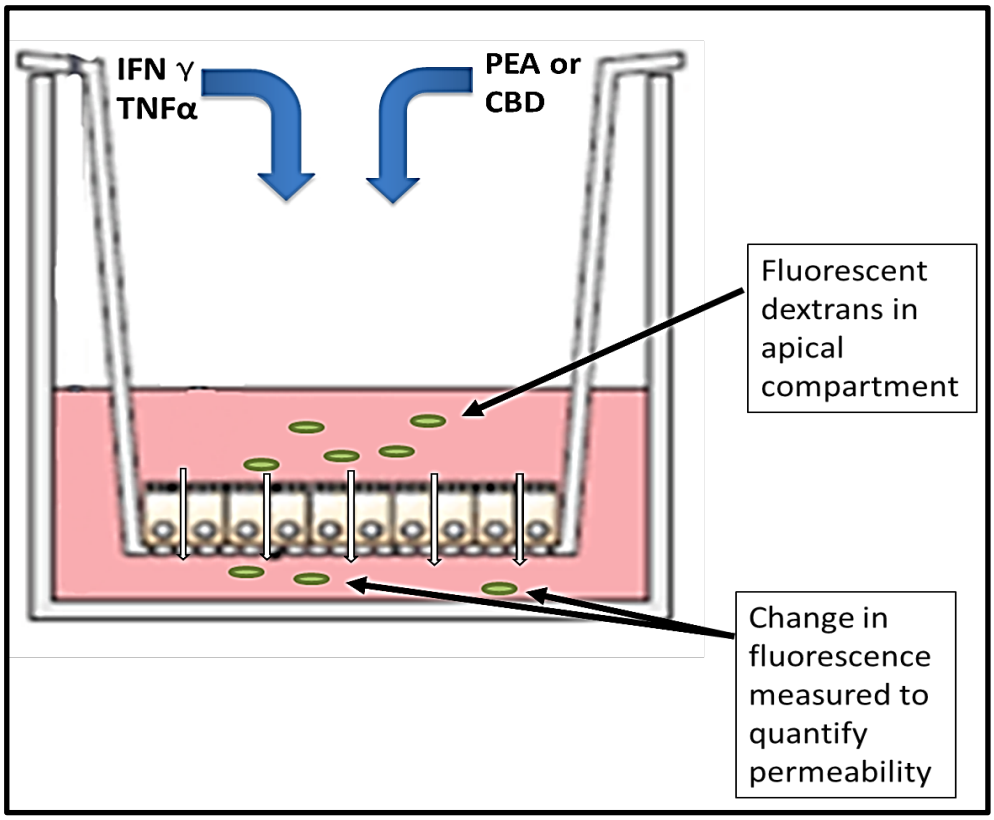


Figure 4.1 Culture of Caco-2 cells on membrane inserts. Membrane inserts are semi-permeable polyester platforms which allow cell culture between an apical and basolateral compartment. When fully confluent, permeability of these cultured cells may be measured by placing fluorescent dextrans in the apical compartment, and measuring the fluorescence of the basolateral media at regular time points.

Randomly assigned wells (n=8) were treated with the following drug treatments: vehicle (0.01% ethanol as appropriate), an inflammatory protocol of IFNγ (basolateral, 10 ng.ml^{-1}, Sigma-Aldrich) for 8 hours, followed by TNFα (basolateral, 10 ng.ml^{-1}, Sigma-Aldrich) for 16 hours (total 24 hours), inflammation (as above) and PEA (basolateral, 10μM, Tocris Bioscience, Bristol UK), inflammation and CBD (apical, 10 μM, Tocris Bioscience, Bristol UK), PEA alone (basolateral, 10 μM), or CBD alone (apical, 10 μM). PEA, CBD or vehicle were added simultaneously with IFNγ at the start of the 24-hour experimental period.

For protein expression experiments, Caco-2 cells were cultured as described in section 3.2.2 in flat bottomed 6-well polystyrene plates (Corning Incorporated, ME, USA) for 14 days until fully confluent. Following 24-hour drug treatments as previously described (all apical), cells were washed twice with ice-cold PBS, and treated with RIPA containing phosphatase inhibitor and protease inhibitor (Sigma-Aldrich) at 4 °C for one hour on a rocking platform to cause cell lysis. Cell lysates were then collected and stored at -80 °C until analysis.

4.2.2 Human colonic culture

Human colonic tissue was collected and treated as described in section 3.2.4.

4.2.3 Transport studies

Confluent monolayers cultured on membrane inserts were washed three times with pre-warmed PBS. Clear, non-fluorescent media (FluoroBrite DMEM, ThermoFisher Scientific MA, USA) supplemented with 1% nonessential amino acids and 1% penicillin/streptomycin were added to both the apical (225 μl) and basolateral compartment (700 μl) as per manufacturer's instructions and left for 30 min to equilibrate. FD4 and FD10 fluorescent dextrans (FDs; 25 mg.ml^{-1}) were then added to the apical compartment. At 37 °C, baseline (0 h) 100 μl aliquot samples were withdrawn from the basolateral compartment. Florescence of aliquots was determined using a fluorometer (Fluoroskan Ascent FL2.5, ThermoFisher Scientific, Waltham, MA), with excitation and emission wavelengths of 485 and 520 nm, respectively. Aliquot fluorescence was determined at regular intervals for 36 h.

Randomly assigned wells (n=8, three readings were taken per well and mean result calculated) were treated as described above with TNFα and IFNγ with or without the presence of PEA (basolateral) and CBD (apical). To identify the target sites of action of PEA and CBD, we co-applied the following antagonists to the apical/basolateral compartment as appropriate with PEA or CBD under inflammatory conditions: AM251 (100 nM, CB_1 antagonist, Tocris Bioscience, Bristol, UK), AM630 (100 nM,

CB_2 antagonist, Tocris Bioscience, Bristol, UK), GW6471 (500 nM, PPARα antagonist, Tocris Bioscience, Bristol, UK), and SB366791 (500 nM, transient receptor potential vanilloid 1 (TRPV1) antagonist, Tocris Bioscience, Bristol, UK).

To identify intracellular signalling mechanisms, we co-applied the following inhibitors to the apical/basolateral compartment as appropriate with PEA or CBD under inflammatory conditions: KT5720 (PKA inhibitor, 1 μM, Tocris Bioscience, Bristol, UK), L-NAME (nitric oxide synthase inhibitor, 10 μM, Tocris Bioscience, Bristol, UK), PD98059 (MEK/ERK inhibitor, 10 μM, Tocris Bioscience, Bristol, UK), SQ22536 (adenylyl cyclase inhibitor, 1 μM, Tocris Bioscience, Bristol, UK) and Gö6983 (PKC inhibitor, 1 μM, Tocris Bioscience, Bristol, UK). Concentrations were based on previous experimentation (Karwad et al. 2017).

4.2.4 Tight junction protein and receptor quantification

To determine levels of mRNA for endocannabinoid receptors and tight junction proteins, undiluted cell lysates and tissue homogenates were thawed on ice and assayed using Quantigene Plex assays (Affymetrix, US). mRNA for receptors PPARγ, PPARα, PPARδ, GPR55, GPR119, CB_1, CB_2 and TRPV1 were determined using kit QP1013, claudin -1, -2, -3, -4, -5, -7 and -8 were determined using kit QP1014. Luminescence per sample was determined using a Magpix plate reader (MAGPX 11326002, Luminex, Texas US). Samples were assayed according to the manufacturer's instructions. Geometric mean mRNA levels were normalised according to levels of the three housekeeping proteins β-actin, peptidyl-prolyl cis-trans isomerase B (PPIB), and hypoxanthine phosphoribosyltransferase 1 (HPRT1), which did not change in expression under the inflammatory protocol.

4.2.5 Aquaporin quantification

To determine levels of membrane-bound aquaporins, cell membrane fractions were obtained from Caco-2 cells and human colonic tissue using Mem-PER Plus kit (#89842, ThermoScientific). Undiluted membrane fractions then underwent ELISA to determine levels of membrane-bound AQP3 and AQP4 as per manufacturer's instructions (#LS-F13079 & #LS-F13078, Lifespan Biosciences, WA USA). Human biopsy AQP levels were normalised by mean protein content or cell-membrane supernatants using a bicinchoninic acid assay (Sigma-Aldrich).

4.2.6 Statistical analysis

Data are presented as mean (or mean percentage change from baseline where indicated) ± SEM. Throughout experiments, from each measured sample three readings were taken, and the mean result calculated. Transport study results were

compared using two-way ANOVA and Dunnett's post hoc test. Caco-2 and human tissue group results were compared using one-way ANOVA. Significant differences between categorised groups were analysed by Dunnett's post-hoc test as appropriate. A p-value of <0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 7.01 for Windows (GraphPad Software, San Diego, USA).

4.3 Results

4.3.1 Caco-2 cell culture

Transport studies

Within Caco-2 cultures, inflammation caused a significant and persistent increase in the transfer of labelled FD4 and FD10 dextrans at 2 and 4 h respectively, until the end of both study periods at 36 h (figure 4.2 A and B).

PEA, in the presence of inflammation, caused a reduction in FD4 transfer compared to inflammatory conditions alone, which became significantly different at 4 h ($p<0.01$, figure 4.2 A). This difference maintained significance with time, with maximal difference seen at 12 h ($p<0.0001$), observed until 36 h. Within FD10 experiments, PEA in the presence of inflammation caused a significant reduction in the transfer of dextrans at 4 h ($p<0.0001$, figure 4.2 B), which was observed until 36 h. Trials with antagonists of TRPV1, PPARα, CB_1 and CB_2 identified that antagonism of the PPARα receptor blocked the ability of PEA to reduce transfer of FD10 dextran over the study period (figure 4.3 A).

Within the experiments using FD4, CBD in the presence of inflammation caused a reduction in dextran transfer compared to inflammatory conditions alone, which became significant at 4 h ($p<0.01$, figure 4.2 A). This difference became more pronounced with time, with a maximal difference seen at 8 hours ($p<0.0001$) until 36 hours. Within the experiments using FD10, CBD in the presence of inflammation caused a reduction in the transfer of dextran at 4 hours ($p<0.0001$, figure 4.2 B), this difference was observed until 36 h. Trials with antagonists of TRPV1, PPARα, CB_1 and CB_2 receptors identified that antagonism of the CB_1 receptor blocked the ability of CBD to cause a reduced transfer of FD10 dextran over the study period (figure 4.3 B).

As PEA and CBD appeared to act through different receptors, we investigated whether these two drugs had an additive effect on permeability. PEA and CBD applied together had no further effect on permeability in the presence of inflammation when compared to PEA and CBD applied independently (figure 4.2 C).

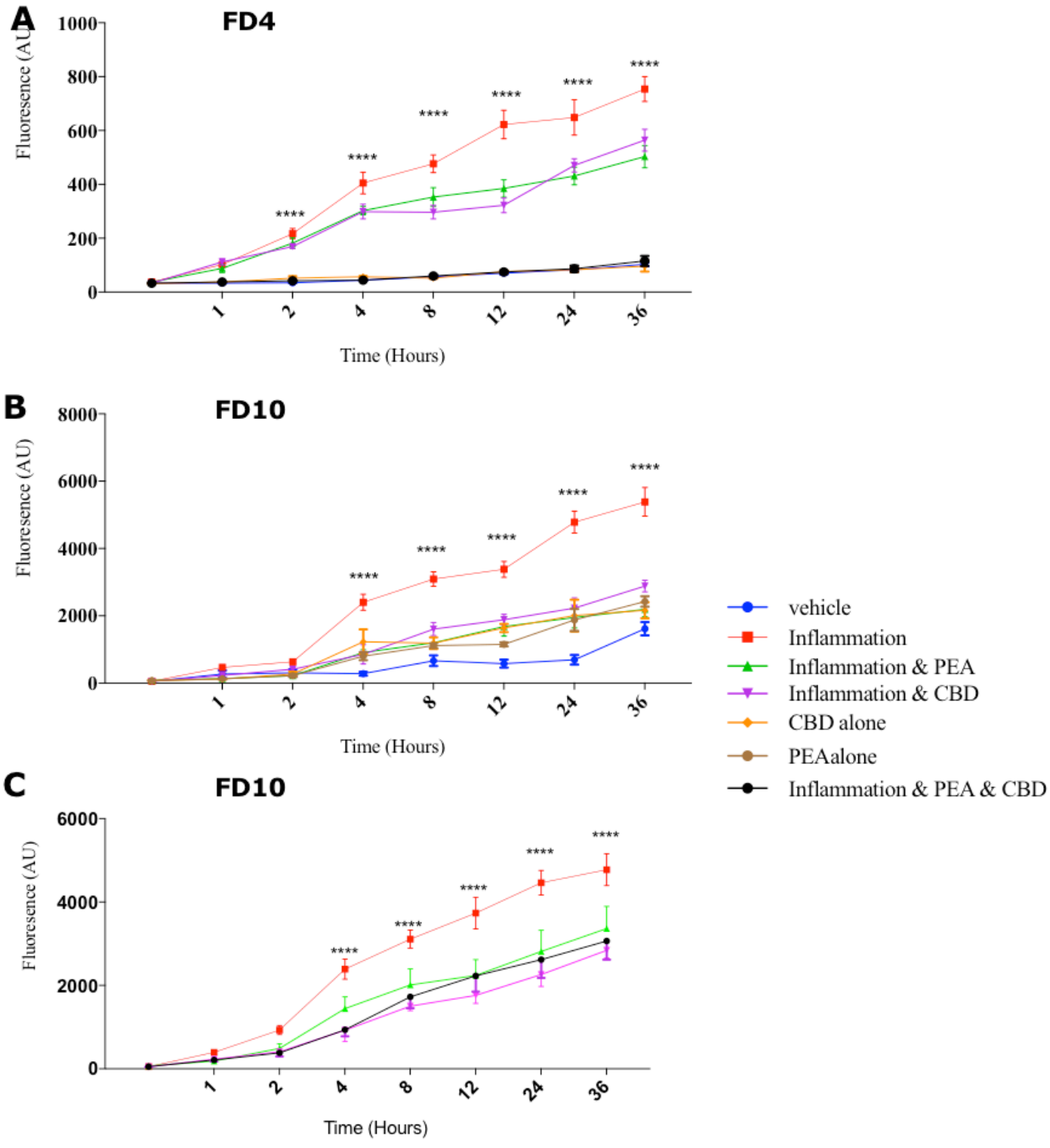


Figure 4.2 The effects of PEA, applied basolaterally, and CBD, applied apically, on the permeability of Caco-2 monolayers in response to 24 h exposure to TNFα and IFNγ measured by transfer of fluorescent dextrans (A:FD4 B: FD10 C: FD10 PEA & CBD). Raw data are expressed as the mean fluorescence per group +/- S.E.M. n=8 per condition. Data were analysed by two-way ANOVA using Dunnett's post hoc test comparing against the vehicle control (****p<0.0001).

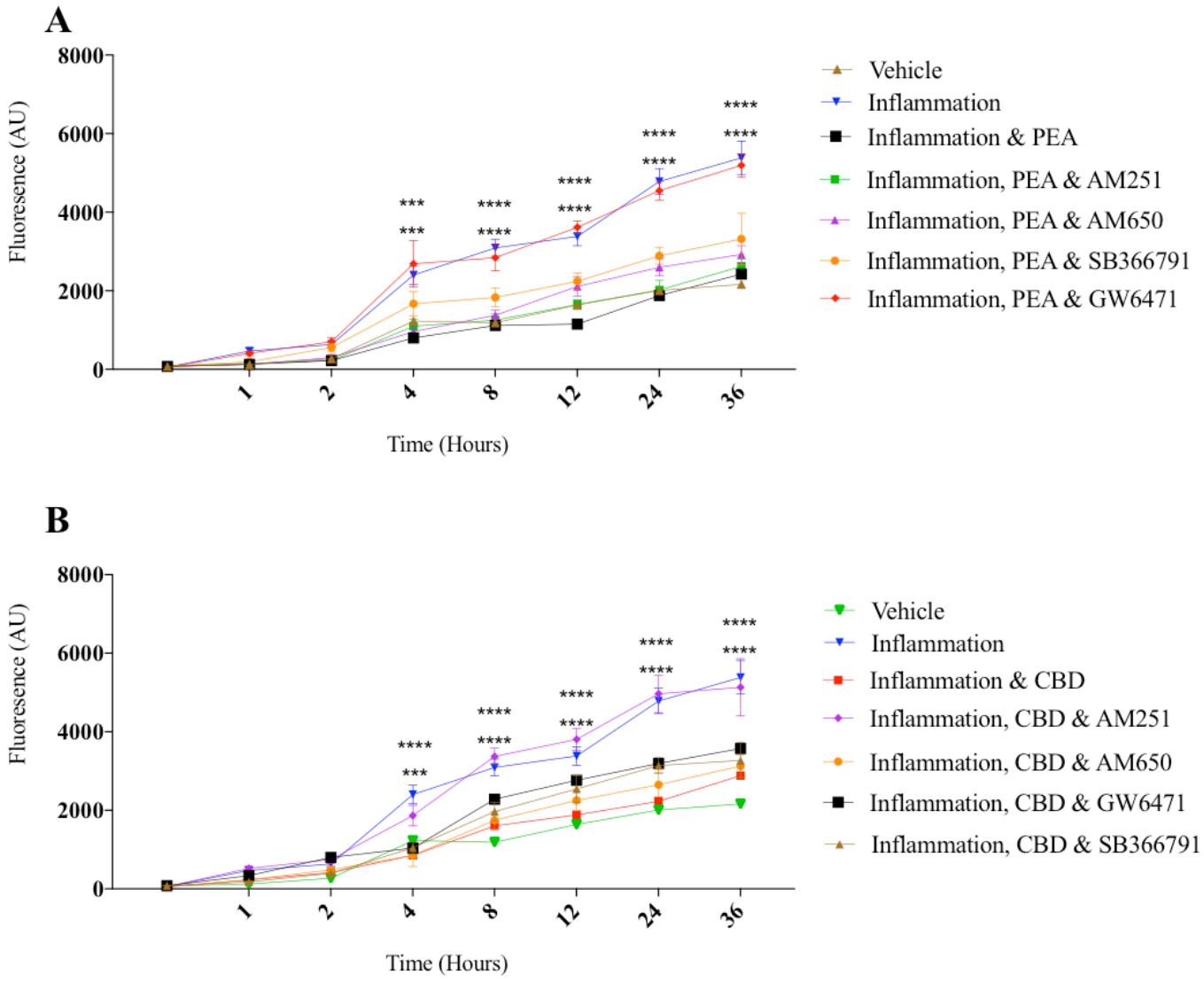


Figure 4.3 The effects of PEA (basolateral, A) and CBD (apical, B) on the permeability of Caco-2 monolayers in response to 24 h exposure to TNFα and IFNγ in the presence of multiple receptor antagonists, measured by ransfer of fluorescent dextrans (FD10). Raw data are expressed as the mean fluorescence per group +/- SEM, n=8 per condition. Data were analysed by two-way ANOVA using Dunnett's post hoc test comparing against the vehicle control (*), ***<0.001, ****<0.0001).

Intracellular signalling mechanisms of action

We investigated the intracellular signalling pathways through which PEA and CBD were acting in the presence of inflammation. In these experiments, over a 36 h time period, inflammation caused an increase in the transfer of FD10 fluorescein-labelled dextrans as before (figure 4.4 and 4.5). The effect of PEA in reducing this transfer of dextrans was blocked by the presence of the PKA inhibitor KT5720, the MEK/ERK inhibitor PD98059 and the adenylyl cyclase inhibitor SQ22563. The effect of CBD on the transfer of dextrans was also blocked by the presence of the PKA inhibitor KT5720, the MEK/ERK inhibitor PD98059 and the adenylyl cyclase inhibitor SQ22563.

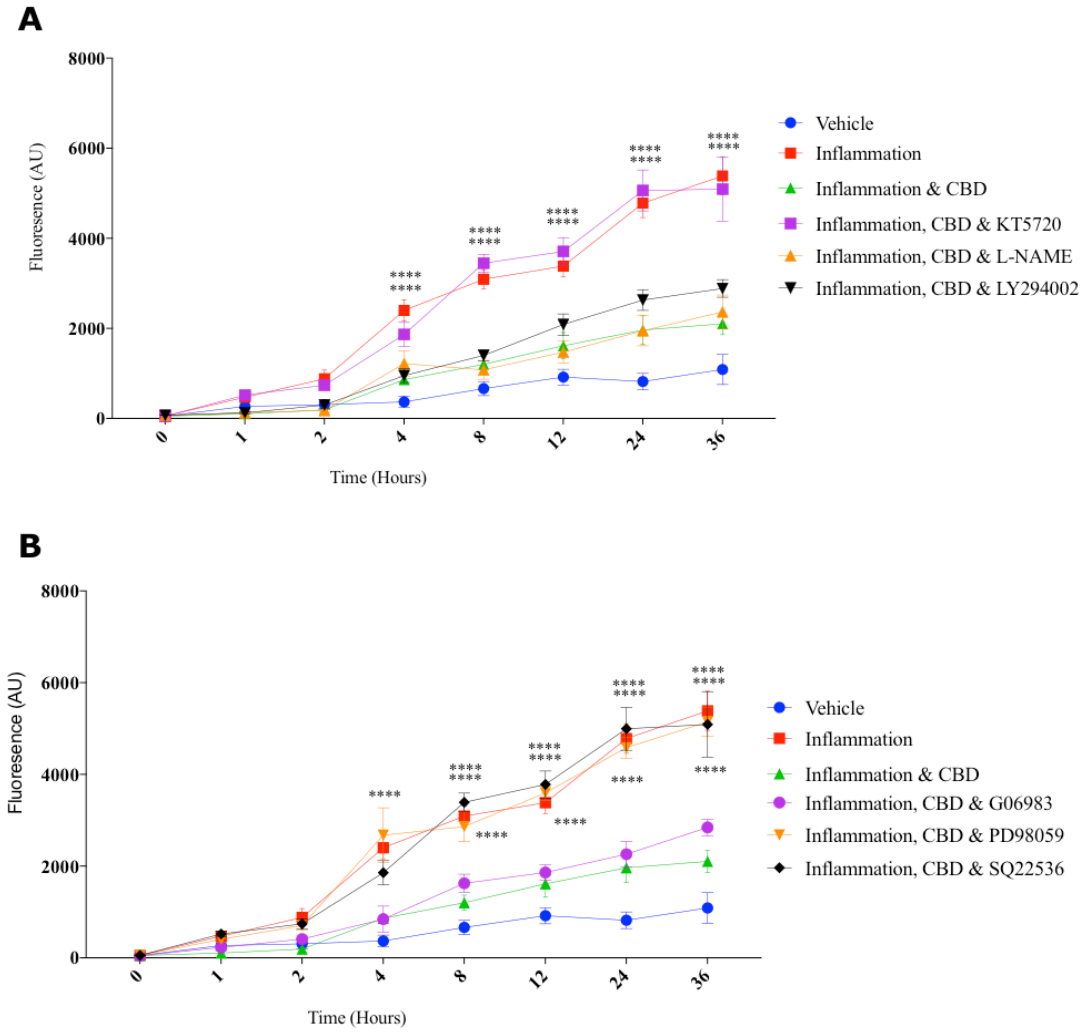


Figure 4.4 The effects of CBD, applied apically, on the permeability of Caco-2 monolayers in response to 24 h exposure to TNFα and IFNγ in the presence of multiple signalling protein inhibitors measured by transfer of fluorescent dextrans (FD10). Raw data are given as mean fluorescence per group +/- S.E.M. n=8 per condition. Data were analysed using two-way ANOVA with Dunnett's post hoc analysis of selected pairs comparing against vehicle (****p<0.0001).

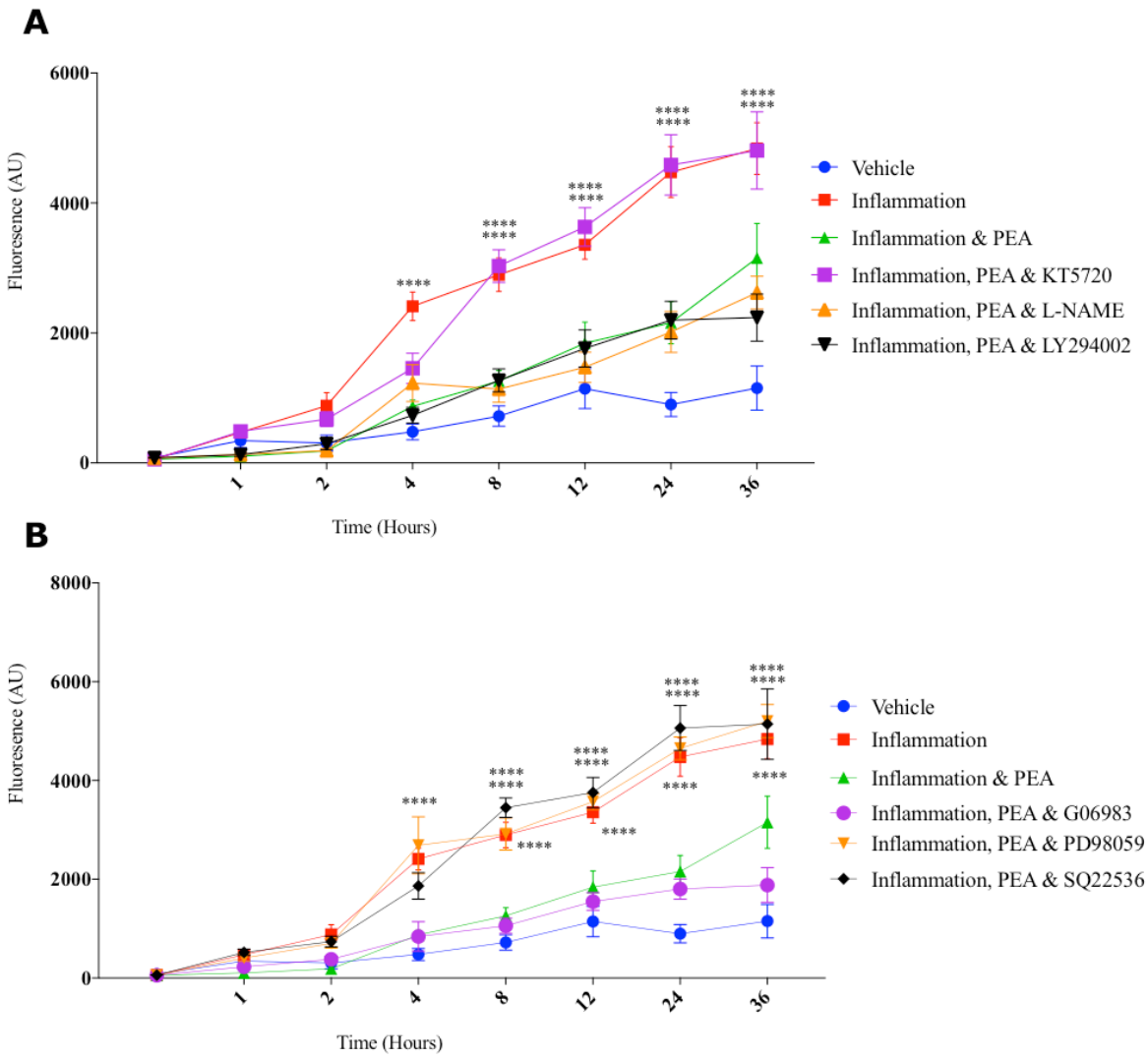


Figure 4.5 The effects of PEA, applied basolaterally, on the permeability of Caco-2 monolayers in response to 24 h exposure to TNFα and IFNγ in the presence of multiple signalling protein inhibitors measured by transfer of fluorescent dextrans (FD10). Raw data are given as mean fluorescence per group +/- S.E.M. n=8 per condition. Data were analysed using two-way ANOVA with Dunnett's post hoc analysis of selected pairs comparing against vehicle (****p<0.0001).

Protein expression

Claudin proteins

Compared to control cultures, inflammation did not significantly change the transcription of claudin-1, -2, -3, -4, -5, 7, and -8, though inflammation did cause a non-significant decrease in the expression of mRNA for claudin-5 (figure 4.6 A-G). PEA and CBD had no effect on the production of these proteins compared to vehicle or control.

Expression of cannabinoid receptors

Treatment with TNFα and IFNγ caused a significant fall in the transcription of TRPV1 ($p<0.00001$) (fig 4.7 D). This change was prevented by PEA and CBD, respectively. Inflammation did not affect the expression of any of the other measured receptors (fig 4.7 A-H).

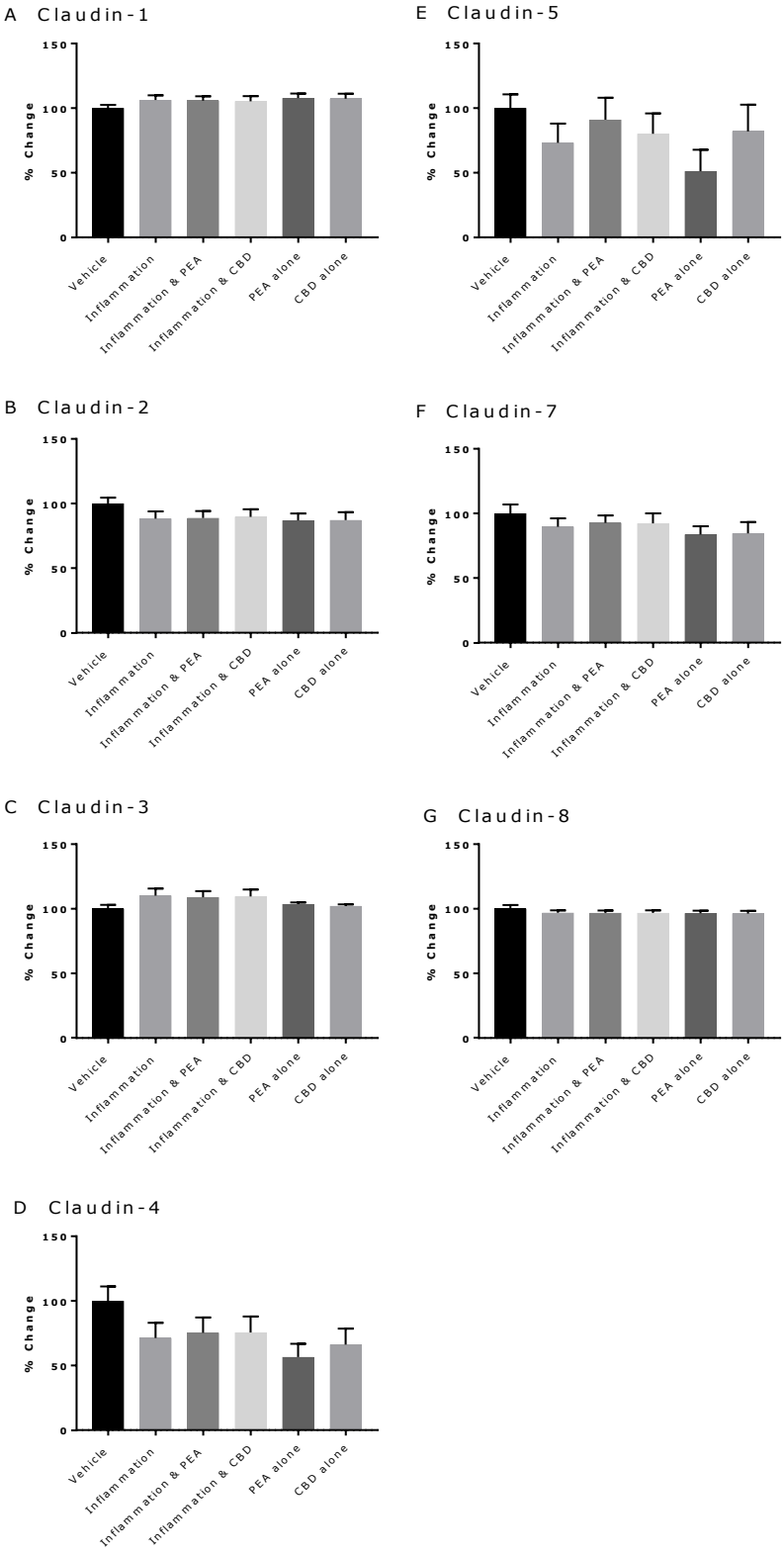


Figure 4.6 The effects of PEA and CBD on mRNA levels for tight junction proteins in response to an inflammatory protocol in cultured Caco-2 cells; A=claudin-1, B=claudin-2, C=claudin-3, D=claudin-4, E=claudin-5, F=claudin-7, G=claudin-8 measured by multiplex. Data are presented as percentage change from vehicle per plate +/- SEM n=8 per condition. Data were analysed by one-way ANOVA comparing against vehicle.

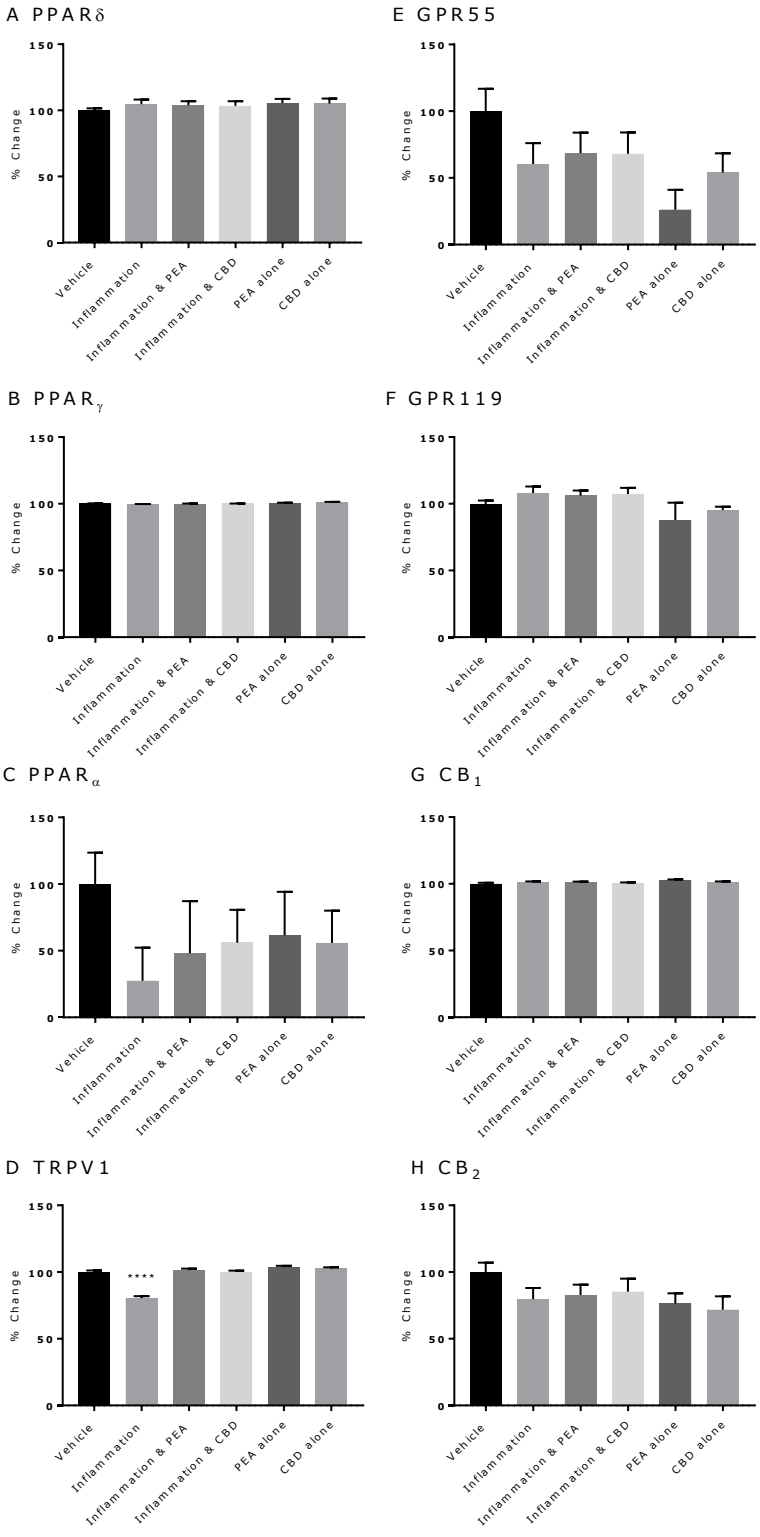


Figure 4.7 The effects of PEA and CBD on the production of mRNA levels for multiple receptors in response to an inflammatory protocol in cultured Caco-2 cells; A=PPARδ, B=PPARγ, C=PPARα, D=TRPV1, E=GPR55, F=GPR119, G=CB1, H=CB2 measured by multiplex. Data are presented as percentage change from vehicle per plate +/- S.E.M. n=8 per condition. Data were analysed by one-way ANOVA comparing against vehicle (****p<0.0001).

Membrane-bound aquaporin channels

Treatment of Caco-2 cultures with IFNγ and TNFα caused an increase in the presence of membrane-bound AQP3 ($p<0.05$) (fig 4.8 A). This increase was prevented by the presence of PEA (apical, $p<0.05$), and CBD (apical, $p<0.05$). PEA or CBD alone did not affect the presence of AQP3 compared to control. Treatment of Caco-2 cultures with the inflammatory protocol did not affect the presence of membrane-bound AQP4, though treatment of Caco-2 cultures with CBD or PEA alone cause increases in the presence of AQP4 (both $p<0.05$) (fig 4.8 B).

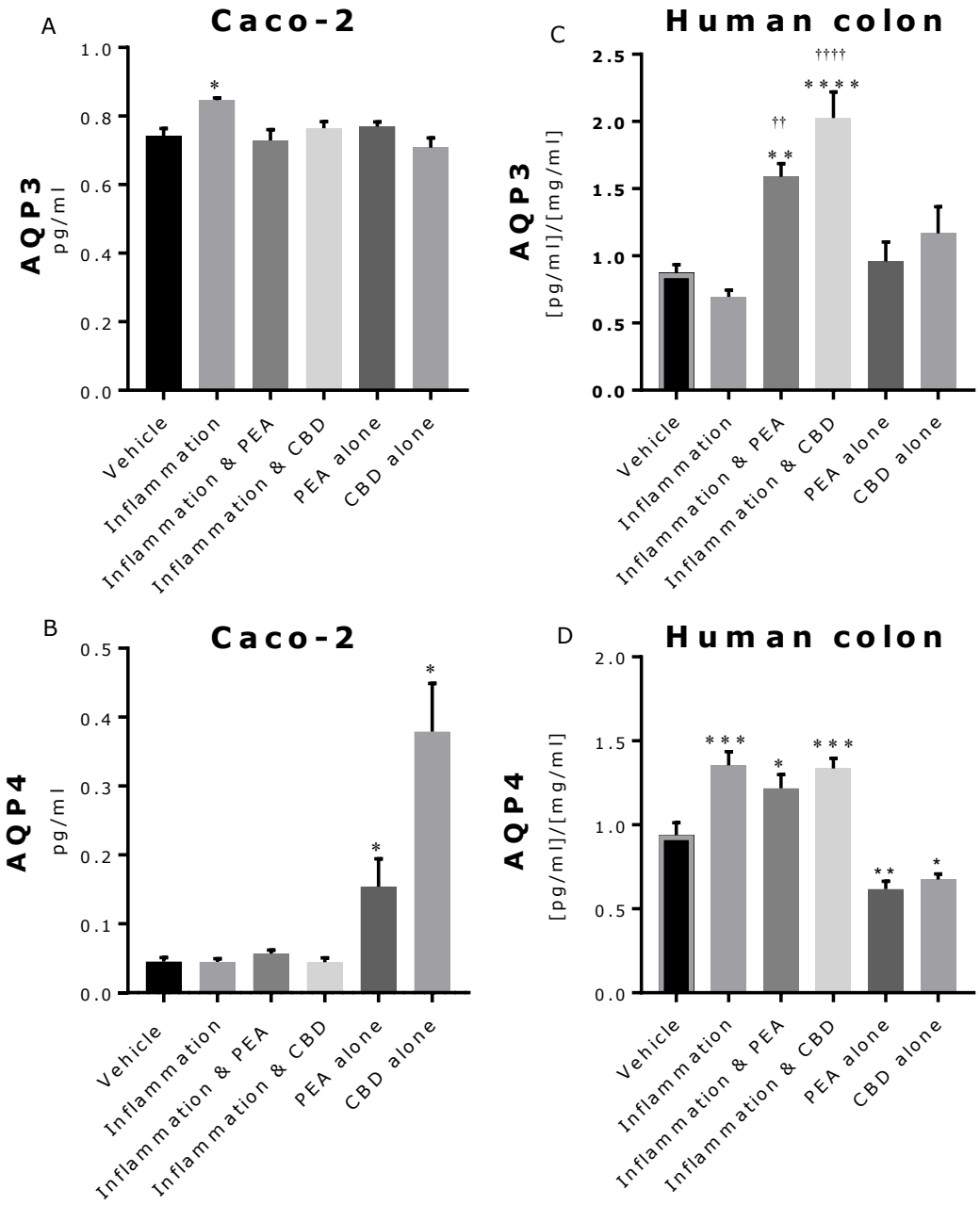


Figure 4.8 The effects of PEA and CBD on the levels of membrane-bound AQP3 in Caco-2 (A) and human colonic mucosa (C) and AQP4 in Caco-2 cultures (B) and human colonic mucosa (D) in response to an inflammatory protocol in cultured Caco-2 monolayers measured by ELISA. Raw data are are expressed as mean protein levels per group +/- S.E.M. n=6-8 per condition. Data were analysed by one-way ANOVA comparing against vehicle (* $p<0.05$, ** $p<0.01$, *** $p<0.0001$, ****$p<0.00001$) or inflammation (†† $p<0.01$, †††† $p<0.00001$).

4.3.2 Human colonic tissue

Protein expression

Claudin proteins

Treatment of human colonic tissue with IFNγ and TNFα caused a fall in the production of claudin-5 mRNA compared to control tissue ($p<0.05$) (figure 4.9 7 E). CBD prevented this fall in the presence of inflammation ($p<0.05$). PEA in the presence of inflammation and alone caused a fall in the production of claudin-3 ($p<0.05$) (figure 4.9 C). Inflammation alone did not change the production of claudin-3. The inflammatory protocol with or without the application of PEA and CBD did not change mRNA production for any other claudin proteins.

Receptors

Inflammation caused a fall in the production of TRPV1 ($p<0.05$), an effect that was prevented by co-administration with CBD ($p<0.05$) (figure 4.10 D). Inflammation also caused an increase in the production of PPARα ($p<0.05$), but this was not prevented by treatment with PEA or CBD (figure 4.10 D). PEA had no effect on the production of mRNA for any of the measured receptors.

Membrane-bound aquaporin channels

IFNγ and TNFα did not significantly change the levels of membrane-bound AQP3, though AQP3 levels were increased in the presence of inflammation and PEA ($p<0.01$), and also inflammation and CBD ($p<0.0001$) (figure 4.8 C). Treatment with PEA or CBD alone did not significantly affect the levels of AQP3 in human mucosal tissue. IFNγ and TNFα increased the presence of membrane-bound AQP4 in human colonic mucosa ($p<0.001$), which was not affected by treatment with PEA or CBD, however treatment of colonic mucosa with PEA or CBD alone decreased the presence of this protein ($p<0.01$ and <0.05 respectively) (figure 4.8 D).

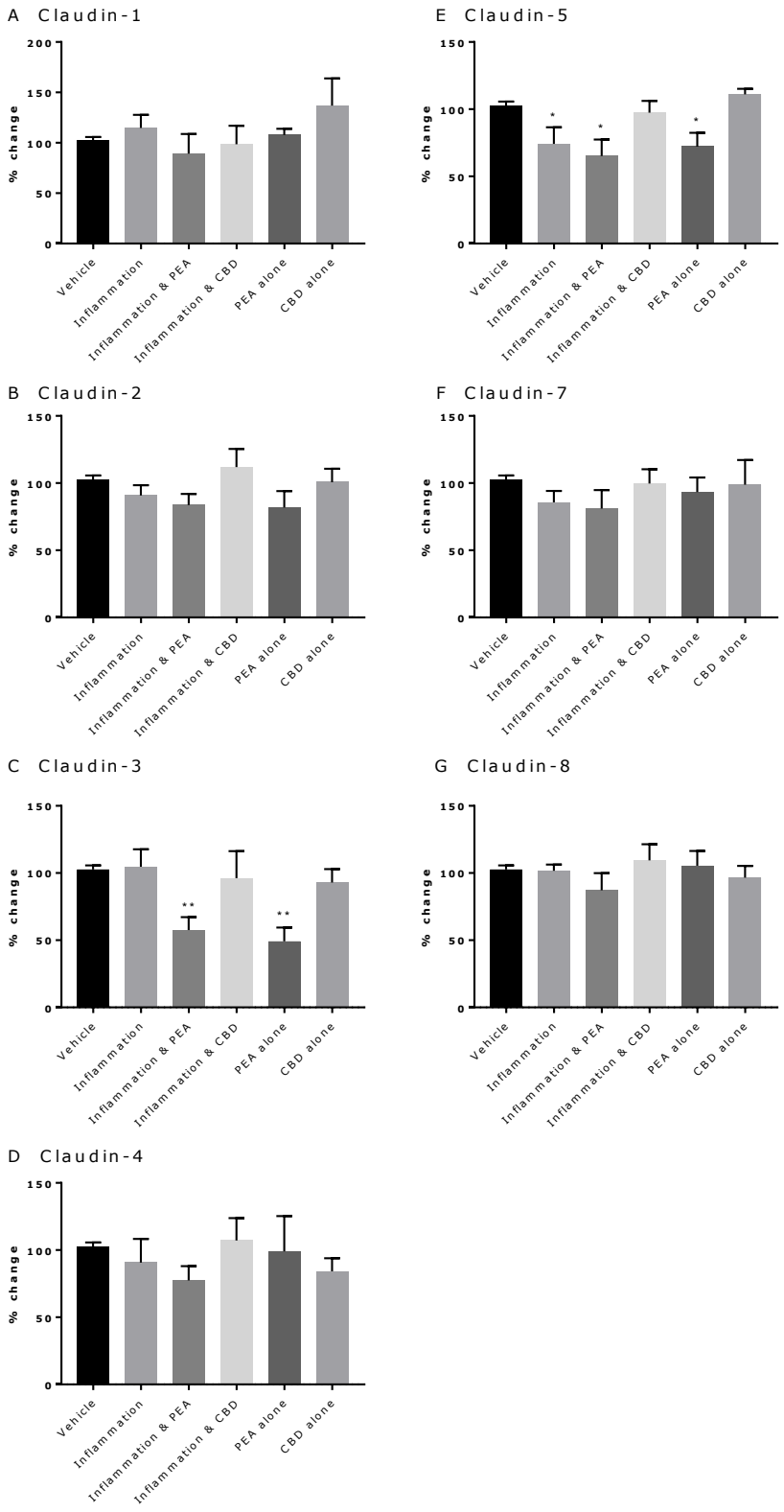


Figure 4.9 The effects of PEA and CBD on mRNA levels for tight junction proteins in response to an inflammatory protocol in cultured human colonic tissue homogenates; A=claudin-1, B=claudin-2, C=claudin-3, D=claudin-4, E=claudin-5, F=caludin-7, G=claudin-8 measured by multiplex. Data are presented as mean percentage change from vehicle +/- S.E.M. n=7 per condition. Data were analysed by one-way ANOVA and Dunnett's post hoc test comparing against vehicle (* $p<0.05$, **<0.01).

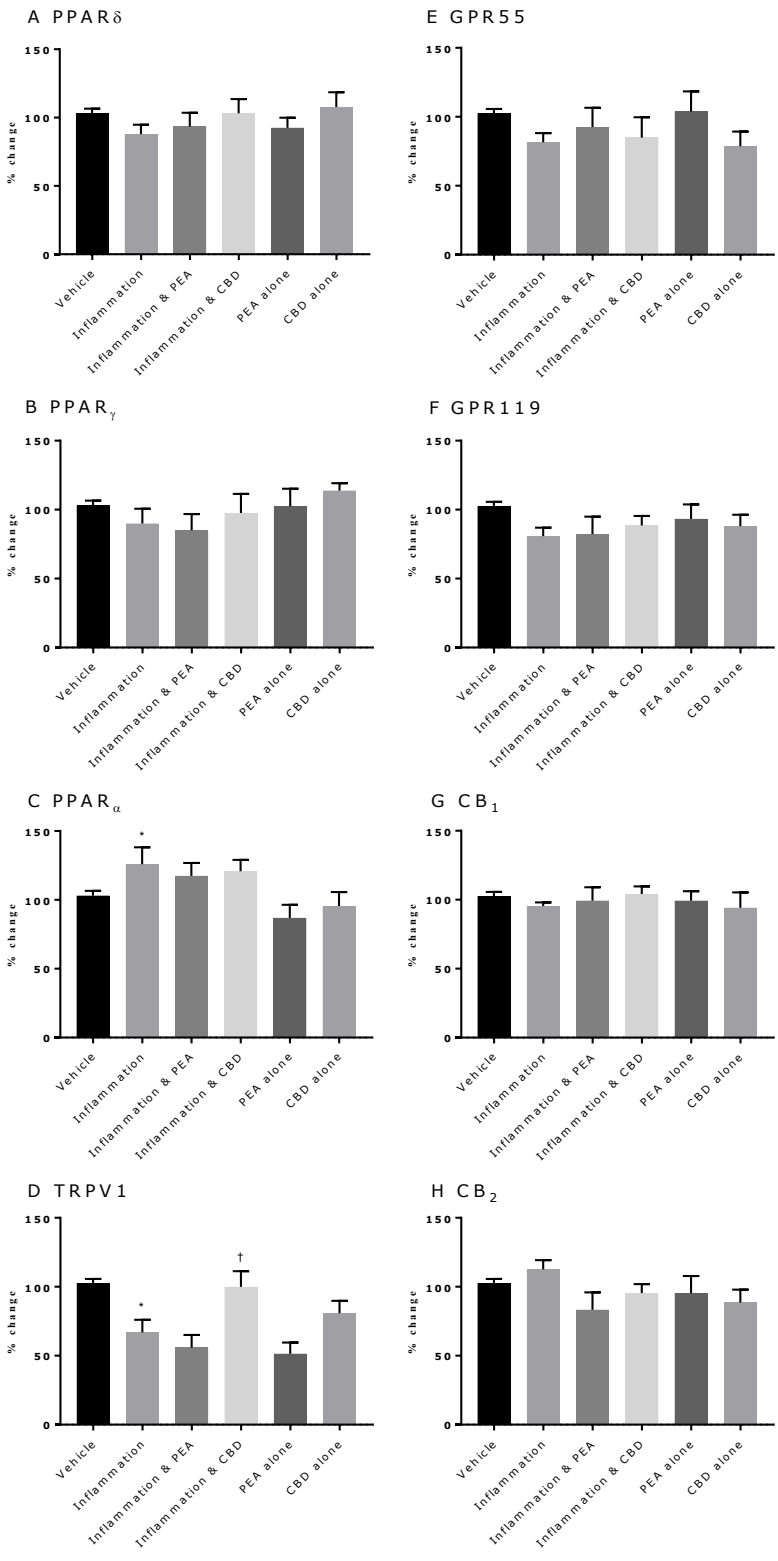


Figure 4.10 The effects of PEA and CBD on mRNA levels for multiple receptors in response to an inflammatory protocol in cultured human colonic tissue homogenates; A=PPARδ, B=PPARγ, C=PPARα, D=TRPV1, E=GPR55, F=GPR119, G=CB1, H=CB2 (n=7) measured by multiplex. Data are presented as mean percentage change from vehicle +/- S.E.M. n=7 per condition. Data were analysed by one-way ANOVA and Dunnett's post hoc test comparing against vehicle (* $p<0.05$) or inflammation († $p<0.05$).

4.4 Discussion

The aims of this study were to investigate in Caco-2 cultures whether PEA and CBD were able to prevent inflammation-induced changes in epithelial permeability to macromolecules, determine receptor and intracellular signalling pathways for these actions, and identify membrane-bound mechanisms by which permeability is affected. We compared these cell culture changes with the effects of PEA and CBD on inflamed human explant colonic tissue. We found that in Caco-2 cultures, PEA and CBD prevented inflammation-induced permeability to large molecules, and that these effects were mediated by different membrane receptors but similar intracellular pathways. PEA and CBD may prevent these changes through preventing down-regulation of TRPV1 expression and membrane-bound AQP3. In human explant tissue, we found that CBD, but not PEA, prevented changes in TRPV1 and claudin-5 expression, and that PEA decreased the production of claudin-3. Stimulation of colonic mucosa with inflammation did not affect the presence of AQP3, but inflammation and PEA or CBD caused it to increase. Inflammation increased AQP4 levels, which was not affected by PEA or CBD. This is really just repetition of the results, not useful when you should be focussing on an explanation

It had been previously demonstrated that PEA and CBD are able to prevent changes in TEER in Caco-2 monolayers caused by inflammation (Alhamoruni et al. 2012; Karwad et al. 2016). We chose to examine the effect of CBD and PEA on the transfer of FD4 and FD10 dextrans as these molecules are of a similar size to that of LPS from *Escherichia coli* and *Pseudomonas* species (20-40Å) (Qiao et al. 2014; Mangoni et al. 2008). PEA and CBD both reduced the transfer of FD4 and FD10 across Caco-2 membranes, these effects of PEA were blocked by antagonism of PPARα. The effects of CBD were prevented by antagonism of the CB_1 receptor, in line with previous reports (Karwad et al. 2016; Alhamoruni et al. 2010). As these drugs appeared to be acting via separate receptors, we hypothesized that there may be additive effects on inflammatory hyperpermeability if PEA and CBD were applied simultaneously. This was found not to be the case, leading us to investigate the downstream intracellular mechanisms of action. We found that non-selective inhibition of PKA, ERK/MEK and adenylyl cyclase pathway prevented the actions of both CBD and PEA, suggesting that although the receptors of CBD and PEA may be different, they may exert their actions through similar intracellular pathways and hence demonstrate no additive effects when applied together. Although PEA signalling pathways in human colonic mucosa has not been previously reported, this is in line with research demonstrating that PEA in murine colitis acts through phosphorylation of ERK (Esposito et al. 2014), and in neuronal tissue acts through

PKA (Lin et al. 2015). Similarly, although the signalling pathway of CBD in the colon had not previously been described, central CBD action at CB_1 has been shown to control signalling through adenylyl cyclase (Matsuda et al. 1990).

We then explored the structural mechanisms by which these permeability changes occur. Initially, we hypothesized that permeability changes caused by inflammation may be due to transcriptional changes in the production of tight junction proteins such as claudin, as changes in the mucosal presence of these proteins have been shown in Crohn's disease (Zeissig et al. 2007). We found the inflammatory protocol had no effect on mRNA levels of claudin proteins in Caco-2 cultures, and this was not influenced by PEA or CBD treatment. As the expression of claudin proteins has been shown to be affected in Crohn's disease, we compared our Caco-2 culture findings with normal human colonic tissue experimentally inflamed with IFNγ and TNFα (Zeissig et al. 2007). We found that inflammation caused a decrease in the production of mRNA for claudin-5, and that this change was prevented by treatment with CBD. Claudin-5 is highly expressed in the human colon, and acts to strengthen the mucosal barrier by decreasing permeability though cysteine residues (Wen et al. 2004). Inflammation caused a decrease in the presence of claudin-5 and promoted increased permeability across mucosal types, hence this may be a key mechanism by which CBD affects permeability (Kanda et al. 2004; Zeissig et al. 2007; Fedwick et al. 2005).

We also found that PEA decreased the production of mRNA for claudin-3, a protein unaffected by the inflammatory protocol. Claudin-3 is present in the healthy colon, providing a barrier function by decreasing permeability to charged ions (Prasad et al. 2005). The finding that PEA may decrease the production of claudin-3 in human colon yet decreases hyperpermeability in Caco-2 cultures suggests that either the role of claudin-3 in the gut is incompletely understood, or that PEA does not simply affect permeability in terms of pore formation. Claudin-3 expression under inflammatory conditions has been investigated by other centres with varying results across models; in support of our findings, Zeissig (2007) found that claudin-3 expression is unaffected by Crohn's disease (Zeissig et al. 2007), however in cell culture experiments expression is reduced by TNFα (Prasad et al. 2005; Mei et al. 2015), but not by IL-13 (Prasad et al. 2005). Claudin-3 has also been shown to be upregulated in colonic carcinoma and inflammatory state (de Oliveira et al. 2005). As no other studies have examined the effect of cannabinoids on claudin expression in human tissue we are unable at this point to determine if the effect of PEA on claudin-3 is a reproducible mechanism by which permeability is affected and requires further study.

As aquaporins have an increasingly important role in both permeability to water and the immune response, we hypothesised that changes in the production of two aquaporins, AQP3 and AQP4 may be a mechanism by which cannabinoids act on permeability. The role of AQP3 in epithelia is not limited to water and ion transport, as previous research has demonstrated that AQP3 contributes to the epithelial content of glycerol, and that, in epidermal models, AQP3 deletion leads to dry skin in mice and an increased susceptibility to inflammation (Hara et al. 2002). Interestingly the study from Hara et al, demonstrated that AQP3 null mice had delayed wound healing which was not due to water loss using selective skin taping. This is further supported by studies of gastric cancer where the presence of AQP3, in transporting glycerol intracellularly, had an anti-proliferative effect acting though the PI3K/Akt pathway (Li et al. 2016). As gastric cancer possesses a significant inflammatory component, and indeed may be caused by the presence of a persistent pro-inflammatory stimulus, we may suggest that the presence of AQP3 may itself limit inflammation, or activate an immune-tolerance response, limiting pro-inflammatory cytokine production. Taken together these findings suggest that AQP3 does not simply contribute to water transport, but plays a role in immune function, which may be dependent on glycerol transport. In other gut studies this hypothesis has been further supported. Thiagarajah et al. demonstrated that AQP3 deficient mice have a decreased mortality, increased severity of disease and decreased cell proliferation in a dextran sulphate sodium model of IBD compared to wildtype controls (Thiagarajah et al. 2007). The authors then demonstrated AQP3-deleted mice had a small but significantly improved survival when co-administered intracolonic glycerol, suggesting that in the gut also AQP3 presence provides an anti-inflammatory role by contributing to glycerol transport. AQP3 expression in the ileum is reduced in IBD which was suggested to be a mechanism to reduce oxidative stress through limiting water loss (Ricanek et al. 2015), although it has been shown that knock-down of AQP3 paradoxically impairs gut barrier function and increases permeability (Zhang et al. 2011). We found that in Caco-2 cells inflammation increased levels of membrane-bound AQP3, which was prevented both by PEA and CBD. Conversely, in human tissue, the inflammatory protocol alone had no significant effect on AQP3 levels, whereas in the presence of the inflammatory protocol and PEA or CBD these levels were increased. This must be taken in context with the findings in Chapter 3, demonstrating that PEA and CBD are not anti-inflammatory in Caco-2 cultures, but do prevent the increased secretion of pro-inflammatory cytokines in human colonic tissue (Couch et al. 2017). Therefore, a potential mechanism of PEA and CBD on the inflammatory response may be through upregulation of functional membrane-bound AQP3 and glycerol

uptake, although this may not be the direct mechanism through which permeability is affected in Caco-2 cultures. Further study is required examining the effect of CBD and PEA on glycerol uptake in conjunction with AQP3 expression in the human colon.

AQP4 is known not to contribute significantly to water transfer in the gut, as knock-down of the channel does not affect permeability (Ma et al. 1997), however there is evidence suggesting AQP4 plays a role in the immune response of the colonic mucosa as AQP4 is upregulated in colonic mucosa of IL-10 knock out mice (Hansen et al. 2009). In Caco-2 cells we found no change in membrane-bound AQP4 expression in response to inflammation, but upregulation in expression caused by PEA and CBD treatment. In comparison with experimentally inflamed human colonic tissue, inflammation increased the presence of AQP4, which was not affected by PEA or CBD, although in the absence of inflammation these levels were reduced compared to vehicle alone. The absence of an immune cell-mediated response to inflammation in Caco-2 cells may explain the difference between the two culture models' response to TNFα and IFNγ. As AQP4 expression was reduced in human tissue compared to vehicle it may be possible that, if CBD and PEA were administered prophylactically, we might witness a change in response to inflammation. However, given the levels of membrane-bound AQP4 it may also be the case that AQP4 expression may not be a mechanism by which CBD or PEA act in the human colon.

It has been previously demonstrated that the expression of the CB_1 and CB_2 receptors on the gut epithelium, immune cells and enteric nervous system change with inflammation (Izzo, Fezza, et al. 2001; Massa et al. 2004; D'Argenio et al. 2007; Marquéz et al. 2009). As PEA had been previously shown to alter the expression of these receptors in mice, we hypothesized that PEA and CBD may demonstrate these effects in experimentally inflamed human colon (Esposito et al. 2014; Borrelli et al. 2015). In both Caco-2 cultures and human colon, we found a significant decrease in the expression of TRPV1 in response to inflammation, in line with previous reports (Borrelli et al. 2015). In both cell culture and explant tissue models, CBD prevented these falls, whereas PEA only prevented falls in TRPV1 expression in Caco-2 cultures. We also found that PPARα expression was increased by inflammation, but not affected by PEA of CBD treatment. No other receptors were affected by inflammation. Interestingly we found that in human colonic explants PPARα expression was increased by inflammation, and unaffected by PEA and CBD, but in Caco-2 cultures, expression of this receptor appeared to non-significantly fall. Additionally, the changes in PPARα expression in Caco-2 cells was

not uniform, and prone to large variation, unlike the expression of the other measured receptors. This reasons for this are not clear from this study, but could be explained by genetic changes across cultures, as Caco-2 cells have been previously shown to demonstrate significant genetic mutations across successive generations, and even individual plates (Cuadras et al. 2002). In addition to changes in genetic composition, this has also been shown to affect the functional phenotype of cultures, measured by permeability and morphology (Volpe, 2008). The absence of change in CB_1 or CB_2 expression is interesting, and contradicts existing evidence that both CB_1 and CB_2 are upregulated in biopsies from IBD patients (Marquéz et al. 2009). One possible explanation for this difference is the role of the enteric nervous system. Peripherally restricted cannabinoid agonists have been shown not to prevent inflammatory changes in murine colitis, suggesting that cannabinoid action at the central nervous system is crucial to their effect on gut inflammation (Cluny et al. 2010). This was supported by a study by Esposito, demonstrating that PEA may act directly on enteric glial cells, rather than on mucosal immunocytes (Esposito et al. 2014). We may suggest that our normal tissue, which is no longer innervated by the enteric nervous system, did not therefore undergo any nerve-mediated changes in receptor expression. This presents further evidence that the gut is dependent centrally on endocannabinoid tone for the immune response and barrier function.

In conclusion the effects of PEA and CBD on inflammation-induced permeability extend to the transfer of macromolecules, acting through similar intracellular pathways. The mechanism underlying these effects may occur through preventing receptor downregulation, tight junction and aquaporin expression.

5 The Sweet PEA study – a randomised, double-blind controlled study investigating the effects of CBD and PEA on the permeability of the human gastrointestinal tract

5.1 Introduction

In the previous Chapter, PEA and CBD were able to prevent inflammation-induced permeability in Caco-2 cultures to macromolecules and the mechanisms by which this may occur were examined. However, given the cell culture or explant nature of these previous experiments, it is not clear if these compounds will affect permeability in humans *in vivo.* The only clinical trial investigating the use of CBD in IBD did not yield easily interpretable results because of poor study design (see section 2.4.2) (Naftali et al. 2017). The aim of the present study was to test whether these compounds have permeability effects *in vivo*.

Permeability is not routinely measured clinically. However, intestinal permeability may be measured using the sugar absorption test (Sequeira et al. 2014b). This test measures the urinary excretion of ingested sugar probes which are able to move from the gut lumen to submucosal space. This test is non-invasive and measures diffusion of solute via the paracellular route, rather than by active absorption (Bjarnason et al. 1995). The choice of probe in these investigations are crucial, as the size and charge of molecules used will affect their absorption and renal excretion. Molecules used as markers or permeability must possess the following qualities:

- Inert substances that do not increase permeability themselves,
- Not be metabolised in humans,
- Be absorbed and excreted in a regular fashion,
- Cannot be harmful,

Previously used probes and their properties are described in table 5.1.

Probe	Description	Size	Site of uptake	Degradation in gut	Absorption time
D-Mannitol	Monosaccharide	182 Da	Small intestine	75% degradation	0-6 hr
Lactulose	Disaccharide	342 Da	Small intestine & colon	75% degradation	0-6 hr
Sucrose	Disaccharide	342 Da	Stomach	50% degradation	0-2 hr
Sucralose	Disaccharide	397 Da	Colon	0%	3-15 hr
^{51}Cr-EDTA	Radio-isotope	341 Da	Small intestine	0%	0-6 hr
PEG 400	Collagen polymer	520 Da	Small intestine and colon	0%	5-24 hr Variable absorption

Table 5-1 Commonly used probes in measuring gastro-intestinal permeability.

Saccharides, which possess no ionising radiation, are preferred over radioisotopes as this limits the exposure of potentially ionising radiation to participants, which may have long-term consequences for development of sporadic cancers (van Elburg et al. 1995, Little et al. 2009). The use of sugars, such as D-mannitol, for measuring permeability has been called the sugar absorption test (Sequeira et al. 2014b). Administering two sugars, with differing rates of absorption in health and disease is preferable to measuring the absorption and excretion of a single probe, as this avoids being affected by factors such as delayed gastric emptying, differing volumes of distribution across participants, intestinal transit time and varying renal clearance (Twiss et al. 2001). D-mannitol is passively absorbed by the small intestine at a steady rate in health. Lactulose, a larger molecule, is not normally absorbed in health, but is passively absorbed during inflammatory intestinal episodes (Benjamin et al. 2008). As both compounds undergo regular and similar degradation in the gut, and are absorbed and excreted within the same timeframe, the excreted lactulose to mannitol ratio (LMR) gives a measure of intestinal permeability (Sequeira et al. 2014b). This ratio has previously been used to calculate intestinal permeability in Crohn's disease, where lactulose absorption proportionally increases to the absorption of mannitol (Benjamin et al. 2008). Sucrose is highly metabolised in humans and therefore an inappropriate choice of probe. Sucralose is not easily paired with another sugar probe, as it requires long experimental duration.

Experimentally increased intestinal permeability can be caused by several methods. Previous studies have induced hyperpermeability with LPS infusion in healthy

volunteers, which gives a reliable and profound increase in intestinal permeability (Hietbrink et al. 2009). However, with this method the authors reported that all 14 participants developed the systemic inflammatory response syndrome, 3 of which required hospital admission. Therefore despite promise of the desired experimental effect, the benefits of further study with LPS in this context may not outweigh the risk to participants. The use of aspirin to cause increased intestinal permeability has been previously well documented (Sequeira et al. 2014a; Sequeira et al. 2015; Sequeira et al. 2014b). In these experiments, a single 600mg dose of oral aspirin reliably increased the absorption of sugar probes. It has been hypothesized that the mechanism by which increased permeability to sugars occurs is through inhibition of cyclooxygenase production, and therefore a decrease in mucosal prostaglandin production (Bjarnason et al. 1995; Bjarnason et al. 1991). This may therefore divert arachidonic acid along the lipoxygenase pathway causing leukotriene production and local free radical production with resultant microvascular and mucosal damage. This action, alongside a reduction in adenosine triphosphate production, may inhibit paracellular junction and enterocyte membrane integrity, therefore promoting the absorption of small probes through denaturation of the lipid bilayer and membrane-bound or para-membranous proteins responsible for permeability. No previous adverse effects were reported from a single 600 mg dose in these studies. Similar studies also used indomethacin and cigarette smoking (Suenaert et al. 2000).

The aims of this study were to examine the effect of PEA and CBD on the permeability of the human gut *in vivo.* In a group of healthy volunteers, we induced a state of increased gut permeability with high-dose aspirin, and used the sugar absorption test, quantifying urinary concentrations of lactulose and D-mannitol to determine whether PEA or CBD would affect this increased permeability.

5.2 Methods

5.2.1 Participant recruitment

All experiments and procedures received prior approval of the University of Nottingham Ethics Committee (REC reference: J16122016). Healthy male participants between the ages of 18 and 65 were recruited following an informed consent process. All participants were screened to exclude those with any gastrointestinal medical conditions of symptoms, regular medications or recreational drugs, heavy alcohol use, previous abdominal surgery, and personal or family history of inflammatory bowel disease. Following recruitment, participants were asked to refrain from the use of any pro or prebiotics the week prior to the study, alcohol or non-steroidal anti-inflammatory use or heavy exercise within the three days leading up to the study.

5.2.2 Sample size

Based on previously published literature, a sample population under the same experimental conditions demonstrated a urinary LMR of 0.81 when treated with aspirin, verses an LMR of 0.053 (SEM=0.02) quantified by high pressure liquid chromatography (Sequeira et al. 2014b). Based on an $\alpha=0.05$ using a paired t-test (effect size = 1.0) assuming a power of 80%, a sample size of 10 should be sufficient to detect a difference between drug and control groups assuming a difference of 0.02 is reached in LMR in the treatment groups compared to placebo should a difference exist between the treatment and placebo groups. Software used was Genstat (Genstat for windows, version 19.1, VSN international, US).

5.2.3 Materials

CBD was obtained from Artelo Biosciences (CA, USA). CBD was manufactured from *C. sativa* extract, and analysed at an independent laboratory to possess >99.65% purity. PEA was obtained from Russel Science (Nicosia, Cyprus). Placebos were manufactured by Artelo Biosciences using the base cellulose also used in the preparation of CBD. Aspirin was obtained from Aspar Pharmaceuticals (London, UK). Lactulose was obtained from TEVA (Castleford, UK). D-Mannitol was obtained from Sigma Aldrich (MO, USA). All products used in the test solution were intended and tested safe for oral consumption. The purity of these sugar probes was reported to be >99.0%.

5.2.4 Randomisation

Using the block method in a 1:1:1 ratio, participants were randomly assigned to receive CBD, PEA or placebo. Both participants and investigators were blinded to the group assignments. Participants were numbered sequentially and were assigned to the treatment groups in the order in which they were recruited. The code assignment was sealed within an envelope until after the samples were analysed. Participant assignment was only revealed at the end of the study.

5.2.5 Experimental Protocol

On the day of study, participants attended at 08:00 after an overnight fast. Aspirin 600 mg was administered orally with 400 ml tap water with either oral CBD 600 mg, PEA 600 mg or placebo. This dose of CBD was chosen because a previous study in IBD had shown that a 10mg oral dose as insufficient to generate a detectable change in primary outcome, and the normally prescribed CBD for epilepsy is between 2mg/kg to 25mg/kg orally (O'Connell et al. 2017). As a previously study had shown in adult males that doses above 700mg orally begins to induce perceived side effects in healthy adults (Bergamaschi et al. 2011), we trialled a 600mg dose as this was felt likely to generate a change in absorption with minimum side effects. In addition, PEA has been shown previously to reduce ocular hypertension at 600mg oral dosing with no side effects, and therefore this dose was chosen (Keppel Hesselink et al. 2015). At 09:00, a solution of 1 g lactulose and 1 g mannitol in 600 ml water was administered orally. These doses were chosen as they were the minimum doses detectable in urine using the sugar absorption test. This dose, below the clinical dose of 10g lactulose, was unlikely to generate a significant laxative effect which may have biased intestinal uptake of either CBD, PEA or the measured sugars. At this time point, a baseline urine sample was collected. At one hour intervals, further urine samples were collected. To facilitate urinary collection, 400 ml water was administered orally at 12:00. Urinary samples were immediately centrifuged at 1,000 g for 7 min at 3 °C and then frozen at -80 °C until analysis. The study protocol is summarised in figure 5.1.

5.2.6 Determination of urinary concentrations of lactulose and D-mannitol

Concentrations of urinary lactulose and mannitol were determined using a liquid chromatography (LC) mass spectrometry (MS). This analytical chemistry technique uses the principles of separation and measurement of different components of a solution. A LC phase passes the solution in question through a heated column, so components of different mass and boiling points emerge from the column at different time points. These different components are then structurally identified

using MS, comparing their mass and charge to known standards. Quantities in solution can then be calculated against a standard curve. All LC MS determinations were performed by Dr Catherine Ortori, Faculty of Science, University of Nottingham, who was blinded to the study. 20 µl aliquots of urine were thawed and diluted to precipitate any excess salt with 980 µl 90% acetonitrile to which internal standards xylitol and raffinose were pre-mixed at 0.5 µg/ml final concentration. These were vortexed, incubated at -20 ^{0}C for 4 h, centrifuged and the supernatant layer decanted into amber high pressure LC vials. Previous work demonstrated the necessity to monitor sucrose to ensure the assay is specific, correctly identifying lactulose (Gervasoni et al. 2016). Therefore, sucrose was included as an analyte, although not quantified here. Calibration standards were made as a dilution series from 2.5 to 250 µg/ml of the analytes mannitol and lactulose from stocks made in water. The method had been previously validated by creating 6 independently prepared dilutions of 5, 50 and 500µg/ml as above (Gervasoni et al. 2016). Standards were injected at regular intervals during the sequence.

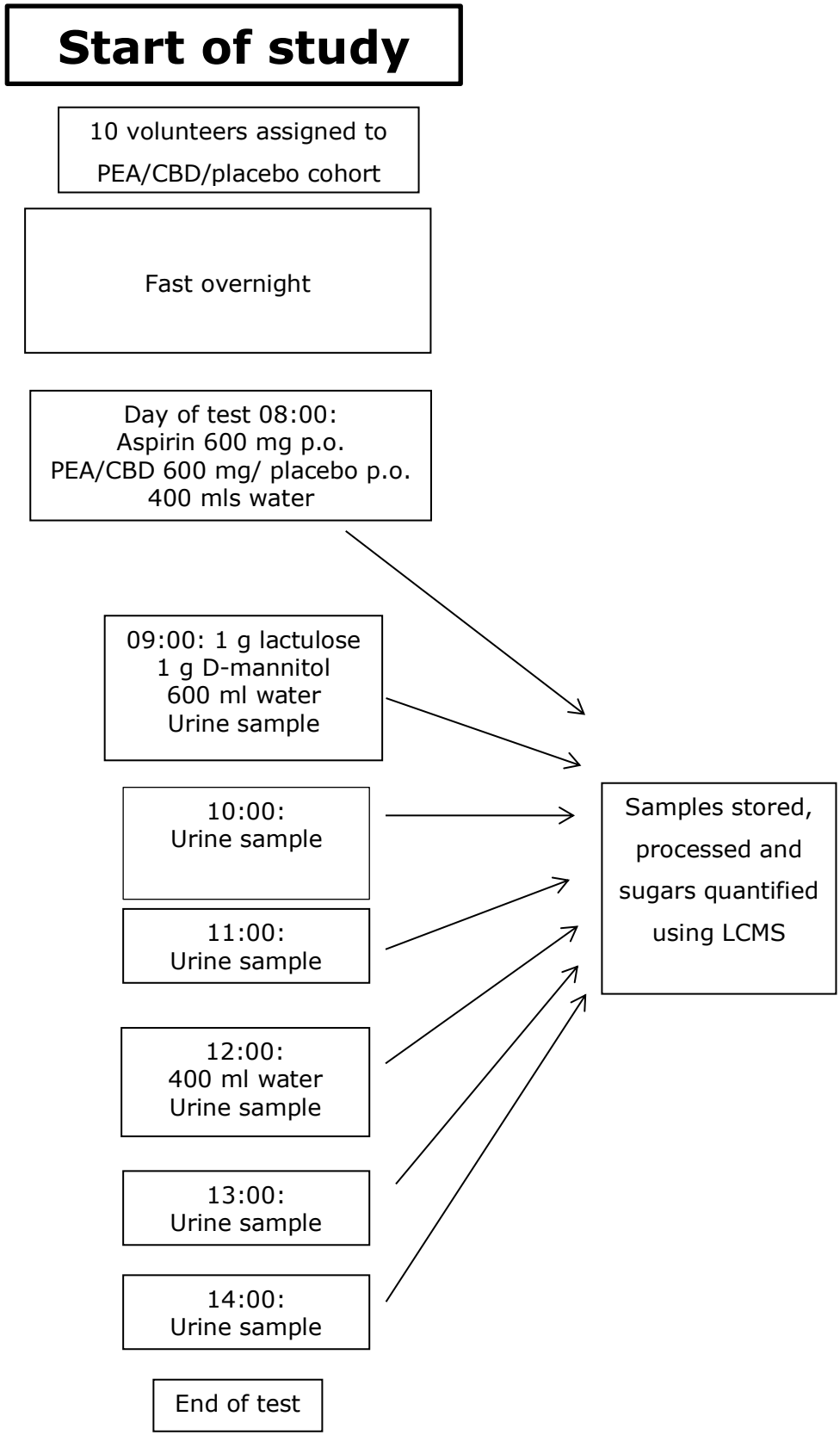


Figure 5.1 Study protocol

The LC method was based on that of Kubica (Kubica et al. 2012). The LC column was a Sequant ZIC-pHILIC pHILIC (150 x 4.6 mm, 5 µm) kept at 40 ^{0} C. The mobile phases were A, acetonitrile and B, 5mM ammonium acetate, adjusted drop-wise to pH 6.85 with 5 mM ammonium hydroxide solution. The LC gradient was as follows:

Time (min)	% B	Flow (ml/min)
0.0	75	0.5
10.0	58	0.5
11	10	0.7
12	85	0.7
15.0	85	0.7

Samples were kept in a chilled auto-sampler and 2 µl volumes were injected for analysis.

The detector was a Sciex 4000 QTrap operating in -ve ion electrospray mode with the source at 450 ^{0} C with curtain, nebuliser and auxiliary gases set to 10, 40 and 20 respectively. The ion-spray voltage was -4200 V. Table 5-2 shows the ions, collision energy and retention time monitored for each compound.

Class	**Name**	**Precursor ion**	**product ion**	**Declustering potential**	**Collision energy**	**Retention time**
Analyte	lactulose main	341.0	161.0	-40	-14	9.1
	lactulose secondary		101.0		-26	
Analyte	Mannitol main	181.0	59.0	-65	-32	8
	Mannitol secondary		89.0		-22	
IS	Raffinose main	503.1	59.0	-110	-72	7.3
	Raffinose secondary		179.1		-32	
	Raffinose secondary		88.9		-110	
IS	xylitol main	151.1	59.0	-60	-28	10.3
	xylitol secondary		71.0		-26	
Confounder-Monitored not quantified	Sucrose main	341.0	179.0	35	-20	9.1
	Sucrose secondary		88.8		-26	
	Sucrose secondary		59.0		-66	

Table 5-2 LC MS parameters for each compound monitored.

Samples were quantified against the appropriate region of the line. A minimum of 5 points was used for each analyte. Xylitol was used as the internal standard to normalise the lactulose signal but the raw peak area was used to calculate the mannitol concentrations. Raffinose was included as an internal standard as it has

been used in previous studies (Gervasoni et al. 2016; D 2018). Significant variations in the raffinose signal, with trends that related to sample codes, were noted during the current analysis and this internal standard was discarded.

Considerable care was required to distinguish sucrose from lactulose as both disaccharides have common precursor/product ions. Product ions 161 and 179 were produced more abundantly by lactulose and sucrose (~10 and 20 fold) respectively and are used to demonstrate that any sucrose signals were insignificant in these samples.

A standard prepared at 50 µg/ml, injected every 20 samples, was used to monitor precision and accuracy. The CVs were 14.1 % and 7.3 % for lactulose and mannitol respectively. The precisions were 107 and 89 %. Calibration lines are linear over a range greater than 2.5 to 250 µg/ml with R^2 >0.9995 for both analytes.

5.2.7 Statistical analysis

The calculated concentration of each sugar was assessed by repeated measures ANOVA for the effect of time within treatment groups, and two-way ANOVA between treatment groups with post hoc comparisons subject to the Bonferroni correction. The ratio differences in excretion between lactulose and D-Mannitol over the study period were compared using two-way ANOVA using Dunnett's post hoc test. A p-value of <0.05 was considered statistically significant. Normality was assessed using the D'Agostino & Pearson normality test. All statistical analyses were performed using GraphPad Prism 7.01 for Windows (GraphPad Software, San Diego, USA).

5.3 Results

30 male participants aged between 22 and 50 years (median age 28.7) were recruited and successfully completed the study. No exclusions were made on the basis of health screening. No participants reported any gastrointestinal or other side effects in any of the experimental sessions. No participants reported any taste disturbance. Urinary concentrations of mannitol excreted over the 6 h study period were normally distributed using the D'Agostino & Pearson normality test.

For participants receiving placebo, aspirin administration caused a significant increase in the urinary concentrations of mannitol and lactulose compared to baseline urine sample, both becoming significant at 3 h from aspirin administration (lactulose $p<0.00001$, mannitol $p<0.01$) (table 5.3 and figure 5.2 and 5.3).

Aspirin & Placebo						
	Lactulose			Mannitol		
	Mean concentration (ug/ml)	Standard deviation	Significance from baseline	Mean concentration (ug/ml)	Standard deviation	Significance from baseline
1 h	7.1	2.013		3.2	3.8	
2 h	139.1	76.511		4.8	5.0	
3 h	313.4	196.387	****	9.2	6.8	**
4 h	290.1	183.454	****	7.0	3.0	***
5 h	194.8	134.162	*	6.2	2.5	*
6 h	182.3	129.543	*	5.9	2.1	

Table 5-3 Urinary concentrations of lactulose and mannitol in healthy volunteers receiving aspirin therapy (n=10). Results are expressed as mean concentrations +/- standard deviation. Concentrations were compared to baseline using repeated measures ANOVA and Dunnett's multiple comparison test (*P<0.05, **P<0.01, ***P<0.001, ****P<0.00001).

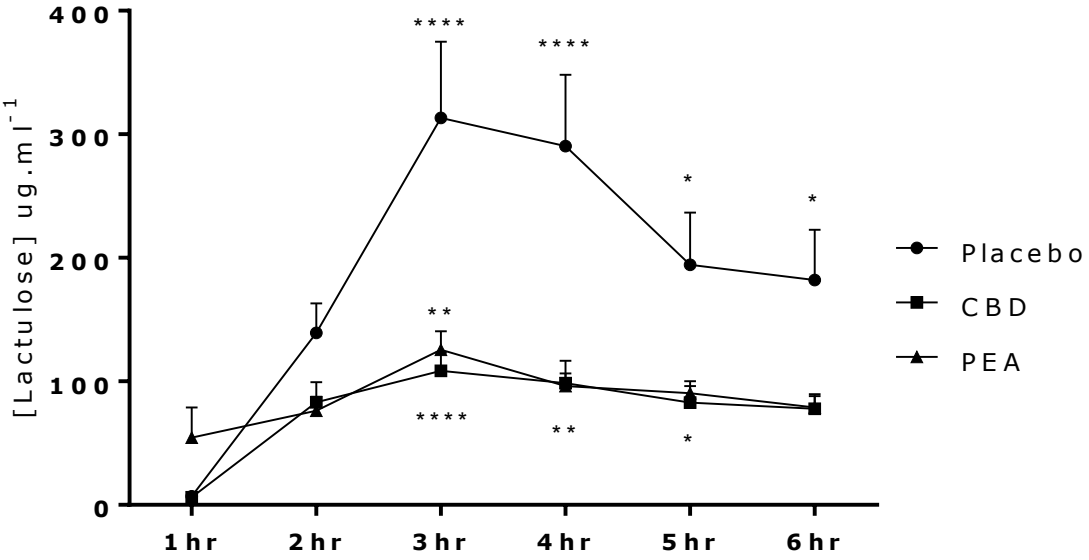


Figure 5.2 The effect of CBD, PEA and placebo on aspirin-induced increases in intestinal absorption and urinary secretion of lactulose in healthy human subjects (n=10). Results are expressed as mean concentrations +/- SEM. Concentrations were compared to baseline sample using repeated measures ANOVA and Dunnett's multiple comparison test (*P<0.05, **P<0.01, ***<0.001, ****<0.00001).

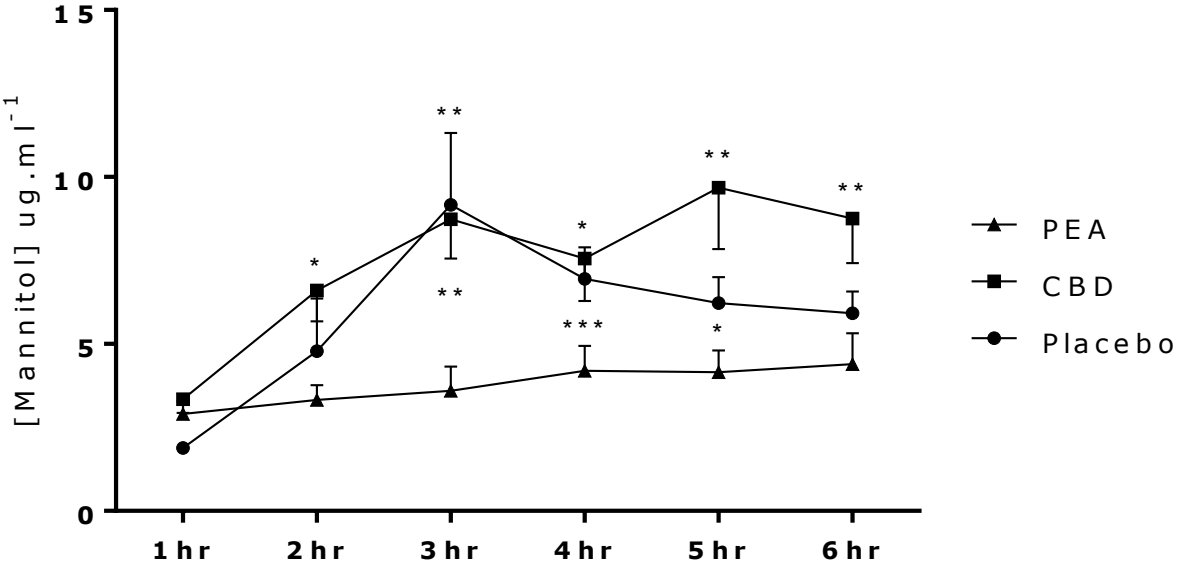


Figure 5.3 The effect of CBD, PEA and placebo on aspirin-induced intestinal absorption and urinary secretion of mannitol in healthy human subjects (n=10). Results are expressed as mean concentrations +/- SEM. Concentrations were compared to baseline sample using repeated measures ANOVA and Dunnett's multiple comparison test (*<0.05, **<0.01, ***<0.001).

In participants receiving CBD or PEA, aspirin also caused a significant increase in the concentration of urinary mannitol and lactulose; data are given in tables 5.4 and 5.5 and figures 5.2 and 5.3.

Aspirin & Cannabidiol						
	Lactulose			Mannitol		
	Mean concentration (ug/ml)	Standard deviation	Significance from baseline	Mean concentration (ug/ml)	Standard deviation	Significance from baseline
0 hr	6.05	1.888		3.19	1.264	
1 hr	83.09	51.92		6.6	2.922	*
2 hr	108.5	46.16	****	8.73	3.752	**
3 hr	98.59	57.42	**	7.56	4.01	*
4 hr	82.84	42.5	*	9.68	5.814	**
6 hr	77.74	32.88		8.76	4.239	**

Table 5-4 Urinary concentrations of lactulose and mannitol in healthy volunteers receiving aspirin and CBD therapy (n=10). Results are expressed as mean concentrations +/- standard deviation. Concentrations were compared to baseline using repeated measures ANOVA and Dunnett's multiple comparison test (*<0.05, **<0.01, ****<0.00001).

Aspirin & Palmitoylethanolamide						
	Lactulose			Mannitol		
	Mean concentration (ug/ml)	Standard deviation	Significance from baseline	Mean concentration (ug/ml)	Standard deviation	Significance from baseline
0 hr	54.4	77.0		2.52	1.171	
1 hr	76.3	35.96		3.32	1.006	
2 hr	125.7	47.61	**	3.98	0.6979	
3 hr	96.12	32.91		4.74	1.408	
4 hr	90.42	30.74		4.9	1.815	
6 hr	78.97	34.51		4.76	2.336	

Table 5-5 - Urinary concentrations of lactulose and mannitol in healthy volunteers receiving aspirin and PEA therapy (n=4-10). Results are expressed as mean concentrations +/- standard deviation. Concentrations were compared to baseline using repeated measures ANOVA and Dunnett's multiple comparison test (*<0.05, **<0.01, ****<0.00001).

As the excretion of single sugar probes is affected by glomerular excretion rates and hydration status, the ratio of lactulose to mannitol concentrations in urinary samples were calculated per participant for each time point. Compared to placebo, both CBD and PEA significantly reduced the ratio of lactulose to mannitol concentration across the study reaching significance at 2 hours from baseline (both $p<0.01$), lasting until the end of the study period (figure 5.4), suggesting that PEA and CBD had caused a decrease in intestinal permeability.

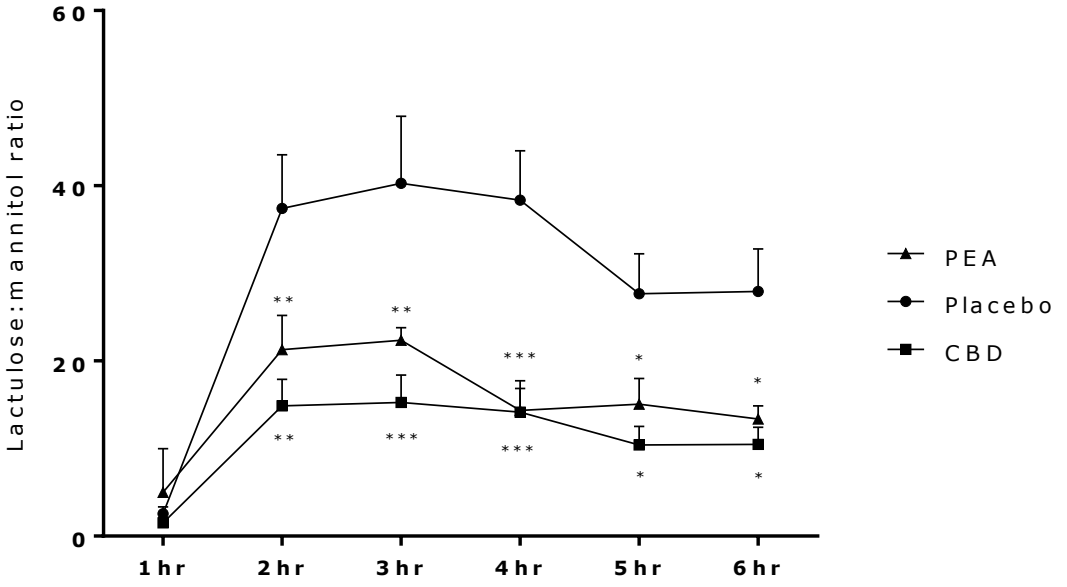


Figure 5.4 The concentration ratios of urinary lactulose and mannitol over time in healthy participants treated with aspirin and either placebo (n=10), CBD (n=10) or PEA (n=4), measured by LC MS. Results are expressed as mean ratios +/- SEM. Time points between groups were compared using two-way ANOVA using Dunnett's multiple comparisons test comparing to placebo at the same time point (*p<0.05, **p<0.01, ***p<0.001).

5.4 Discussion

The aims of this study were to assess the effect of PEA and CBD on the permeability of the human gastrointestinal tract *in vivo*. We used a randomised, double blinded controlled trial to test this. We measured the absorption and excretion of sugar probes in an aspirin-induced pro-inflammatory model, with or without treatment with PEA or CBD. We found that aspirin treatment caused an approximately 10-fold increase in the LMR, suggesting that aspirin had caused an increase in the permeability of the gastrointestinal tract in line with previous studies (Sequeira et al. 2014a; Sequeira et al. 2014b). CBD and PEA both reduced this increase. Taken together with results from chapter 4, this provides further evidence that PEA and CBD may have merit as potential novel agents to prevent or reduce the consequences or symptoms Crohn's disease, ulcerative colitis or other inflammatory states.

The mechanism by which aspirin increases intestinal permeability is incompletely understood, though may occur through COX inhibition with resulting free radical generation and microvascular damage. The resultant generation of free radicals may induce increases in permeability through denaturation of membrane-bound proteins, or the integrity of the lipid bilayer itself. However, as there has been limited investigation into the mechanism by which this occurs this can only be suggested. In order to develop this model of IBD, and other disorders of increased gut permeability such as Coeliac disease, further a deeper of understanding of this effect of aspirin on the gut mucosa should be investigated. This could be achieved by examining the effect of aspirin on the lipid bilayer, expression or presence of the paracellular apparatus, production of inflammatory mediators and COX activity in cell culture and explant models.

We found in this study that CBD reduced the effect of aspirin on increased permeability, which may have been caused by inhibition of COX-2. Other centres have examined the effects of CBD on COX with conflicting findings. An early study demonstrated that CBD inhibits COX production in a murine arthritis model, with associated decreases in free radical production (Costa et al. 2007). However, more recently in a cell culture model CBD was reported to be a COX-2 inducer, rather than an inhibitor, and a weak inhibitor of COX-1 (Ruhaak et al. 2011). Another study from the same centre reported in a murine model that PEA was also an inhibitor of COX (Costa et al. 2004), supported with evidence that PEA inhibits the production of COX-2 (D'Agostino G et al. 2009). However, these data was not supported more recently examining the effect of PEA on COX-2 activity in RAW264.7 cells (Gabrielsson et al. 2017). Although in this study we did not investigate

mechanisms of action, further *in vitro* and *ex vivo* experimentation using aspirin, PEA and CBD may demonstrate their relationship more clearly. For example, using Caco-2 cell and ex vivo colonic cultures, we could investigate the inducing or inhibiting effect of CBD or PEA on COX-1 and COX-2 using commercially available enzyme assays. Although we have used colonic explants in this centre previously, these tests could be extended to gastric, jejunal and ileal tissue, alongside investigation of the effect of aspirin in these tissues.

We have hypothesized that these changes in urinary sugar concentration are secondary to the effects of PEA or CBD on the gut barrier. However, these results could also be explained by a decrease in glomerular filtration or preventing the renal excretion of these sugars. Previous reports have demonstrated that these compounds are able to limit the effects of ischaemic renal injury, in the case of CBD (Fouad et al. 2012), or hypertensive and contrast induced nephropathy, in the case of PEA (Mattace Raso et al. 2013; Cordaro, Impellizzeri, Bruschetta, et al. 2016), on glomerular filtration. However, in these reports, both compounds prevented falls in glomerular filtration, rather than causing a decrease. This suggests that falls in LMR are unlikely to be due to renal effects but could be examined further by measuring CBD and PEA effects on lactulose and mannitol renal excretion in the explant animal kidney, or the excretion of other similar sugars.

There are several limitations to this study. Although using high dose aspirin was used to increase the absorption and excretion of ingested probes, this may not be a reliable simulation of increased intestinal permeability secondary to clinical disease. The mechanisms of increased permeability with aspirin use may be different when compared with endogenous inflammation. Therefore, the effects of PEA and CBD may not translate into a clinical effect. Alternative models have been used to induce a state of hyperpermeability which may resemble clinical disease more closely. Secondly, this study is limited by small group numbers. This may have exaggerated or under-estimated differences between groups. In particular, 6 of 10 samples in the PEA cohort were found to contain mannitol levels which were below the level of quantification using our LC MS method from baseline sample onwards. Therefore, ratios of lactulose to mannitol were not possible to calculate for these 6 participants and were excluded from the analysis in figure 5.4. Effect sizes were calculated from the remaining samples in the group, although confidence intervals remained narrow. Additionally, lactulose and mannitol are preferentially absorbed in the small intestine rather than the colon. This suggests that CBD and PEA in this experiment were likely to be acting primarily in the proximal gut rather than in the colon. Suggestions therefore that PEA and CBD could be used in

ulcerative colitis, which does not primarily affect the proximal small intestine, may not be true in subsequent clinical experimentation. Lastly, the oral bioavailability of CBD is low because of its lipophilic nature, which may underestimate the effect of CBD on permeability, and should be further examined with the administration of CBD via a different route or preparation. For example, the lipophilic nature of CBD is suggested to prevent its rapid oral uptake. Administration of a yet unavailable IV preparation would allow insight into if CBD exerts is effect at a mucosal, topical level, or via serum concentrations.

In summary, we have demonstrated that CBD and PEA attenuate induced hyperpermeability in the human gut. This raises the possibility of these drugs becoming novel therapeutic agents in the future. In addition to permeability effects, examination of their effects on patient-reported symptomatology, dependence on systemic immunosuppression or serum inflammatory markers may also be measured. These should be measured through randomised, controlled phase 2 clinical trials.

6 Discussion and future directions

Inflammatory bowel disease is a serious, life limiting chronic disease without a definitive treatment in clinical practice. A component of IBD is an increase in mucosal permeability, and it has been suggested that therapies targeted at this aspect of the disease may provide improved outcomes. Manipulation of the endocannabinoid system, by activation or antagonism of its receptors, or inhibition of its synthesizing and metabolic enzymes, may provide novel therapies for IBD or other inflammatory disorders of the gut. This is supported by a growing collection of preclinical and clinical studies examining the effect of these substances and pathways on gut inflammation. The aims of this thesis were to identify cannabinoid compounds with potential for use in clinical practice, quantify their effect on permeability and inflammation in the colon, and investigate their effects on permeability in vivo.

Previously published data had not been systematic reviewed or pooled for meta-analysis, although a collection of narratives on their action in the gut had previously been published (Izzo & Sharkey 2010; Izzo & Camilleri 2009; Izzo & Camilleri 2008). In Chapter 2, a systematic literature search collated the existing data. Meta-analysis of commonly used endpoints demonstrated that two particular compounds, PEA and CBD, had been well investigated, yielding consistently positive results in experimentally induced colitis. However, there was shown to be a lack of clinical studies. Meta-regression found that prophylactic administration of cannabinoid or related compounds may be more effective to therapeutic, and that the existing preclinical data may overestimate the effect of cannabinoids. This study also demonstrated that almost all cannabinoids produced positive outcomes, regardless of model, supporting the further examination of cannabinoid agents in humans.

Of interest in chapter 2 was the diversity of animal models used to simulate IBD. Currently there are in excess of twenty animal models of IBD in regular experimental use, which may be reflective of the failure of any individual model to accurately represent the disease (Jurjus et al. 2004). For example, IL-10 knock-out mice spontaneously develop colitis after 10 weeks of life, however do not develop ileal inflammation, as is seen in Crohn's disease. The previous lack of sufficient animal models is likely to be due to the uncertain aetiology of IBD, and therefore difficultly in replicating the disease. As animal models of disease are important to allow investigation into the mechanisms behind pathology and propose candidate treatments for phase 1 clinical use, further efforts to develop models of Crohn's disease and ulcerative colitis may prove a valuable use of resources. This

process, given the lack of understanding behind IBD, may be difficult but necessary, as the alternative is more invasive experimentation on human IBD subjects, which may not be ethically tolerable without a body of pre-existing experimental cell culture and animal model data.

It has been suggested that PEA and CBD are beneficial in treating colitis, and Chapters 3 and 4 investigated their mechanism of action in cultured Caco-2 monolayers and explant human colonic tissue. Chapter 3 disproved the hypothesis that the effects of PEA and CBD on TEER in Caco-2 cells were due to local suppression of inflammation (Alhamoruni et al. 2012; Karwad et al. 2016). Although an anti-inflammatory effect of cytokine secretion was found in colonic tissue, this was not seen in Caco-2 cultures, which lack a secondary immune response. Therefore, any permeability effects in Caco-2 cells, and possibly human tissue, cannot be solely due to an anti-inflammatory action.

Over the last 20 years, multiple animal inflammatory models have been developed in order to study IBD, including those analysed in Chapter 2. Although these models are well reported, they are labour intensive and may not be an accurate model of endogenous human colonic disease (Kiesler et al. 2015; Mizoguchi 2012). The use of animal models to study IBD in this way have been criticised previously (Nicotra et al. 2013). We found that when comparing the secreted cytokine response of healthy human colonic tissue with that of IBD and appendicitis explants, all three tissue types were able to generate a response to experimental inflammation. This was the first report of such data and suggests that the use of TNFα and IFNγ on healthy explant colonic tissue may be an appropriate model for future study of acute colonic inflammation. This may allow a move away from the use of animals in the preclinical study of IBD. We found that, in human tissue, PEA and CBD were able to reduce inflammation both in experimentally inflamed normal tissue, and clinically inflamed tissue. In normal, uninflamed tissue, baseline levels of cytokines were also non-significantly reduced. These findings are supported by other reports in animal models of PEA and CBD, demonstrating that when given before the onset of colitis, both agents were able to cause falls in baseline levels of disease activity (Alhouayek et al. 2015; McKernan & Finn 2013; de Filippis et al. 2011). This is in line with studies on the brain which have shown that the prophylactic administration of CBD is able to reduce infarct size in experimental stroke (Sultan et al. 2017). The implication of these data on the potential use of PEA and CBD in IBD are unclear, further clinical study could examine their prophylactic and therapeutic effects in patients.

Following these findings, we confirmed in Chapter 4 that the effects of PEA and CBD on inflammation-induced changes in TEER (Alhamoruni et al. 2012; Karwad et al. 2016), which is a measure of ion flow, also translated into the transfer of macromolecules. If this had not been the case the hypothesis that PEA and CBD might treat IBD by maintaining the gut barrier, preventing translocation of noxious material into the submucosa space, would have been disproved. We found that PEA and CBD had non-additive effects on permeability when administered together. Although we found that PEA and CBD acted at different receptors, PPARα and CB_1 respectively, they were both then identified to act along similar intracellular pathways of adenylyl cyclase, MEK/ERK and PKA, likely explaining why there were no additive effects. This is in line with previous data which showed that, in murine colitis, PEA acts through phosphorylation of ERK (Esposito et al. 2014), and in neuronal tissue through PKA (Lin et al. 2015). Although the signalling pathway of CBD in the human colon has not previously been described, CBD has been shown to act at the $5\text{-}HT_{1A}$ receptor (Russo et al. 2005), activation of which affects the activity of adenylyl cyclase (Ghavami et al. 2004). This is the first report demonstrating the intracellular pathways through which CBD and PEA act in the human gut, and that the intracellular pathways are the same, despite different receptor targets.

The actual mechanism by which permeability may be affected may not be due to the prevention of downregulation of TRPV1, upregulation of AQP3, and upregulation in the production of claudin-5 in the case of CBD, or downregulation of claudin-3 in the case of PEA. The permeability experiments reported here are based on explant tissue and *in vitro* Caco-2 cultures, which are not in connection with visceral neurons. Previous work has demonstrated that activation of cannabinoid receptors in the brain are able to limit systemic inflammation (Lunn et al. 2006), and also decrease gastrointestinal inflammation (Massa et al. 2004). PEA prevents intestinal inflammation through action at the enteric nervous system (Esposito et al. 2014), and CBD was shown to prevent the amplification of the intestinal immune response by action at enteric glial cells (de Filippis et al. 2011). Furthermore SAB378, a peripherally restricted CB_1 and CB_2 agonist, is reported not to have any significant beneficial effects on intestinal inflammation (Cluny et al. 2010). Taken together this suggests that, although PEA and CBD are shown here to control the production of proteins and expression of mRNA which govern paracellular permeability the effects *in vivo,* in the presence of a functioning neuro-immune axis these effects may be different. Further study examining the relationship between the enteric nervous system and the paracellular and membrane-bound apparatus which control

permeability might allow a clearer description of how cannabinoid agents affect intestinal inflammation and permeability.

Lastly, as the permeability effects of PEA and CBD had not previously been examined in the human colon *in vivo,* the effect of these compounds on the permeability of the inflamed human colon were measured using a randomised, double blind, placebo-controlled trial. This study used the urinary concentration of ingested sugar probes as a marker of permeability in healthy participants talking high-dose aspirin. PEA and CBD both prevented increased gut permeability to these sugars, suggesting that they decreased the experimentally-induced hyperpermeable state. Although previous data suggested that CBD and PEA may limit hyperpermeability caused by inflammation both in animal and *in vitro* cell culture models (Cassol-Jr et al. 2010; Alhouayek et al. 2011; Esposito et al. 2014; Alhamoruni et al. 2012; Karwad et al. 2016), this is the first study to demonstrate that these two agents may limit increased gastrointestinal permeability in humans *in vivo.* There are no previous reports demonstrating the effect of PEA on permeability in the human colon *in vivo*, and the two previous clinical studies examining the effect of THC and CBD did not demonstrate a change in their primary endpoint and were limited by study design (Naftali et al. 2013; Naftali et al. 2017). These data therefore provide evidence that, in humans, PEA and CBD may be of clinical use in the future.

Further work arising from this thesis may address several deficits in current experimental knowledge. Firstly, as we have shown evidence that PEA and CBD are anti-inflammatory and decrease hyperpermeability experimentally, the clinical effect of CBD and PEA in appropriately sized, prospective clinical trials should now be investgated. Gastrointestinal permeability *per se* may not be useful clinical measure, as patients do not present with "hyper-permeability," but symptoms secondary to it. The clinical effect of PEA and CBD may be more appropriately measured with the Crohn's disease activity index, or one of the other validated questionnaires to assess disease currently in use such as the short-form 16 (SF-16). For example, endpoints such as serum CRP levels, frequency of diarrhoea, abdominal pain and the incidence of salvage surgery may be appropriate experimental and pragmatic endpoints. These studies could examine either the effect of CBD and PEA in acute flares of IBD, or their ability to maintain disease remission.

One factor to consider when assessing the use of cannabinoids in colitis is that many of these compounds have an anxiolytic, orexigenic and analgesic effect, such as in the case of THC, in addition to any anti-inflammatory action. Data from clinical

trials of CBD use in Dravet syndrome, have shown that CBD may actually decrease appetite, rather than stimulate it (Devinsky et al. 2018). The effect of PEA on appetite has also shown a negative association, which may potentially be caused by an increase in leptin signalling (Mattace Raso et al. 2014). This aside, as THC independently increases appetite and cognitive effect, other agents within *cannabis sativa* may independently bring about the same changes. If quality of life be found to be significantly improved with the use of either PEA, CBD or other cannabinoid drugs, careful study design must be able to differentiate between effects on inflammation and symptomatology. This is important because a previous study found that *Cannabis* use amongst IBD patients saw improvements in symptoms, but a five times greater risk of surgery over the 6 month study period (Storr et al. 2014). It is possible that, although a cannabinoid agent may improve the short-term quality of life of IBD patients and wean individuals from steroid dependence, we may increase their risk of surgery or in-patient immunosuppression.

Beyond PEA and CBD, the systematic review in Chapter 2 also highlighted the potential benefits of other cannabinoid compounds which may be clinically useful in the future, such as CBG, CBC and β-caryophyllene. Therefore, although the work in this thesis has examined the effects of CBD and PEA, other potential cannabinoid agents have not yet been examined which may also provide future clinical benefit. From the existing literature CBG, following PEA and CBD, is persistently effective in reducing inflammation in animal models, and has already been shown to be safe in human use colloquially through the ingestion of *cannabis sativa* and in experimental models (Brierley, et al. 2016). Experimentation in a similar fashion to the protocols used in this thesis could examine the effects of CBG (and other cannabinoids as resource allows) on Caco-2 and explant human colonic models of inflammation and permeability, with progression to phase 1 clinical trials. In addition, clinical trials might examine the use of cannabinoid agents either alone or in combination with other agents. Although we found no additive effect of CBD and PEA on permeability in Chapter 4, combination of other cannabinoid compounds or enzyme inhibitors may have potent effects on permeability, inflammation or symptomatology. Clinically it is common practice to combine several immunosuppressive agents to obtain remission, and the use of a cannabinoid drug, such as CBD, used in combination with contemporary immune modulators should also be investigated.

In addition to the design of phase 2a and 2b trials examining the effects of CBD and PEA in acute IBD, further work from this thesis could focus on other clinical areas. For example, PEA, CBD and other cannabinoid agents should also be examined for their effects in human rheumatoid arthritis, uveitis, dermatitis and systemic sepsis.

Although not focussed on in this thesis, there is already a body of existing work examining these effects in preclinical models, use of these otherwise well tolerated agents should also be investigated through relevant clinical trials. These trials could be accomplished by bringing expertise in these areas into the research group, with the design of appropriate clinical endpoints.

Secondly, PEA and CBD are currently only available as oral preparations, however the development of intravenous preparations are likely to be in progress in the pharmaceutical industry. Available of such preparations would allow quantification of their effects compared to conventional oral administration, giving insight into the anatomical site of their action, being either mucosal or via serum concentrations. Interestingly one pharmaceutical manufacturer is attempting to bring to market an oral CBD-based medicine which is inactive until reaching the terminal ileum, and hence may improve the effectiveness of CBD in the gut (Vitality Biopharma, 2018). Advances in the delivery systems of these drugs will allow design of trials to define the most effective mode of delivery in IBD.

The pharmacokinetics of CBD and PEA in humans are also poorly understood, with specific reference to the formation of further compounds after metabolism through cytochrome p450, COX-1/COX-2. We have suggested that it is the CBD and PEA themselves which are responsible for effects on inflammation and permeability, although this may not be the case. CBD and PEA may be acting as pro-drugs, with secondary metabolites responsible for the observed effects of decreased mucosal permeability and reduced inflammation. Although it has already been described that CBD and PEA are metabolised into smaller metabolites, the quality and time scale by which this occurs is not well understood. Further characterisation of the metabolites of CBD and PEA by way of time-dependent sequential sampling of serum, plasma and urine after oral administration using mass spectrometry, and then secondary *in vitro* and *ex vivo* testing of these metabolites on permeability and inflammation could demonstrate if this hypothesis is true or not. Should these metabolites be more clinically effective, it could lead to the development of more soluble drugs for experimental and clinical use.

In conclusion, this thesis demonstrates that CBD and PEA reduce inflammation in the human colon using an explant model, reduce permeability both *in vitro* and in *in vivo*. Their clinical use in IBD should now be assessed using appropriately-sized phase I and II clinical trials.

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8 Appendices

8.1 Appendix 1 – PRISMA statement

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	59
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	58
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	58
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	58
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	59
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	59
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	59
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	59
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	59
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	59
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	59-60
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	59-60
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	59-60
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	60

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	60
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	59
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	61
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	66-68
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	66-68
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	66-68
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	66-68
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	66-68
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	66-68
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	85
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	89
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	90
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	NA

8.2 Appendix 2 - NHS Ethics

8.2.1 NHS participant consent form

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Derby Teaching Hospitals NHS
NHS Foundation Trust

Centre Number:
Study Number:
Patient identification number for this trial:

CONSENT FORM

Title of Project: Genetic and Vascular Studies on Colorectal Patients
Chief Investigator : Mr. Jon Lund
Assistant Investigators: Dr. Saoirse O'Sullivan, Dr Daniel Couch, Dr. Cristina Tufarelli, Dr. Christodoulos Neophytou and Dr. Elena Theophilidou.

Please tick boxes

1	I confirm that I have read and understand the information sheet **version 9, dated 1** for the above study and have had the opportunity to ask questions.	☐
2	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected	☐
3	I understand that sections of any of my research records or medical notes where appropriate may be looked at by responsible individuals from the University of Nottingham or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.	☐
4	I agree to take part in the above study and approve the use of my tissue **and blood samples** in this study.	☐
5	I agree to my DNA, RNA **and proteins** being stored and I am happy for them to be in future research.	Yes* ☐ No* ☐

* Please delete

Name of patient	date	signature
Name of person taking consent	date	signature
Researcher	date	signature

1 copy for patient, 1 copy for researcher, 1 copy to be kept with hospital notes

05/05/2015
Version13

8.2.2 NHS participant information sheet

Derby Hospitals NHS
NHS Foundation Trust

Genetic and Vascular Studies of Colorectal Patients

Derby City General Hospital
Uttoxeter Road
Derby
DE22 3NE

Tel: 01332 340131
Minicom: 01332 254944
contactus@derbyhospitals.nhs.uk
www.derbyhospitals.nhs.uk

You are invited to take part in a research study. It is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with us if you wish. Please ask us if there is anything that is not clear or if you would like more information. Thank you for reading this.

What is the purpose of this study?

This study is in three parts. The first part involves looking at what changes in genes and protein expression occur in colorectal cancer within the primary tissues and in the blood. We hope to understand more about how these genes are controlled and how these control mechanisms are changed in the development of colorectal cancer. This would hopefully increase our knowledge of how and why these genes are involved in the development of colorectal cancer and may ultimately lead to targeted treatments and sensitive diagnostic tests. The second part of this study is to investigate a number of chemicals that might potentially cause blood vessels to relax, and so may help to control the circulatory system**. The third part is to examine how intestinal epithelial cells (cells that make up the inside lining of the bowel) react in the presence of inflammation and infection (for example appendicitis) and the chemicals produced.** Results collected from this study will contribute to dissertations for undergraduate and postgraduate degrees.

Why Have I Been Chosen?

You have been chosen because you are to undergo colorectal surgery.

Do I Have To Take Part?

It is up to you whether you wish to take part or not. If you do decide to take part you will be asked to sign a consent form and be given this information sheet. You may decide to withdraw from the study at any time and you do not have to give us any reason for this. A decision not to take part or to withdraw from the study will NOT affect the standard of care you receive. Should you withdraw from the study your tissue sample and any data collected will be destroyed.

What Will Happen If I Decide to Take Part?

Once you are admitted to hospital you will have a blood sample taken for this study before your operation and whilst you are still in hospital after your operation.

During your operation some of your bowel is removed (this is a standard part of the operation). We propose to take a very small part of this removed tissue for laboratory analysis. The tissues will be destroyed after laboratory analysis, your details will be coded and none of your details will be available in results or publications. A small amount of demographic data such as date of birth and sex will be included in the trial master file only.

What Do I Have To Do?

If you wish to take part then we need your permission to collect these samples of tissue for later analysis in the laboratory. You can do this verbally and by signing the consent form.

Are there any side effects in taking part and are what are the possible disadvantages and risks in taking part?

No. We are not proposing to give you any new medication or drugs. No additional tissue will be removed – only that which would be removed during your surgery anyway. There will be no direct benefit to you but the results may help future treatments.

Are there any ethical issues?

All Research in the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing

and dignity. This study has been reviewed and given a favourable opinion by Derbyshire Research Ethics Committee.

What if I have any complaints?

If for any reason you wish to make a complaint about this study, you can contact the Patient Advice & Liaison Services at any time on 01332 785557.

What will happen to the results of the study?

The results of this study will form part of undergraduate and postgraduate students' dissertations and will be published in National/International meetings and publications.

What if I have any questions?

We would be delighted to provide further information and answer any questions you might have. You may contact the research team.

Mr. Lund	01332 724704
Dr. Neophytou	01332 724681
Dr. Theophilidou	01332 724681
Mr Couch	01332 724681

You will be given a copy of this information sheet and your signed consent form for you to keep if you decide to take part in this study.

8.2.3 NHS ethics approval

Derby Hospitals NHS
NHS Foundation Trust

RESEARCH & DEVELOPMENT OFFICE

Royal Derby Hospital
Uttoxeter Road
Derby
DE22 3NE

Tel: 01332 340131
Minicom: 01332 785566
www.derbyhospitals.nhs.uk
Follow us on Twitter @DerbyHospitals

☎ **Please telephone on**: 01332 724639
Fax No: 01332 724714
E-mail: dhft.randdadmin@nhs.net

04 February 2016

Mr J Lund
Division of GI Surgery
Royal Derby Hospital
Uttoxeter Road, DE22 3NE

Dear Mr Lund

Re: Genetic and Vascular studies of human colorectal tissue
Ref: DHRD/2008/060

I acknowledge the receipt of your amendment dated 21.06.2015 enclosing the following revised documentation for the above study

- Ethics Approval letter dated 11.01.2016 for Amendment #6 dated 21.06.2015
- Protocol V1.6 dated 08.06.2015
- ICF V14 dated 08.06.2015
- PIS V9 dated 08.06.2015
- Notice of substantial amendment dated 21.06.2015

I have reviewed the revised documents and can confirm that there are no Trust issues. You may therefore use the above documents with Trust approval.

Please do not hesitate to contact any member of the Research and Development staff if you feel we can be of assistance.

Yours sincerely

Josie Towle

Josie Towle
Research Management and Governance Facilitator
Research & Development

8.3 Appendix 3 – University of Nottingham permeability study ethics

8.3.1 Participant consent form

CONSENT FORM

Version 1.0: 07/12/2016

Title of Study: **The Effect of PEA, CBD and Aspirin on the Absorption and Secretion Of Ingested Sugars**

REC ref: J16122016

Name of Researchers: Dr Daniel Couch Clinical Research Fellow/PhD Student, Dr Saoirse O'Sullivan, Associate Professor/Chief Investigator and Supervisor, Mr Jon Lund, Associate Professor/Supervisor, Division of Medical Sciences & Graduate Entry Medicine (MSGEM)

Name of Participant: **Please initial**

1. I confirm that I have read and understand the information sheet version number 1 dated 07/12/2016 for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my legal rights being affected. I understand that should I withdraw then the information collected so far cannot be erased and that this information may still be used in the project analysis. ☐

3. I understand that relevant sections of data collected in the study may be looked at by authorise individuals from the University of Nottingham, the research group and regulatory authorities where it is relevant to my taking part in this study. I give permission for these individuals to have access to these records and to collect, store, analyse and publish information obtained from my participation in this study. I understand that my personal details will be kept confidential. ☐

4. I understand and agree that blood and urine samples will be taken for analysis of content of several sugars including lactulose and mannitol. ☐

5. I understand the blood and urine samples are not stored for more than 7 days before being rendered acellular or destroyed (this is a requirement of the Human Tissues Act). ☐

6. **Optional:** I agree to the storage of my urine and blood samples for any future, unspecified research relating to inflammation and permeability of the gut. These samples will be stored for 2 years and then destroyed. Such stored samples will fall under the Human Tissue Act, and sample records will be stored under the HTA Research Licence and will be maintained for 25 years. ☐

☐

7. I understand that some of these studies may be carried out by researchers other than current team of Dr Couch, Dr O'Sullivan and Mr Lund, who ran the first study, including researchers working for commercial companies. Any samples or data used will be anonymised, and I will not be identified in anyway.

8. I understand that the tests or procedures associated with my participation in this study are carried out for research only, and not for clinical diagnostic purposes. However, if the study investigator should feel it necessary to inform my GP of my participation in the study, an adverse event or abnormal test result, I understand I am giving my consent to do so. ☐

9. I have not been a subject in any other research study in the last three months which involved: taking a drug; being paid a disturbance allowance; having an invasive procedure (eg blood sample >50ml) or exposure to ionising radiation. ☐

10. I voluntarily agree to take part in the above study.

Name of Participant	Date	Signature
Name of Person taking consent (if different from Principal Investigator)	Date	Signature
Name of Principal Investigator	Date	Signature

8.3.2 Participant information sheet

Participant Information Sheet

Draft version 1.0:07/12/2016

The Effect of Palmitoylethanolamide or Cannabidiol and Aspirin on The Absorption and Secretion Of Ingested Sugars

Study ID – SweetPEA

Name of Researcher(s): Dr Daniel Couch

Dr Saoirse O'Sullivan

Mr Jon Lund

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. Talk to others about the study if you wish. Ask us if there is anything that is not clear.

What is the purpose of the study?

Death from infection accounts for 20,000 deaths in the UK alone per year. Part of the problem with infection is an increase in the leakiness of the gut. This allows transfer of dangerous compounds from the gut into the bloodstream, which worsens the severity of the infection in the body. Palmitoylethanolamide (PEA) is produced normally in the body, and also used as a food supplement. Cannabidiol (CBD) is a drug derived from the cannabis plant, which has already been used to treat epilepsy in children. When given under laboratory conditions to samples of inflamed intestine and test tube-grown cells, both PEA and CBD prevent this increase in leakiness. In this study we aim to find out if PEA or CBD will prevent the leakiness increases in the gut in humans.

Why have I been invited?

You are being invited to take part because you have/are responded to a poster calling for healthy volunteers to enter our study. We are inviting 30 participants like you to take part.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

What will happen to me if I take part?

This study will require you to attend the University of Nottingham Medical School Derby Campus on one occasion for 6 hours. Prior to your attendance we will ask you to attend the university to take your consent to take part in the study, and also attend a further time go through a questionnaire. We will ask you not to take any ibuprofen or similar medications, or alcohol for the week prior to the test. We would also request that you do not exercise the day before the test.

We will ask you to fast from midnight the night before the test (do not eat or drink anything overnight or in the morning), and attend the Medical School at 0800 on the day of the test. At this time we will ask you to take a tablet, along with a dose of aspirin and water. We are asking you to take aspirin because it increases the permeability of the gut. One hour later we will give you a sugar drink (600mls) and ask you to produce a urine sample. We will ask you to produce several further urine samples through the following 6 hours and take 4 blood tests during the test until 1400. You will also get another drink of water mid-morning.

We will not require access to any medical records for this study.

Expenses and inconvenience allowance

Participants will be given a £10 Amazon gift voucher to participate in the study if requested.

What are the possible disadvantages and risks of taking part?

PEA has been used in several clinical trials, including treatments for glaucoma and kidney damage, and has not demonstrated any side effects or adverse events. CBD has been used to treat epilepsy, and can occasionally cause nausea. As part of the study we will be asking you to take a dose of aspirin (600mg). This is a widely used drug and is taken on a daily basis by hundreds of thousands of people in the UK. It has a low incidence of side effects, though prolonged use may result in stomach ulcer formation and bleeding problems.

Part of the sugar drink we will ask you to drink will contain lactulose, which can be used clinically as a mild laxative. It is unlikely that with the small dose we administer (which is smaller than the clinical dose) you will experience any symptoms, but it is possible that you may experience loose stools the next day.

During the test days we will be taking several blood tests from the arm, in a method similar to blood tests that you may have had at the GP in the past. There is a risk that you may experience some minor bruising from this, as if you had a blood test from you GP.

In this study we are including healthy adult volunteers. We will exclude anyone presenting with any of the following:

- Any disorder of the stomach, small or large intestine (as assessed by a questionnaire).
- Regular heavy alcohol use (more than 14 units per week).
- Recent use (within 7 days) of ibuprofen or similar drugs or alcohol.
- Use of vitamin supplementation or probiotics within the last 4 weeks.
- If you have been a subject in any other research study in the last three months which involved: taking a drug; being paid a disturbance allowance; having an invasive procedure (e.g. blood sample >50ml) or exposure to ionising radiation.

What are the possible benefits of taking part?

The information we get from your participation in this study may help develop a novel way to prevent the gut leakiness seen in illness. This would help reduce the death and illness rate from severe infection.

Will my taking part in the study be kept confidential?

Your information will be maintained in accordance with the Data Protection Act 1998.

We will follow ethical and legal practice and all information about you will be handled in confidence. If you join the study, some parts of the data collected will be looked at by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by authorised people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty. All information which is collected about you during the course of the research will be kept **strictly confidential**, stored in a secure and locked office, and on a password protected database. Any information about you which leaves the institution will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it.

Your personal data (address, telephone number) will be kept for 24 months after the end of the study so that we are able to contact you about the findings of the study *and possible follow-up studies* (unless you advise us that you do not wish to

be contacted). All other data (research data) will be kept securely for 7 years. After this time your data will be disposed of securely. During this time all precautions will be taken by all those involved to maintain your confidentiality, only members of the research team will have access to your personal data.

What will happen if I don't want to carry on with the study?

Your participation is voluntary and you are free to withdraw at any time, without giving any reason.

Involvement of the General Practitioner/Family doctor (GP)

This study does not require us to routinely contact your GP. However, should you demonstrate symptoms of previously undiagnosed conditions then we may advise that you attend your GP with this information. Should you fall ill in the month following your participation in this trial you should inform your GP that you have taken part in this study.

What will happen to any samples I give?

Urine and blood samples obtained from this study will be processed and then analysed for sugar content. Following this process samples will be stored in the Medical School securely for up to 36 months (should the tests need to be repeated), at which point they will be destroyed. Samples may be re-analysed if required by the investigators.

We would also like to seek your consent so that any remaining samples may be stored and used in possible future research – this is optional (please indicate you agree to this on the consent form). The samples will be stored with a code unique to you and securely at the University of Nottingham under the University's Human Tissue Research Licence (no 12265).

Some of these future studies may be carried out by researchers other than current team of Dr Couch, Dr O'Sullivan and Mr Lund, who ran the first study, including researchers working for commercial companies. Any samples or data used will be anonymised, and you will not be identified in anyway. If you do not agree to this any remaining samples will be disposed of in accordance with the Human Tissue Authorities codes of practice.

Will any genetic tests be done?

No genetic studies will be performed in this research.

What will happen to the results of the research study?

The results of this study will be collated and may be published in a scientific journal in an anonymised fashion. No personal data will be published in this way. If so the results will be likely to be published within 24 months of the study. If requested we can contact you with a copy of these published results. Furthermore results from this study may form part of an educational qualification for one of the investigators.

Who is organising and funding the research?

This research is being organised and funded by the University of Nottingham.

Who has reviewed the study?

All research in the University of Nottingham is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by the Faculty of Medicine & Health Sciences (FMHS) Research Ethics Committee (FMHS REC ref no: J16122016).

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. The researchers contact details are given at the end of this information sheet. If you remain unhappy and wish to complain formally, you should then contact the If you remain unhappy and wish to complain formally, you should then contact the FMHS Research Ethics Committee Administrator, c/o The University of Nottingham, Faculty PVC Office, B Floor, Medical School, Queen's Medical Centre Campus, Nottingham University Hospitals, Nottingham, NG7 2UH. E-mail: FMHS-ResearchEthics@nottingham.ac.uk

Further information and contact details

We would be delighted to provide further information and answer any questions you might have. You may contact the research team.

Dr O'Sullivan	01332 724643
Mr Lund	01332 724704
Dr Couch	01332 724681

8.3.3 University of Nottingham Ethical approval

The University of Nottingham

E-mail: FMHS-ResearchEthics@nottingham.ac.uk

Faculty of Medicine and Health Sciences

Research Ethics Committee
C/o Faculty PVC Office
School of Medicine Education Centre
B Floor, Medical School
Queen's Medical Centre Campus
Nottingham University Hospitals
Nottingham
NG7 2UH

30th March 2017

Dr Daniel Couch
PhD Student
c/o Dr Saoirse O'Sullivan
Associate Professor
Room 4107, Division of Medical Sciences & Graduate Entry Medicine
School of Medicine
University of Nottingham Royal Derby Hospital Centre
Uttoxeter Road
DE22 3DT

Dear Dr Couch

Ethics Reference No: J16122016 **– please always quote**	
Study Title: The Effect of Palmitoylethanolamide (PEA) or Cannabidiol (CBD) and Aspirin on the Absorption and Secretion Of Ingested Sugars. **Short Title:** SweetPEA	
Chief Investigator/Supervisor: Dr Saoirse O'Sullivan, Associate Professor, Division of Medical Sciences & Graduate Entry Medicine (MSGEM)	
Lead Investigators/student: Dr David Couch Clinical Research Fellow/PhD Student, MSGEM	
Other Key Investigators: Mr Jon Lund, Associate Professor, MSGEM	
Type of Study: PhD, physiology, Aspirin, PEA, CBD	
Proposed Start Date: 02/2017	**Proposed End Date:** 31 May 2017 2 mths
No of Subjects: 30	**Age:** 18+years
School: Medicine	

Thank you for submitting the above application which was considered by the Committee at its meeting on 16th December 2016 and the following documents were received:

Short Title: SweetPEA

- FMHS Research Ethics Committee Application form and supporting documents version 1.0, 07/12/2016.

These have been reviewed and are satisfactory and the study is approved.

Approval is given on the understanding that the conditions set out below are followed:

1. You must follow the protocol agreed and inform the Committee of any changes using a notification of amendment form (please request a form).
2. You must notify the Chair of any serious or unexpected event.
3. This study is approved for the period of active recruitment requested. The Committee also provides a further 5 year approval for any necessary work to be performed on the study which may arise in the process of publication and peer review.
4. An End of Project Progress Report is completed and returned when the study has finished (Please request a form).

Yours sincerely

pp

Professor Ravi Mahajan
Chair, Faculty of Medicine & Health Sciences Research Ethics Committee