

**THE EFFECT OF DEFINED FORMULAE LIQUID DIETS ON
INFLAMMATORY BOWEL DISEASE AFFECTED TISSUE**

Doris HT Meister

Thesis submitted for the degree of PhD

University of Edinburgh

May 2002



Declaration

I declare that this thesis has been composed by me and the work performed is original. The work contained within was performed by me. The entire thesis was completed at the Gastrointestinal Laboratory, Western General Hospital University of Edinburgh. The thesis, or part of it, has not been submitted for any other degree or professional qualification.

Doris HT Meister

Acknowledgements

I am most grateful and indebted to my supervisor Dr Subrata Ghosh. He encouraged and supported me doing this thesis. He took up his current post as Professor of Gastroenterology at the Hammersmith Hospital, London in February 2002. The work contained in the thesis was initiated under the late Professor Anne Ferguson's guidance whose untimely death in 1998 was a major blow to the gastrointestinal laboratory. I also thank Dr David Wilson my co-supervisor in reviewing and commenting on the manuscript.

I would like to thank John Bode for cutting the paraffin tissue sections, creating and helping me with the computer program for TGF- β 1 detection and teaching me the laboratory skills of immunohistochemistry, tissue processing and embedding of the tissue. I thank Dr Gordon Brydon for reviewing the manuscript and Norman Anderson for technical advice. I am grateful to Dr Sami Hoque, as a friend and colleague during my time in the gastrointestinal laboratory and for his helpful advice. I acknowledge Dr Marian Aldhous for showing me how to perform ELISAs.

Lastly, I thank my parents for their constant encouragement and help during my 4 years away from Germany.

Publications based on this thesis

D. Meister, A. Shand, J. Bode, S. Ghosh

Anti-inflammatory effects of enteral diet components on Crohn's disease affected tissues *in vitro*

Digestive and Liver Disease, 2002 ; 34: 430-438

M.C. Aldhous, **D.Meister**, S.Ghosh

Modification of enteral diets in inflammatory bowel disease

Proceedings of the Nutrition Society 2001; 60: 457-461.

D.Meister, J.Bode, S.Ghosh

TGF- β 1 expression is upregulated in Crohn's disease (CD) but not in ulcerative colitis (UC) mucosa after incubation with elemental diet-whey enriched with TGF- β (EWT) and elemental diet-colostrum (ECO)

British Society of Gastroenterology Annual Meeting, Birmingham 2002.

Gut 2002; 50 (suppl.2): A81

D.Meister, MC.Aldhous, J.Bode, A.Shand, W.Johnson, S.Giffen, S.Ghosh.

Anti-inflammatory effect of elemental diet *in vitro* depends on fatty acid composition.

British Society of Gastroenterology Annual Meeting, Glasgow March 2001.

(Oral Presentation)

Gut 2001; 48 (suppl.1): A2

D.Meister, MC. Aldhous, J.Bode, A.Shand, W.Johnson, S.Giffen, S.Ghosh

Fish oil substituted elemental diet in ulcerative colitis

British Society of Gastroenterology Annual Meeting, Glasgow March 2001

Gut 2001; 48 (suppl.1): A73

D.Meister, MC Aldhous, A.Shand, J.Bode, W.Johnson, S.Giffen, N.Anderson, S.Ghosh.

Anti-inflammatory effect of elemental diet on Crohn's disease affected tissue *in vitro*.
Annual BAPEN Meeting (British Association for parenteral and enteral nutrition),
Harrogate 2000
Proceedings of the Nutrition Society; 2001; 60: 17A

A.Shand, **D.Meister**, MC.Aldhous, N.Anderson, S.Ghosh
Direct anti-inflammatory effect of elemental diet on Crohn's tissue *in vitro*
British Society of Gastroenterology Annual Meeting, Birmingham 2000.
(Oral Presentation)
Gut 2000; 46 (suppl.2): A43

MC. Aldhous, **D.Meister**, A.Shand, J.Bode, N.Anderson, W.Johnson. S.Ghosh
Modifications of enteral diet in inflammatory bowel disease
Annual Congress of the British Society of Immunology, Harrogate 2000.
(Oral Presentation)
Annual Congress of the British Society of Immunology, 2000; 101 (suppl.1): IS45

Abbreviations

5-ASA	5-amino salicylic acid compounds
5-HETE	5-hydroxy-6,8,11,14-eicosatetraenoic acid
5-HPETE	5-hydroperoxy-6,8,11,14-eicosatetraenoic acid
AA	Arachidonic acid
AI	Adequate Intake
ATP	Adenosine-Triphosphate
cAMP	cyclic Adenosine Monophosphate
CD	Crohn`s disease
CDAI	Crohn`s Disease Activity Index
cGMP	cyclic Guanosine Monophosphate
COC	Enteral diet EO28 modified with coconut oil
COCF	Enteral diet EO28 modified with fractionated coconut oil
Con A	Concanavalin A
CRP	C-reactive protein
d	day
DAG	Diacylglycerol
DGLA	Di-homo- γ -linolenic acid
DHA	Docosahexaenoic acid
DP	Prostaglandin-receptor D
DPA	Docosapentaenoic acid
EAA	Elemental diet EO28
EC	Enteral diet EO28 modified with casein protein
ECA	Enteral diet EO28 modified with canola oil
ECM	Extracellular Matrix
ECO	Enteral diet EO28 modified with colostrum
ED	Enteral diet
EFI	Enteral diet EO28 modified with fish oil
EGF	Epidermal growth factor
ELISA	Enzyme Linked Immuno-Sorbent Assay

EOL	Enteral diet EO28 modified with olive oil
EP1, 2, 3, 4	Prostaglandin-receptor E1, 2, 3, 4
EPA	Eicosapentaenoic acid
ER	endoplasmic reticulum
ESA	Enteral diet EO28 modified with safflower oil
ESR	Erythrocyte sedimentation rate
ESU	Enteral diet EO28 modified with sunflower oil
ESY	Enteral diet EO28 modified with soya oil
EW	Enteral diet EO28 modified with whey protein
EWT	Enteral diet EO28 modified with whey protein and TGF- β
F	Female
FA	Fatty acid
FABP	Fatty Acid Binding Protein
FO	Fish oil diet
FP	Prostaglandin-receptor F
GI	Gastrointestinal
Gi	inhibitory G protein
gm	gram
Gs	secretory G protein
IBD	Inflammatory bowel disease
IEL	Intraepithelial Lymphocyte
I κ B	inhibitor protein kappa B
IL1 β	Interleukin 1 beta
IL1ra	Interleukin 1 receptor antagonist
IL2	Interleukin 2
IL4	Interleukin 4
IL5	Interleukin 5
IL6	Interleukin 6
IL8	Interleukin 8
IL10	Interleukin 10
IL12	Interleukin 12
InsP ₃	Inositol-tri-phosphate

IFN- γ	Interferon gamma
IP	Prostacyclin-receptor I
iv	intravenous
kcal	Kilocalorie
LA	Linoleic acid
LCPUFA	Long chain polyunsaturated fatty acids
LNA	α -Linolenic acid
LPMC	Lamina propria mononuclear cells
LPS	Lipopolysaccharide
LTA ₄	Leukotriene A ₄
LTB ₄	Leukotriene B ₄
LTB ₅	Leukotriene B ₅
LTC ₄	Leukotriene C ₄
LTE ₄	Leukotriene E ₄
M	Male
MHC	Major-histocompatibility complex
mRNA _{AOX}	messenger Ribonucleic Acid Acyl Co-Oxidase
MUFA	Monounsaturated fatty acid
nd	not detected
NF-AT	Nuclear Factor activated T cells
NF- κ B	Nuclear factor kappa B
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drugs
OO	olive oil diet
P/S	Polyunsaturated to saturated fatty acid Quotient
PAF	Platelet derived stimulatory factor
PBMC	Peripheral blood mononuclear cells
PEG	Percutaneous Endoscopic Gastrostomy
pg	picogram
PGD ₂	Prostaglandin D ₂
PGE ₂	Prostaglandin E ₂
PGF _{2α}	Prostaglandin F _{2α}

PGG ₂	Prostaglandin G ₂
PGH ₂	Prostaglandin H ₂
PGI ₂	Prostacyclin I ₂
PHA	Phytohaemagglutinin
PKC	Proteinkinase C
PL	Phospholipid rich diet
PLA ₂	Phospholipase A ₂
PLC	Phospholipase C
PLD	Phospholipase D
PPAR	Peroxisome Proliferator Activator Receptor
PUFA	Polyunsaturated fatty acid
RDI	Dietary Reference Intake
SAS	Sulfasalazine
SCFA	Short-chain-fatty acids
SEM	Standard error of the mean
TcR	T cell Receptor
TGF-β	Transforming growth factor beta
Th1	T helper cell subset 1
Th2	T helper cell subset 2
TNF-α	Tissue necrosis factor alpha
TNF-β	Tissue necrosis factor beta
TP	Thromboxane-receptor
Tris	tris (hydroxymethyl) amino methane
Tween 20	polyoxyethylene-sorbitan monolaurate
TXA ₂	Thromboxane A ₂
UC	Ulcerative Colitis
μg	microgram

Table of Contents

<i>THE EFFECT OF DEFINED FORMULAE LIQUID DIETS ON INFLAMMATORY BOWEL DISEASE AFFECTED TISSUE.....</i>	<i>1</i>
<i>Declaration</i>	<i>2</i>
<i>Acknowledgements.....</i>	<i>3</i>
<i>Publications based on this thesis</i>	<i>4</i>
<i>Abbreviations.....</i>	<i>6</i>
<i>Table of Contents</i>	<i>10</i>
<i>List of Figures</i>	<i>19</i>
<i>List of Tables</i>	<i>23</i>
<i>Abstract.....</i>	<i>25</i>
<i>CHAPTER ONE.....</i>	<i>27</i>
<i>1. Background and Aims.....</i>	<i>27</i>
<i>CHAPTER TWO</i>	<i>29</i>
<i>2. Literature review</i>	<i>29</i>
<i>2.1. Inflammatory bowel disease.....</i>	<i>29</i>
2.1.1. Nutritional morbidity in inflammatory bowel disease	35
2.1.2. Nutritional treatment in inflammatory bowel disease.....	37
2.1.2.1. Total parenteral nutrition	40
2.1.2.2. Polymeric and Oligopeptide diet	40
2.1.2.3. Elemental diet	42
2.1.2.4. Polyunsaturated fatty acids in enteral diets and as dietary supplementation.....	43
2.1.2.5. Proteins and amino acids in enteral diets	44
<i>2.2. The mucosal immune system of the intestine</i>	<i>47</i>
2.2.1. Lymphocytes	48

2.2.1.1. Cytokines	51
2.2.2. The cytokine pattern in inflammatory bowel disease	57
2.3. Involvement of lipids in inflammation and immunoregulation	58
2.3.1. Eicosanoids and Fatty acids	62
2.3.2. Intracellular Signalling	67
2.3.3. Lipid derived mediators in inflammatory bowel disease	69
2.3.4. Immunomodulatory effects of fatty acids	70
2.3.5. Clinical Implications of PUFA	74
2.3.6. Occurrence of PUFA in the Diet	75
 CHAPTER THREE	 78
3. Medical management of inflammatory bowel disease	78
3.1. Course of disease	78
3.2. Goals of therapy	80
3.3. The strategies	80
3.4. The armamentarium	80
3.4.1. 5-Aminosalicylates	81
3.4.2. Corticosteroid therapy	82
3.4.3. Immunosuppressive therapy	84
3.4.3.1. Azathioprine	84
3.4.3.2. Methotrexate	87
3.4.3.3. Cyclosporine	88
3.4.3.4. Mycophenolate mofetil	89
3.4.3.5. Tacrolimus	90
3.5. Biological Therapy	91
3.5.1. TNF α inhibitors	91
3.5.1.1. Alpha-4 integrin antibody	94
3.5.1.2. Other biologicals	94
3.5.1.3. Combination immunomodulatory therapy	94
3.5.1.4. Probiotic therapies	95
3.5.2. Antibiotic therapies	96
3.5.3. Rectal Administration	96
3.6. Other novel therapies	98
3.6.1. Heparin	98

3.6.2. Nicotine	98
3.6.3. Thalidomide.....	99
3.6.4. Fish oil.....	99
3.6.5. Growth hormone.....	100
3.6.6. Leukotriene inhibitors	100
3.6.7. Thromboxane synthetase inhibitor	101
3.6.8. Chloroquine	101
3.7. Quality of life and pharmacoeconomics.....	101
CHAPTER FOUR.....	104
SECTION I.....	104
4. Study Plan: Experimental design	104
4.1. Enteral diets modified in protein composition	108
4.1.1. Enteral diets modified for casein and whey.....	108
4.1.2. Enteral diet modified for bovine colostrum.....	109
4.1.3. Enteral diet modified for TGF- β enriched whey extract	109
4.2. Enteral diet modified in oil / fatty acid composition.....	110
4.3. Organ culture model.....	116
4.3.1. Technique of Organ culture for enteral diet incubation.....	117
4.4. Histological Method.....	120
4.4.1. Bromodeoxyuridine	120
4.4.1.1. BrdU staining method.....	121
4.4.1.2. Examination of BrdU stained tissue	122
4.4.2. TGF- β staining method	125
4.4.2.1. Examination of TGF- β 1 stained tissue	126
4.5. Cytokine Assays	127
4.5.1. IL1 β Assay	130
4.5.2. IL1ra Assay	130
4.5.3. IL10 Assay	131
4.5.4. TGF- β 1 Assay	132
4.5.5. Interference of enteral diet with ELISA assay.....	133
4.5.5.1. Recovery of cytokines after incubation in enteral diet formulae	134
4.6. Statistics	137

SECTION II	138
Study Plan: Experiments and Subjects	138
4.7. Enteral diet protein modifications	139
4.7.1. Biopsies for protein incubation.....	140
4.8. Enteral diet fatty acid modifications	140
4.8.1. Biopsies for fatty acid incubation.....	142
RESULTS	146
SECTION I	146
CHAPTER FIVE	147
5. Effects after incubation of inflamed and non-inflamed tissues with elemental diet (EAA), enteral diet- casein (EC) and enteral diet-whey (EW).	147
5.1. IL1ra / IL1β ratios after incubation with amino-acid based elemental diet (EAA) enteral diet containing casein (EC) and enteral diet containing whey (EW)	150
5.1.1. Crohn's disease.....	150
5.1.2. Ulcerative colitis.....	151
5.1.3. Control patients	153
5.2. IL10 / IL1β ratio after incubation with EAA, EC and EW	156
5.2.1. Crohn's disease.....	156
5.2.2. Ulcerative colitis.....	156
5.2.3. Control patients	156
5.3. Discussion	157
CHAPTER SIX	161
6. Effect of growth factors: TGF-β1 expression after incubation with enteral diet- colostrum (ECO), enteral diet-whey extract enriched with TGF-β (EWT) and amino-acid based enteral diet (EAA)	161
6.1. Expression of TGF-β1 immunostaining after incubation with different enteral formulae	164

6.1.1. Crohn's disease.....	164
6.1.2. Ulcerative colitis.....	166
6.1.3. Control patients	167
6.2. Discussion: TGF-β1 expression after incubation	172
 CHAPTER SEVEN.....	 174
7. TGF-β1 immunostaining expression after incubation with enteral diet - casein (EC) and enteral diet- whey (EW)	174
7.1. Percentage of TGF- β 1 staining expression	176
7.1.1. Crohn's disease.....	176
7.1.2. Ulcerative colitis.....	177
7.1.3. Control patients	178
7.2. Discussion: TGF-β1 staining after EC and EW incubation	178
 CHAPTER EIGHT	 181
8. Summary: TGF-β1 expression in IBD affected tissues after incubation with enteral diets	181
 CHAPTER NINE.....	 186
9. Cytokine response after incubation with enteral diet - colostrum (ECO) and enteral diet diet-whey enriched with TGF-β (EWT) in CD, UC and control patients tissues and correlation with TGF-β1 immunostaining.....	186
9.1. IL1ra / IL1 β ratios after incubation with ECO and EWT	187
9.1.1. Crohn's disease.....	187
9.1.2. Ulcerative colitis.....	188
9.1.3. Control patients	189
9.2. Interleukin 10 concentration in supernatant after incubation with ECO and EWT and TGF-β1 concentration as % of medium control.....	190
9.2.1. Crohn's disease.....	191
9.2.2. Ulcerative colitis.....	191
9.2.3. Control patients	191

9.2.4. Crohn`s disease.....	193
9.2.5. Ulcerative colitis.....	193
9.2.6. Control patients	193
9.3. Discussion of cytokine supernatant response assayed in the supernatant after incubation with ECO and EWT	195
9.4. Correlation between TGF-β1 expression and cytokine concentration after incubation with ECO and EWT	198
9.4.1. Correlation between TGF- β 1 expression by immunostaining and IL1ra / IL1 β ratio	198
9.4.1.1. Crohn`s disease.....	198
9.4.1.2. Ulcerative colitis.....	199
9.4.1.3. Control patients.....	200
9.4.2. Correlation between TGF- β 1 expression by immunostaining and percentage IL10 of medium control.....	200
9.4.3. Discussion	201
<i>RESULTS</i>.....	202
<i>Section II</i>.....	202
<i>CHAPTER TEN</i>.....	203
<i>10. Effects after incubation of inflamed and non-inflamed tissues with enteral diet-fish oil (EFI).....</i>	203
10.1. IL1ra / IL1β ratio after EFI incubation.....	205
10.1.1. Ulcerative colitis.....	205
10.1.2. Crohn`s disease.....	205
10.1.3. Control patients	206
10.2. Changes in individual cytokine production after incubation with enteral diet-fish oil (EFI) as a percentage of incubation with medium control.....	207
10.2.1. IL1 β	207
10.2.2. IL1ra.....	208
10.3. Discussion.....	210
<i>CHAPTER ELEVEN</i>.....	213
<i>11. Effects after incubation of inflamed and non-inflamed tissues with enteral diet</i>	

<i>sunflower oil (ESU) and enteral diet safflower oil (ESA)</i>	213
11.1. IL1ra / IL1β ratios after incubation with ESU and ESA	215
11.1.1. Crohn's disease.....	215
11.1.2. Ulcerative colitis.....	216
11.1.3. Control patients	217
11.2. IL10/IL1β ratios after ESU and ESA incubation	218
11.2.1. Crohn's disease.....	218
11.2.2. Ulcerative colitis.....	218
11.2.3. Control patients	219
11.3. Changes in IL1β after incubation of CD, UC and Control patients tissue with ESU and ESA, as percentage of medium control	220
11.3.1. Crohn's disease.....	220
11.3.2. Ulcerative colitis.....	220
11.3.3. Control patients	220
11.4. Changes in IL1ra concentration of CD, UC and Control patients tissues as percentage of medium control after incubation with ESU and ESA	222
11.4.1. Crohn's disease.....	222
11.4.2. Ulcerative colitis.....	223
11.4.3. Control patients	223
11.5. Discussion	225
 CHAPTER TWELVE	 227
12. Effects after incubation of inflamed and non-inflamed tissue with enteral diet olive oil (EOL) and enteral diet canola oil (ECA)	227
12.1. IL1ra / IL1β ratios after incubation with EOL and ECA	229
12.1.1. Crohn's disease.....	229
12.1.2. Ulcerative colitis.....	229
12.1.3. Control patients	230
12.2. Changes in IL1β after incubation of inflamed and non-inflamed tissues in EOL and ECA as a percentage of medium control	231
12.3. Changes in IL1ra concentration in the supernatant after incubation in EOL and ECA, as a percentage of medium control	233

12.4. Discussion.....	235
CHAPTER THIRTEEN.....	237
<i>13. Effects of incubating inflamed and non-inflamed tissues with enteral diet fractionated coconut oil (COCF) and enteral diet coconut oil (COC).....</i>	<i>237</i>
13.1. IL1ra / IL1β ratios after incubation with COCF and COC.....	239
13.1.1. Crohn's disease.....	239
13.1.2. Ulcerative colitis.....	239
13.1.3. Control patients	240
13.2. Discussion.....	241
CHAPTER FOURTEEN.....	243
<i>14. Effects after incubation of inflamed and non-inflamed tissues with enteral diet soya oil (ESY).....</i>	<i>243</i>
14.1. IL1ra / IL1β ratios after incubation with ESY.....	244
14.1.1. Crohn's disease.....	244
14.1.2. Ulcerative colitis.....	245
14.1.3. Control patients	245
14.2. Discussion.....	247
CHAPTER FIFTEEN.....	249
<i>Summary and Discussion</i>	<i>249</i>
<i>15. Principal observations.....</i>	<i>249</i>
15.1. Intestinal biopsy cytokine responses after incubation with elemental diet and enteral diets modified in their amino acid composition.....	249
15.2. Cytokine response after incubation with enteral diet modified in fatty acid composition.....	251
15.3. TGF-β1 tissue expression by immunostaining after incubation with enteral diet modifications	255
15.4. Summary.....	256

15.4.1. Incubation with enteral diets modified in protein composition – cytokine ratios	256
15.4.2. Incubation with enteral diets modified in protein composition – TGF- β 1 expression	257
15.4.3. Incubation with enteral diets modified in fatty acid composition- cytokine ratios	258
15.5. Comparison of our findings with other published data.....	260
15.5.1. Strengths of the study	271
15.5.2. Weaknesses of the study.....	273
15.5.3. Proposals for further work.....	275
<i>Appendix</i>	<i>277</i>
<i>Bibliography</i>	<i>280</i>
<i>Published papers</i>	<i>310</i>

List of Figures

Figure 1	Proposed factors involved in IBD.....	30
Figure 2	(left) Endoscopic image of CD.....	32
Figure 3	(right) Endoscopic image of UC.....	32
Figure 4	Downregulation of lymphocyte activation in the intestine.....	51
Figure 5	Cytokines in Th1 response, as in Crohn`s disease.....	56
Figure 6	Cytokine-balance in IBD.....	58
Figure 7	Structure of Arachidonic acid.....	59
Figure 8	Polyunsaturated Fatty acid metabolism.....	61
Figure 9	Prostaglandins of COX pathway with specific receptors and signal route.....	63
Figure 10	Cyclooxygenase pathway.....	64
Figure 11	Lipoxygenase pathway.....	65
Figure 12	Eicosanoid release from COX and LOX pathway.....	67
Figure 13	(A) Signalling via G protein coupled receptor and (B) enzyme linked receptors.....	68
Figure 14	Mediators of inflammatory response.....	70
Figure 15	Study design with modified diets.....	105
Figure 16	EAA - fatty acid profile.....	107
Figure 17	Protein experiment design – different enteral diet compositions used.....	110
Figure 18	Fatty acid experimental design – the different compositions of enteral diets modified in fatty acid compositions.....	111
Figure 19	(left): COC - fatty acid profile.....	113
Figure 20	(right): COCF - fatty acid profile.....	113
Figure 21	(left): EOL - fatty acid profile.....	113
Figure 22	(right): ESA - fatty acid profile.....	113
Figure 23	(left): ECA - fatty acid profile.....	114
Figure 24	(right): ESU - fatty acid profile.....	114
Figure 25	(left): ESY - fatty acid profile.....	114
Figure 26	(right): EFI - fatty acid profile.....	114
Figure 27	Organ culture chamber with triangular metal grids and organ culture dishes.....	119
Figure 28	BrdU labelled cells after incubation with ED-fish oil in UC (x250).....	124
Figure 29	BrdU labelled cells after incubation with medium in CD (x250).....	124
Figure 30	BrdU labelled cells after incubation with medium in control patients (x250).....	124
Figure 31	Graphs A to D showing % recovery of cytokines by ELISA up to 24-hours. The two different lines indicate two different initial spiking concentrations.....	136
Figure 32	Patient design for experimental set up.....	138
Figure 33	Patient design for protein experiment.....	139
Figure 34 A+B	(A) IL1ra / IL1β ratio after 24-h EAA incubation in CD. (B) IL1ra/IL1β showing	

<i>median and % of medium control of IL1ra + IL1β</i>	150
Figure 35 A+B (A) IL1ra / IL1β ratio after 24-h EC incubation in CD. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	150
Figure 36 A+B (A) IL1ra / IL1β ratio after 24-h EW incubation in CD. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	151
Figure 37 A+B (A) IL1ra / IL1β ratio after 24-h EAA incubation in UC. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	152
Figure 38 A+B (A) IL1ra / IL1β ratio after 24-h EC incubation in UC. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	152
Figure 39 A+B (A) IL1ra / IL1β ratio in EW after 24-h incubation in UC. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	152
Figure 40 A+B (A) IL1ra /IL1β ratio after 24-h EAA incubation Control patients. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	153
Figure 41 A+B (A) IL1ra / IL1β ratio after 24-h EC incubation in Control patients. (B) IL1ra / IL1β showing median and % of medium control of IL1ra + IL1β.....	154
Figure 42 A+B (A) IL1ra / IL1β ratio after 24-h EW incubation in Control patients. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	154
Figure 43 %TGF-β staining in CD after 24- h incubation with ECO.....	164
Figure 44 % TGF-β1 staining in CD after 24-h incubation with EWT.....	165
Figure 45 % TGF-β1 staining in CD after 24-h incubation with EAA.....	165
Figure 46 % TGF-β1 staining in UC after 24-h incubation with ECO.....	166
Figure 47 % TGF-β1 staining in UC after 24-h incubation with EWT.....	166
Figure 48 % TGF-β1 staining in UC after 24-h incubation with EAA.....	167
Figure 49 % TGF-β1 staining in Control patients after 24-h incubation with ECO.....	168
Figure 50 % TGF-β1 staining in Control patients after 24-h incubation with EWT.....	168
Figure 51 % TGF-β1 staining in Control patients after 24-h incubation with EAA.....	168
Figure 52 TGF-β1 staining in CD.....	170
Figure 53 TGF-β1 staining in UC.....	171
Figure 54 (left): % TGF-β1 staining in CD after 24-h incubation with EC.....	176
Figure 55 (right): % TGF-β1 staining in CD after 24-h incubation with EW.....	176
Figure 56 (left): %TGF-β1 staining in UC after 24-h incubation with EC.....	177
Figure 57 (right): % TGF-β1 staining in UC after 24-h incubation with EW.....	177
Figure 58 A+B (A) IL1ra/IL1β ratio after incubation with ECO in CD. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	187
Figure 59 A+B (A) IL1ra /IL1β ratio after incubation with EWT in CD. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	187

Figure 60 A+B	(A) IL1ra /IL1β ratio after incubation with ECO in UC. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	188
Figure 61 A+B	(A) IL1ra /IL1β ratio after incubation with EWT in UC. (B) IL1ra/ IL1b showing median and % of medium control of IL1ra + IL1β.....	188
Figure 62 A+B	(A) IL1ra /IL1β ratio after incubation with ECO in Control patients. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	189
Figure 63 A+B	(A) IL1ra / IL1 ratio after incubation with EWT in Control patients. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	189
Figure 64	% IL10 after ECO in CD, UC and Control patients	192
Figure 65	% IL10 after EWT in CD, UC and Control patients	192
Figure 66	% IL10 in after ECO and EWT in UC.....	192
Figure 67	% TGF-β1 release after ECO incubation compared with medium control.....	194
Figure 68	% TGF-β1 release after EWT incubation compared with medium control.....	194
Figure 69	% TGF-β1 release after EAA incubation compared with medium control	194
Figure 70	(left) % TGF-β1 staining vs. IL1ra /IL1β ratio after incubation with ECO in CD	199
Figure 71	(right) % TGF-β1 staining expression vs. IL1ra /IL1β ratio after incubation with EWT in CD.....	199
Figure 72	(left) % TGF-β1 staining vs. IL1ra /IL1β ratio after incubation with ECO in UC.....	199
Figure 73	(right) % TGF-β1 staining vs. IL1ra / IL1β ratio after incubation with EWT in UC.....	199
Figure 74	(left) % TGF-β1 staining expression vs. IL1ra /IL1β ratio after incubation with ECO in Control patients	200
Figure 75	(right) % TGF-β1 staining expression vs. IL1ra / IL1β ratio after incubation with EWT in Control patients	200
Figure 76 A+B	(A) IL1ra / IL1β ratio in UC after 24-h incubation with EFI . (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1 β.....	205
Figure 77 A+B	(A) IL1ra / IL1β ratio in CD after 24-h incubation with EFI. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	206
Figure 78 A+B	(A) IL1ra / IL1β ratio in Control patients after 24-h incubation with EFI . (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	206
Figure 79	% IL1β in UC, CD and Control patients after 24-h incubation with EFI.....	208
Figure 80	% IL1ra in UC, CD and Control patients after 24-h incubation with EFI.....	209
Figure 81 A+B	(A) IL1ra / IL1β ratio in CD after 24-h incubation with ESU. (B) IL1ra/IL1β showing median and % of medium control IL1ra + IL1β.....	215
Figure 82 A+B	(A) L1ra / IL1β ratio in CD after 24-h incubation with ESA. (B) IL1ra/IL1β showing median and % of medium control of IL1ra+IL1β.....	215
Figure 83 A+B	(A) IL1ra / IL1β ratio in UC after 24-h incubation with ESU. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	216

Figure 84 A+B (A) IL1ra / IL1β ratio in UC after 24-h incubation with ESA. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	216
Figure 85 A+B (A) IL1ra / IL1β ratio in Control patients after 24-h incubation with ESU. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	217
Figure 86 A+B (A) IL1ra / IL1β in Control patients after 24 h incubation with ESA. (B) IL1ra/IL1β showing median and % of medium control IL1ra + IL1β.....	217
Figure 87 % IL1β of control after incubation with ESU in CD, UC and Control patients.....	221
Figure 88 % IL1β of control after incubation with ESA in CD, UC and Control patients.....	221
Figure 89 % IL1ra after incubation with ESU in CD, UC and Control patients.....	224
Figure 90 % of IL1ra after incubation with ESA in CD, UC and Control patients.....	224
Figure 91 A+B (A) IL1ra / IL1β ratio in CD after 24-h incubation with EOL. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	229
Figure 92 A+B (A) IL1ra / IL1β ratio in UC after 24-h incubation with EOL. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	230
Figure 93 A+B (A) IL1ra / IL1β ratio in Control patients after 24-h incubation with EOL. (B) IL1ra/IL1b showing median and % of medium control of IL1ra + IL1b.....	230
Figure 94 % IL1β after incubation with EOL in CD, UC and Control patients.....	232
Figure 95 % IL1β after incubation with ECA in CD, UC and Control patients.....	232
Figure 96 % IL1ra after incubation with EOL in CD, UC and Control patients.....	234
Figure 97 % IL1ra after incubation with ECA in CD, UC and Control patients.....	234
Figure 98 A+B (A) IL1ra/IL1b ratio in CD after 24-h incubation with COCF. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	239
Figure 99 A+B (A) IL1ra / IL1β ratio in UC after 24-h incubation with COCF. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	240
Figure 100 A+B (A) IL1ra / IL1β ratio in Control patients after 24-h incubation with COCF. (B) IL1ra / IL1β showing median and % of medium control of IL1ra + IL1β.....	240
Figure 101 A+B (A) IL1ra / IL1β ratio in CD after 24-h incubation with ESY. (B) IL1ra/ IL1β showing median and % of medium control of IL1ra + IL1β.....	244
Figure 102 A+B (A) IL1ra / IL1β ratio after 24-h incubation with ESY in UC. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	245
Figure 103 A+B (A) IL1ra / IL1β ratio in Control patients after 24-h incubation with ESY. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β.....	245

List of Tables

Table 1	<i>Differences between Crohn's disease and Ulcerative colitis</i>	33
Table 2	<i>Elemental diet (EO28, SHS), referred in text as Elemental diet amino acid composition (EAA)</i>	106
Table 3	<i>Differences in amino acid composition of EAA, EC and EW</i>	108
Table 4	<i>Enteral diet modified in protein composition</i>	109
Table 5	<i>Defined lipid composition of EAA, EC and EW in percent</i>	110
Table 6	<i>ED Fatty acid composition of enteral diets modified according</i>	112
Table 7	<i>Saturated Fatty acids</i>	115
Table 8	<i>Mono-unsaturated Fatty acids</i>	115
Table 9	<i>Polyunsaturated Fatty acids</i>	115
Table 10	<i>Correlation between graded standard concentrations of cytokines added to enteral diet composition and results of ELISA performed on these solutions</i>	134
Table 11	<i>Clinical characteristics of patients for protein incubations</i>	139
Table 12	<i>Biopsies in protein experiment</i>	140
Table 13	<i>Patient design for fatty acid experiment</i>	141
Table 14	<i>Clinical characteristics of patients for fatty acid experiments</i>	141
Table 15	<i>Biopsies in fatty acid experiments</i>	142
Table 16	<i>Table of the 117 patients in whom incubation experiments have been performed with different defined formulae diets</i>	143
Table 17	<i>IL1ra / IL1β after 24h incubation with EAA, EC and EW</i>	155
Table 18	<i>IL10 / IL1β ratio after 24 h incubation with EAA, EC and EW</i>	157
Table 19	<i>Percentage of TGF-β1 staining expression after 24-h incubation with EAA, ECO and EWT</i>	169
Table 20	<i>% TGF-β1 staining expression in EC and EW</i>	178
Table 21	<i>IL1ra / IL1β ratios after 24-h incubation</i>	190
Table 22	<i>Percentage of TGF-β1 release of control in CD</i>	195
Table 23	<i>IL1ra / IL1β ratio in EFI</i>	207
Table 24	<i>Percentage of IL1β and percentage of IL1ra</i>	209
Table 25	<i>IL1ra/IL1β ratio for ESU and ESA incubation</i>	218
Table 26	<i>IL10/IL1β ratio for ESU and ESA in CD, UC</i>	219
Table 27	<i>Percentage of IL1β of control for ESU</i>	222
Table 28	<i>Percentage of IL1ra of control in</i>	225
Table 29	<i>IL1ra / IL1β ratio in EOL and ECA for CD, UC and Control patients</i>	231
Table 30	<i>Percentage of IL1β of MC in CD, UC</i>	233
Table 31	<i>Percentage of IL1ra of control in CD, UC</i>	235
Table 32	<i>IL1ra / IL1β ratio for COCF and COC</i>	241

Table 33	<i>IL1ra / IL1β ratio for ESY in CD, UC and Control patients</i>	246
Table 34	<i>A summary of IL1ra / IL1β response after fatty acid incubations</i>	259

Abstract

Inflammatory bowel disease is an incurable disease of the gastrointestinal tract of unknown aetiology. The two distinct types are Crohn's disease and ulcerative colitis. Both diseases have an imbalance of pro-and anti-inflammatory cytokines, such as interleukin-1 β , interleukin-1ra and interleukin 10. Transforming growth factor beta (TGF- β) is upregulated in active disease, though upregulation also might contribute to mucosal healing and downregulation of inflammation. A treatment option which has been successfully used in Crohn's disease, alone or with corticosteroids, is nutritional therapy in the form of enteral defined formula diets, elemental or polymeric. This improves inflammatory activity as well as the nutritional status of the patient with Crohn's disease. In paediatric Crohn's disease patient's growth retardation may also be reversed. The actual mechanism by which liquid enteral diets improve inflammatory symptoms and heal the intestinal mucosal wall is not known. Hypotheses include exclusion of dietary antigens, improvement of nutritional status, alteration of bacterial flora, low lipid content and their low residue, reducing intestinal obstruction. The current study attempted to answer the question whether enteral diet and its modifications might directly influence the inflammatory response in intestinal tissue affected by inflammatory bowel disease.

Intestinal endoscopic biopsies from patients with Crohn's disease, ulcerative colitis and control patients were incubated in an *in-vitro* organ culture model for 24 hours with elemental diet and modified enteral diets. The diets used were modified in nitrogen composition using casein or whey instead of amino acids in elemental diet. Other enteral diets were modified in fatty acid composition using fish oil, sunflower oil, safflower oil, canola oil, olive oil, fractionated coconut oil, unfractionated coconut oil and soya oil, but keeping the amino acid composition of elemental diet. The viability of tissue after 24 hours culture was established by bromodeoxyuridine uptake. Anti-and pro-inflammatory cytokines were measured in culture fluid by enzyme-linked immunoassay (ELISA) and anti-and pro-inflammatory cytokine ratios of IL1ra /IL1 β and IL10/IL1 β were used as a marker of *in vitro* inflammatory balance. In a further

experiment biopsies were incubated with enteral diets containing colostrum and enteral diet containing whey extract enriched with TGF- β . Immunohistochemistry for TGF- β was performed to detect differences in TGF- β 1 expression as a percentage of immunostained tissue area per mm².

The results of these experiments show that enteral diets have a direct anti-inflammatory response *in-vitro* with an increase in anti-/pro-inflammatory cytokine ratio of IL1 α / IL1 β and to some extent in IL10 / IL1 β . This response was different in Crohn's disease and ulcerative colitis affected tissue and was also dependent on the specific modified diet. Amino acid modified diets had an immuno-modulatory effect in Crohn's disease but not in ulcerative colitis and specific fatty acid modulated diets also produced disease and fatty acid specific response. Enteral diet modified by sunflower oil showed significant increases in the anti-/pro-inflammatory ratio in Crohn's disease and to some extent in ulcerative colitis, whereas incubation with enteral diet modified by fish oil was only anti-inflammatory in ulcerative colitis but not in Crohn's disease. The results also showed that diets containing growth factors, as in the case of colostrum and whey extract enriched with TGF- β , led to a significant upregulation in TGF- β expression in tissues affected with Crohn's disease but not with ulcerative colitis.

These experiments have shown that amino acid and fatty acid modified diets can have a direct and disease specific impact on *in-vitro* intestinal inflammatory response in inflammatory bowel disease. Hence, by dietary modulation, it is possible to downregulate intestinal inflammatory response, and further adaptation of defined formulae diet is possible. It may also be possible to use some formulations to treat ulcerative colitis such as fish oil enriched diet, but further research into precise mechanisms of action and palatability is required.

CHAPTER ONE

1. Background and Aims

Inflammatory bowel disease (IBD) is an inflammation of the gastrointestinal tract of unknown aetiology. The two distinct categories of IBD are known as Crohn's disease (CD) and ulcerative colitis (UC). A small percentage of patients cannot be categorised definitely and are labelled as indeterminate colitis.

Though the aetiology of IBD is still obscure, many factors have been suggested, both host and environmental, which may predispose or trigger the onset of the disease. These include environmental factors, genetic predisposition, bacterial and viral infections, vaccination and diet. The gut is one of the largest immunologic systems in the human body and is the main target of the disease. Intestinal involvement can profoundly affect food processing and nutrient uptake resulting in malnutrition. Disturbance of the delicate balance of commensal intestinal flora is postulated to be important in the perpetuation of disease.

Numerous reports have confirmed that enteral nutrition in the form of elemental and polymeric formulae diets lead to significant improvement of the inflamed intestinal mucosa and a decrease in clinical inflammatory parameters. This is accompanied by an improvement of the nutritional status of the patient, solely or in combination with steroid therapy. Such diets are helpful in CD but in UC dietary therapy has a negligible role. These defined formula diets are now used extensively in the treatment of CD, especially in children.

The mechanism of action of elemental and polymeric diets in improving the symptoms of CD are uncertain. Proposed theories include alteration of bacterial flora, low fat content, reduced antigenicity, improved nutrition and tolerability in the presence of mechanical obstruction. Whether these defined formulae diets have a direct anti-inflammatory effect on the intestinal mucosa is difficult to assess *in vivo* due to many

confounding factors, and so in this thesis, a reductionist, *in vitro* assessment system has been employed.

In the present study, endoscopic biopsies from patients with IBD and non-inflamed non-IBD patients (control patients) were incubated with elemental diet and with enteral diets specifically modified in their fat and protein composition in an organ culture model for 24 hours. The enteral diet modifications included replacement of its amino acid composition by casein and whey proteins and manipulation of its fatty acid composition using a number of oils such as fish oil, canola oil, sunflower oil, olive oil, unfractionated coconut oil, fractionated coconut oil, soya oil and safflower oil.

The work presented has investigated changes in inflammatory response by measuring surrogate markers of inflammation such as pro- and anti-inflammatory cytokines after direct contact of intestinal tissue with different enteral diets over a 24-hour period. Pro- and anti-inflammatory cytokines, such as interleukin 1 β (IL1 β) and interleukin 1 receptor antagonist (IL1ra), interleukin 10 (IL10) and transforming growth factor β (TGF- β) were measured by ELISA. The inflammatory cytokine response was expressed as the ratio of IL1ra / IL1 β and IL10 / IL1 β , compared with control (incubation with medium alone) incubations and also as a percentage of cytokine response with reference to medium control.

In addition this study investigated the presence and possible enhancement of TGF- β 1 in the intestinal mucosa after incubation with enteral diets. This was performed by incubating tissues with formulae diets enriched with TGF- β . Such preparations have been marketed recently, for example Modulen IBD®, Nestle, UK. Differences in immune response were estimated by immunohistochemical staining for TGF- β 1. The results were expressed by measuring the cytokine production during incubation phase and as percentage of TGF- β 1 staining per unit area of tissue.

Such understanding of nutrient – inflamed intestine interactions may lead to development of better formulations to treat CD and also possibly UC.

CHAPTER TWO

2. Literature review

Inflammatory bowel disease, both UC and CD are chronic relapsing and remitting diseases. Despite remarkable advances in management, the aetiology of IBD is still not known, though a variety of initiating triggers have been suggested. The incidence of UC has been estimated to be approximately 10-20 per 100,000 per year with a prevalence of 100-150 per 100,000. The incidence of CD is approximately 5 per 100,000 with a prevalence of 50 per 100,000 (Binder 1998).

As the aetiology of the disease is not known a curative therapy is not possible. Therefore the treatment is generally symptomatic, and ideally aims to maintain a long-term remission phase, whereas treatment in severe exacerbations may include surgical intervention to deal with severely affected areas of affected bowel or to avoid possible life threatening complications.

This literature review will discuss differences between CD and UC and their nutritional therapies. Further it will give a brief overview of the immune system and the involvement of lipids in immunoregulation. Chapter 3 will concentrate on the broad spectrum of medical therapy in IBD, quality of life and pharmacoeconomics.

2.1. Inflammatory bowel disease

IBD refers to two distinct manifestations of chronic intestinal inflammation, known as CD and UC. This disease can develop during childhood or adolescence in 20-25% of patients (Griffiths, 1998). The incidence of CD in children and adolescents seems to be higher in Scotland (Armitage et al., 2001; Armitage et al., 1999) compared with the European mainland and continues to rise, whereas in Germany the incidence of CD has

not been increasing in the last 15 years (Timmer et al., 1999). It appears that IBD is more common in the Western world compared with the developing countries, though this geographical distribution seems to change as these countries become more influenced by western life style. These life style changes involve improved sanitation, increased uptake of vaccination, increasing dietary fat/refined sugar and exposure to enteric infections at a later age. The actual cause is still obscure, despite the fact that some factors are widely implicated in the pathogenesis, including genetic predisposition, environmental factors, commensal bacterial flora and immunological dysfunction leading to chronic inflammation. In UC smoking appears to convey a protective effect, as only 10% of UC patients are smokers, a significantly lower proportion compared with CD and normal population (Rampton D.S. and Shanahan, 2000). The effect of diet is discussed in a separate paragraph. Specific infections such as mycobacterium paratuberculosis or measles and vaccination have been implicated but do not seem to be involved (Feeney M. et al., 1997),(Ghosh et al., 2001). Further, a loss of immunological tolerance and disturbance of enteric flora, appendectomy (protective in UC) and stress might influence the development of the disease. Figure 1 pictures the presumed aetiological factors involved in IBD. Recently the NOD2 / CARD 15 mutations have been associated with the development of CD, especially ileal CD (Ahmad et al., 2002).

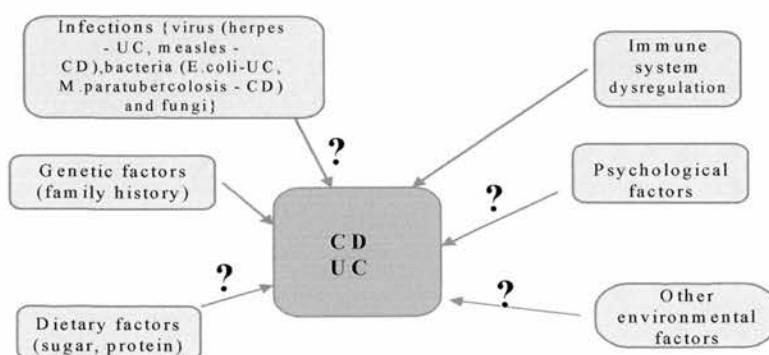


Figure 1 Proposed factors involved in IBD

IBD causes inflammation of the gastrointestinal tract (GI-tract) and extra-intestinal manifestations. In CD this can affect the whole GI-tract from the mouth to the anus. However in UC only the colon is involved. The macroscopic features of CD include aphthous ulcers, progressing to deeper ulceration, cobblestoning, fibrosis, strictures and fistulation. The combination of inflammation and fibrosis results in intestinal strictures accompanied by obstructive symptoms or local perforation of the intestinal wall, which can lead to abscess formation. The histological appearance in CD is characterised by transmural chronic inflammation, ulceration and microabscesses. Non-caseating granulomas are characteristic, but not always present. An increased risk of cancer is present in the chronically inflamed small intestinal, colorectal and anorectal mucosa. Active CD is accompanied by abdominal pain, rectal bleeding, anorexia and weight loss.

UC presents differently in many respects, normally being confined to the rectum or spreading more proximally. In severe UC the distal ileum can also become involved and is described as backwash ileitis. The appearance of the colon is characterised by diffuse mucosal inflammation with hyperaemia and granularity. In severe cases this can lead to appearance of pus on the mucosal surface and blood loss due to extensive ulceration. Histologically the disease is characterised by acute and chronic inflammatory infiltrates in the lamina propria and crypts, which may result in the typical crypt abscesses. This is usually followed by distorted architecture of the crypts and goblet cell depletion due to loss of mucin. The mucosa is oedematous and ulceration of the epithelium occurs. The common clinical features in UC are rectal bleeding, passage of mucus, urgency, bloody diarrhoea and abdominal pain.

In both CD and UC, extra- intestinal complications may occur. These include joint and bone problems such as arthropathy and arthritis, ankylosing spondylitis and osteoporosis; eye and skin problems such as iritis, episcleritis and uveitis, erythema nodosum, and other complications such as uric acid stones, oxalate stones, fibrosing alveolitis, hepatic steatosis, chronic active hepatitis, sclerosing cholangitis and cirrhosis.

Involvement of the biliary tract is also characterised by cholesterol gallstones in terminal ileal CD or after resection of diseased bowel. The mucous membrane of the mouth may be affected by aphthous ulcerations or cobblestoning of the buccal mucosa. Haematinic abnormalities include iron, serum B₁₂ and folate deficiency. Weight loss is very common and in children growth retardation is associated with severe disease (Rampton D.S. and Shanahan, 2000; Kamm, 1996). The characteristic features of CD and UC and their differences are summarised in Table 1. Figure 2 and Figure 3 demonstrate endoscopic images of CD and UC.

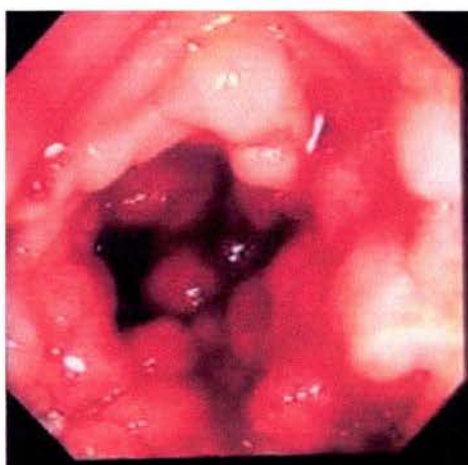


Figure 2 (left) Endoscopic image of CD
Figure 3 (right) Endoscopic image of UC

Table 1 Differences between Crohn's disease and Ulcerative colitis

Disease features	Crohn's disease	Ulcerative colitis
Smoking	Smokers	Non-smokers or ex-smokers
Long latent period	yes	No
Genetic susceptibility	++, non-major histocompatibility complex, NOD 2	+, HLA class II
Osteopenia at diagnosis	Yes	No
Oral and perianal disease	Yes	No
Growth failure	++	±
Anatomy of involvement	Entire gut, rectal sparing	Colon
Histology and Immunology		
Granuloma, structure, fistula	Yes	Generally no
Transmural inflammation	Yes	No
Cytokines	Normal IL4 and IL5; increased IFN- γ and IL12	Increased IL4 and IL5, normal IFN- γ and IL12
Associated autoimmune disease	No	Yes
Mucosal IgG subclass	IgG2	IgG1
Autoantibodies	+	+++
Management		
Response to antibiotic	Yes	No
Nutritional therapy	Effective	Not effective
Maintenance with 5-aminosalicylic acid	Small benefit	Effective
Ileoanal pouch	Generally no	Yes
Recurrence after surgery	Yes	No (apart from pouchitis)
Extraintestinal manifestations		
Sclerosing cholangitis	+	+++
Autoimmune hepatitis	+	+++

± equivocal associated; + weakly associated; ++ moderately associated; +++ strongly associated (Adapted from (Ghosh et al., 2000).

The severity of the disease can be estimated in CD and UC by the so-called Clinical Disease Activity Indexes, which have been established by several investigators. These include the activity index in UC, simplified by Walmsley *et al* (Walmsley RS *et al.*, 1998); the Crohn's Disease Activity Index (CDAI) by Best *et al* (Best *et al.*, 1976); the Harvey Bradshaw index (Harvey R.F. and Bradshaw J.M., 1980), the van Hees Index (van Hees *et al.*, 1980) and newer approaches to establish disease activity (Walmsley RS *et al.*, 1998). In the paediatric population separate indices are used to assess disease activity in CD (van Beers-Schreurs *et al.*, 1998). The main parameters recorded for adult CDAI are summarised in brief as follows. The CDAI (Best *et al.*, 1976) involves recording on a weekly diary card a number of clinical features - the number of stools of loose consistency, abdominal pain graded from none to severe and general well being. In addition, existing fistulas, abscesses, anal fissures as well as extra-intestinal complications are counted. Further drugs to counter diarrhoea, presence of abdominal mass, haematocrit for males and females, body weight, standard weight and deviation of actual weight from ideal weight in percentage are recorded. These single clinical parameters are multiplied by weighted factors to obtain the final score as an index. It is customary to consider the CDAI score above 150 points as active and reaching above 300 points as severe activity. The activity index of Best *et al* is also generally widely used in practice in a modified form by Hees *et al* (van Hees *et al.*, 1980). The severity index of Harvey and Bradshaw (Harvey R.F. and Bradshaw J.M., 1980) is simple, based on a 5 item scoring system of general well being, graded 1 to 4; abdominal pain, graded 1 to 3; abdominal mass and intestinal and extra-intestinal manifestations. These latter indices do not include laboratory criteria of inflammation, such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and plasma viscosity.

2.1.1. Nutritional morbidity in inflammatory bowel disease

Nutrition in IBD and the nutritional status of the patient with IBD are major issues in this disease. Nutritional disturbances in the form of weight loss, diarrhoea, malabsorption and anorexia with inadequate intake of nutrients are common features leading to malnutrition. Malnutrition influences the morbidity due to the disease and also affects the immune system, which may already be impaired by steroids and immunosuppression. An increase in energy expenditure might be caused by inflammatory cytokine production and fever (Griffiths, 1998). This nutritional depletion and depleted body stores of essential nutrients and impaired defence mechanisms can have extensive short and long term effects in patients with IBD. Inadequate nutrition leads to loss of growth velocity in paediatric IBD patients (McCartney and Ballinger, 1999; Ballinger et al., 2001). Decreased bone density is multifactorial and is well recognized in IBD, especially CD (Ghosh and Meister, 2000). Careful dietary supervision and estimation of the nutritional status in patients with IBD are essential components of the monitoring of these patients. This should include weight and height measurement in all, and also height velocity, which is especially important in paediatric IBD (McCartney and Ballinger, 1999). Body composition measurements, by caliper skin fold measurements or bioelectric impedance analysis (BIA) measurements are easy to perform (Ghosh et al., 1997), while more sophisticated scans, such as Dual-Energy X-ray Absorptiometry (DEXA) may be used for investigating bone density to estimate the risk for developing osteopenia and osteoporosis. (Ghosh and Meister, 2000; Boot et al., 1998). The haematological and biochemical investigations to assess nutritional status involve full blood count, Prothrombin Time, plasma iron, albumin, folic acid, B₁₂, and Vitamin A, D and E measurements. A total nutrition screen involving vitamins, macro – and micro- nutrients should be performed at least annually in vulnerable patients, such as those with extensive small bowel involvement or extensive surgical resections or more frequently if artificial nutritional support is required.

Defined formulae diets such as polymeric or enteral diets can be given as primary therapy or combined with steroids and this will be discussed in details separately. In

general, no specific diet plans or generalised diet recommendations are established for patients with IBD as there are for diabetes mellitus, lipid disorders or gout. Suggestions concerning which food might need to be avoided have been obtained out of experience of patients, or out of food exclusion or food reintroduction trials after courses of enteral diets (Pearson et al., 1993). Since in CD the entire gut can be involved, including the duodenum and small bowel, malnutrition is a more common feature of the disease. Fat uptake can be disturbed as a result of disturbance of bile acid metabolism, fat emulsification and mucosal damage. Malabsorption of protein, carbohydrate, minerals and vitamins also occur. Decreased uptake of fat can lead to steatorrhoea accompanied by loss of fat-soluble vitamins, such as Vitamin K (important for blood clotting), Vitamin D (Calcium homeostasis, bone density), Vitamin E (important antioxidant), Vitamin A (visual acuity) and minerals. Lactose intolerance may be present, as the enzyme lactase may be deficient in the inflamed mucosa. The terminal ileum is frequently involved, where Vitamin B₁₂ is reabsorbed and this can result in megaloblastic anaemia and neuropathy. Due to inflammation, protein is lost across the gastrointestinal mucosa, contributing to weight loss and a depressed immune system. In the active stage, mineral loss occurs as a result of diarrhoea. Depleted zinc (Zn) stores are more common in CD than in UC and Zn has a positive benefit on the inflammatory process. Since only the colon is affected in UC, these patients are seldom as malnourished or anorectic compared with CD patients, although fluid and mineral losses occur in association with diarrhoea. High fibre diets should be avoided in the presence of intestinal strictures, but processed fibres in softly cooked forms, or peeled and blended fruits can be attempted (Mueller, 2001). Some studies have proposed the use of milk free diets in UC (Wright R. and Truelove S.C., 1965). Some authors have suggested that a high intake of monounsaturated fat and polyunsaturated fat and Vitamin B₆ may enhance the risk of developing UC (Geerling et al., 2000), while other authors have found that fish oil, a major source of polyunsaturated fatty acids (PUFAS) of omega 3 (ω -3) type, improves inflammatory symptoms (Grimble and Tappia, 1998) (Meydani and Dinarello, 1993). A high intake of ω -3 PUFAS may be desirable rather than monounsaturated fatty acids (MUFAS), as these have been found to play a beneficial role in increasing the concentrations of certain eicosanoids, which assist inflammatory downregulation (Hunter, 1998). The medical treatment of IBD is

discussed in details in chapter 3 and the next section concentrates on nutritional treatment. Most of the medical treatment of IBD is associated with considerable side effects and hence the attraction of nutritional therapy. For example, dietary therapy is associated with a reduced risk of osteoporosis compared with steroid therapy (Dear KL et al., 2001).

2.1.2. Nutritional treatment in inflammatory bowel disease

Nutritional treatment is one of the major therapies in IBD. It improves and boosts the immune system, helps to increase the depleted body stores caused by high cell turn over and can help to restore body weight (Ferguson et al., 1998). Enteral nutrition can lead to decreased intestinal permeability and reduced inflammation (Teahon et al., 1991). Just how enteral diets improve the inflammatory activity is still unknown. Teahon *et al* (Teahon et al., 1995) summarised various hypotheses including withdrawal of food antigens, reduction of fat intake, decrease and alteration in luminal bacteria, reduction in pancreatic and biliary secretion, bowel rest and improvement in nutritional status.

In animal studies comparing enteral and parenteral nutrition, loss of intestinal barrier was greater in rats fed parenterally than with enteral diet, indicating that host immune function is better preserved with enteral feeding (Deitch et al., 1995) with less bacterial translocation. However a study by Xu *et al* (Xu et al., 1998) investigated the effect of enteral and parenteral diets on bacterial translocation in rats. The results confirm that both enteral and parenteral nutrition promote bacterial translocation and an impairment of the systemic and intestinal immune response with an improvement in immune response by the addition of dietary fibres. Nutritional intervention should always be considered, especially in childhood CD, where enteral nutrition has been shown not only to reduce relapse rate but also to improve linear growth in the growth retarded child (Wilschanski et al., 1996), associated with a decrease in linear growth inhibitory cytokines (Ballinger et al., 2001).

Teahon *et al* (Teahon et al., 1995) investigated whether the benefits of growth improvement with nutritional therapy in paediatric IBD might be due to the improved nutrition per se, or to a reduction in inflammation. The results illustrate a reduction of inflammatory symptoms within two weeks of treatment, which did not correlate significantly with nutritional variables. Independently and after disease remission the nutritional status improved in most cases.

Although the definition of remission and relapse after enteral diet treatment can often be a subject of controversy (Teahon et al., 1990), remission is usually defined as decrease of the CDAI to less than 150 or the Harvey-Bradshaw index to less than 3 (Giaffer et al., 1990). Remission is associated with a decrease of specific laboratory indices such as ESR and CRP, normalisation of plasma albumin and Haemoglobin (Hb), or decrease of IL1 β and IgG in whole gut lavage fluid (Ferguson et al., 1998). The duration of treatment with enteral diets is generally accepted to be three to four weeks, with improvement in inflammation in the first two weeks (Teahon et al., 1991). Enteral nutrition can be taken orally by drinking, but is better taken chilled because of lack of palatability. Another form of administration is by naso-gastric tube, often used for children in overnight feeds, patients who cannot tolerate the taste or in patients who are too exhausted and ill to drink. For long-term enteral feeding a feeding tube can be inserted, using minimal surgical intervention, either as a percutaneous endoscopic gastrostomy (PEG) or as a jejunal feeding tube. These require special aftercare for maintenance and prevention of infections. Supplemental enteral feeding with elemental or polymeric diet is also effective in the maintenance of remission in CD and this argues against it being solely an exclusion diet (Verma et al., 2000). Several studies have been carried out to determine whether enteral nutrition is superior in the treatment of active IBD to medical treatment with corticosteroids. The results are controversial. Gorard *et al* (Gorard et al., 1993) have shown that elemental enteral nutrition can be as effective as prednisolone in short-term treatment of active CD. They further showed, in this small cohort, that there was no difference in positive treatment response of elemental diet in well-nourished and poorly nourished patients, which would suggest that improvement of nutritional status may not be the only explanation. The results of the European Co-operative Crohn's disease study IV (Lochs et al., 1991) postulated that enteral nutrition,

which was in this case a protein-hydrolysed diet containing oligopeptides rather than free amino acids as in elemental diet, was less effective in active CD compared with drug treatment using corticosteroids and sulphasalazine. These results were summarised in a meta-analysis by Griffiths *et al* (Griffiths et al., 1995) (which includes also the two previously mentioned citations), who concluded that liquid diets given enterally or orally combined with bowel rest, were consistently less effective than conventional oral steroid treatment. However these trials excluded paediatric studies, where the benefits of nutritional therapies have been demonstrated in a meta-analysis of only paediatric papers suggesting that nutritional therapy was as effective as corticosteroids (Heuschkel, 2000).

In CD nutritional therapy may be used as primary therapy to modulate inflammatory activity, while in all IBD patients nutritional support is important in correcting undernutrition and in maintaining nutrition. As mentioned above, meta-analysis of defined formula liquid diet in CD vs. corticosteroids consistently confirm superior efficacy of the latter. Griffiths *et al* considered eight randomised controlled trials of enteral nutrition against corticosteroids, involving 413 patients, and reported that enteral nutrition was inferior to corticosteroids (pooled odds ratio 0.35; 95% confidence interval 0.23-0.53) (Griffiths et al., 1995). Fernandez-Banares *et al* {Fernandez-Banares, Cabre, et al. 1995 ID: 1749} considered 16 randomised trials that fulfilled defined entry criteria, and calculated the pooled odds ratio for all types of enteral diets compared with steroid therapy as 0.35 (95% confidence interval 0.23-0.53). Therefore, meta-analysis of eligible trials confirms the superiority of corticosteroid therapy over defined formula liquid diets. However, these meta-analyses have combined studies in which a variety of chemically defined elemental and polymeric, diets have been used. Adult and paediatric patients have been combined. Griffiths *et al* also reported on five trials including a total of 134 patients showing no difference in the efficacy of elemental versus non-elemental formulae (pooled odds ratio 0.87; 95% confidence interval 0.41-1.83) (Griffiths et al., 1995). There is no doubt that defined formula nutritional therapy may be invaluable in CD children with poor nutritional status and impairment of growth and development. Whether modulation of enteral formulae can lead to better efficacy will be an important area of future research.

In UC, nutritional support is important, but nutritional therapy has little role to play in modulating inflammatory activity. Intestinal rest with total parenteral nutrition (TPN) is not superior to enteral nutrition, as shown in a prospective randomised trial on 42 patients (Gonzalez-Huix et al., 1993). Though TPN offers no additional benefit in UC patients treated with corticosteroids, it may have primary effects on acute Crohn's colitis (Seo et al., 1999).

2.1.2.1. Total parenteral nutrition

Total parenteral nutrition (TPN), *par enteron*, is a nutritional therapy, which excludes nutrients from the gastrointestinal tract. TPN requires the application of chemically defined sterile nutrients via an intra venous (i.v.) access. The i.v. access can be either 'peripheral' or 'central'. The central option is often chosen if the patient needs high volumes of high osmolality¹ solutions, which are not suitable for peripheral access. TPN is often used in major gastrointestinal surgery to ensure wound healing, short term nutritional therapy and adequate fluid intake. TPN is an optional treatment in IBD when bowel rest is indicated to prevent further nutritional deterioration. This may be a useful adjunct to the treatment of CD, especially severe, complicated and fistulising disease.

2.1.2.2. Polymeric and Oligopeptide diet

Polymeric diets are defined formula diets, where the ingredients are prepared commercially by a designated procedure to ensure that their composition is established fairly well but not necessarily with a 100% chemical precision. Polymeric diets contain macronutrients in the form of proteins, triglycerides and carbohydrate polymers. The range of polymeric diets supplied by different manufacturers is considerable. These

¹ Osmolality: measures the concentration of molecular and ionic particles in a solution.

solutions contain whole proteins, generally isolated from casein, lactalbumin, whey, egg white or a combination. The carbohydrates are mostly glucose polymers such as starch and its hydrolysates. The fat content derives from vegetable oil, such as corn oil, safflower oil, sunflower oil, or others. Vitamins and essential minerals and trace elements are present to provide the appropriate quantities according to the recommended daily allowances (RDA). The osmolality varies between 300 and 450 mosmol/L in solutions containing 1 kcal/ml. (Shike, 1994). In a study by Raouf *et al* (Raouf et al., 1991) the effect of a whole protein / polymeric diet vs. amino acid diet as a sole treatment in active CD was tested. This confirmed a therapeutic effect of both forms of enteral feeds in CD, but showed that the low residue of elemental feeds might be important in patients with intestinal strictures.

Other forms of enteral nutrition are semi-elemental or oligopeptide diets, which contain oligopeptides rather than short chain (whole) proteins. The carbohydrates are dextrin maltose polymers and the lipids are long-chain and medium-chain triglycerides, approximately 12-40% (Cezard and Messing B., 1993).

Mansfield and his group (Mansfield et al., 1995) investigated the effectiveness of oligopeptide diet vs an elemental diet in active CD. They showed that both diets induced remission in active CD, and seemed to be effective regardless of site of disease and duration of disease. The oligopeptide diet used was from hydrolysed whey proteins with 4-5 amino acids. In this study it was also confirmed that oligopeptide and amino acid diets were exerting an anti-inflammatory effect by reduction of leukocyte migration. Verma *et al* (Verma et al., 2000) reported a double-blinded randomised study comparing polymeric vs. enteral diet, and found both diets effective in inducing remission in CD. The authors suggest that the molecular or peptide structure of the nitrogen source is unlikely to influence the therapy in acute CD. The study also showed that newly diagnosed patients with untreated CD were more likely to respond to dietary treatment. Polymeric diets often contain more than 30% of total calories as fat with different proportions of fatty acids components such as oleic and linoleic acid, with a hypothesis that diets high in oleic acid were producing higher remission rates than those diets high in linoleic acid. Finally the study by Verma *et al* proposes the use of polymeric diets as primary therapy especially in newly diagnosed CD (Verma et al.,

2000). A recent study by Fell *et al* (Fell J.M.E. et al., 2000) investigated the effect of a casein based polymeric diet, which was rich in transforming growth factor $\beta 2$ (TGF- $\beta 2$) on inflammatory cytokine mRNA production in colonic biopsies from paediatric CD patients before and after treatment. The results showed a decrease in mucosal IL1- β mRNA, in IFN- γ mRNA and in IL8 mRNA after treatment but no treatment response in IL10 mRNA. Mucosal biopsies after treatment showed significant improvement and healing. An increase in TGF- $\beta 1$ mRNA expression in the ileum was observed, which could be a result of TGF- β added to the diet.

2.1.2.3. Elemental diet

Elemental diet is also categorised as a defined formula diet. These liquid diets contain protein in the form of free amino acids, which have their origin from casein, whey or other protein hydrolysates. The absorption of these 'nitrogen carriers' is rapid and but the osmolality is high. Since elemental or monomeric diets require little or no digestion, these have some physiological advantages. The carbohydrates in these solutions are partly hydrolysed starches such as maltodextrine and glucose oligosaccharides. The fat content is generally a mixture of medium chain and long chain triglycerides of plant origin. The osmolality of monomer solutions ranges between 400 and 700 mosmol/kg (Shike, 1994). In a long-term follow up study Teahon *et al* (Teahon et al., 1990) investigated the outcome of elemental diet therapy with and without steroids. The results suggest that elemental diet is a successful treatment in acute CD, giving remission rates comparable with steroid therapy. Patients with jejunal and ileal disease had a more rapid and symptomatic response than those with ileocolonic, colonic or perianal disease. Although these studies show that elemental or polymeric diets decrease inflammatory parameters and increase remission rate, the mechanism of this effect is not yet defined. Serizawa *et al* (Serizawa et al., 1994) has investigated the influence of long-term ingestion of elemental diet on lymphocyte transport and immune response of the gut-associated-lymphoid tissue (GALT) in rats. There were no significant morphological changes in Peyer's patches or intestinal villi of the small intestine after four weeks of treatment. No bacteriological differences were found between elemental

and control group. In conclusion, the group proposed that the effectiveness of achieving remission in CD using these diets might, in part, be due to an immunological effect.

2.1.2.4. Polyunsaturated fatty acids in enteral diets and as dietary supplementation

Modification of enteral diets with polyunsaturated fatty acids, especially ω -3 fatty acids, has become a subject of great interest with their benefits being demonstrated in several allergic, cardiac or autoimmune diseases. Lorenz *et al* (Lorenz et al., 1989) studied the influence of ω -3 fatty acids derived from fish oil on chronic IBD in a prospective, randomized, double-blind, placebo-controlled cross-over trial. Thirty-nine patients (CD n=29, UC n=10) were entered into the 7-months trial. The fish oil was given as capsules (Maxepa, Germany), containing 19 % eicosapentaenoic acid (EPA) 14 % docosahexaenoic acid (DHA), and 25 % and 30 % monounsaturated and saturated fatty acids, respectively. This provided about 1.8 gm EPA and 1.3 gm DHA. Three capsules per day were taken over 3 months following a wash-out period of 4 weeks and were switched to the alternate preparation taken in phase II of the trial, such as placebo 75 % oleic acid, and 8 % and 15 % ω -6 PUFA and saturated fatty acids. During fish oil supplementation plasma phospholipid fatty acid analysis showed a threefold increase in EPA and a twofold increase in DHA, associated with a significant decrease in ω -6 fatty acids of linoleic acid and arachidonic acid. Overall the results indicated a more obvious beneficial effect of ω -3 fatty acids in UC. The study confirmed an alteration of the eicosanoid profile and a small improvement of the morphological appearance after the treatment period in both diseases. A study by Lorenz-Meyer *et al* (Lorenz-Meyer et al., 1996) investigated the effect of ω -3 fatty acids capsules (55 % EPA, 30 % DHA) and a low carbohydrate diet of less than 84 gm carbohydrate per day in patients with CD. 23 centres recruited a total of 204 patients. Patients were considered for admission to the trial and were included once they reached remission (CDAI \leq 150) with conventional steroid therapy over a 3-month period. The proportion of patients without relapse over a year was similar in the placebo group and active treatment group (intention-to-treat

analysis: placebo 30%; active treatment 30%; protocol-adhering patients 29% vs. 28%). Patients gained benefit (53%, $p=0.023$) for as long as they maintained the diet. However, using intention-to-treat analysis (diet group 40%) this did not show a significant difference when compared with placebo. In summary, ω -3 fatty acids did not extend the remission in CD. French *et al* (French et al., 1997) designed a study to investigate whether intake of a diet with a high polyunsaturated to saturated fatty acid ratio would influence the absorption-oxidation of polyunsaturated fatty acids in CD. It has been shown that difference in fatty acid pattern of polyunsaturated and saturated fatty acids (P/S – Quotient) may influence intestinal function, transport processes and transporter activity as reported in rats (Esteve-Comas et al., 1993). A high PUFA diet increased the transport of saturated and monounsaturated fatty acids, such as palmitic, stearic and oleic acid in brush border membrane of rats, whilst the uptake of medium-chain fatty acids was unaffected. The results indicated that the ω -6/ ω -3 ratio of 60 in this diet had a more beneficial effect than P/S ratio itself in CD (Esteve-Comas et al., 1993).

2.1.2.5. Proteins and amino acids in enteral diets

The addition of nitrogen components, in the form of oligopeptides, proteins or single amino acids in enteral polymeric and elemental diets, respectively, were dealt with in the above paragraph `polymeric and oligopeptide diets`. The following paragraph will give a short summary of the general importance of proteins and amino acids in enteral diets.

Proteins and amino acids are essential in all mammalian cells. Twenty L-amino acids, known as proteinogenic amino acids can be incorporated into proteins. Eight of these amino acids are essential and have to be supplied by diet, as the body is unable to synthesise them. Dietary proteins are essential luminal growth factors for intestinal mucosa in terms of growth and development. Prolonged feeding of protein deficient diets results in significant hypoplasia of small intestinal and colonic mucosa in rats

(Loser et al., 1999). Dietary glutamine, glutamate, aspartate and arterial blood glutamine are the major fuels for the small intestinal mucosa. These are needed to provide energy for intestinal adenosine-tri-phosphate (ATP) dependent processes necessary for the active transport of nutrient and for high rates of intracellular protein turn over (Wu, 1998). Peptides in the GI-tract have different functions and can be categorised according to their specific roles. These can act as *mucosal integrity peptides*, such as transforming growth factor α (TGF- α) and pancreatic secretory trypsin inhibitor, which are expressed in the mucosa of the whole GI-tract and help to maintain normal mucosal integrity. In addition there are *luminal surveillance peptides*, such as epidermal growth factor (EGF) and *rapid response peptides* such as spasmolytic polypeptides. The latter are upregulated in mucosal damage and are most likely to be important in early mucosal repair (Playford, 1995). Playford *et al* have suggested that addition of specific proteins to enteral diets such as casein may prevent intestinal atrophy after TPN and preserve the intestinal mucosa (Playford et al., 1993). The results showed that human EGF and TGF- α were protected from digestion by fasting human intestinal juice in the presence of casein and in the presence of soybean trypsin inhibitor. This effect was only achieved by whole proteins and not by the equivalent amino acid composition in elemental diets. However Wu *et al* (Wu, 1998) have shown the importance of specific dietary amino acids in the maintenance of the intestinal mucosal mass and integrity, e.g. ornithine, the immediate precursor of polyamine synthesis, which is essential for proliferation, differentiation and repair of intestinal epithelial cells. Also arginine the physiologic precursor of NO is important in regulating blood flow, integrity, secretion and epithelial cell migration while glutamate, glycine and cysteine precursors of glutathione, are critical for intestinal mucosal defence against toxic and peroxidative damage.

Special interest has increased in recent years in milk-derived proteins and growth factors and attention was given to the effects of colostrum protein, casein and whey proteins. Whey, derived out of the production of cheese, attracted considerable interest especially in food producing industry as so called functional foods (physiologically functional foods, nutraceuticals, pharmafoods or designer foods), which have health benefit when consumed (McIntosh GH et al., 1998). Lactoferrin and lactoperoxidase are two major milk proteins and can be purified out of milk, especially whey and have

attracted attention as by-products in enteral diets with their immunological effects. Lactoferrin is also present in biological fluids (such as colostrum breast milk, mature breast milk, tear fluid, seminal plasma, synovial fluid, saliva, cow's colostrum whey and cows milk). *In vivo* and *in vitro* properties of lactoferrin have shown (a) effects on iron absorption, (b) antioxidant effect, (c) antimicrobial, (d) antiviral, (e) anti-inflammatory and immune modulating effects, (f) even possible anti-cancer properties (Steijns JM and van Hooijdonk ACM, 2000). Kuwata *et al* have shown that lactoferrin is resistant and survives proteolytic degradation in the small intestine of adult rats (Kuwata H et al., 2001). Casein, a main component of milk, constituting about 80% of total milk protein fraction (Shah, 2000) consists of four major fractions, alpha s1 (α_s1), alpha s2 (α_s2), beta (β) -casein and kappa (κ)- casein. Beta-casein and its opioid peptides are known as β -caseomorphins and have shown having immunomodulatory activities, such as promoting antibody synthesis and phagocytosis (Wong et al., 1996). All of these milk-derived proteins have bioactive properties, but further research is required to fully exhaust the possibilities of these natural components.

2.2. The mucosal immune system of the intestine

The GI tract is the largest immunologically active system in the body. Several lymphatic organs such as spleen, lymph nodes, liver, the thymus gland in children for T-cell maturation, as well as bone marrow, contribute to the body's defence mechanism. The GI-tract is covered by a mucous membrane, and this mucosal surface reaches a size of 400 m² in adults (Nicoletti, 2000).

The epithelium acts as a physical barrier and the cell membrane prevents bacteria and non-invasive virus from entering the underlying lymphoid tissue. Further mechanisms of protection are inter-enterocyte cell – cell connections, so called tight junctions, which can prevent small peptides from passing through the epithelium (Mayer, 2000). The epithelial cell layer is followed by a loose overlying lymphoid tissue, the lamina propria, which lies directly beneath the epithelium and acts not only as a supportive tissue, but also contains immune competent cells and the gut-associated lymphoid tissue (GALT) (Myrvik, 1994; Mayer, 2000) (Nagura et al., 2001). The epithelial barrier is composed of glycocalyx, glycoproteins and mucin overlying the epithelial layer as a thick mucus rich cover where it acts as an important first barrier as well as an interface between the intestinal lumen and the intestinal internal environment. The mucus layer is rich in water and contains glycoproteins, serum proteins and immunoglobulins and can be altered in the diseased state. For example, a thinner mucus layer and an altered mucin composition was found in UC compared with CD (Nagura et al., 2001) (Corfield et al., 2000).

In the GI-tract, all mucosal surfaces and their associated lymphatic structures come into contact with antigens or potentially toxic particles, and are equipped with a specially designed lymphatic structure called Mucosa-Associated-Lymphoid Tissue (MALT) (Myrvik, 1994). The MALT in the intestine is also called gut-associated lymphoid tissue (GALT), and it is the largest lymphoid organ in the body with more than 10¹² lymphocytes and more antibody production than any other site in the body. This

extensive size is because of the length of the GI-tract and the surface structure of Kerkring's folds and villus in the small intestine. The usual one layered columnar cell epithelial layer is derived from the basal crypts, which differentiate into villus or surface epithelium, goblet cells and neuroendocrine cells or Paneth cells. Intraepithelial lymphocytes (IELs) occur mainly in the villi of the small intestine and are dominated by CD3⁺ (present on all mature T-cells) and CD8⁺ T-cells (described in section below) (Brandtzaeg, 1998). An unusual specialised epithelial cell type in the gastrointestinal mucosa are the so-called M cells with many controversial aspects of their origin, biology and interaction in the mucosa defence system. M cells display a dome like appearance, with a poorly organised brush border, short irregular microvilli and an absent glycocalyx. These features allow access to the apical domain of M cells, where microorganism and macromolecules are transported to the underlying lymphoid tissue (Nicoletti, 2000). The basolateral surface of M-cells is invaginated and extends into the lamina propria, forming intraepithelial pockets to give a close contact to lymphocytes from the lymphatic tissue (Nicoletti, 2000). These cells also overlie the so-called Peyer's patches (PP), which are the only true organised lymphoid structure in the GALT. The function of M cells is to sample especially large antigen particles from the gut lumen including food antigens, bacteria and viral antigens. Antigens pass through the M cell and are taken up by macrophages in the M cell pocket. From there the antigen is presented by the macrophages and dendritic cells to the underlying lymphocytes to generate an active immune response (Mayer, 2000). The morphological feature of M cells distinguishes them from the surrounding enterocytes, which is designated the follicle associated epithelium (FAE). The follicle associated epithelium, overlying the MALT, allows a far more restricted transport of antigens and microorganisms into the intestinal wall.

2.2.1. Lymphocytes

Lymphocytes are part of the body's immune defence mechanism, divided into (a) cellular and (b) humoral components. The former includes the T-cell system, which

differentiates and develops in the foetal thymus. T-cells functions include helping B-cells to make antibody, killing virally infected cells, regulating the level of immune response and stimulating the microbicidal and cytotoxic activity of other immune effector cells, including macrophages. Communication between these cells is controlled either direct by cell-to-cell contact or by antibodies and cytokines. T-cells recognise antigen and MHC (major histocompatibility complex) molecules via the T-cell receptor (TCR) (Male David et al., 1996). T-cells have two different classes of T-cell receptors, which consist of and are distinguished by either γ and δ chains or α and β chains. Mature $\alpha\beta$ T-cells are subdivided into helper/inducer CD4+ and suppressor/cytotoxic CD8+ T-cell subpopulations (McCoy, 1998). CD (cluster designation) markers are cluster of differentiation markers and are responsible for antigen or antigen/MHC binding. MHC is responsible for the rapid rejection of grafts between individuals and has important functions as a signal transmitter between lymphocytes and cells expressing antigens. Both are complementary molecules, which permit T-cells to recognise antigens. Class II MHC (two MHC-encoded polypeptides which are non-covalently associated and are present on antigen presenting cells) are mostly associated recognition of antigens by CD4+ T-cells, whereas CD8+ T-cells generally recognise antigen and class I MHC (Male David et al., 1996). More than 90 % of mature peripheral T-cells express $\alpha\beta$ TCR. This receptor is composed of one (not dimerized) chain of α and β . The TCR does not mediate cell activation by itself but associate with several molecules forming the CD3 complex. The CD3 complex binds to the $\alpha\beta$ TCR during assembling of the receptor in the endoplasmic reticulum. Four CD3 chains form ϵ - γ , ϵ - δ and ζ - ζ dimers. The first two dimers are in mature T-cells functional equivalent to Ig α/β in mature B-cells (McCoy, 1998). Th1 and Th2 are intestinal T-helper subsets of CD4+ lymphocytes. Th1 cells activate macrophages and promote production of opsonizing antibodies for clearing infection by intracellular organisms. Th1 cells produce IL1 and IFN- γ . Th2 cells secrete cytokines such as IL4, IL5, IL6 and IL10, downregulate macrophages, and promote isotype switching to cells producing IgE and IgG, stimulate mast cells and induce eosinophilia. The relevance of these Th1 and Th2 system in intestinal inflammation is that probably the balance of these subsets determines activity and susceptibility to chronic inflammation. The balance of Th1 and Th2 system could be altered by therapeutic intervention and therefore could change the

inflammatory response (Herfarth and Sartor, 1994). CD is considered a Th1 disease whereas UC resembles a Th2 disease in many, but not all, aspects.

The humoral mechanism is composed of the B-cells, and the production of B-cells starts during foetal development in the liver and begins to shift between 5 and 6 month into the bone. After birth and throughout life the B-cells are solely produced in the bone marrow. The B-cell receptors are immunoglobulins (Igs) (McCoy, 1998).

Immunoglobulins are active as membrane surface Igs, which act as B-cell receptor and occurs as integral membrane protein. Immunoglobulins are IgM, IgG1, IgG2, IgG3, IgG4, IgA1, IgD and IgE; these are divided into these different classes and subclasses according to the types of heavy chains these contain (Male David et al., 1996). IgM as the first functional mature antigen receptor is composed of a heavy μ chain and a light κ or λ chain by dimerizing two heavy and two light chains together. The antigen-binding site is formed by a variable domain of the heavy and light chain and determines the antigen specific binding of IgM. Antigens, which are recognised by IgM, include proteins, carbohydrates or DNA. After antigen engagement with the B-cell surface immunoglobulin these cells are stimulated. Immunoglobulins have to associate with some accessory chains to transmit and activate signals. Accessory chains, which are in the endoplasmatic reticulum, are Ig α and Ig β . They are also known as CD79a and CD79b and are required for cell surface expression of immunoglobulin. Ig α and Ig β are essential for B-cell activation through the antigen receptor by linking IgM to the signal transduction mechanism. The surface IgM is only expressed in immature B-cells. Mature B-cells express both surface IgM and surface IgD. Only fully mature B-cells are capable of antigen response (McCoy, 1998).

Therefore for the recognition of a specific immune response by antigen binding molecules three binding molecules are involved. The B-cell antigen receptor in form of immunoglobulins, the T-cell antigen receptor and the class I and class II molecules of the MHC (Male David et al., 1996). Figure 4 describes a simple model of lymphocyte downregulation on Th1 response, influenced by anergy, cell apoptosis and downregulatory cytokines such as IL10 and TGF- β .

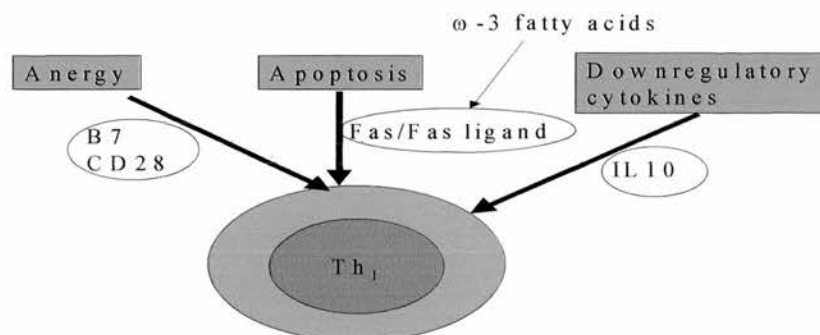


Figure 4 Downregulation of lymphocyte activation in the intestine.

The intestine is heavily exposed to antigenic load and has multiple mechanisms to downregulate lymphocyte activation and proliferation. These include anergy by the absence of co-stimulatory molecules B7 and CD28, apoptosis by Fas/FasL interaction which can be promoted by ω -3 fatty acids and finally IL10 and TGF- β mediated downregulation.

2.2.1.1. Cytokines

Cytokine is a generic term for messenger molecules (polypeptides) which are secreted by lymphoid and non-lymphoid cells and form a mediator network, regulating growth, differentiation and function of cells involved in immunity, haemopoiesis and inflammation. Cytokines are produced as a host response to injury, inflammation and infection. Different types of cells produce cytokines and, in addition to oxygen radicals and eicosanoid production, these are major mediators of physiological and pathological changes in the immune response. These are associated with an increase in resting energy expenditure, increase in eicosanoid production, induction of free oxygen radicals and induction of stress hormones. Some cytokines are associated with a pro-inflammatory response, such as IL1, IL6 and TNF (Gonzalez Z.A., 2000). More than a dozen and half

cytokines, which can act in defence and also as disease mediators, have been characterised so far (Meydani and Dinarello, 1993) and the number is constantly increasing. In the following section, cytokines which play a major role in T-cell function, B-cell function and both T- and B-cell function are discussed such as IL1, IL2 and IL4. Lastly, cytokines which are known to modulate inflammation and chemokines that attract immune cells and are important in modulating immune response (Male David et al., 1996) by altering immune cells are also discussed.

Interleukin-1 (IL1) and Interleukin-1 receptor antagonist (IL1ra): IL1 is a 13-17kd molecule and exists in two isoforms as IL1 α and IL1 β . There is only a minimal amino acid homology between the two and IL1 β is the dominant IL1 in humans. It is also one of the key mediators in immunologic reactions, microbial invasion and inflammatory response. Although IL1 is produced by fibroblasts, endothelial cells, keratinocytes and smooth muscle cells; the major source of IL1 is the macrophages. IL1 regulates T- and B-lymphocyte development and also induces other cytokines and their receptor synthesis, affects the central nervous system and has metabolic and pro-inflammatory effects (Meydani and Dinarello, 1993). If IL1 is released locally, it induces oedema, neutrophil tissue infiltration and T- and B-cell activation. Both forms of IL1 bind to the two IL1 receptors (Herfarth and Sartor, 1994). Only the type I receptor is capable of signal transduction (Male David et al., 1996). The IL1ra receptor antagonist has structural homology with IL1 β and originates from the same cells where IL1 is released. It blocks the biological activity of IL1 β , by binding to either type I or types II IL1 receptor without agonist activity. It has also been shown that tissue concentration of IL1ra correlates with the degree of inflammation. In an animal model using Lewis rats, which are susceptible to chronic inflammatory conditions, an increase in the IL-1 β /IL1ra ratio during induced inflammation could be shown compared with the acute phase in low responding Fischer rats. It is thought that the relative balance of IL-1 β /and IL1ra may play an important role in modulating inflammatory response, which may be influenced by genetic factors (Herfarth and Sartor, 1994). In experimental models of induced intestinal inflammation it has been shown that IL1ra reduces inflammation (Bhan et al., 1994).

IL2 is known to have activity on a variety of cells such as natural killer cells (NK) macrophages, monocytes and B- and T-cells. It is involved in T-cell proliferation and only produced by T- cells (Male David et al., 1996). In IBD contradictory levels have been reported with enhanced, normal, or decreased mucosal levels by different laboratories. In spite of this controversy, a more uniform picture is emerging regarding dissimilarities in the production of and response to IL2 between the two forms of IBD. Levels of IL2 bioactivity or mRNA are significantly greater in CD than in UC (Fiocchi et al., 1994)

IL4 is produced by mast cells and by subpopulations of T-cells. It promotes the growth of T-cells, B-cells, mast cells, myeloid cells and erythroid progenitors (Male David et al., 1996).

IL5 is a glycoprotein and produced by activated T-cells. T-cell production of this cytokine is stimulated by parasitic infections. IL5 also plays a key role in allergic diseases (Male David et al., 1996).

IL6 is produced during the immune response and can influence both T-and B-cell growth and differentiation. It acts with IL1 together synergistically in T-cell proliferation. It also causes differentiation of B-cells to antibody secreting cells. It has been found to be elevated in patients with IBD and AIDS (Meydani and Dinarello, 1993). IL6 combines with its receptor to signal via binding to gp-130.

IL7 has promoting effects on the growth of T-and B-cell progenitors and mature T-cells. IL7 is produced only by stromal cells and induces the secretion of IL1 α , IL1 β , IL6 and TNF- α by monocytes (Male David et al., 1996).

IL9 is a T-cell growth factor and is produced by some activated T-cells.

IL10 is produced by Th0 and Th2 subsets of murine T-cells and its production is inhibited by IFN- γ . It has important biological effects on T-cell function, downregulates MHC class II expression on macrophages and inhibits LPS induced production of IL1

and TNF- α . It also inhibits IFN- γ induced production of reactive oxygen intermediate and NO (nitric oxide) by macrophages. It appears that IL10 may have anti-inflammatory properties by down regulating Th1 response (Male David et al., 1996).

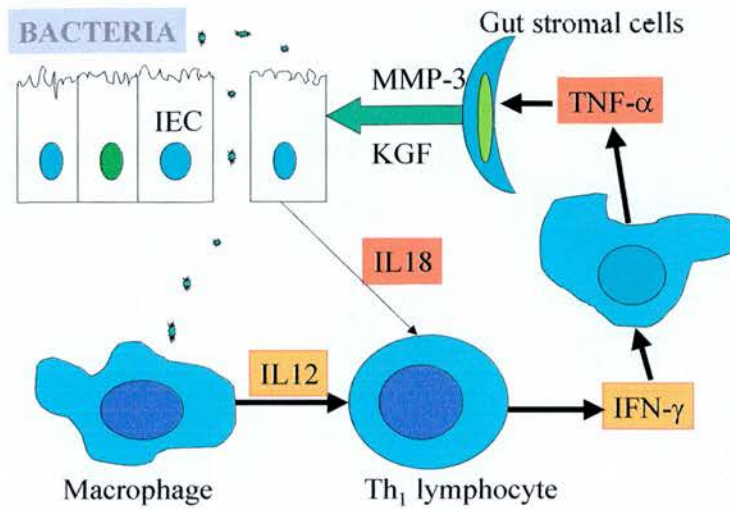
IL12 is synthesised by B-cells, monocytes and macrophages. It depresses IgE production and increases IFN- γ . IL12 acts also as a key cytokine in directing the T-cell response (Male David et al., 1996). IL12 is important in promoting Th1 differentiation acting in concert with other co-stimulatory molecules such as IL18 secreted by intestinal epithelial cells (Figure 5).

Tumour Necrosis Factor (TNF) is used to describe two polypeptides TNF- α and TNF- β . Macrophages and/or monocytes produce the former; the latter is derived from lymphocytes. TNF- α is cytotoxic or cytostatic for several tumour cell lines *in vitro*. Receptors for TNF- α can be found in a variety of cells. TNF- α decreases phagocyte activity of human neutrophils and is involved in activating eosinophils. Like IL1, TNF- α decreases appetite and inhibits lipoprotein lipase, which leads to cachexia (Meydani and Dinarello, 1993). TNF- α as the product of macrophages acts as a multipotent mediator by sharing many biological activities with IL-1 β and IL6. The importance of TNF- α in inflammation is paramount and it has been found in significantly high concentrations in IBD (Fiocchi et al., 1994).

Transforming Growth Factor beta (TGF- β) belongs to a polypeptide family, which occurs in mammalian tissue in the three isoforms of TGF- β 1, TGF- β 2 and TGF- β 3. These are 25 kDa proteins of identical or nearly identical subunits (di Mola et al., 1999). The isoforms share 70 to 80 % sequence homology and are practically indistinguishable at their functional levels. TGF- β 1 appears in humans as the predominant form (Lionetti et al., 1999). Each of the three isoforms has its specific transmembrane TGF- β receptor. TGF- β receptor type I (T β R-I) subtype ALK5 and TGF- β receptor type II (T β R-II) are the signalling receptors and must be simultaneously present to achieve a signal transduction. TGF- β binds directly to T β R-II, as an active serine/threonine kinase. This composite is recognised by T β R-I_{ALK5} and becomes phosphorylated by T β R-II. The

phosphorylation allows T β R-I_{ALK5} to transmit the signal downstream to the substrate. TGF- β receptor type III (T β R-III) also called betaglycan, participates in storage of TGF- β s and in the presentation of the various isoforms to their signalling receptors (di Mola et al., 1999). TGF- β is produced by lymphocytes, macrophages and other cells such as intestinal epithelial cell lines. TGF- β inhibits the proliferation of B- and T-cells, intestinal epithelial cells and other cells. It inhibits the endothelial cell proliferation by arresting these cells in G₁ phase of the cell cycle. Intestinal epithelial cell lines have receptors for TGF- β (McGee et al., 1992). TGF- β promotes intestinal restitution after mucosal injury, stimulates the growth and differentiation of mesenchymal cells such as fibroblasts and dendritic cells. It regulates the extracellular matrix (ECM) deposition, increases the synthesis of ECM proteins such as collagens, fibronectin and elastin. It controls the immune response at the local level and acts as chemotactic signal for neutrophils, monocytes and lymphocytes at the site of inflammation (Lionetti et al., 1999). TGF- β is a potent inhibitor of crypt cell proliferation *in vitro* and may play an important role as a regulator of intestinal cell proliferation and differentiation. In rats it has been shown that TGF- β 1 expression is low in the crypt cells which are actively dividing and high in cells which went through terminal differentiation at the villus tip. It inhibits the proliferation of rat intestinal crypt cells by blocking cell cycle at the middle of the G₁ phase (Ko et al., 1994).

IFN- γ influences the growth, development and activity of T-cells, Natural killer cells (NK) and B-cells. It is made by activated T-cells (Th0, Th1 and CD8+) and is a monomeric glycoprotein. It is a powerful enhancer of MHC class I expression on different cell types and also induces MHC class II expression. IFN- γ is also a powerful activator of macrophages inducing NO synthase, TNF- α and IL-1. This cytokine increases the ability of macrophages to kill intracellular parasites and tumour cells. It also works synergistically with other cytokines, for example with TNF- α , whereby it mediates cytotoxicity in a variety of cell types (Male David et al., 1996). Figure 5 shows a cartoon of Th1 differentiation and secretion of IFN- γ after exposure of the mucosa to bacterial antigen.



MMP-3: Matrix metalloproteinase-3

KGF: Keratinocyte Growth factor

IEC: Intestinal epithelial cell

Figure 5 Cytokines in Th1 response, as in Crohn's disease.

Macrophages in the lamina propria are activated by bacterial antigen. IL12 secretion by macrophages with the help of IL18 derived from IEC results in Th1 differentiation. The resulting IFN-γ secretion promotes TNF-α production by macrophages. IEC damage and proliferation is mediated by MMP-3 and KGF produced by gut stromal cells stimulated by TNF-α.

Chemokines (chemotactic cytokines) belong to a family of small molecular weight cytokines that are secreted by monocytes, macrophages and T-cells, some of which have important functions in innate immune response. They influence leukocyte trafficking and attract leukocytes to sites of tissue inflammation or infection (Male David et al., 1996). IL8 is a member of the chemokine family and is perhaps the most potent inducer of polymorphonuclear neutrophil chemotaxis, with some activity for T-cells and its role in IBD is justifiably the focus of intense investigation. In IBD elevated levels of IL8 has been found in the mucosa of UC patients as well as CD patients (Fiocchi et al., 1994).

2.2.2. The cytokine pattern in inflammatory bowel disease

IBD is characterised by an imbalance in cytokines, produced by a variety of cells such as monocytes, endothelial cells and neutrophils. One of the major cytokines involved is IL-1 β , released from mononuclear phagocytes (McAlindon et al., 1998) (Ligumsky et al., 1990). Other pro-inflammatory cytokines, contributing to IBD are IL6, IL8, IL12 and TNF- α , which are produced by the innate immune system, such as macrophages and monocytes (Papadakis and Targan, 2000). This is reflected by an upregulation of IL-1 β , reduced or normal secretion of IL2 (Nakamura et al., 1992), a decrease of IL10 in LPMC (Lamina propria mononuclear cells) of inflamed colonic mucosa and an over expression of TNF- α and IFN- γ (Gasche et al., 2000); (Akagi et al., 2000). Several studies have found a decrease in IL1ra / IL1 β ratio in inflamed mucosa of IBD patients, due to an increase in IL-1 β (Hyams et al., 1995); (Andus et al., 1997); (Casini-Raggi et al., 1995); (Dionne et al., 1998). IL1ra competes with IL-1 β for the same receptor and can therefore inhibit IL-1 β by blocking its receptor. The involvement of cytokines in the pathogenesis appears to be different in CD and UC, with a dysregulated and excessive Th1 T-cell response to an initiating antigenic stimulus in CD. It manifests itself as an increased level of IFN- γ and IL12 besides decreased levels of IL4. UC is more consistent with a Th2 T-cell response. IL5 production is consistently increased and IL4 levels are elevated (McClane and Rombeau, 1999). These different cytokine profiles, with two different immune responses are reflected also in targeted therapeutic strategies. In addition to cytokines, a range of lipid derived inflammatory mediators and chemokines are produced as part of the immune reaction, which are discussed in the following section. Figure 6 shows a schematic diagram of cytokine balance of pro- and anti-inflammatory cytokines involved in pathogenesis of IBD.

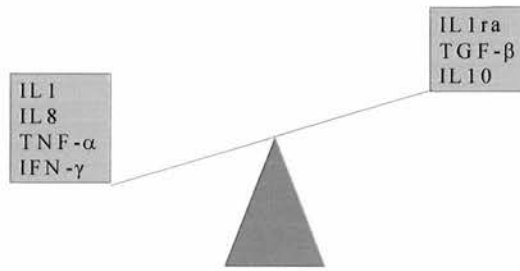


Figure 6 Cytokine-balance in IBD.

There is a preponderance of pro-inflammatory cytokines and chemokines and a relative deficiency of downregulatory or anti-inflammatory cytokines. The ratio of anti-inflammatory to pro-inflammatory cytokines denotes the inflammatory status of the tissue.

2.3. Involvement of lipids in inflammation and immunoregulation

Lipids or triacylglycerides are essential components of human nutrition. Lipids, which are formed by an ester binding to glycerol, have multiple functions in the body. The human body needs fatty acids to accumulate fat stores, to line organs for protection and as a carrier for the essential lipid soluble vitamins (retinol, calciferol, tocopherols, and phyllochinons). Lipids act as templates for steroid hormones and the lipids are incorporated into all cells as a main structural component (in the form of phosphoglycerides, sphingolipids, and cholesterol) of the intracellular membrane of mitochondria, liposomes and the endoplasmic reticulum, as well as of the plasma membrane of the cell itself.

Fatty acids are classified into short chain fatty acids (SCFA), medium chain fatty acids (MCFA) and long chain fatty acids (LCFA). The chemical structure for these fatty acids is $C_nH_{2n+1}COOH$. The carbon chain length can be from two to 26 carbons.

Fatty acids can have one, two or multiple double bonding with the carbon chain, and therefore called mono-unsaturated fatty acids (MUFA) or poly-unsaturated fatty acids (PUFA), respectively. Fatty acids are numbered in arabic numerals starting from the carbon of the carboxylgroup as number one continued to number two etc. The latter can be also named as alpha (α) followed by beta (β) and so on, up to the last carbon on a methyl group as omega (ω). Omega ω -3 or ω -6 defines the location of the first double bond, counting from the methyl end of the fatty acid molecule that distinguishes these classes of PUFA. Double bonds in the chain are pronounced as delta (Δ) (e.g. Δ^9 for a double bond between C atom 9 and C atom 10). The double bond also defines the conformation of the fatty acids, if in *cis* or *trans* form. Most of the natural fatty acids occur as *cis*-Isomer. Unsaturated fatty acids have generally the first double bond between C atom 9 and C atom 10. In PUFA the double bond generally occurs within a distance of 3 C atoms, so called isolated double bonds (Koolman J and Roehm KH, 1994). Figure 7 shows the molecular structure of the PUFA arachidonic acid.

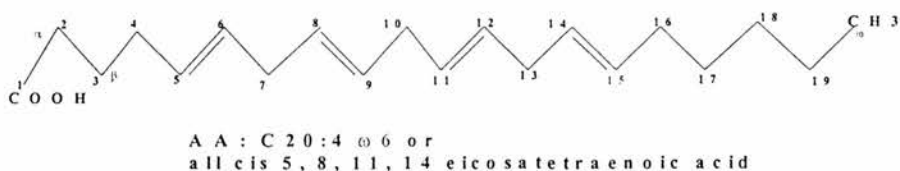
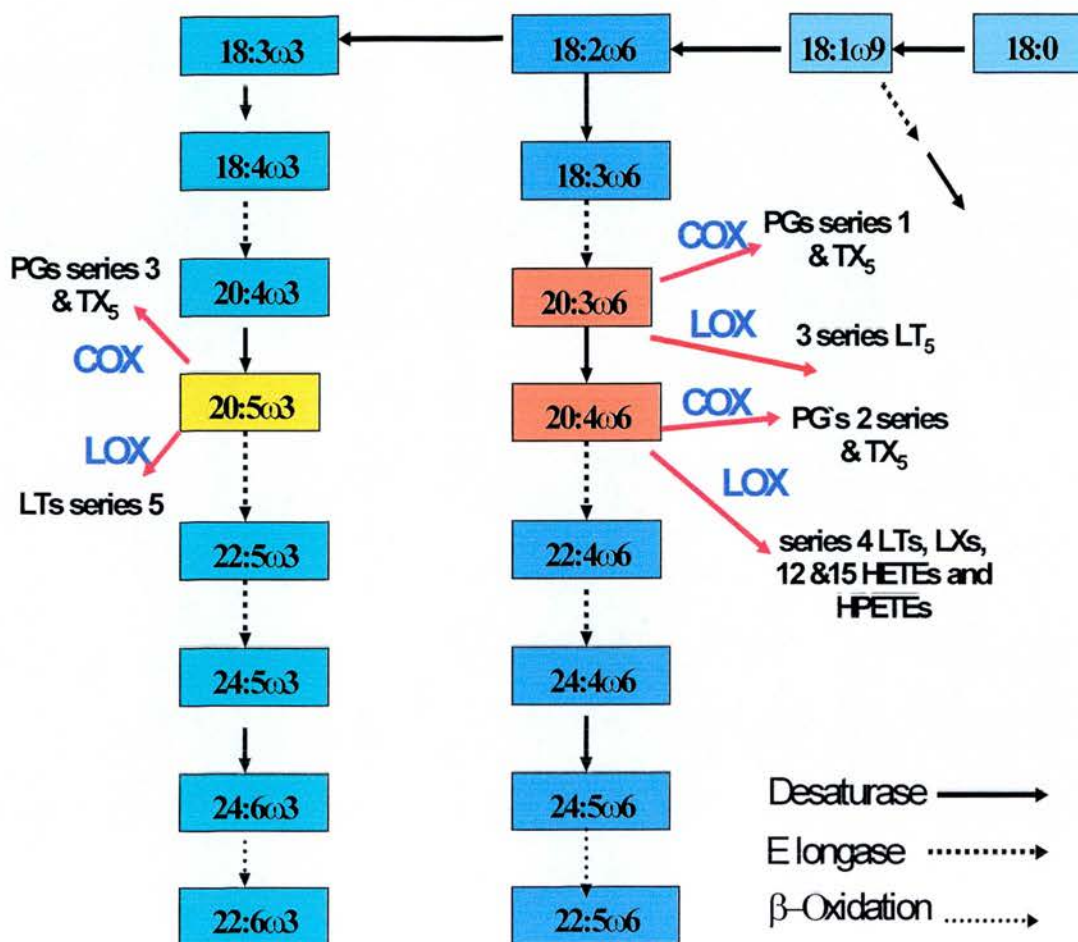


Figure 7 Structure of Arachidonic acid

The short form for fatty acid formulae with double bonds is e.g. for linoleic acid 18:2 ω -6. It means that linoleic acid consist of 18 carbon atoms with two double bonds where ω -6 defines the last double bond at position 6 counting from methyl end of carbon chain. The following double bonds continue with 3 C atom distance, and are found for linoleic acid on positions 9 and 12.

Some fatty acids are essential for the body such as linoleic acid (18:2 ω -6) and eicosapentaenoic acid (EPA) (20:5 ω -3). The latter originates out of the ω -3 fatty acid group. Omega 3 fatty acids occur as docosapentaenoic acid (22:5 ω -3) and docosahexaenoic acid (DHA) (22:6 ω -3), which are only found in high concentrations in fish living in cold water. This high concentration in fish originates out of the food chain where α -linolenic acid (18:3 ω -3) acts as a precursor of these ω -3 fatty acids. Alpha-linolenic acid is found in canola-, flaxseed- and soya oil, but can be metabolised in humans only in low amounts because of the lack of sufficient enzymes.

Omega-6 fatty acids are mostly found in the food in form of linoleic acid (18:2 ω -6). Linoleic acid (18:2 ω -6) is metabolised via reduction and elongation into γ -linolenic acid (GLA) (18:3 ω -6), dihomo- γ -linolenic acid (DGLA) (20:3 ω -6) and eicosatetraenoic acid (20:4 ω -6), which is also known as arachidonic acid (AA). Figure 8 depicts FA metabolism for ω -3 and ω -6 fatty acids.



COX: Cyclooxygenase
 LOX: Lipoxygenase

Figure 8 Polyunsaturated Fatty acid metabolism

2.3.1. Eicosanoids and Fatty acids

Eicosanoid is the generic term of a group of short-lived lipid-derived mediators, which modulates hormone and other biochemical signals. These are generated in different tissues and cells and are derivative of polyunsaturated C 20 fatty acids. The name originates from the Greek word *eicosa* meaning twenty (Krause and DuBois, 2000) (Alexander, 1998). These metabolites are released out of the lipid cell membrane, especially out of arachidonic acid (AA) (20:4 ω -6), which is esterified in all membrane phospholipids. Arachidonic acid is a major eicosanoid precursor. To this mediator group belongs the prostanoids: prostaglandins (**PG**) which occur in the form of PGE₂, PGD₂, PGF_{2a}, prostacycline (**PGI**), thromboxane (**TXA**) and leukotriene (**LT**) as LTA₄, LTB₄, LTC₄, LTD₄ and LTE₄. AA is incorporated into the cell membrane preferably into phospholipid phosphatidylinositol. At least three enzymatic pathways can release AA out of cellular phospholipids. (a) Direct action by phospholipase A₂ (PLA₂), (b) combined action of phospholipase C (PLC) and diacylglycerol (DAG) lipase or PLC, DAG kinase and PLA₂; and (c) combined action of phospholipase D (PLD) and PLA₂. This pathway is schematically shown in Figure 10 A. There is evidence that an AA specific PLA₂ is responsible for AA release from membrane, which leads to eicosanoid formation. (Yang, 1996). After AA is released from the lipid bilayer it can be metabolised via three enzymatic pathways: the cyclooxygenase (COX) (Figure 10 B), lipoxygenase (LOX) (Figure 11) and finally the cytochrome P450 monooxygenase pathway (Krause and DuBois, 2000). A nonenzymatic pathway is capable of controlling AA by a free radical-catalysed peroxidation into F₂-isoprostanes. These products are produced in significant amounts following oxidative stress for, for example associated with liver injury. (Awad et al., 1996). The cyclooxygenase enzyme (COX) or so-called prostaglandin H synthase (PGHS; also known as prostaglandin endoperoxide synthetase) (Kulmacz, 1998) occurs in two isoforms of COX-1 and COX-2, where both isoforms possess cyclooxygenase and peroxidase activities, but it has also been suggested that these isoforms have unique and separate physiological functions (Garavito and DeWitt, 1999). Both cyclooxygenase enzymes are glycosylated, integral

membrane proteins, found in the endoplasmatic reticulum (ER) and COX-2 also is found in the nuclear envelope. These have a molecular mass of 67-72 kDa and exist as homodimers. COX-2 and COX-1 have a sequence identity of 60-65% within a species. The major differences in the primary structure are that COX-2 has a truncated signal peptide and an 18-amino acid C terminal insertion. COX-1 is constitutively expressed in most tissues and is often referred to as 'housekeeping enzyme', that helps maintain homeostasis. COX-2 is only expressed in a few tissues, notably brain and kidney. Expression is induced and increased at sites of inflammation, in stimulated macrophages, fibroblast synoviocytes and is rapidly induced by inflammatory mediators such as IL1 β and LPS. On the other hand the anti-inflammatory cytokines such as IL4, IL12 and glucocorticoids repress the expression of COX-2, but not the expression of COX-1. (Garavito and DeWitt, 1999). The three-enzymatic pathways result in different metabolic end products, targeting specific prostaglandin receptors and activating receptor specific signalling pathways, which is schematically shown in Figure 9 (Austin and Funk, 1999) (Krause and DuBois, 2000). Therefore, the availability of AA in membrane lipids, the activity of enzymes such as PLA₂ and PLC and activity of COX and LOX pathways determine the amount of synthesized eicosanoid mediators. (Alexander, 1998).

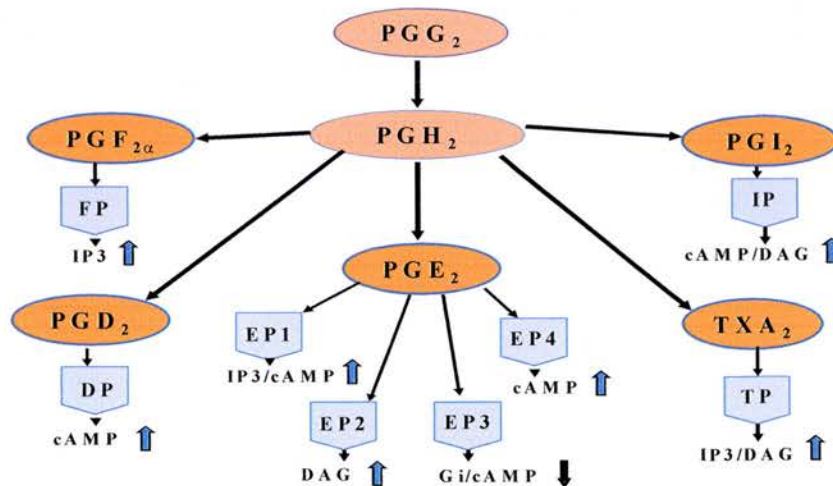


Figure 9 Prostaglandins of COX pathway with specific receptors and signal route

Cyclooxygenase pathway: The conversion of AA starts at the COX active site and a bicyclic peroxide intermediate, PGG₂ is formed (Marnett, 2000). This is followed by peroxidation of PGG₂ to diffusible prostaglandin H₂ (PGH₂), a common substrate or intermediate in the prostaglandin synthesis (Krause and DuBois, 2000). From the unstable PGH₂ form originates the prostaglandin derivatives PGE₂, PGF_{2α}, PGI₂ or prostacyclin (PI) and thromboxane A₂ (TXA₂) through their specific PG synthases (PGE-synthase, PGF-synthase, PGI-synthase and TXA-synthase) (Figure 10 B). These eicosanoid-derived mediators belong to the PG of series 2 and are descendents of AA. Prostaglandin's of series 1 are descendents of dihomo- γ -linolenic acid (DGLA) (systematic name eicosatrienoic acid), which all belongs to the ω -6 fatty acids. Prostaglandins PG₃, TXA₃ and PGI₃ of series 3 are descendents of eicosapentaenoic acid (EPA) and therefore of ω -3 fatty acids (Alexander, 1998). Figure 8 again displays these eicosanoid derived mediators within the PUFA metabolism process.

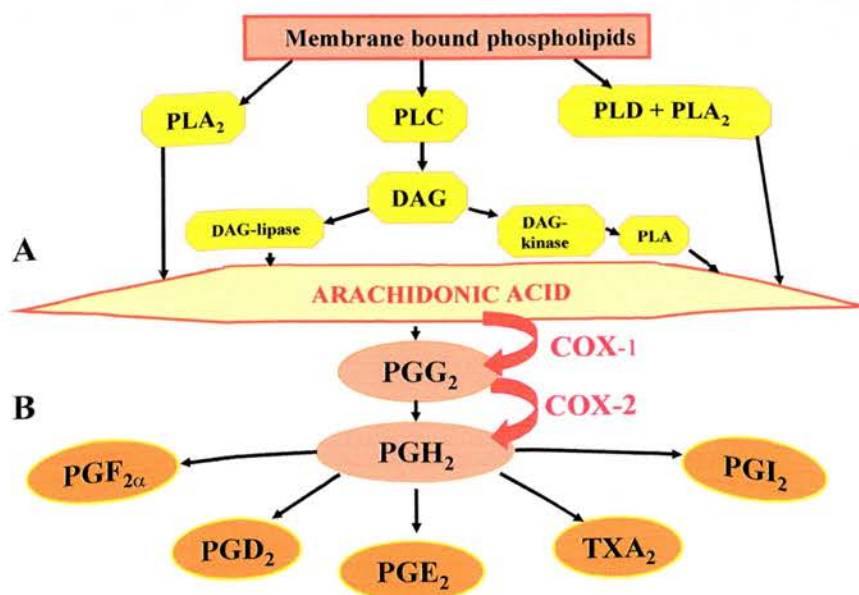


Figure 10 Cyclooxygenase pathway

Lipoxygenase pathway: The lipoxygenase enzyme is present as 12-lipoxygenase, 15-lipoxygenase and 5-lipoxygenase, named for their ability to add molecular oxygen at a specific carbon of AA. 5-lipoxygenase plays a central role in the biosynthesis of leukotrienes, which constitute an important class of inflammatory mediators.

The enzyme 5-lipoxygenase catalyse the conversion of AA to products 5-HETE (5-hydroxy-6, 8,11,14-eicosatetraenoic acid) and leukotriene A₄ (LTA₄) from AA via the metabolic intermediate 5-HPETE (5-hydroperoxyeicosatetraenoic acid). The eicosanoid LTA₄ of series 4 is hydrolysed into leukotriene B₄ (LTB₄). LTA₄ can be combined with the tripeptide glutathione into leukotriene C₄. Cleaving the glutamyl –residue leads to leukotriene D₄ (LTD₄), which changes after elimination of glyceryl into leukotriene E₄ (LTE₄). The leukotriene (LT) of the lipoxygenase pathway for eicosapentaenoic acid (ω -3) gives rise to LT of series 5 (LT₅) (Yang, 1996). This lipoxygenase pathway is illustrated in Figure 11.

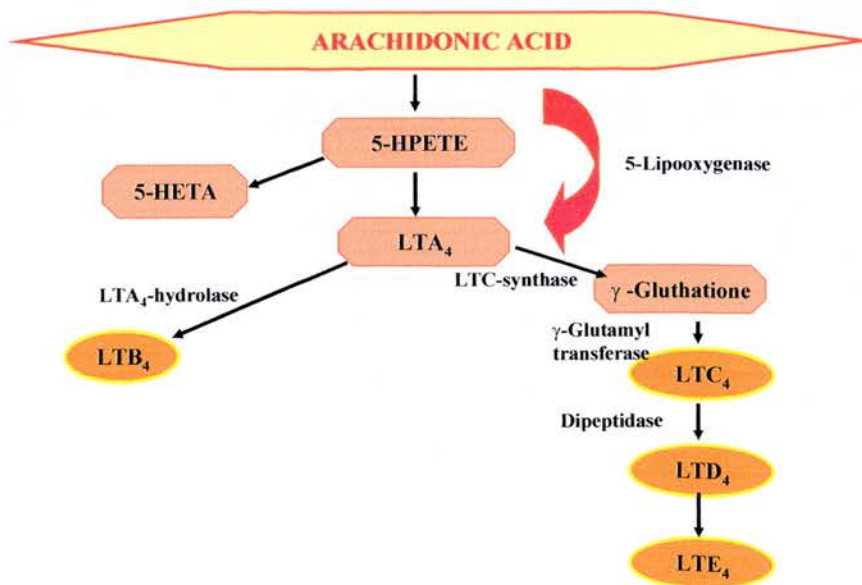


Figure 11 Lipoxygenase pathway

Eicosanoids have a variety of modulating influences, especially on cell signal transmission. The biological activity of eicosanoids depends on the type of fatty acids from which these are released. The details are given in Figure 8 and Figure 12.

Therefore eicosanoids released from AA have different biological functions and liberate eicosanoids preferentially of series 2, known as more inflammatory than those released from dihomo- γ -linolenic acid (DGLA or eicosatrienoic acid ω -6) or eicosapentaenoic acid (ω -3) of series 1&3 and series 3 respectively. Omega-3 poly-unsaturated fatty acids especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are major PUFAs incorporated into the cell membrane. Eicosapentaenoic acid is also a substrate for the cyclooxygenase and lipoxygenase, but not DHA, and can compete as a substrate with AA to induce the production of the less inflammatory eicosanoids of series 3 by COX- pathway. In general, eicosanoids originating from ω -3 fatty acids have a reduced biological influence on evoking cellular response compared with eicosanoids derived from AA. Therefore intake of fish oil, which is high in ω -3 PUFAs, will increase PUFAs and decrease AA membrane levels. This will enforce the production of eicosanoids derived from eicosapentaenoic acid of PG series 3 and LT series 5, respectively (Hillier et al., 1991) as shown in Figure 12.

Aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) interfere with the COX enzymes by inhibiting both COX-1 and COX-2 enzyme activity. The active site of both enzymes appears to be at the end of a long, tubular channel in the molecule, with COX-2 having a site branch to the channel. Aspirin and NSAIDs block the opening of this channel to the entrance of arachidonate. Aspirin does this by irreversibly acetylating a specific serine molecule. NSAIDs block the channel by reversible binding. When these channels are blocked by aspirin or NSAIDs, both COX-1 and COX-2 are unable to convert arachidonate to prostaglandine H₂, the necessary precursor of other prostaglandins and thromboxane (Bjorkman, 1999).

functions, eg. the light and dark adaptation in the visual cycle. Binding of the α -unit to an ion channel opens the channel for potassium or calcium ions. The activation of PLC by the α -unit is important. PLC hydrolyses membrane lipids, such as phosphatidylinositol, which is phosphorylated by ATP to phosphatidylinositol-4-phosphate (PI4P) and phosphatidylinositol-5,4-bis-phosphate (PI(2,4,5)P₃). The substrate for PLC is therefore PI(2,4,5)P₃ which is cleaved to inositol-1,4,5-tri-phosphate (IP₃) and diacyl glycerol (DAG). Both products have a second messenger function. The hydrophilic IP₃ provokes the endoplasmic reticulum (ER) to liberate calcium ions. The lipophilic DAG stays in the membrane and activates protein kinase C (Silbernagel A and Despopoulos A, 1991). Figure 13 is a simple scheme of signal activation by G-protein receptor (A) and enzyme-linked receptor (B).

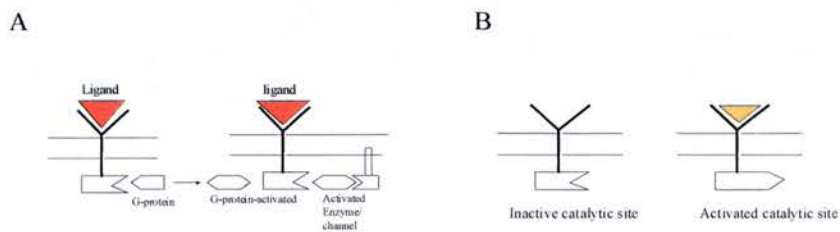


Figure 13 (A) Signalling via G protein coupled receptor and (B) enzyme linked receptors

2.3.3. Lipid derived mediators in inflammatory bowel disease

Fatty acids have a wide range of biological roles and cellular functions. These act as integral components in the phospholipid layer of cellular membranes, affecting membrane fluidity and lipid-protein interactions. Fatty acids can affect transport proteins and cellular receptors for hormones and neurotransmitters, act as substrate for eicosanoid synthesis, affect the production of biologically active compounds such as platelet-activation factor (PAF) and cytokines. These can affect the expression of genes encoding for enzymes involved in lipid metabolism and can interact with nuclear receptor proteins that bind to DNA, where in turn fatty acids can alter the transcription of regulatory proteins. The following paragraphs will therefore focus on the importance of fatty acids in respect of their influence and modulator properties of lipid mediators, cytokine production and clinical applications with special relevance to IBD.

Omega-6 Arachidonic acid (AA) is metabolised via cyclooxygenase and lipoxygenase pathway leading to eicosanoids of series 2 i.e. PGE₂, PGI₂, TXA₂, TXB₂ or series 4 LTB₄ or LTA₄, known as having pro-inflammatory properties. Eicosanoids derived from ω -3 fatty acids EPA or DHA have less inflammatory activities.

IBD has been shown to increase AA levels in phospholipids in colonic mucosa compared with normal controls. The fatty acid profile for palmitic (C16), stearic (C18), oleic (C18: 1) and linoleic acid (C18: 2) showed no differences between IBD and normal controls, but tended to be lower in IBD for C18: 1 (Pacheco et al., 1987). Nielsen *et al* (Nielsen et al., 1987) confirmed an increased liberation of LTB₄ in IBD. Measurement of eicosanoids using a filter paper method in rectal mucosa of UC patients confirmed that in addition to a significant increase in pro-inflammatory cytokines, there was also a significant increase of TXB₂ and PGE₂ correlated with disease activity (Carty et al., 2000). Figure 14 depicts the interplay of inflammatory mediators, namely eicosanoids, cytokines and oxygen radicals, influenced by amino acids, nucleotides, lipids and trace elements.

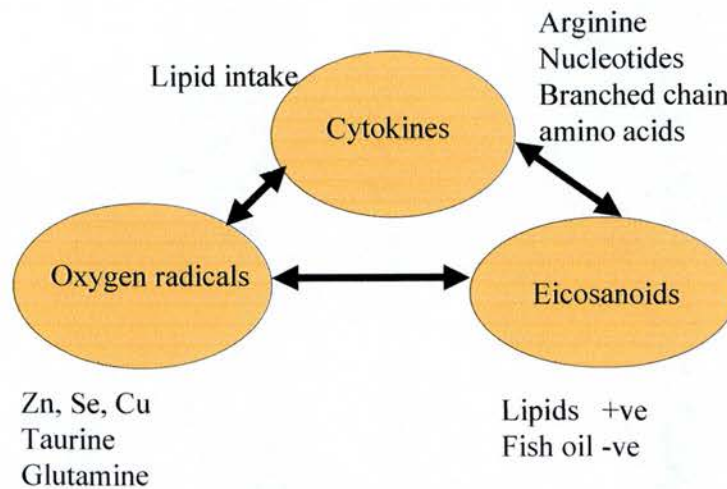


Figure 14 Mediators of inflammatory response
Cytokines, eicosanoids and oxygen radicals play key roles, are inter-related and may be modulated by nutrient intake.

2.3.4. Immunomodulatory effects of fatty acids

Omega 3 and omega 6 fatty acids are fatty acids of special importance, as these can alter cell membrane structure in respect of fluidity, rotation, formation of receptors, binding of ligands to their receptors and therefore activation of intracellular signal pathways. After stimulation and release of fatty acids out of membrane phospholipids, where PUFA are usually bound at the second carbon of glycerol, PUFA of ω -3 and ω -6 are stepwise saturated and elongated resulting in lipid derived mediators, eicosanoids, (Alexander, 1998), as previously shown in Figure 12. Inflammation results from the release of inflammatory mediators, predominantly released from activated leukocytes that migrate into the target area. To this key inflammatory mediator group belong eicosanoids derived from ω -6 fatty acid arachidonic acid. The eicosanoid PGE₂ released

from AA is known to have pro-inflammatory effects. Additionally prostaglandins and other eicosanoids have a role in regulating differentiation and functions of T cells, B cells, natural killer cells and macrophages. However, eicosanoids freed from EPA do not always have the same biologic properties as the analogues produced from AA. LTB₅ is markedly less chemotactic than LTB₄ towards human neutrophils (Calder, 1998). PGE₂ can inhibit leukocyte migration and activity. Fatty acids and their mediators can regulate expression of adhesion proteins, such as integrins and selectins in leukocytes and endothelial cells (Pompeia et al., 2000).

Each prostaglandin has its own specific receptor with characteristics of the G-protein receptor family, containing seven membrane-spanning domains. Eicosanoids act as intracellular signal mediators by increasing or decreasing signal enzymes such as phosphatidylinositol 3-phosphate (PIP₃), cyclic adenosine monophosphate (cAMP), (Krause and DuBois, 2000; Alexander, 1998) as illustrated previously in Figure 9. Eicosanoids can alter intracellular signalling. PGE₂ significantly increases intracellular cAMP levels, preventing an increase in intracellular calcium as an early event in T-cell activation. Eicosanoids change cytokine production as PGE₁ and PGE₂ are potent inhibitors of IL2 and IL2 receptor production and can modulate protein kinase C (PKC) activation. Eicosanoid LTB₄ enhances generation of IFN- γ and IL2 (Alexander, 1998).

The biological effects of eicosanoids differ depending on which of the ω -3 and ω -6 fatty acids it is derived from. Generally eicosanoids that are derived from ω -3 fatty acids are known to have less inflammatory effects. Fatty acids can modulate cytokine production through the effect of plasma membrane modulation. It is possible that fluid changes can alter G-protein activity, which in turn changes adenylate cyclase, phospholipase A₂ (PLA₂) and phospholipase C (PLC) activity. This would lead to a rise in cAMP, prostaglandin, leukotriene and DAG production, which again can modulate cytokine production having pro- and anti-inflammatory effects (Grimble and Tappia, 1998).

The released eicosanoids leave the cell and act locally in an autocrine or paracrine manner on cell surface receptors, which are linked, to G-proteins. Again, activation of G-proteins leads to changes in intracellular cAMP or calcium levels. These changes

serve as second messengers, activating signalling mechanisms, which can further affect transcription factor functions, such as NF- κ B. This acts through the presence of the Peroxisome-Proliferator Activator Receptor (PPAR), which is involved in lipid transport and metabolism similar to acylCoA. PPARs are divided into three subfamilies of α , γ , δ . Whereas PPAR α is expressed in hepatocytes, cardiomyocytes, renal proximal tubule cells and enterocytes, PPAR γ is expressed in adipose tissue, spleen, retina, hematopoietic cells, endothelial cells of the colon, prostate and mammary gland. It is reported that eicosanoids binding to PPAR γ can target gene expression either through plasma membrane G-proteins or through nuclear receptors (Jump and Clarke, 1999).

The mechanism by which PUFA may influence lipid mediator and cytokine production has been investigated in several studies. The ratio of ω -3 to ω -6 fatty acids may suppress eicosanoid biosynthesis from AA, suggesting that the ratio of ω -3 to ω -6 seems to be the determining factor in inhibiting eicosanoid synthesis rather than the amount of ω -3 fatty acids in the diet (Boidreau et al., 1991).

Furthermore, it might be possible, that after signal transduction, ω -3 fatty acids could intracellularly influence immune response by altering the level of phospholipid derived second messengers, such as DAG and ceramide. A disturbance of normal signalling in the T cell could result in a failure of NF- κ B or NF-AT (nuclear factor-activated T cells), which are needed for cytokine production such as IL2. It was reported that in ω -3 supplemented subjects a possible disturbance at the transcription level could also happen, such as a failure to secrete active IL2 or a failure in IL2 receptor binding (McMurray et al., 2000). In a study by Wallace *et al* (Wallace et al., 1999b) there was decreased lymphocyte proliferation and IL2 production measured in splenic lymphocyte supernatant of mice fed with olive oil, safflower oil and fish oil compared with coconut oil rich diet. IFN- γ production was reduced by safflower or fish oil feeding compared with coconut or olive oil rich diets, showing that PUFA decreased Th1 lymphocyte response and did not markedly influence Th2 response. A further fatty acid feeding experiment with mice demonstrated that after feeding with EPA and DHA there was a decrease in AA content in total spleen leukocyte lipids compared with beef tallow fed group. In addition it was demonstrated that DHA had a greater ability to suppress IL1 β

RNA induction in LPS stimulated mouse spleen lymphocytes compared with EPA (Watanabe S. et al., 2000).

How ω -3 fatty acids may alter T cell function is not known, although some mechanisms have been proposed, such as (McMurray et al., 2000)

- (a) Altered accessory cell functions for antigen presentation
- (b) Co-stimulation of PGE₂ production
- (c) An imbalance of T-cell subsets of CD4 vs. CD8, Th1 vs. Th2, naïve vs. memory cells
- (d) A change in T-cell membrane structure
- (e) Modification of intracellular signalling and post-translational events.

Further the following observations were made in a mouse model fed short term with purified EPA and DHA:

- (a) Reduction in mitogen lymphoproliferation (induced by concanavalin A), IL2 secretion and IL2 receptor α -chain mRNA expression.
- (b) Dietary effects seem to be exerted directly on the T-cells themselves as opposed to indirect effects through assessor cells (dendritic cell, macrophages).
- (c) Diet did not seem to induce significant alterations in relative proportions of CD4 and CD8 T-cells.
- (d) Reduction in important intracellular second messengers such as DAG and ceramide.
- (e) Dietary effects on T-cell activation may depend on the precise nature of the agonist (McMurray et al., 2000).

The potent anti-inflammatory influence of PUFA has attracted attention in several diseases as discussed later. PUFA have potential as anti-atherogenic agents.

Inflammation and arteriosclerosis share the same basic mechanism involved in leukocyte adhesion to vascular endothelium in early phase. The actual modulation of this endothelial activation is not known. Regarding structural requirements for inhibition of cytokine induced endothelial activation, long chain saturated fatty acids inhibited cytokine induced endothelial adhesion molecule expression, whereas unsaturated fatty acids inhibited adhesion molecules. In a study by de Caterina *et al* (De Caterina et al., 1998). DHA inhibited endothelial activation most potently. In addition

they showed that degree of unsaturation but not chain length influenced inhibition of cytokine induced endothelial activation, which was independent of *cis* or *trans* fatty acid conformation. The same group of de Caterina *et al* (De Caterina et al., 1999) investigated the inhibitory influence of PUFA on endothelial activation. They showed that, (a) saturated fatty acids are inactive; (b) potency of polyunsaturated fatty acid increases with degree of unsaturation; (c) potency does not depend on chain length; (d) the single double bond present in the monounsaturated fatty acid oleic acid is indeed sufficient to produce all the effects obtainable with higher unsaturated FA, albeit at higher concentrations; (e) for such an effect to occur even the configuration (*cis* vs. *trans*) of the double bond does not really matter. Inhibition of NF- κ B could also be reproduced upon incubation of endothelial cells with oleic acid. Fatty acids containing more double bonds in the membrane lipid bilayer lead to diminished activation of the NF κ B system in response to cytokines sufficient to reduce the subsequent start of transcription of genes encoding for endothelial leukocyte adhesion molecules. One possible explanation relates to the intracellular mediators of NF κ B activation, namely reactive oxygen species formed through the activation of NADH or NADPH oxidase after cytokine activation (De Caterina et al., 2000).

2.3.5. Clinical Implications of PUFA

The main focus of attention has been on fish oil therapy and its beneficial effects in UC (Ross, 1993; Aslan and Triadafilopoulos, 1992; Hawthorne et al., 1992; Lorenz et al., 1989) and to a lesser extent in CD (Kim, 1996). It has been shown that ω -3 PUFA may exert protective effects in some common cancers such as breast cancer, colon cancer and prostate cancer (Rose and Connolly, 1999).

Although oleic acid is not a PUFA, this monounsaturated fatty acid (MUFA) has attracted attention due to the low prevalence of arteriosclerosis and cardiovascular diseases in Mediterranean populations, known for high consumption of olive oil. In a study by Yaqoob *et al* (Yaqoob et al., 1998) there was a significant decrease of

intracellular adhesion molecule 1 expression after consumption of a diet high in MUFA.

In other inflammatory disorders such as rheumatoid arthritis, clinical improvement was found after 12 weeks of 3g EPA and DHA along with a reduction in the inflammatory mediator release of LTB_4 from stimulated neutrophils and IL1 from monocytes (Kremer, 2000). Belch and Hill (Belch and Hill, 2000) summarised the positive effects of essential fatty acids in the form of increased intake of di-homo- γ -linolenic acid (DGLA) in rheumatologic conditions. Intake of DGLA shifts the production into release of the less inflammatory PGE_1 eicosanoids. Similar effects were also reported in the inflammatory skin condition psoriasis (Ziboh et al., 2000; Burton, 1989).

These studies indicate that nutritional modulation of fatty acid composition, especially of ω -3 and ω -6 can modulate and influence the inflammatory mediator production. This can take place through increased incorporation of these fatty acids into the plasma lipid membrane and thereby acting as substrate for the less inflammatory mediator production derived from ω -3 FA, or through a change of the enzymatic pathway into production of PGs of series 1 as in the case of DGLA. The advantage of dietary intake of ω -3 fatty acids is that because these fatty acids do not have to go through the processes of elongation and saturation these can therefore immediately be incorporated into plasma membrane.

2.3.6. Occurrence of PUFA in the Diet

PUFA are important fatty acids in the diet of the young and old as well of the healthy and diseased subjects. The first oral contact the human has with PUFA is in breast milk of the suckling new born. Several studies have shown the importance of both ω -3 and ω -6 PUFA in term and preterm infants in visual development, mental development and brain development via a balanced supply of ω -3 DHA and ω -6 AA (Innis, 2000).

The mammal is able to synthesise fatty acids but is not able to incorporate a double

bond after carbon 9 (C9). Such fatty acids are therefore essential and must be taken in the diet. The two most important fatty acids are linoleic acid (C18:2 ω -6) with a double bond at C9 and C12 and α -linolenic acid (C18:3 ω -3) with a double bond at C9, C12 and C15. The human body can produce more polyunsaturated fatty acids by elongation and saturation of these two precursors. Further, these fatty acid precursors can be replaced if higher homologues of these fatty acids are supplied with the diet.

Polyunsaturated fatty acids and their different higher homologues of ω -3 and ω -6 PUFA can be found in animal and plant products. Fish are known for their high content of ω -3 fatty acids, mainly EPA, C20:5 ω -3 and DHA, C22:6 ω -3. Fish, which are rich in fat with a fatty acid pattern high in ω -3 FA are salmon, tuna, mackerel, kippers, trout, sardines but not eel, though eel is known as a fatty fish. It is also important to note that fish can have differences in fatty acid profile, depending on whether the fish are harvested naturally or are farmed.

Oil producing seed plants are rich in PUFA. The ω -3 fatty acid in plants is α -linolenic, whose main source is linseed oil, rapeseed oil, soya oil and walnut oil. Other sources for α -linolenic acid are from grass-fed animals (beef) and green leafy vegetables (spinach) (Conference Report, 2000). Oil seed plants such as sesame, soya, safflower, corn, sunflower and wheat germ oils are also major sources of PUFAs. Alpha-linolenic acid (LNA) alone is relatively ineffectual in raising plasma EPA levels. Supplementation of LNA (10g/d or more) alters cellular phospholipid DHA levels only minimally (de Deckere et al., 1998). Therefore it is important to supply fatty acids, especially ω -3 and ω -6 fatty acids in a well-balanced manner (Boidreau et al., 1991).

Present recommendations are a daily intake of PUFA containing ω -3 and ω -6 in a ratio of 1:4, based on activity equality which does not reflect biological reality (Whitney et al., 1998; Crawford, 2000). In a recent Workshop Meeting in Bethesda, USA expert recommendations with regard to adequate intake (AI) of ω -3 and ω -6 was established (Anonymous 2000). Summarised statements of the recommendations are as follows:

(a) Reduction of saturated and of *trans* fatty acids intake (chemically manufactured fats, main occurrence in bread spread e.g. margarine) with an upper limit for saturated fats of

less than 8 % of energy intake (Galli, 2000) (Simopoulos, 2000).

(b) Separate PUFA recommendations for linoleic acid (LA) (C18:2 ω -6), α -linolenic acid (LNA) (C18:3 ω -3) as well as for the highly unsaturated PUFA such as EPA (C20:5 ω -3), DPA (C22:5 ω -3) and DHA (C22:6 ω -3) present in fish.

(c) An agreement was reached that AI can be established in gm/day based on 2000kcal/day and percentage energy of LA and for LNA giving a ratio of LA:LNA of 3-2 : 1. No AI was given for arachidonic acid (AA), as there were no data available (Simopoulos, 2000). EPA and DHA intake should be at least 0.22gm/d or 0.1% of energy intake. (Simopoulos et al., 2000).

It must be stressed that these recommendations are based on an average adequate intake for a healthy adult of 2000 kilocalories/day (kcal/d). These data do not determine recommended dietary reference intakes (RDIs). Specific recommendations are available for pregnant or breast feeding woman as well as for compositions of infant formulae diets. As a consequence of the positive effects on immune system and inflammation, modulation of ω -3 and ω -6 fatty acid is reasonable and it may be probable that the intake of these PUFA should be increased in IBD. However definite recommendations are not available. This also depends on the individual dietary preferences and absorptive capacity of the IBD patients.

CHAPTER THREE

3. Medical management of inflammatory bowel disease

UC and CD is chronic, relapsing inflammatory disorders of the gastrointestinal tract of unknown aetiology. Both genetic factors and environmental triggers are involved in disease initiation, as mentioned in the previous chapter, while gut commensal bacterial flora is required for disease perpetuation. At present both diseases are medically incurable. Only UC, but not CD is surgically curable. However, development of pouchitis after colectomy and ileal-pouch anal anastomosis may be a recurrence of UC in the ileal mucosa of the pouch. The two diseases are distinct, based on risk factors, immunological features, genetic factors, histology and response to medical and surgical therapy (Ghosh et al., 2000), though undoubtedly they share a number of features in common too. As nutritional therapy forms only small component of the general management of IBD, this chapter reviews the available medical therapy of IBD.

3.1. Course of disease

A prospective study from Norway on 496 UC patients showed a cumulative 1-year relapse rate of 50% (Moum et al., 1997), with 11% of patients with relapses following a chronic relapsing course. In the same study 232 CD patients showed a cumulative 1-year relapse rate of 47%, and 10% of patients had a chronic relapsing course. In UC patients the risk of colectomy was significantly higher in patients with extensive colitis compared with left-sided colitis and patients younger than 50 years had a higher risk of relapse. In CD patients the risk of resectional surgery was significantly higher in patients with small bowel involvement compared with colonic involvement alone. In a recent survey, the outcome after steroid therapy in Olmsted County, Minnesota has been reported after the first course of steroids in IBD (Faubion et al., 2001). Out of 173

patients with CD, only 43% were ever treated with steroids resulting in complete remission in 58%, partial remission in 26% and no response in 16%. Out of 185 patients with UC, only 34% received steroids with complete remission in 54%, partial remission in 30% and no response in 16%. One-year outcome in CD was 32% prolonged response, 28% steroid dependence and 38% surgery while that in UC was 49% prolonged response, 22% steroid dependence and 29% surgery. The outcome data are in agreement with those reported previously by Munkholm *et al* (Munkholm et al., 1994) on a Scandinavian cohort of CD patients prescribed their first steroid course in whom 48% achieved complete clinical remission, 32% partial remission and 20% remained steroid refractory 30 days after commencement of therapy. Forty-six percent of patients in complete and 43% of patients in partial remission had relapsed when evaluated 30 days after cessation of treatment, suggesting steroid dependency. Therefore a standard course of steroid treatment in unselected patients with CD results in prolonged steroid response in 44% of the patients, steroid dependency in 36% and steroid resistance in 20%.

Different subgroups of patients may have different natural histories. Serum Anti-Neutrophil Cytoplasmic Antibody with perinuclear staining (pANCA) is associated with UC and with 10-15% of CD patients. On the other hand, CD patients are characterised by the presence of anti-*Saccharomyces cerevisiae* antibody (ASCA). The CD patients with UC like serology, i.e. positive pANCA and negative ASCA have a more UC like disease with less fibrosis or fistulisation and lower requirement for surgery (Hoffenberg et al., 1999; Vasiliasuskas et al., 2000) CD patients who express high titters of serum ASCA with negative pANCA have aggressive course of disease with fibrosis and fistulisation requiring surgery more frequently

3.2. Goals of therapy

Only colectomy in UC has cure of disease as a realistic goal. Generally, both UC and CD are characterised by episodes of clinical relapse caused by exacerbation of intestinal inflammation and treatment is aimed at reducing inflammation and subsequently maintaining remission. However clinical illness at a particular time is a composite of the effects of a number of variables and disease mechanisms. The endpoint of treatment of a relapse is symptomatic remission, but it has been demonstrated that many patients with clinically quiescent IBD have low grade mucosal inflammation as demonstrated by faecal excretion of ^{111}In labelled granulocytes (Saverymuttu, 1986) or by whole gut lavage fluid (WGLF) granulocyte elastase (Handy et al., 1996).

3.3. The strategies

The choice of therapy depends on severity of disease, the presence of complications, the site of macroscopic disease involvement and patient preferences. A multidisciplinary team consisting of physicians, surgeons, pharmacists, nurse practitioners, dieticians, psychologists and psychiatrists is required for optimum care of IBD patients. The usual strategy is to start with the least toxic drug appropriate to the clinical severity and step-up, though an alternative strategy to start with the most powerful and effective drug and step-down requires further evaluation as effective (but expensive) and safe biological agents are becoming available.

3.4. The armamentarium

The range of medical therapies available for management of IBD is widening and reviewed below, but appropriate timing and their use for the correct indication dictate success. Choice of these drugs depends on an understanding of their mechanism of

action, efficacy, side effects and long term safety profile. The patient needs to be involved in the decision regarding therapeutic choices. Appropriate and timely surgical interventions are crucial in managing complications and medically refractory disease.

3.4.1. 5-Aminosalicylates

The value of 5-aminosalicylate (5-ASA) therapy is different in UC and CD. While 5-ASA therapy is useful in UC to treat mild to moderate active inflammation and in maintenance of remission, the value of 5-ASA in CD is much more limited. The azo-bonded preparations (Sulfasalazine, olsalazine, balsalazide) rely on bacterial azo-reduction for release, whereas the enteric-coated and delayed release mesalamine preparations are dependent on luminal pH, intestinal transit time and gastric emptying. Although the newer 5-ASA preparations in a dose of at least 2 gm/day are more effective than placebo in the treatment of UC, a meta-analysis could not find enough evidence that these are clearly superior to sulfasalazine (Sutherland et al., 1993). There is some evidence to suggest that balsalazide 6.75g/day is more effective than mesalamine 2.4g/day in the treatment of acute UC (Green et al., 1998) (Sinha et al., 2001). Both balsalazide 3g/day and mesalamine 1.2g/day are equally effective as maintenance therapy, though balsalazide might control nocturnal symptoms somewhat better (Ewe et al., 1999). Sulfasalazine, the earliest available therapy for UC is associated with 20-40% incidence of adverse effects including nausea, anorexia, skin rashes, blood dyscrasias and male infertility. Most side effects are associated with the sulfapyridine, and therapeutic efficacy is ascribed to 5-ASA, so that expectedly, all 5-ASA preparations tend to be better tolerated, and are currently used by most gastroenterologist in preference to sulfasalazine. However, if a patient is well on sulfasalazine, there is no necessity to change to the newer 5-ASA preparations. In mildly or moderately active CD, sulfasalazine or 5-ASA might induce remission, but adequate doses (at least 3gm/day or more) must be used. The efficacy of 5-ASA in maintaining remission in inactive CD is controversial. A meta-analysis in 1994 suggested that 5-ASA significantly reduced the relapse frequency in patients with

inactive CD {Odds ratio (OR) 0.56, 95% CI 0.37-0.84 at 6 months; OR 0.47, 95% CI 0.33-0.67 at 12 months; OR 0.53, 95% CI 0.38-0.73 at 24 months) (Messori et al., 1994). In a further meta-analysis including 10 eligible trials (n=1022) on CD patients it was shown that mesalamine but not sulfasalazine therapy used for 1 year was effective in reducing the risk of relapse. (Steinhart et al., 1994). Further meta-analysis (15 RCTs of mesalamine maintenance in CD, n=2097) showed that the risk reduction of symptomatic relapse was significant in the post-surgical setting (pooled risk difference -13.1%; 95% CI -21.8% to -4.5%) but not medical setting (pooled risk difference -4.7%; 96% CI -9.6% to 2.8%) (Camma et al., 1997). Multivariate analysis predicted reduction of the risk of symptomatic relapse in postsurgical patients, in patients with ileitis and in patients with prolonged disease duration. There is considerable heterogeneity in the above studies, including site of disease and the drugs used to induce remission. The data suffers from post-hoc subgroup analysis and efficacy analysis (rather than intention to treat). Currently the data is not sufficient to support the routine use of 5-ASA in prevention of relapse in either the post-surgical or in medically induced remission in CD. The rather modest benefit has to be weighed against the inconvenience of taking a large number of tablets and the cost, and detailed discussion with a patient is necessary prior to institution of therapy. Even in the prevention of post-operative relapse, the benefit of 5-ASA is very modest, (Lochs et al., 2000) but this is the setting in which 5-ASA therapy is most often used in CD.

3.4.2. Corticosteroid therapy

Corticosteroid therapy provides the mainstay of therapy in acute severe UC and CD. In paediatric severe CD nutritional therapy is often a more popular first choice therapy. In severe UC hospitalisation and intravenous therapy is required. In moderately active UC oral corticosteroids may be used in an outpatient setting. Oral-controlled release budesonide has also been used in mild to moderately active UC with the advantage of little suppression of plasma cortisol levels (Lofberg et al., 1996). A pilot trial has also suggested that oral pH-modified-release formulation of budesonide may be used in

steroid dependent UC to reduce side effects (Keller et al., 1997). Corticosteroids induce remission in 70% of patients with active CD compared to approximately 30% on placebo (Summers et al., 1979) (Malchow H et al., 1984). In severely ill, hospitalised patients, intravenous therapy is appropriate. After adequate response in 5-7 days, oral prednisolone is commenced. For distal ileal or ileocolonic CD, budesonide, a locally active steroid with high first pass metabolism, is an alternative to prednisolone with remission rates of 52-60% in 8 weeks (Greenberg et al., 1996) (Rutgeerts et al., 1994) (Campieri et al., 1997) (Gross et al., 1996). A recent meta-analysis however confirmed that budesonide is significantly less effective than conventional steroids in inducing remission in active CD (pooled rate difference -8.5%; 95% CI -16.4 to -0.7%; p=0.02), but is associated with less steroid side effects (Papi et al., 2000). Controlled ileal release budesonide 9mg/day however was more effective than 4g/day of slow-release mesalamine in active CD after treatment for 8 weeks (Thomsen et al., 1998).

Corticosteroids are not effective in maintaining remission in IBD (Steinhart et al., 2000). This has to be distinguished from a small proportion of chronic active IBD patients who are steroid dependent and require a small dose to maintain remission. Budesonide (3mg or 6mg/day or placebo), with its lower incidence of side effects, was evaluated for maintenance of remission in ileal and ileocaecal CD. Though there was some prolongation of the time to relapse in the budesonide groups, the rates of relapse in all groups were similar by 1 year (Greenberg et al., 1996) (Lofberg et al., 1996). A further study of budesonide as maintenance treatment in CD was completely negative (Ferguson et al., 1998). A Cochrane review concluded that oral budesonide therapy at 6mg/day is not effective in preventing relapses of CD (Simms et al., 2001). Budesonide does not prevent endoscopic recurrence of CD after ileocaecal resection though a slight beneficial effect was noted in those who underwent resection due to uncontrolled inflammatory activity compared with those who underwent surgery due to fibro-stenotic disease (Hellers et al., 1999). The beneficial effect of budesonide in preventing post-operative recurrence of CD has been consistently shown to be very small and budesonide cannot be recommended for this indication (Ewe et al., 1999). This has been confirmed by a recent meta-analysis (Papi et al., 2000).

Though not effective in maintaining remission, controlled ileal release budesonide may be used in steroid dependent CD patients. In a double-blind multicenter trial, 120 steroid-dependent ileal or ileocolonic CD patients were randomised to 6mg/day of budesonide or placebo (Cortot et al., 2001). Prednisolone was tapered off during the first 4-10 weeks. Relapse rates in the budesonide group was 17% and 32% after one and thirteen weeks without prednisolone compared to 41% and 65% in the placebo group. Glucocorticosteroids side effects were reduced by 50% by switching from prednisolone, and were similar in the budesonide and placebo groups.

3.4.3. Immunosuppressive therapy

Steroid resistant patients are considered for immunomodulatory therapy. Prolonged corticosteroid therapy has unacceptable side effects. A significant proportion of patients who are steroid dependent should also be considered for immunomodulatory therapies unless the dose of steroids is low (<7.5 mg/day) and side effects are few. The immunomodulatory therapies used in IBD are azathioprine/6-mercaptopurine, methotrexate, cyclosporin, mycophenolate mofetil and biologicals such as infliximab.

3.4.3.1. Azathioprine

Azathioprine (AZA) is the pro-drug of 6-mercaptopurine (6MP). AZA is rapidly converted to 6MP by a non-enzymatic pathway dependent on glutathione and other sulphhydryl containing proteins. AZA is about 55% 6MP by weight, and since over three-quarters of AZA is converted to 6MP, 1mg of AZA is equivalent to 0.5 mg 6MP (Lennard, 1992). Bioavailability of these drugs is low (less than 20%) due to the extensive catabolism by xanthine oxidase found in enterocytes and hepatocytes (but not haemopoietic tissue (Lennard, 1998). 6MP can then be metabolised by 2 remaining pathways; either converted by thiopurine methyltransferase (TPMT) to an inactive form 6-methyl mercaptopurine (6MMP) or metabolised by hypoxanthine guanine phosphoribosyl transferase (HPRT) to its active metabolites 6-thioguanine nucleotides

(6TG) and 6-methylmercaptopurine ribonucleotides (6-MMPRs) (Elion GB, 1969) (Elion, 1989). The mode of action of 6MP rests on the incorporation of these thiopurine nucleotide metabolites into cellular nucleic acids and thus inhibiting *de novo* purine synthesis (Tidd DM and Paterson, 1974). These enzymatic pathways are distinct and competitive and thus a lack of enzymatic activity at one point will result in an excess product being produced from a competing pathway.

There is a paucity of good randomized, double blinded studies into the use of azathioprine and 6-mercaptopurine in IBD. Problems have included small numbers of patients, non-randomized trials, sub therapeutic doses, inadequate duration of treatment and differing concurrent drug therapy. In addition, trials in CD patients have generally been better represented than for similar trials in UC. Meta-analysis of the trials provides positive support for efficacy of azathioprine and 6MP in IBD patients. It should be noted that there have never been any direct comparative studies between azathioprine and 6-mercaptopurine – but it is reasonably assumed that both drugs have similar effects after taking into account obvious dose differences mentioned earlier.

In CD a few studies have addressed treatment in active disease exclusively (Ewe et al., 1993) (Klein et al., 1974) (Present et al., 1980) (Rhodes et al., 1971). These studies were quite heterogeneous in as far as their outcomes (steroid sparing effect, development of remission etc.) and study design (crossover vs. non-crossover), but all compared placebo with azathioprine. Rhodes *et al* found no difference in response, but the study was flawed by the short duration of therapy (7 weeks). All of the other studies found varying degrees of favourable response (odds ratio 1.0-10.45). They also found a steroid sparing effect. A meta-analysis was by Pearson *et al* (Pearson et al., 2000) included multiform studies that looked at active disease and steroid sparing effect. The estimated overall common odds ratio was 3.09 (95% CI, 2.45 to 3.91) in favour of azathioprine or 6-mercaptopurine therapy. Despite favourable results in an open study, a placebo-controlled trial of an intravenous loading dose of azathioprine (40mg/kg/36 hours) could show no decrease in time to response, despite achieving early higher 6-TG concentrations (Sandborn et al., 1999).

Two studies have looked exclusively at treatment with azathioprine in quiescent CD as maintenance therapy (O'Donoghue et al., 1978) (Rosenberg et al., 1975), while there have been several others that have included maintenance therapy as part of a multiarm study (Candy et al., 1995) (Summers et al., 1979) (Willoughby et al., 1971). Again, all of these exhibited varying degrees of benefit (odds ratios 1.20-4.48), and meta-analysis by Pearson *et al* estimated a common odds ratio of 2.27 (95% CI, 1.76 to 2.93) in favour of azathioprine therapy (Pearson et al., 2000).

In active UC the use of both azathioprine and 6MP in active colitis is limited due to the protracted time to maximal onset (approximately 3 months). There is no evidence that azathioprine plus steroids versus steroids alone increases remission rates, but there is evidence of a steroid sparing effect within this time frame (Adler and Korelitz, 1990) (Kirk and Lennard-Jones, 1982). Initial report of a pilot study using 3 regimens of intravenous azathioprine loading dose to reduce the time to response in UC is more promising (Mahadevan et al., 2000) than the results in CD.

As maintenance therapy azathioprine has been studied more extensively than 6MP in UC. There is good evidence that AZA/6MP allow steroid dose reduction, a conclusion found in several studies to date (AZA dose 1.5-2.5 mg/kg (Adler and Korelitz, 1990) (Caprilli et al., 1975) (Jewell and Truelove, 1974) (Kirk and Lennard-Jones, 1982) (Rosenberg et al., 1975). It should be noted that there is no clear evidence from studies to show that AZA or 6-MP are of benefit in the chronic active UC patient who is steroid resistant. Jewell *et al* studied such patients for 12 months and found no clear benefit (Jewell and Truelove, 1974), although uncontrolled data from others has shown beneficial effects (George et al., 1996). Further evidence can be drawn from withdrawal studies, which although unable to comment on the magnitude of the effect of AZA, have shown that AZA withdrawal during remission leads to increased relapse rates (Hawthorne et al., 1992). There are no controlled randomised trials that have looked exclusively at 6MP and maintenance therapy in UC. However, in clinical practice, both AZA and 6MP are widely used in chronic steroid resistant or steroid dependent ulcerative colitis.

3.4.3.2. Methotrexate

This has been used less frequently in IBD than AZA, and gastroenterologists are often wary of its side effects. MTX inhibits dihydrofolate reductase (DHFR) and its metabolites inhibit folate dependent enzymes downstream to DHFR. This results in impairment of purine production, inhibition of methionine production and local accumulation of adenosine. Theories of efficacy of MTX in IBD are based on inhibition of leukocyte proliferation, induction of apoptosis of activated T-cells, inhibition of pro-inflammatory cytokine production, decreased leukotriene B4 production, impaired immunoglobulin synthesis and local accumulation of adenosine. The efficacy of MTX in inducing remission and in steroid sparing for refractory CD was initially described in small, uncontrolled studies (Kozarek et al., 1989) (Baron et al., 1993). The evidence from randomised, controlled trials of the efficacy of oral methotrexate have been conflicting (Arora et al., 1999). However, parenteral methotrexate in a dose of 25 mg/week resulted in remission and steroid withdrawal in 39% of steroid dependent CD patients compared with 19% in the placebo group (Feagan et al., 1995). Methotrexate for the treatment 1995) A randomised, controlled comparison of methotrexate (15 mg/kg IM weekly) with placebo for the maintenance of remission in steroid refractory CD showed 65% remission rate for methotrexate compared with 39% for placebo over a 40 week study period ($p=0.04$) (Faubion et al., 2001). In addition, 28% in the methotrexate group Vs 58% in the placebo group used corticosteroids for relapse ($p=0.01$). As remission was induced in this study by the use of methotrexate initially, the maintenance trial had selected out a group of methotrexate responders who had tolerated the drug. A recent report of long term methotrexate use in 76 steroid-refractory CD patients (mean methotrexate duration 55 weeks; mean dose 20mg/week) suggests that improvement and remission were highest with parenteral methotrexate use and was dose dependent (Chong et al., 2001). Improvement and remission were more likely for younger patients under the age of 40. The steroid-sparing effect of methotrexate was apparent in 78% of patients with complete discontinuation achieved in 40% Intramuscular administration is painful and inconvenient and weekly subcutaneous administration may be more convenient, especially in children (Mack et al., 1998). 1mg

folic acid per day decreases both the stomatitis and gastrointestinal side effects without reducing drug efficacy. Folic acid supplementation may also prevent MTX-induced rise in plasma homocysteine and promotion of arterosclerosis. Lemann et al recently reported probabilities of relapse of 29%, 41% and 48% at 1,2 and 3 years in a cohort of CD patients maintained on methotrexate for a median of 18 months (Lemann et al., 2000).

3.4.3.3. Cyclosporine

Cyclosporine is a lipophilic cyclic peptide that interrupts the cellular immune response by blocking interleukin 2 production by T cells. Adding intravenous cyclosporine to corticosteroids has successfully treated steroid refractory cases of UC. Uncontrolled and open labelled studies have reported improvement with intravenous cyclosporine (Lichtiger and Present, 1990) (Lichtiger, 1990) (Santos et al., 1995) (Carbonnel et al., 1996). A small controlled trial has influenced clinical practice (Lichtiger et al., 1994). In this randomised, double blind controlled trial 4mg/kg of cyclosporine or placebo was administered by continuous infusion to 20 patients with severe UC, unresponsive to at least a week of intravenous corticosteroids. Nine of 11 patients (82%) treated with cyclosporine had a rapid response, as compared with 0 out of 9 patients who received placebo ($p < 0.001$). Restorative proctocolectomy after failure of rescue with intravenous cyclosporine is not associated with any apparent increase in complication rate, though this study had used a dose of 2mg/kg/day dose (Pinna-Pintor et al., 2000). A recent report has shown impressive efficacy of intravenous cyclosporin in steroid-refractory pyoderma gangrenosum in UC and CD (Friedman et al., 2001). Intravenous cyclosporine can also be used as first line therapy in severe UC. In a double blind, controlled trial, 30 severe UC patients were randomised to 4mg/kg/day of IV cyclosporine or 40mg/day of methylprednisolone. After 8 days, those responders received the same medication orally with azathioprine (D'Haens et al., 2001). After 8 days, 53% of methylprednisolone group and 64% of the cyclosporine group responded, but after 12 months 78% of those initially responding to cyclosporine maintained their remission compared with 37% of those responding to corticosteroids.

In CD, beneficial effect of short-term treatment using high dose cyclosporine (median 7.6 mg/kg) has been reported (Brynskov et al., 1989), but such doses have considerable toxicity. In a large randomised, double blind placebo controlled trial, a lower dose of oral cyclosporine (2.5mg/kg increased to 5mg/kg after 2 weeks and then dose adjusted to maintain whole blood trough concentration of 200ng/ml) was added to conventional but standardised treatment of CD patients whose disease had been active within the previous two years. The results were disappointing and addition of cyclosporine did not improve symptoms or reduce requirements for other forms of therapy (Feagan et al., 1994). Therefore long term treatment with cyclosporine is not recommended, though undoubtedly, some patients with severe Crohn's colitis refractory to steroid therapy may respond to intravenous cyclosporine, similar to severe UC.

Most experience in IBD is with the oil-based formulation, but the bioavailability of the microemulsion-based formulation is 20% higher, and the pharmacokinetic parameters of the latter preparation in IBD patients is broadly similar to those measured in healthy volunteers (Latterie et al., 2001). Oral microemulsion based formulation (5mg/kg/day) may be convenient and efficacious in severe UC refractory to steroids (Ortiz et al., 2000) and act as a steroid sparing agent (Actis et al., 1999) but randomised, controlled trials are essential.

3.4.3.4. Mycophenolate mofetil

Mycophenolate mofetil (MMF) is an ester prodrug of mycophenolic acid. It inhibits inosine monophosphate dehydrogenase and suppresses lymphocyte proliferation. It has been shown to be an effective immunosuppressive therapy in organ transplantation and in rheumatoid arthritis, pemphigus vulgaris and psoriasis. In a randomised controlled study of 70 patients with moderately active CD, treatment with MMF (15 mg/kg)/prednisolone (50 mg oral) led to a comparable reduction in disease activity to azathioprine (2.5 mg/kg)/prednisolone (50 mg oral). In patients with severe CD (CDAI >300) treatment with MMF/prednisolone resulted in significant suppression of disease

activity more rapidly than azathioprine/prednisolone. Treatment with MMF/prednisolone was associated with few adverse effects (Neurath et al., 1999). This randomised trial was over 6 months and in a further retrospective study open study azathioprine and MMF (25-35 mg/kg) were compared in CD over one year. The onset of action of MMF appeared to be shorter than azathioprine, but MMF patients had almost twice as many flare ups as azathioprine (80% vs. 47%) (Miehsler et al., 2001). Both drugs have steroid-sparing properties. MMF was prescribed to 15 patients intolerant of azathioprine and hence this study is not truly comparative.

3.4.3.5. Tacrolimus

Tacrolimus (or FK506) shares many of the pharmacological properties of cyclosporin but is 50-100 times more potent as an immunosuppressive. Most reports of the use of tacrolimus in IBD, both CD and UC are anecdotal. A particularly useful indication is low concentration (0.5mg/gm) topical therapy in oral and perianal CD. Such use appears to be effective in small, open series, with little systemic absorption (Casson et al., 2000) unlike oral tacrolimus in perineal fistulae. Oral tacrolimus is well absorbed in CD and may act as a rapidly acting immunomodulatory 'bridge' in complicated small bowel and fistulizing CD before 6-MP or methotrexate becomes effective (Sandborn, 1997). It may also be effective as monotherapy in CD refractory to steroids (Ierardi et al., 2001). In 13 children with severe UC or Crohn's colitis, 69% responded to oral tacrolimus 0.1mg/kg/dose given twice a day, but fewer than 50% achieved a long-term remission (Bousvaros et al., 2000).

3.5. Biological Therapy

3.5.1 TNF α inhibitors

Anti-TNF α therapy has been studied most. The murine chimeric monoclonal antibody infliximab has been shown to be effective in the treatment of moderate to severe active and fistulizing CD (van Dullemen et al., 1995) (Targan et al., 1997) (Present et al., 1999) (Rutgeerts et al., 1999) (Ricart et al., 1999). The principal use of infliximab is in treating patients not responding to conventional therapies or developing unacceptable side effects. A single infusion of infliximab resulted in impressive short-term response in a 12-week multicentre, double blind placebo controlled trial of infliximab (Targan et al., 1997). A dose of 5mg/kg was most effective. Longer term therapy using 10mg/kg given every 8 weeks for four infusions to initial responders to infliximab was effective in maintaining clinical benefit throughout the re-treatment period and 8 weeks after the last infusion (53% at week 44), but the placebo group had only 20% in remission at week 44 (Rutgeerts et al., 1999). Reports of the usefulness of infliximab in clinical practice outside trials confirm its efficacy and safety (Ricart E et al., 2001). Infliximab is steroid sparing, and in the Mayo clinic experience steroids could be discontinued in 73% of patients (Ricart E et al., 2001). Approximately one-third of patients receiving a second infusion of infliximab after not responding to a first dose may improve clinically. In a small open-label study of 15 children (mean age 13 years), a single 5mg/kg intravenous infusion of infliximab resulted in prolonged response in 3/6 patients with early disease, but in none with late disease (Kugathasan et al., 2000). There is increasing data on the short-term safety and efficacy of infliximab in paediatric IBD patients (Serrano et al., 2001). Reactivation of the mucosal and systemic immune system preceded clinical relapse. Patients who relapsed were characterised by a rise in TNF α secretion capacity and by increase of mucosal nuclear NF- κ B p65 before reactivation of clinical symptoms (Nikolaus et al., 2000).

Mouse/human chimeric antibodies such as infliximab induce formation of human anti-chimeric antibodies, anti-DNA antibodies leading in some cases to drug-induced lupus

and infusion reactions. Humanised monoclonal antibodies to TNF α such as CDP571 (95% human residues) is potentially less immunogenic than chimeric antibodies. A short-term placebo-controlled trial with a single infusion of infliximab 5mg/kg in refractory CD demonstrated beneficial effect on disease activity (Targan et al., 1997). This was followed up by a 24-week randomised placebo controlled trial in 169 patients. The patients were initially randomised to 10mg/kg or 20mg/kg of CDP571 or placebo. Subsequently patients were retreated with 10mg/kg CDP571 or placebo every 8 or 12 weeks (Sandborn et al., 2001). At week 2, clinical response occurred in 45% in the CDP571 group compared with 27% in the placebo group (p=0.02). There was no clear superiority of either of the two doses. Patients also appeared to benefit from re-treatment over 24 weeks, but not all secondary endpoints reached statistical significance. The frequency of anti-idiotypic antibodies was 7% and anti-DNA antibodies and antinuclear antibodies developed in 5%. No direct comparison between CDP571 and infliximab is available.

Etanercept is a fusion protein consisting of the p75 TNF (R2) receptor and the Fc portion of IgG₁. Though preliminary open trials have suggested efficacy, early results from randomised, controlled trials have been less promising.

Oral TNF α inhibitor therapy with oxpentifylline does not improve inflammation in CD (Bauditz et al., 1997). Thalidomide also has anti-TNF α properties and is discussed later.

The use of biological agents in UC has lagged behind CD. Subcutaneous injected interferon α -2A for 12 weeks was compared with 30 days of prednisolone enemas in 32 patients with active left sided UC (Madsen et al., 2001). This open labelled randomised study suggested improvement in clinical disease activity, IBDQ score and histological disease activity. The effect of interferon treatment in IBD remains conflicting and blinded, randomised controlled trials are required. In UC, anti-TNF α therapy with both infliximab and CDP571 is reported as promising in open trials (Evans et al., 1997), (Chey, 2001) but large randomised controlled trials are ongoing. Initial reports from a prematurely terminated randomised, double blind, placebo-controlled study (due to slow enrolment) suggested clinical benefit for some patients with steroid-refractory UC

(Sands et al., 2001). In patients with restorative panproctocolectomy for UC with ileal-pouch anal anastomosis who developed findings compatible with CD affecting the pouch, administration of infliximab proved effective after conventional therapy failed (Ricart et al., 1999)..

Biologicals targeted against other inflammatory regulators have been tried. Though a pilot trial of an antisense oligonucleotide directed against mRNA of intercellular adhesion molecule 1 (ICAM-1) was promising, a German multicentre, placebo-controlled trial of subcutaneous antisense ICAM-1 (ISIS-2302) was ineffective in achieving the primary endpoint of steroid free remission at week 14 (Schreiber et al., 2001). Further evaluation of ICAM-1 as a therapeutic target in CD is required.

IL10 inhibits effector functions of activated macrophages and monocytes and down regulates the production of pro-inflammatory cytokines. In a 24-week multicenter prospective, randomised, double blind study in 95 active CD patients were treated with subcutaneous, recombinant human IL10 (rhuIL-10: 1,5,10 or 20 $\mu\text{g}/\text{kg}$) or placebo once daily for 28 consecutive days. Intent-to-treat analysis showed that 23.5% of patients receiving 5 $\mu\text{g}/\text{kg}$ rhuIL-10 experienced clinical remission and endoscopic improvement compared with 0% of patients in the placebo group (Fedorak et al., 2000) rhu IL10 administered subcutaneously (4 $\mu\text{g}/\text{kg}$ once daily X 12 weeks or 8 $\mu\text{g}/\text{kg}$ twice weekly or placebo) within 2 weeks after ileal or ileocolonic resection did not prevent endoscopic recurrence of CD (Colombel et al., 2001). In a prospective, multicentre double blind, placebo-controlled trial on 329 therapy refractory CD patients, a tendency towards clinical improvement but not remission was observed in those receiving 8 $\mu\text{g}/\text{kg}$ dose (but not 1, 4 or 20 $\mu\text{g}/\text{kg}$ doses). Responders to rhu IL10 showed inhibition of NF- κB p65 activation (Schreiber et al., 2000).

3.5.1.1. Alpha-4 integrin antibody

α 4-integrins mediate leukocyte trafficking across the vascular endothelium in the intestine and administration of a recombinant humanised monoclonal antibody to α 4-integrins (Natalizumab) in patients with mild to moderately active CD resulted in elevated circulating lymphocytes and a modest decrease in CDAI at 2 weeks (Gordon et al., 2001). This pilot study used a rather low dose of natalizumab (3 mg/kg) and phase II trials with higher doses have been completed.

3.5.1.2. Other biologicals

A number of targets related to the intestinal immune response have been identified and in the next few years a number of biologically engineered molecules should be available to favourably manipulate intestinal inflammatory response. In an uncontrolled open trial, monoclonal anti-CD4 B-F5 antibody was not successful in severe CD, (Canva-Delcambre et al., 1996) though other phase I studies have showed potential efficacy. Sands *et al* (Sands et al., 1999) evaluated IL11 in a multicentre study on active CD patients and a higher rate of response was noted in the treatment group (16 μ g/kg dose) compared with the placebo group.

3.5.1.3. Combination immunomodulatory therapy

A combination of immunotherapy theoretically may have additional benefit, but controlled trials are few. After cyclosporin therapy in UC, it is common to introduce azathioprine/6-mercaptopurine in combination for a period before withdrawal of cyclosporin. Serious infections have been reported and co-trimoxazole prophylaxis for *Pneumocystis carinii* pneumonia is necessary. Concurrent immunosuppression with

azathioprine or methotrexate might be desirable when treatment with infliximab is instituted, both for increased efficacy (Rutgeerts et al., 1999) and for reduced adverse reactions to infliximab infusion. In the multicentre retreatment trial referred to above, 75% of patients receiving retreatment with infliximab who were on concurrent azathioprine/6 mercaptopurine had a clinical response at 44 weeks after 4 infusions, whereas only 50% not receiving concurrent immunosuppression had a clinical response, but the numbers were small and the difference was not statistically significant. Difficult perianal CD, however, may be effectively treated with a combination of oral tacrolimus and AZA/6-MP, though the side effects may be considerable and potentially serious (Lowry et al., 1999). Without further randomised-controlled trials, combination immunomodulatory therapy cannot be generally recommended in IBD with the possible exception of infliximab and AZA/6-MP. Cyclosporin induced remission of severe UC should also be followed up with AZA (D'Haens et al., 2001).

3.5.1.4. Probiotic therapies

Probiotics are living microorganisms that belong to the natural flora and are important to the health and well being of the host. Various strains of probiotics have different metabolic and immunology activities. Unlike virulent organisms, which may amplify inflammation, probiotics may downregulate inflammation in the intestinal mucosa. In two controlled studies, patients with UC were treated with oral mesalamine or capsules containing non-pathogenic *E.coli* as maintenance treatment and no difference in relapse rates were observed (Kruis et al., 1997). VSL#3 is a probiotic preparation containing 5×10^{11} /gm of viable lyophilised bacteria of 4 strains of lactobacilli, 3 strains of bifidobacteria and 1 strain of *Streptococcus salivarius* subsp. *Thermophilus*. In an open study, VSL#3 was effective in the prevention of UC relapsers who were intolerant of sulfasalazine or mesalazine (Venturi et al., 1999). In a randomised, double blind placebo controlled study on 40 patients with clinical and endoscopic remission of chronic pouchitis, all patients on placebo relapsed within 9 months of follow up period, while only 15% receiving VSL#3 had relapsed over the same period (Gionchetti et al., 2000b).

3.5.2. Antibiotic therapies

Though serological evidence of the presence of *Mycobacterium sp* in CD has been reported, reports of definitive detection of bacterial DNA by PCR from CD affected tissue have been conflicting (Green et al., 1989), (Chiba et al., 1998), (Cellier et al., 1998). The two reported clinical trials of antimycobacterial therapy in CD have produced conflicting results (Gui et al., 1997) (Thomas et al., 1998). A Cochrane review concluded that anti-tuberculous therapy might be effective in maintaining remission in patients with CD when remission has been induced with steroids combined with anti-tuberculous therapy. The results, however, which support this conclusion, come from a subgroup of only two trials with small numbers of patients and routine use of this therapy cannot be recommended on the basis of current evidence (Borgaonkar et al., 2000). Though many practising gastroenterologists would add an antibiotic such as metronidazole to an intravenous steroid regimen for severe UC, objective support for such practice is scant (Dickinson et al., 1985), (Chapman et al., 1986), (Burke et al., 1990), (Mantzaris et al., 1994). A recent prospective randomised double-blind placebo controlled trial addressed this question using ciprofloxacin. In a study including 55 severe UC patients treated with intravenous hydrocortisone, hydrocortisone enemas and TPN, 23/29 patients (79%) treated with intravenous ciprofloxacin and 20/26 patients (77%) treated with placebo showed substantial improvement and could be converted to oral steroids. Six patients in each group did not improve and 9/12 of these patients underwent colectomy (Mantzaris et al., 2001). On the basis of these trials, concurrent antibiotic therapy when treating severe UC patients with intravenous steroids cannot be supported.

3.5.3. Rectal Administration

Scintigraphic studies have shown that a suppository delivers the drug to the rectum, whereas a foam enema can reach the proximal sigmoid colon. A liquid enema can reach

as far as the splenic flexure. Patients find the foam enema more comfortable and easier to retain than liquid enemas. Distal UC involving the rectum or rectosigmoid can be effectively treated with topical corticosteroids or 5-ASA agents. Clinical remission can be achieved in two-thirds of patients, though some may require systemic therapy (Ardizzone and Porro, 1998). Conventional steroid enemas may suppress endogenous cortisol, but not budesonide enema that are also effective in treating distal colitis (Danielsson et al., 1987). Budesonide and prednisolone retention enemas have comparable efficacy in active distal UC (Lofberg et al., 1994). In left-sided UC and in proctitis, meta-analysis suggests topical mesalamine to be superior to topical steroids and oral therapies (Marshall and Irvine, 1997),(Cohen et al., 2000). Therefore rectal 5-ASA should be the first line therapy in distal UC, though sometimes it may be usefully combined with rectal steroids. Recently, a 60ml mesalazine gel enema has been reported to coat the colon as far as the splenic flexure (Gionchetti et al., 1997a). Though uncontrolled studies of short chain fatty acid (SCFA) enemas suggested clinical and endoscopic response (Patz et al., 1996), controlled trials have been conflicting (Scheppach, 1996), (Steinhart et al., 1996), (Vernia et al., 1995). Similarly, though cyclosporin enemas showed some response in left sided colitis in an open trial (Sandborn et al., 1993) a controlled trial by the same group failed to confirm efficacy (Sandborn et al., 1994). Sucralfate enemas in active distal colitis are of little value though recently conflicting results have been reported (Wright et al., 1999). The efficacy of lidocaine (Bjorck et al., 1992), ropivacaine (Arlander et al., 1996) and ecabet sodium (Kono et al., 2001) require confirmation by controlled trials. Nicotine tartrate liquid enemas may be better tolerated than systemic administration and a pilot study has some efficacy in left-sided UC unresponsive to first line therapy (Sandborn et al., 1997). Nicotine complexed with polyacrylic carbomer and administered as an enema is another formulation with predominantly local action (Green et al., 1997).

3.6. Other novel therapies

3.6.1. Heparin

Heparin belongs to the glycosaminoglycan (GAG) family of poly-anionic carbohydrates with variably sulphated disaccharide repeats. GAGs have diverse biological interactions affecting tissue architecture and function mediated by specific and non-specific ionic interactions. The anticoagulant effect, enhancement of mucosal repair and anti-inflammatory effects all contribute to its beneficial effects in UC (Papa et al., 2000). Most studies demonstrating beneficial effects of heparin are small and uncontrolled, but suggest that both unfractionated heparin (UH) and low molecular weight heparin (LMWH) might be effective in severe and even steroid resistant UC. However, the two small randomised controlled trials of unfractionated heparin vs. corticosteroids reported remarkably different results. Panes *et al* (Panes et al., 2000) had no remission in the UH group compared with 69% remission in the corticosteroid group; while Ang *et al* (Ang et al., 2000) had 75% remission in the UH group and 67% remission in the corticosteroid group. All patients in the latter study had received a 5-ASA compound. Heparin monotherapy as first line in severe UC in place of steroid therapy is inadvisable, but undoubtedly some steroid resistant patients may respond. Significant bleeding complications were noted in the Spanish study (Panes et al., 2000).

3.6.2. Nicotine

Interest in nicotine stemmed from the observational studies on the protective effects of smoking on UC. Beneficial effect of transdermal nicotine in UC may result from decreased IL-8 at the transcriptional level (Louvét et al., 1999). Though a number of trials have found nicotine patches effective in inducing remission in UC (Pullan et al., 1994), (Guslandi and Tittobello, 1996), (Sandborn et al., 1997) it is not as effective as corticosteroid (Thomas et al., 1996). Nicotine patches are not as effective as 5-ASA (Bonapace and Mays, 1997) and nicotine patches do not maintain remission in quiescent

UC (Thomas et al., 1995). Conflicting data has been reported in a small randomised controlled study in which patients with mild to moderate clinical relapses of left sided UC maintained on mesalamine 1 gm twice daily received additional treatment with transdermal nicotine or prednisolone for 5 weeks (Guslandi and Tittobello, 1998). During a 6-month follow up period, 20% of patients treated with nicotine relapsed, whereas 60% of patients treated with prednisolone relapsed ($p < 0.03$). Side effects such as headaches, tremor and sleeplessness are common with nicotine therapy, and the place of nicotine therapy in UC remains unclear.

3.6.3. Thalidomide

Thalidomide inhibits production of TNF- α by monocytes. Thalidomide may heal severe refractory oral ulceration caused by CD in a dose of 0.7 mg/kg (Weinstein et al., 1999). Open-labelled 12 weeks trials of thalidomide in refractory CD have reported symptomatic benefit (Ehrenpreis et al., 1999), (Vasiliauskas et al., 1999). In addition to anti-TNF α effect, thalidomide has other potential mechanisms of action including inhibition of neovascularization (Sands and Podolsky, 1999).

3.6.4. Fish oil

Leukotriene B₄ is an important final message mediating cellular inflammation and chemotaxis in inflamed intestinal mucosa of IBD. Supplementation with ω -3 fatty acids (+antioxidants) significantly changed the eicosanoid precursor profile with a decrease in arachidonic acid and increase in EPA and DHA (Geerling et al., 2000). Despite this, the clinical efficacy of ω -3 fatty acids in the form of fish oil is modest. In a small 7-month double blind, placebo-controlled crossover trial of dietary supplementation with fish oil (3.2 gm n-3 fatty acids per day), clinical activity was unchanged in CD but declined modestly in UC (Lorenz et al., 1989). The efficacy of sulfasalazine is superior to that of fish oil (5.4 gm n-3 fatty acids per day) (Dichi et al., 2000). In CD, a relatively small

Italian study (n=78) found fish oil supplementation (2.7 gm n-3 fatty acids in a novel enteric-coated preparation) to reduce relapses compared to placebo (Belluzzi et al., 1996), but a larger study (n=204) found similar relapse rates using an ethyl ester fish oil concentrate compared with placebo (Lorenz-Meyer et al., 1996). Different trials have used varying formulations, dosages and study designs and are not directly comparable. Further studies are required with a more palatable formulation of ω -3 fatty acids, but currently the use of fish oil formulations cannot be recommended in IBD.

3.6.5. Growth hormone

In a 16-week study, recombinant human growth hormone (GH) was compared with placebo in 37 patients with moderate to severe CD (Slonim et al., 2000). Diets were adjusted to provide at least 2g/kg daily protein intake. Treatment with GH resulted in a significant reduction in mean clinical scores compared with placebo within 4 weeks of therapy and this improvement continued throughout the study period. After 16 weeks of therapy, serum IGF-1 increased by 50% in GH-treated patients. IGF-1 is known to promote intestinal wound healing and mucosal restoration.

3.6.6. Leukotriene inhibitors

Despite the obvious key role of leukotrienes (LT) in the pathogenesis of UC, inhibition of LT biosynthesis with a 5-lipoxygenase inhibitor (MK-591) was not effective as a single therapeutic modality in active UC compared with placebo (Roberts et al., 1997). Furthermore, zileuton, a 5-lipoxygenase inhibitor was also not significantly better than placebo in the maintenance of remission in UC (Hawkey et al., 1997). At present it would appear that LT is not a useful therapeutic target in UC.

3.6.7. Thromboxane synthetase inhibitor

Ridogrel, a thromboxane synthetase inhibitor and receptor antagonist was not shown to be any more effective than placebo in a double blind placebo controlled trial at a dose of 5mg/day in active CD (Carty et al., 2001). More potent agents may require development and assessment.

3.6.8. Chloroquine

The immunomodulatory effects of chloroquine and hydroxy-chloroquine have been utilised in a number of immunological diseases. A randomised controlled trial of chloroquine phosphate 500mg/day versus sulfasalazine 3g/day in mild to moderate UC showed no significant difference in response rate (60% vs. 55% complete remission at 4 weeks; $p=ns$) (Goenka et al., 1996). Side effects were minor.

3.7. Quality of life and pharmacoeconomics

The major therapeutic goal for most patients with chronic illness is, rather than a cure, an improvement in function and life quality resulting from alleviation of the illness or from a limitation of the progression of the disease. Moreover, assessment of quality of life (QoL) in IBD is important because the major disease events (death, surgery, and hospitalization) occur relatively infrequently and the burden of symptoms together with social and psychological morbidity is life long. There are 4 major components when assessing quality of life. These comprise physical function, emotional and social well being, ability to work and freedom from disease related symptoms. There are generic and disease specific methods of assessing of QoL. These vary in size and complexity from a visual analogue scale to a 100-item questionnaire. Generic measures, such as the Sickness Impact Profile and the Short Form-36, are particularly useful to compare

relative health differences between diseases, whereas disease specific measures, such as the IBD questionnaire (IBDQ), address problems encountered within a specific disease. Patients with IBD have a lower HRQoL than normal individuals and although disease activity is a major determinant of QoL adverse effects are also present in remission (Markowitz et al., 2000). The relative contribution of the health domains in active and inactive disease differs, with social impairment becoming the less damaged dimension of the IBDQ in active IBD as compared to digestive and systemic symptoms. The IBDQ has been validated to be a reliable and reproducible method of assessing IBD and is being increasingly used in clinical trials and the assessment of other treatments of IBD. It has been suggested that surgery improves HRQoL but more recent data suggests that the induction of remission, whether medical or surgical, is the main determinant of improvement. Corresponding use of QoL measures in clinical trials have shown improvements with budesonide (Irvine et al., 2000), cyclosporine (Irvine et al., 1994) methotrexate (Egan LJ et al., 2000). QoL assessment provides invaluable information about the effect of IBD and its treatment on patients with IBD. However, as financial implications are increasingly important in treatment of IBD, more sophisticated data is required on value for money and to this end the cost benefit analysis and analysis of utility are being increasingly used.

The exact costs of IBD are difficult to assess, as attributing a cost to pain and suffering is inaccurate. Costs that can be attributed are either direct, i.e. those incurred by the patient during delivery of healthcare, or indirect, i.e. the loss of earnings or productivity. There is evidence that indirect cost may be considerably in excess of direct costs (Blomqvist and Ekbom, 1997). There are 3 methods to estimate the costs of the disease. In a prospective manner, usually in association with a clinical trial, data can be collected to estimate health care costs. Although accurate for the examined patients, clinical trials represent highly selected groups of patients and extrapolation to the general population may not be accurate. Secondly, data may be collected retrospectively from claims made to insurance companies or other databases. Extrapolation to a wider population is easier although the dataset is often incomplete, with those who have not claimed being omitted. The last method uses a medical decision algorithm, using the published data to estimate proportion of patients requiring different therapeutic interventions, to estimate

the cost of a theoretical group of IBD patients. Estimations of the annual cost of CD in 1990 was \$6561 and of UC \$1488 (Hay and Hay, 1992). More recent estimations using medical claim data show the mean annual cost of CD to be \$12417 (Feagan et al., 2000). The major costs are involved with hospitalizations occupying 56% of CD charges and 60% of UC charges. Surgery accounts for 40% of hospital admissions and accounts for 75% of costs (Cohen et al., 2000). Medical admissions were similarly costly for CD and UC but surgery was more expensive in UC (Bernstein et al., 2000). In addition the cost data are skewed with median values being somewhat lower than means. This is illustrated by the observation that the most severely affected 25% of the population accounted for 80% of the charges (Feagan et al., 2000). Pattern of expenditure appears to be similar in a public as well as private healthcare setting. Therefore, newer therapies, which may be more expensive, have the potential to be cost effective if they reduce hospitalization and especially the need for surgery. A simple pharmacoeconomic assessment indicates that the cost for preventing each relapse using 5-ASA for CD is at least \$4,000 to \$10,000. Cost-utility analysis has shown that mesalamine maintenance therapy in CD was associated with a cost of about \$5000 per quality-adjusted life year (QALY) gained (Trallori and Messori, 1997).

Despite the need for accurate cost benefit assessments, conventional cost-effectiveness analyses are of limited use in IBD as major disease events are relatively infrequent. To this end cost-utility assessment is increasingly used. Utility is a generic measure that places a value on HRQoL that lies between 1 (perfect health) and 0 (death). Quality adjusted life years (QALYs) are calculated by multiplying the time in a health state by the utility score. Thus, differences in potentially expensive new treatments may be expressed as incremental cost per QALY. Infliximab, a new and relatively expensive treatment for CD has been the subject of cost-benefit analysis with some conflicting results. Infliximab reduces hospital costs in the year after infusion when compared to the year prior to infusion. The outcome of utility analysis has depended on the duration of remission and indication for use. The use in luminal disease may well be cost effective but a cost utility analysis examining the use in perianal fistulas found that the additional benefit over conventional treatments might not justify the cost. Cost utility analysis will be used increasingly in the coming years to attempt to clarify the value and position of new treatment in the armamentarium available against CD and UC.

CHAPTER FOUR

SECTION I

4. Study Plan: Experimental design

The enteral diet and enteral diet modifications used in this study were supplied by Scientific Hospital Supply (SHS, Liverpool, UK). The enteral diet (ED) study was based on elemental diet (EO28). EO28 is widely used to treat CD and is the main defined formula diet used at the Western General Hospital Edinburgh. This is an amino acid based enteral diet, which is referred to in the text as EAA. Although the use of ED in CD has recently declined because of new drug treatments, such as infliximab, ED is an effective treatment especially in paediatric CD. A number of modifications of ED were made by SHS to our specifications, and these are described in details later. This was an investigator-initiated study and the dietary modifications were made by SHS after discussion with us. All the diets formulations supplied by SHS were modified from the basis formulation of EO28. Therefore the emulsifiers used were the same in all formulae used in this experiment.

Colonic and, to a lesser extent ileal tissue biopsies from patients with IBD (CD and UC) were incubated in an organ culture system for 24 hours. Tissue samples from non-inflamed non-IBD patients (control patients) suffering from constipation, irritable bowel syndrome, diarrhoea or polyps were also incubated as control. This group of patients is referred to as control patients.

Biopsy tissue was incubated with a culture fluid medium (Waymouth's medium) and enteral diet. The objective of this study was to measure inflammatory responses after incubation with the different modified enteral diets. This was investigated by measuring

the pro-inflammatory cytokine IL1 β and the anti-inflammatory cytokines IL1ra and IL10 in the supernatant. TGF- β was also assayed.

Detail of the design and plan of experiments with modified diets is shown in Figure 15.

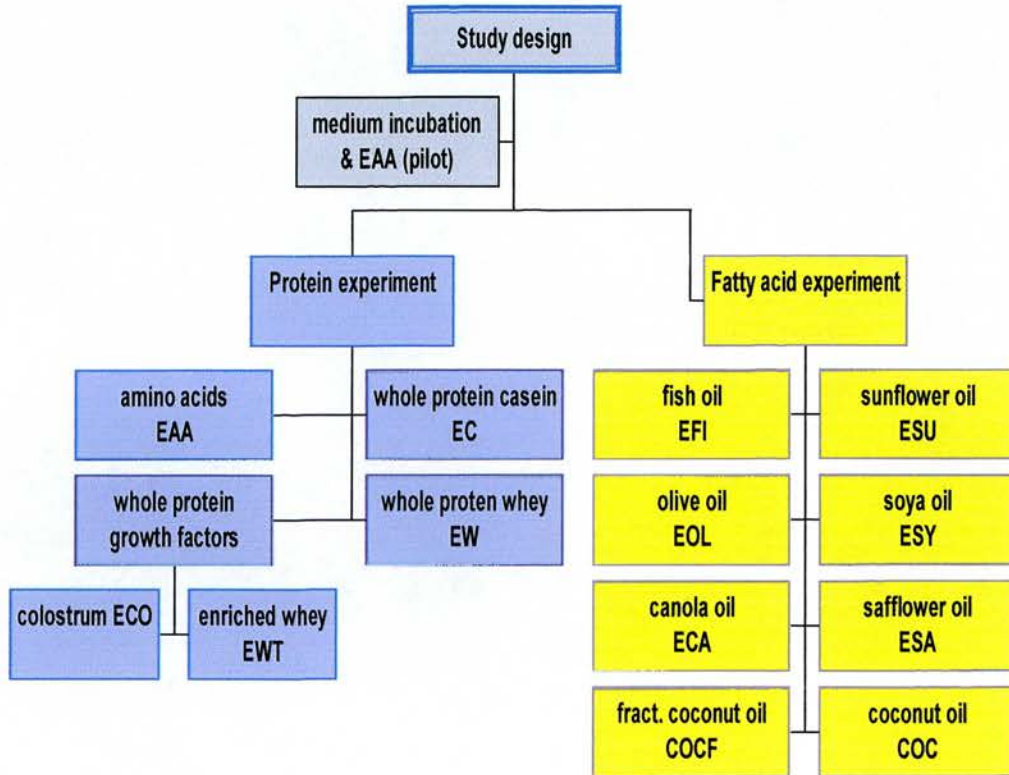


Figure 15 Study design with modified diets

The different modifications were made to the original elemental diet EO28 formulae, keeping the rest of the formulae unchanged.

The basic composition of ED was equivalent to commercial EO28 including vitamins, macro – and micronutrients but fat and protein composition were modified. Table 2 shows the composition of EO28, referred to in the text as elemental diet amino acid composition (EAA). The osmolality of EO28 was 300 mosmol, which approximates the physiological osmolality of plasma (290 mosmol). ED was diluted with culture fluid at three different concentrations of 1:5, 1:10 and 1:20.

Table 2 Elemental diet (EO28, SHS), referred in text as Elemental diet amino acid composition (EAA)

Nutrient	Per 100g powder
Energy (kcal)	443
Protein equivalent (g)	12.5
Carbohydrate (g)	60
Fat (g)	17.45
Sodium (mg)	305
Pottasium (mg)	466
Chloride (mg)	333
Calcium (mg)	187.5
Phosphorus (mg)	200
Magnesium (mg)	81.6
Iron (mg)	4.2
Zinc (mg)	4.2
Iodine (μg)	33.3
Manganese (mg)	0.6
Copper (mg)	0.4
Molybdenum (μg)	33.3
Selenium (μg)	15
Chromium (μg)	15
Vitamin A (μg)	330
Vitamin E (mg)	8.3
Vitamin C (mg)	28.3
Thiamine (mg)	0.6
Riboflavin (mg)	0.6
Pyridoxine (mg)	0.8
Niacin (mg)	4.2
Pantothenic (mg)	2
Inositol (mg)	9.2
Choline (mg)	91.6
Vitamin D (μg)	1.9
Vitamin B12 (μg)	1.8
Biotin (μg)	58.3
Vitamin K (μg)	25

Patients were informed about the study prior to endoscopy, and asked if willing to give additional biopsy sample in addition to the specimens obtained for clinical reasons. Ethical approval was obtained for the study from the Lothian Ethics in Research Medicine and Oncology subcommittee. The consent for biopsies was always obtained by the clinician performing colonoscopies.

Prior to this study, a small pilot study was performed in three patients where twenty-one biopsies were obtained and incubated with Waymouth's medium and EAA. The experimental design of the study, dilution factors for enteral diet with culture medium, and viability of the tissue during culture period (24-h and 48-h) were investigated and BrdU staining and assays for cytokines in culture supernatant performed. It was shown that 3 dilutions of enteral diet (1:5, 1:10 and 1:20) with culture medium were equivalent to plasma osmolality. Further, the biopsies were viable over the 24-hour culture period and the cytokine assay with culture supernatant could be done without interference by ELISA (protein recovery satisfactory). Figure 16 displays the fatty acid profile of elemental diet EAA.

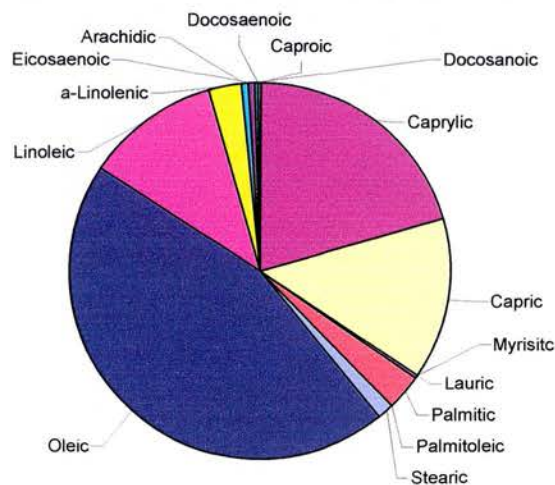


Figure 16 EAA - fatty acid profile

4.1. Enteral diets modified in protein composition

4.1.1. Enteral diets modified for casein and whey

Enteral diet was supplied in 50gm packets in powder form. The aim was to investigate different inflammatory response to the elemental and enteral diets modified in nitrogen content and composition. Enteral diets were modified and specially manufactured to contain whole proteins of casein (EC) and whey (EW). These modified enteral diets were compared with commercial elemental diet EO28 (EAA) containing amino acids only. Differences of amino acid composition of these diets are presented in Table 3.

Table 3 Differences in amino acid composition of EAA, EC and EW

gm/100gm Amino acid	EAA	EC	EW
Protein Equivalent	12.63	13.57	12.13
Nitrogen	2.31	2.12	1.9
L-Alanine	0.51	0.45	0.69
L-Arginine	1.16	0.52	0.33
S-Aspartic Acid	1.00	1.05	1.49
L-Cystine	0.07	0.07	0.27
L-Glutamic Acid	0.00	3.22	2.40
Glycine	0.87	0.26	0.24
L-Histidine	0.63	0.40	0.23
L-Isoleucine	0.86	0.76	0.87
L-Leucine	1.48	1.40	1.43
L-Lysine	1.10	1.10	1.25
L-Methionine	0.50	0.42	0.25
L-Proline	1.01	1.48	0.87
L-Phenylalanine	1.22	0.70	0.39
L-Serine	0.62	0.84	0.76
L-Threonine	0.72	0.60	1.03
L-Tryptophan	0.29	0.17	0.23
L-Tyrosine	0.25	0.78	0.38
L-Valine	0.94	0.97	0.80
L-Carnitine	0.025	0.00	0.00
Taurine	0.047	0.00	0.00
L-Glutamine	1.72	0.00	0.00

4.1.2. Enteral diet modified for bovine colostrum

The study further investigated a different nitrogen source, high in immunoglobulins. This was bovine colostrum protein (ECO) made up by SHS into enteral diet by modulating the basic ED composition of EO28. The bovine colostrum contained 0.9mg of TGF- β per 1 gm of colostrum powder. The enteral formula of ED-colostrum contained 15 gm of colostrum per 100 gm ED, with a TGF- β content of 13.5 mg per 100 gm. The main TGF- β content of this diet was TGF- β 2, although colostrum normally contains some TGF- β 1 (approximately 10-20% of TGF- β 2 content).

4.1.3. Enteral diet modified for TGF- β enriched whey extract

A further diet composition consisted of TGF- β enriched whey extract. This whey extract itself contained 300 mg of TGF- β per 1 gm. 50mg of this TGF- β enriched whey extract were manually mixed into 100gm of enteral diet EAA, and this final diet composition is referred in text as EWT. The resultant composition was afterwards equivalent to a TGF- β content of 200 gm enteral diet containing colostrum protein. Again, the TGF- β subform was TGF- β 2. Table 4 gives an overview about ED modifications using whole proteins from different sources and Table 5 shows the lipid composition of EAA, EC and EW. Figure 17 displays the design of the protein experiments.

Table 4 Enteral diet modified in protein composition

	EAA	EC	EW	ECO	EWT
ED base	EO28	EO28	EO28	EO28	EO28
Nitrogen source	Amino acids	Casein	Whey	b ovine colostrum	Whey extract
TGF- β 2 enriched	-	-	-	0.9mg / 1gm colostrum	300mg / 1gm whey extract

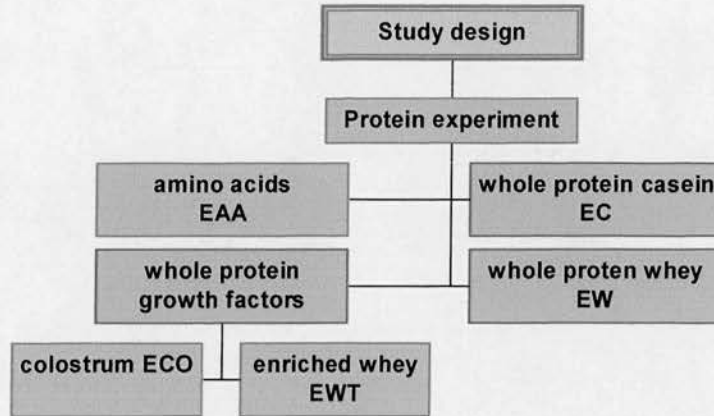


Figure 17 Protein experiment design – different enteral diet compositions used

Table 5 Defined lipid composition of EAA, EC and EW in percent

EAA	EC	EW
10.8 % fract. Coconut oil	10.8 % fract. Coconut oil	10.8 % fract. Coconut oil
10.03 % Safflower oil	10.03 % Safflower oil	10.03 % Safflower oil
10.03 % Canola oil	10.03 % Canola oil	10.03 % Canola oil

4.2. Enteral diet modified in oil / fatty acid composition

A further series of experiments investigated the cytokine response in enteral diet EO28 modified in its fatty acid (FA) content. Details of experimental designs are shown in Figure 15. The diets were supplied in 50 gm powder form batches. Eight different kinds of oil combinations were used in this study: (a) Fractionated coconut oil, (b) unfractionated coconut oil, (c) olive oil, (d) fish oil, (e) sunflower oil, (f) soya oil, (g) safflower oil and (h) canola oil. The natural occurrence of some of these oils containing

fatty acids is described in chapter 2. The variation of the ED-oils in respect of their FA composition is shown in Table 6 and in the pie chart description. These enteral diets were diluted with sterile distilled water and culture medium in the same way as the ED protein incubations. The FA content is described in gram per 100gm of ED. The total amount of fat in all compositions was 17.65 gm, (approximate 30 % of enteral diet) the same as in commercial EO28. A description of the FA with their trivial and systemic names, in respect of their saturated and non-saturated structure composition is shown in Table 7, Table 8 and Table 9. Generally if possible, were two diets for one patient incubated in pairs, as shown in Figure 18 of the fatty acid experiment.

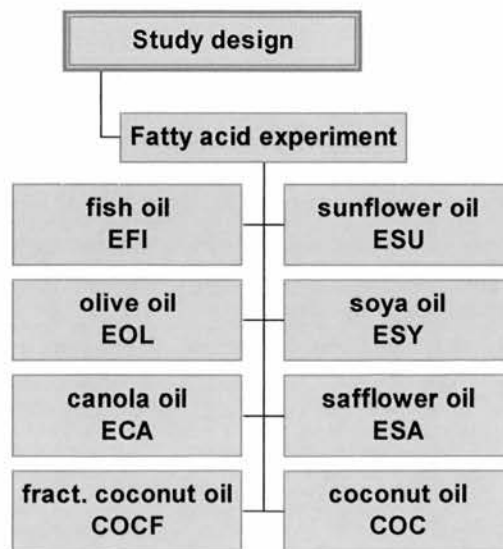


Figure 18 Fatty acid experimental design – the different compositions of enteral diets modified in fatty acid compositions

Table 6 ED Fatty acid composition of enteral diets modified according to different oils.

gm/100 gm Fat	Trivial name	Coconut fract. COCF	Coconut not fract. COC	Olive EOL	Safflower ESA	Canola ECA	Sunflower ESU	Soy ESY	Fish EFI
Total Fat		17.65	17.65	17.65	17.65	17.65	17.65	17.65	17.65
C6:0	Caproic	0.09	0.47						
C8:0	Caprylic	55.39	6.69						
C10:0	Capric	38.15	5.65						
C12:0	Lauric	0.09	44.37						
C14:0	Myristic		17.43		0.08				6.60
C16:0	Palmitic		8.57	13.15	4.84	4.40	6.69	10.04	13.67
C16:1	Palmitoleic			1.67	0.38	1.43	4.78	3.30	8.32
C18:0	Stearic		2.64	1.91	2.19	58.7	21.99	23.41	2.58
C18:1	Oleic		6.41	67.4	72.42	19.12	61.18	50.67	10.33
C18:2 ω 6	Linoleic		1.79	11.23	14.4	8.41		7.17	3.44
C18:3 ω 3	α Linolenic		0.09	0.72	0.10	0.48			0.57
C20:0	Arachidic			0.24	0.83	1.34			
C20:1	Eicosaenoic			0.10	0.41	0.38			1.34
C20:4	Arachidonic								1.62
C20:5 ω 3	EPA								18
C22:0	Docosanoic			0.10	0.31	0.76			
C22:1	Docosaenoic			0.05					
C22:5 ω 3	DPA								17.30
C22:5 ω 6	DPA								1.91
C22:6 ω 3	DHA								7.27

The following two pages illustrate the fatty acid profile of the diets shown in Table 6 as pie chart figures (Figure 19 to Figure 26), for ease of comparison.

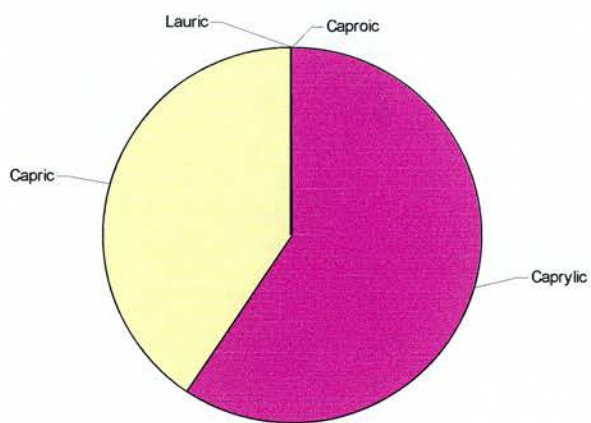
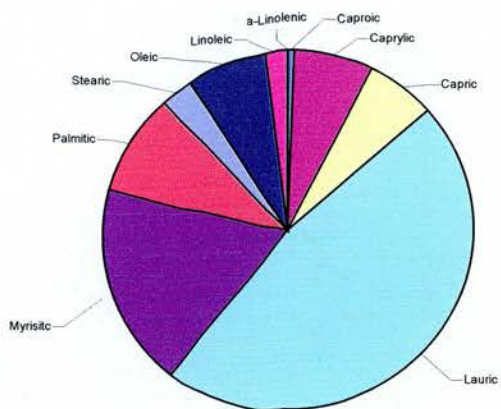


Figure 19 (left): COC - fatty acid profile
 Figure 20 (right): COCF - fatty acid profile

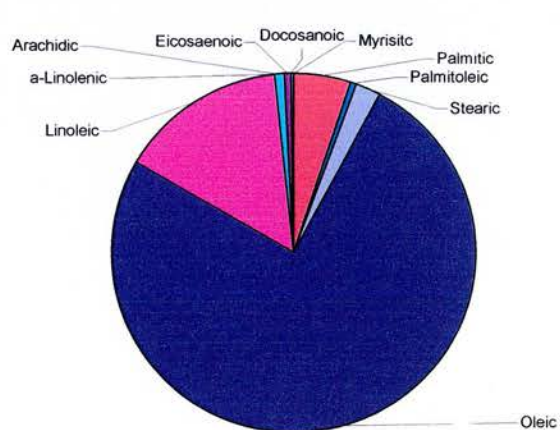
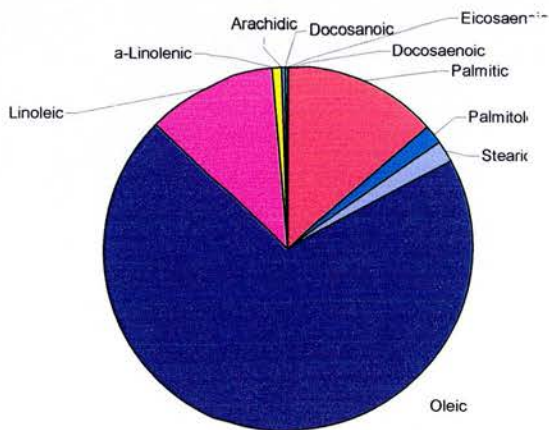


Figure 21 (left): EOL - fatty acid profile
 Figure 22 (right): ESA - fatty acid profile

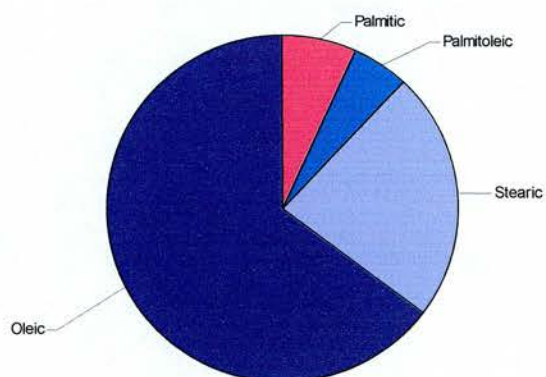
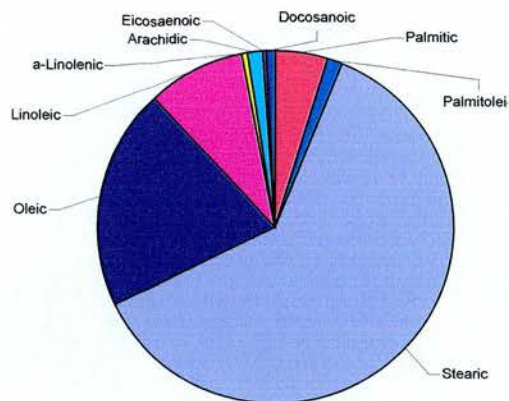


Figure 23 (left): ECA - fatty acid profile
 Figure 24 (right): ESU - fatty acid profile

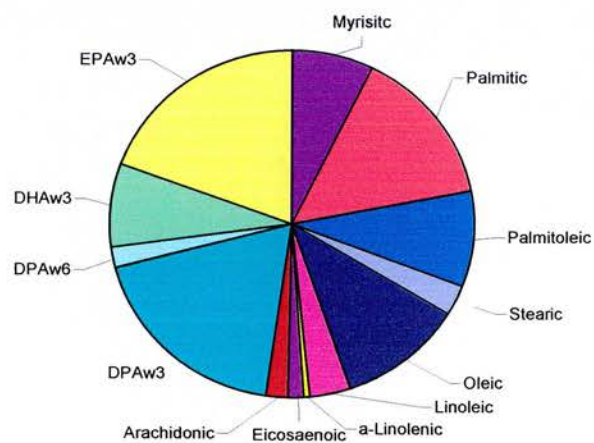
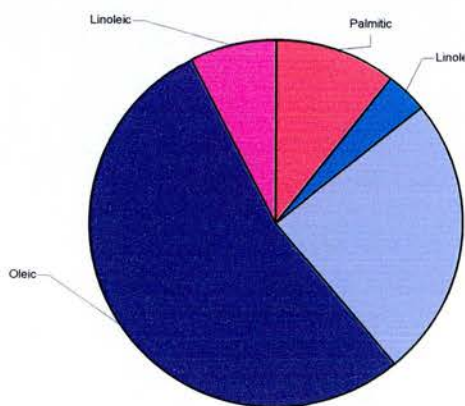


Figure 25 (left): ESY - fatty acid profile
 Figure 26 (right): EFI - fatty acid profile

Table 7 Saturated Fatty acids

Shorthand notation	Trivial name	Systemic name
C:6:0	Caproic	Hexanoic
C:8:0	Caprylic	Octanoic
C10:0	Capric	Decanoic
C12:0	Lauric	Dodecanoic
C14:0	Myristic	Tetradecanoic
C16:0	Palmitic	Hexadecanoic
C18:0	Stearic	Octadecanoic
C20:0	Arachidic	Eicosanoic
C22:0	Behenic	Docosanoic

Table 8 Mono-unsaturated Fatty acids

Shorthand notation	Trivial name	Systemic name
C16:1	Palmitoleic	Cis-9-hexadecenoic
C18:1	Oleic	Cis-9-octadecenoic
C20:1	Gadoleic	Cis-11-eicosenoic
C22:1	Cetoleic	Cis-11-docosenoic

Table 9 Polyunsaturated Fatty acids

Shorthand notation	Trivial name	Systemic name
C18:2 ω 6	Linoleic	Cis, cis-9, 12-octadecadienoic
C18:3 ω 6 // ω 3	γ -Linolenic (GLA) // α -Linolenic	All cis-6,9,12-octadecatrienoic // all cis-9, 12, 15-octadecatrienoic
C20:4	Arachidonic (AA)	All-cis-5,8,11,14-eicosatetraenoic
C20:5	Timnodonic (EPA)	All-cis-5, 8, 11, 14, 17-eicosapentaenoic
C22:5 ω 6 // ω 3	Osmond // Clupanodonic	All-cis-4,7,10,13,16-docosapentaenoic // All cis-7, 10, 13, 16, 19-docosapentaenoic
C22:6	Cervonic (DHA)	All-cis-4,7,10,13,16,19-docosahexaenoic

4.3. Organ culture model

The organ culture technique is a method to study cell proliferation, metabolic pathways and nutritional requirements of organs in a controlled environment. With this technique it is possible to maintain the structure of the tissue. It is generally accompanied by a minimum of outgrowth and preserves the histological tissue structure for further investigations. The main principle of an organ culture is the maintenance of a tissue specimen from an organ between fluid phase i.e. the medium and a high oxygen environment i.e. the gas phase. An oxygen level of 95% is necessary for culturing human colon to prevent a decrease in the pH due to production of lactic acid (Autrup et al., 1978). The ability to culture cells in a chemically defined medium provides the opportunity to determine the appropriate medium content in quality and quantity for the tissue specific nutritional demand. This method has been used successfully by several investigators for cell kinetic studies after 18 hours incubation of colorectal tumours (Pritchett et al., 1985) and in mouse colon with supplemented Waymouth's medium for 3 days (Defries and Franks, 1977). Gregory *et al* reported viability in human rectal mucosa after 24 hours incubation with Trowel T-8 medium (Gregory L et al., 1973). Human colon could be kept viable for up to 4 to 7 days, showing viable epithelium in 60% after 7 days. Tissue could be kept for up to 20 days by adding glucose to the culture fluid, the glucose being highly utilised within 48 hours (Autrup et al., 1978). Senior *et al* kept tissue alive for up to 336 hours with supplemented Waymouth's medium (Senior et al., 1982).

Waymouth's MB 752/1 medium is used as the fluid phase and has been described by Hodges and Melcher (Hodges and Melcher, 1976). We used in our study the organ culture method of Wilson *et al.* previously performed in our laboratory (Wilson et al., 1990) and essentially based on the method described by Pritchett *et al* (Pritchett et al., 1985). The medium was supplemented with 10% foetal calf serum, 300 µg/ml ascorbic acid, 0.45 µg/ml ferrous sulphate, 50 µg/ml gentamycin, 5000 µg/ml streptomycin, 5000 IU/ml penicillin and 1.5mM L-Glutamine with the addition of 100 µmol BrdU per 100 ml of complete medium.

4.3.1. Technique of Organ culture for enteral diet incubation

For each experiment, culture medium was freshly prepared prior to the experiment and the design is described in the following paragraph.

The possibility of weighing the biopsies to obtain tissue weight was considered. As the biopsies had to be prevented from desiccation, weighing was only possible in a fluid phase without manipulating biopsies too much. Therefore transport universal containers were weighed prior to and after biopsy collection. Although weights were determined as the average of 3 measurements, the between-measurement variations were large, even using a sensitive digital balance (Oertling, Smethwick, UK), mainly as the endoscopic biopsies were small. The degree of hydration considerably affected measurement of such small weights. The alternative option of indexing results against protein or DNA content was not possible as the tissues were processed for BrdU uptake at the end of each incubation to ensure viability. Therefore, weighing of biopsies was omitted and final estimation of cytokine supernatant was expressed as a ratio of anti and pro-inflammatory cytokines. This enabled expression of results without taking into account the weight, which was the same for both cytokine assays in the supernatant. After the initial experiments with EAA, EC and EW, later experiments were carried out using the same biopsy forceps by the same investigator, allowing uniformity of biopsy sizes.

Two grams of ED were diluted with 15 ml of sterile distilled water to obtain an isomolar solution with an approximate osmolality of 300 mosmol as described on package for a 1:7.5 dilution. The ED fluid was added to the prepared medium in 1:4 dilution. Out of this stock solution, serial dilutions were performed for experiments using dilutions of 1:5, 1:10 and 1:20. To each experiment culture solution the synthetic thymidine analogue BrdU was added in a concentration of 100 μ l/100ml, giving the final culture fluid.

BrdU is incorporated into dividing cells and is used as a marker to indicate the viability of the tissues cultured.

A steel triangular grid was sterilised over an open gas fire, briefly cooled in Waymouth's medium and placed over a sterile centre well of the organ culture dish. The dish contained 1.5-ml culture fluid, so that it just touched the underside of the grid. For each experimental set up for each patient a tissue was included for incubation using only medium as control.

The Waymouth's medium was pipetted into transport universal containers and all containers were pre-gassed in an incubation chamber to enrich transport fluid with oxygen, which could be observed as a colour change of medium as a result of oxidation of ascorbic acid. To preserve humidity of the sealed chamber, a dish with sterile water was placed into the chamber base. The chamber was closed and sealed with its metal ring, gassed with 95% O₂/5% CO₂.

After being notified by telephone, transport containers were transferred into an ice beaker and tissue was collected from the endoscopy suite. The prepared organ culture dishes were kept in the incubation chamber during tissue collection, and also pre-gassed to enrich it with oxygen. After collection, the tissue was orientated under a dissection microscope to ensure that the explant luminal surface was uppermost and then gently transferred onto the triangular grid suspended on the culture well. A thin layer of culture fluid was drawn over the biopsy by capillary action and the lid carefully placed over. After all tissues were dealt with in the same way, the incubation chamber was closed. Before the chamber was finally gassed, it was briefly 'flushed' with the oxygen/carbon dioxide gas mixture, before outlet ports were closed with their clamps and chamber set under pressure with an oxygen flow rate of 15 litres/min. Finally the organ culture chamber was placed into an incubator at 37° C for 24 hours. Figure 27 shows an image of the modular incubator chamber with culture dishes and metal triangular grids used in this study.

After 24 hours incubation period the pressure was carefully released from the chamber. Triangular grids with tissues were placed into labelled glass jars containing Carnoy's fixative (60% absolute alcohol, 30% chloroform and 10% acetic acid) for a maximum of

16 hours. Following this, the tissue was stored in alcohol (IMS99), awaiting further histological processing.

The culture supernatant was collected in APEX 1.5ml tubes and immediately frozen and stored at -70° C for ELISA assay.

For immunohistological staining of BrdU labelled cells and TGF- β 1 tissue expression, tissue was embedded into paraffin wax, via an automatic 24-hour long cycle of IMS 99, HistoClear/alcohol, HistoClear and finally 56°C paraffin wax. The processed tissue blocks were cut on a Microtome (Leica 2045 Multicut, Nussloch, Germany) in sections of 5 μ m and placed on 0.1% poly-L-lysine coated histology slides for further staining.



Figure 27 Organ culture chamber with triangular metal grids and organ culture dishes

4.4. Histological Method

The basic staining performed is an immuno-histochemistry method. The principle depends on an enzyme marker, where the enzyme is conjugated to a specific antigen attached to the tissue section and then visualised with a substrate. Horseradish peroxidase can be used as an enzyme marker and the method is generally called an immuno peroxidase method. Different ways of detection are possible, such as direct and indirect methods, which are similar to those used in ELISA detection methods (Graham Robinson, 1982). To detect BrdU labelled cells in the incubated organ culture tissue, a three stage indirect method was used. The sections were incubated with the primary antibody, which is unlabelled and directed against the antigen under investigation (Anti-BrdU, Becton & Dickinson). The second antibody, biotinylated F(ab)₂ fragment of rabbit anti-mouse immunoglobulins (DAKO) is directed against the primary antibody. A conjugated horseradish peroxidase (StreptABCComplex/HRP, DAKO), was bound to the biotinylated antibody. Finally the binding was visualised with the substrate 3,3-Diaminobenzidine-Substrate (DAB) (Sigma), which reacts histochemically with HRP for a brown reaction product, which can be detected under the microscope.

4.4.1. Bromodeoxyuridine

Detection of replicating cells can be performed by labelled [³H] thymidine incorporated into DNA by autoradiography or scintillation. This is time-consuming and scintillation counting, although rapid, is a 'batch' method, it cannot provide quantitative information on the frequency of replicating cells within a population. Therefore an immunological method using monoclonal antibodies specific for Bromodeoxyuridine (BrdU) to detect BrdU incorporation into replicating DNA has shown to provide a sensitive method to detect DNA replication in single cells (Gratzner, 1982) and is now a well established alternative to [³H] thymidine incorporation (Green et al., 1998). BrdU is a pyrimidine analogue, which is incorporated into DNA-synthesizing nuclei (during S-phase of cell

cycle) and can be immunohistochemically detected using antibodies against BrdU (Sugihara et al., 1986). It is a method widely used by investigators for measurements of *in vivo* proliferation in human colorectal mucosa (Potten CS et al., 1992), proliferation measurement of gastrointestinal epithelium (Potten et al., 1992), cell kinetic studies in animals (Wynford-Thomas and Williams, 1986) and abnormal cell proliferation in colonic mucosa (Wilson et al., 1990). BrdU uptake was used in the current study to determine the viability of the explants after organ culture for 24 hours in different enteral diet formulae incubations.

4.4.1.1. BrdU staining method

In the present study, staining for BrdU labelled cells was performed using a method modified after the protocol of Wilson *et al* (Wilson et al., 1990) and Green *et al* (Green et al., 1998). To prepare the slides for staining, these had to be de waxed and rehydrated by transfer into HistoClear for 2x5 minutes and into IMS99 for 2x5 minutes. Blocking of endogenous peroxidase was performed by placing slides into 1 % Hydrogen peroxide in Methanol for 20 minutes. After washing in running tap water, sections were incubated for 8 minutes in 1molar hydrochloric acid at 60°C, to denature and release purine base (adenine and guanine) of the DNA double helix and expose bound BrdU to make it accessible for anti-BrdU antibody. After a further wash in running tap water sections were surrounded with a hydrophobic pap pen to prevent antibody solution running off the slide. Subsequently sections were incubated with normal rabbit serum (SAPU, UK) in Tris buffered saline (pH7.6) (20%, NRS/TBS) for 10 minutes to prevent non-specific antibody binding. Incubations were performed in a humid box to prevent the sections from drying out. Murine monoclonal antibody anti-BrdU mouse was applied for 60 minutes (dilution 1/300 in NRS/TBS). Following rinsing and washing for 10 minutes in Tris-buffer saline bath (TBS) mouse immunoglobulin/biotinylated rabbit IgG, was applied for 40 minutes (dilution 1/250 in NRS/TBS). After repeated rinsing and washing for 10 minutes StreptABCComplex/HRP Peroxidase Conjugate was applied to the sections for 30 minutes (9µl A+B / 1ml TBS). Final visualising of binding was

detected with DAB (dilution in 10ml TBS: 10 μ g DAB + 60 μ l of 6% Hydroperoxide) for 10 minutes. Sections were washed in running tap water and then treated for 5 minutes in copper enhancement solution to enhance the staining density. Slides were again washed under running tap water and counterstained in Harris Haematoxylin (Sigma) for 5 minutes. Subsequently slides were washed in running tap water, dipped in 1% acid alcohol to remove excess haematoxylin, washed in running tap water, and dipped into saturated aqueous lithium carbonate until the cell nucleus assumed a blue colour. After a final wash in running tap water sections were dehydrated through graded alcohol of 70% alcohol, IMS 99, Isopropanol, cleared in HistoClear and Xylene, mounted with DPX and covered with cover slip. BrdU stained cells were brown/black stained. To confirm staining, a positive control of mouse jejunum, which has been labelled by intravenous injection of BrdU was used in each staining run.

4.4.1.2. Examination of BrdU stained tissue

Slides were examined in a blinded fashion under a light microscope (Leitz, Wetzlar, Germany) in magnifications of 100X and 250X. Intact morphological appearance of the tissue, epithelial surface and BrdU labelled cells in horizontal and vertical crypts were assessed.

The Labelling Index (number of labelled cells in crypt / total number of cells in crypt) is a measurement of colonic proliferation. Regions exhibiting a greater proliferation have higher values of labelling index (Roe et al., 1996). The focus of the present study was not on colonic tissue proliferation, and so BrdU labelled cells were not scored according to labelling index as in the study of Gregory *et al* (Gregory L et al., 1973).

Staining and detection of BrdU labelled cells was performed to confirm tissue viability during culture period of 24 hours and no quantitative threshold was employed. BrdU labelled cells were estimated in horizontal and vertical (U-shaped) crypts. BrdU labelled cells occurred in vertical crypts at the base of the crypts and along the sides of the crypts and intact epithelium. Morphology of the tissue and detection of BrdU

labelled cells confirmed tissue viability over the culture period. Tissues which expressed these criteria, i.e. intact morphology and BrdU uptake were included in the final analysis. Representative tissue sections after BrdU staining are shown in Figure 28, Figure 29 and Figure 30.

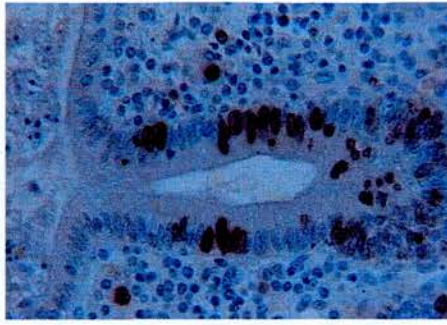


Figure 28 BrdU labelled cells after incubation with ED-fish oil in UC (x250)

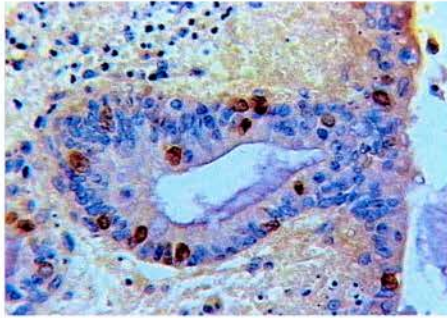


Figure 29 BrdU labelled cells after incubation with medium in CD (x250)

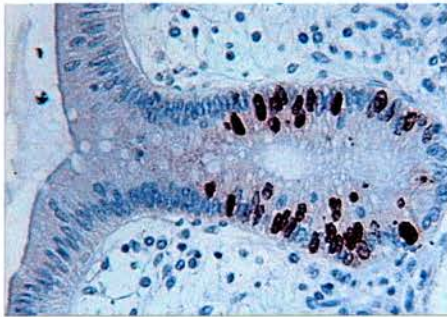


Figure 30 Brdu labelled cells after incubation with medium in control patients (x250)

All explants required to have intact morphology and BrdU to be included for analysis in the study.

4.4.2. TGF- β staining method

The antibody used for TGF- β 1 staining was a polyclonal IgG antibody supplied by Santa Cruz Biotechnology (Heidelberg, Germany). This antibody is specific for TGF- β 1, and is not cross-reactive with TGF- β 2 or TGF- β 3, as confirmed with the company. It reacts with TGF- β 1 of mouse, rat and human origin. It can be further used either for Western blotting or, as in the experiments performed in this thesis, for immunohistochemistry in paraffin-embedded tissue sections. Several investigators have used this antibody in different studies, eg. in wound healing in mice (Frank et al., 1996), in human pancreatic carcinoma tissue (Bellona G et al., 1999) or in human prostate tissue (Royuela M and Paniagua R, 1998).

Sections stained for TGF- β 1 followed a three-step immuno-peroxidase ABC method as described for BrdU staining. Differences in details were in the steps of non-specific binding blocking, use of serum and acid denaturation at 60° C.

Blocking of non-specific binding was performed with normal sheep serum (NSS) in TBS (20%) for 10 minutes. The sections were drained and primary antibody rabbit-polyclonal IgG TGF- β 1 (Santa Cruz Biotechnology) was applied for 60 minutes (diluted 1/50 in NSS/TBS). Dilution of primary antibody was determined empirically. Sections were rinsed and washed as above and the secondary antibody a sheep anti-rabbit IgG biotinylated (Santa Cruz Biotechnology) (diluted 1/250 in NSS/TBS) was applied for 60 minutes. Subsequent steps were similar to BrdU staining procedure. Positive TGF- β 1 staining was seen as brown staining. TGF- β 1 expression was found at the epithelium surface and or at the crypt epithelium and occasionally in lamina propria. A positive control for TGF- β 1 and a negative control without addition of primary antibody were included in each staining run to confirm positive staining. Representative TGF- β 1 stained tissue sections are shown in chapter 6 (Figure 52 and Figure 53).

(Details and catalogue reference numbers of reagents used are found at the end of the Thesis in 'Appendix').

4.4.2.1. Examination of TGF- β 1 stained tissue

Stained slides were investigated in a blinded manner under a Video-Image-Analyser Leica, Q500 MC (Leica, Cambridge, UK). Some workers have used a scoring system for TGF- β positive cells as - strongly positive (+), weakly positive (\pm) or negative (-) (Avery et al., 1993). However, inter-observer variation can be a problem as seen in a study by Roe *et al* (Roe et al., 1996), who investigated measurements of colonic proliferation by BrdU across laboratories showing that counting crypts and labelled cells may vary among different laboratories. In this study TGF- β 1 staining expression was estimated as a percentage of actual tissue area in mm^2 , which gave a quantitative value for comparison.

Section staining was quantified using a Leica Video-Image-Analyser Q500MC (Leica UK, Cambridge). The TGF- β 1 staining was detected by a specially designed computer program, which minimised observer variation. The microscope was set up for Kohler illumination calibrated for a magnification of x10 and the motorised stage was initialised. After initialising, each section was examined under the microscope under a calibrated x10 objective. Automatic colour detection was set to ensure that all positive staining was recorded. The stage was set to move automatically in a pattern that covered the whole section but did not encroach on previously visited areas. The total area of tissue was also measured and the results expressed as area of TGF- β 1 staining per mm^2 of tissue and 1 pixel size on the monitor was equal to 0.000832 mm. Measurements were also made using a calibrated x25 objective, which gave identical results but required many more fields to be examined and yielded no obvious benefit. This automated method was largely independent of the operator and permitted high throughput.

4.5. Cytokine Assays

The collected and stored frozen culture supernatant (at -70°C) was analysed by an Enzyme Linked Immuno-Sorbent Assay (ELISA) and this was based on the indirect sandwich ELISA technique. This technique consisted of 10 general steps:

1. Passive absorption of the antibody directed against antigen
2. Washing
3. Addition of the antigen
4. Washing
5. Addition of the antibody from different species vs. antigen
6. Washing
7. Addition of enzyme-labelled anti species (directed against antibody)
8. Washing
9. Addition of colour development substrate
10. Reading plate on ELISA reader

(Crowther, 1995)

Four different cytokines were detected using ELISA: IL1 β , IL1ra, IL10 and TGF- β 1. The cytokine ELISAs did not differ from each other in their general procedure and individual descriptions are therefore abbreviated. For the TGF- β ELISA, samples prior to assay were activated with 1 N hydrochloride acid and after 10 minutes neutralised with 1.2 N NaOH. Assay block and diluent solutions were specific for individual cytokine assays and specific for TGF- β 1 assay. Washing buffer was the same for interleukin and TGF- β 1 assays.

Material

Phosphate buffer saline tablets (PBS)

ELISA plates

IL1 β capture antibody

IL1 β protein

IL1 β biotinylated detection antibody

Streptavidin-horse-radish peroxidase conjugate (HRP)

∪

IL1ra capture antibody

IL1ra protein

IL1ra biotinylated detection antibody

Streptavidin-horse-radish peroxidase conjugate (HRP)

∪

IL10 capture antibody

IL10 protein

IL10 biotinylated detection antibody

Streptavidin-horse-radish peroxidase conjugate (HRP)

Wash buffer

0.05 % Tween 20 in PBS Ph 7.4

Interleukin diluent (for 200ml):

0.2 % Bovine Serum albumin

20 ml of Tris buffer saline Ph 7.6

180 ml of Sodium chloride, 0.9%

0.002 Tween 20

Interleukin blocking buffer (for 200ml):

2% Bovine serum albumin

10 % Sucrose

∪

Substrate

O-phenylene-edamine-dihydrochloride (Fast-OPD) tablets

Tetra-methylbenzidine (TMB) substrate.

∪

TGF β 1 Duoset kit containing:

TGF β capture antibody

TGF β protein

TGF β biotinylated detection antibody

Streptavidin – HRP conjugate

∪

TGF β 1 diluent (for 100ml)

1.4 % delipidized bovine serum

0.05% Tween 20 in PBS, pH 7.3, 0.2 μ m filtered

TGF β Block solution (for 100ml):

5 % sucrose

5 % Tween 20 in PBS

1 N sodium chloride

1.2 N NaOH/0.5 M HEPES

∪

(Details and catalogue reference numbers of reagents used are found at the end of the thesis in 'Appendix').

4.5.1. IL1 β Assay

IL1 β was measured in culture supernatants using matched antibody pairs obtained from R&D Systems. Each antibody had been titrated for use at each stage of the ELISA. IL1 β capture antibody was reconstituted with 1 ml of Phosphate buffer saline (PBS). ELISA plates were coated with 100 μ l per well of the capture antibody at a concentration of 3.5 μ g / ml in PBS (adding 70 μ l of capture antibody to 10 ml of PBS). The plate was incubated overnight at 24°C. After 3x washing the next morning with interleukin wash and blotting the plate on a paper towel, it was blocked with block buffer 300 μ l/well for a minimum time of 1 hour. Defrosted culture supernatant samples were added in duplicates of 100 μ l/well to the plates, either undiluted or diluted with culture medium, depending on amount of sample available. A standard curve was constructed using recombinant IL-1 β in culture medium with concentrations double diluted out from 250 – 3.9 pg/ml. A blank sample of culture medium alone was included in the standard curve. After adding the samples, the plate was incubated at 24°C for 2 hours. A biotinylated detection antibody was used at a concentration of 90 ng/ml in diluent, (adding 18 μ l of detection antibody to 10 ml of diluent) and 100 μ l were added per well. Subsequently plates were repeatedly incubated at 24°C for 2 hours. After washing, HRP conjugate peroxidase was added to the plate (100 μ l/well) at a dilution of 1:20.000 and incubated at 24°C for 30 minutes. Substrate OPD was added at 100 μ l per well and incubated for 30 minutes at 24°C. The colour development was stopped with 3M sulphuric acid by adding 50 μ l per well, and plates were read at 490nm on an ELISA reader.

4.5.2. IL1ra Assay

IL-1ra was measured in culture supernatants using matched antibody pairs obtained from R&D Systems. As in the IL1 β assay, each antibody had been titrated for use at

each stage of the ELISA. The plate was coated at a concentration of 9µg/ml in PBS (adding 90µl of capture antibody to 10 ml of PBS) and coated with 100 µl per well. The IL1ra plate was incubated overnight and 3x washed the next morning and blotted on a paper towel. The processing steps for IL1ra are the same as the IL1β ELISA method. The plates were blocked with 300µl/well, using the block buffer for the Interleukin assays. A standard curve was constructed using recombinant IL-1ra in culture medium with concentrations double-diluted out from 5000 – 78 pg/ml. A blank sample of culture medium alone was included. Culture supernatant samples were added to plate (100µl/well) in duplicate. Samples were incubated at 24°C for 2 hours. An IL1ra detection antibody was used at a concentration of 60 ng/ml in Interleukin diluent (100µl/well), (adding 12 µl of detection antibody to 10 ml of PBS) and the plate was incubated at 24°C for 2 hours. A HRP conjugate was added to the plate (100µl/well) at a dilution of 1:20,000 and incubated at 24°C for 30 minutes. TMB substrate was used and finally incubated for further 30 minutes at 24°C. Colour development was stopped with 3M sulphuric acid and plates were read at 450nm on an ELISA reader.

4.5.3. IL10 Assay

IL-10 was measured in culture supernatants by following the same steps as for IL1β and IL1ra. The IL10 capture antibody was obtained from R&D and reconstituted with 1ml of PBS at a concentration of 4µg/ml in PBS. Plates were coated with 100µl per well, by adding 80µl of IL10 coat to 10 ml of PBS. Plates were incubated overnight at 24°C. After washing, blotting and blocking for a minimum of 1 hour, 100µl /well culture supernatant samples were added to plate. A standard curve was constructed using recombinant IL-10 in culture medium with concentrations double diluted out from 2000 – 31.3 pg/ml. A blank sample of culture medium alone was included. After 2 hours incubation period, a biotinylated detection antibody was used at a concentration of 420 ng/ml in diluent (adding 42µl of detection antibody to 10 ml of diluent). After the incubation period of 2 hours, repeated washing and blotting, a HRP conjugate was added to the plate (100µl/well) at a dilution of 1:20,000 and incubated at 24°C for 30

minutes. TMB was used as substrate, and the plate was incubated for further 30 minutes. The colour development was stopped with 3M sulphuric acid and plates were read at 450nm on an ELISA reader.

4.5.4. TGF- β 1 Assay

TGF- β was measured in culture supernatants using a DuoSet kit from R&D. The method was carried out according to the manufacturer's instructions, using ELISA plates from Corning Costar. The plate was coated with capture antibody anti-TGF- β 1, which was reconstituted with 1 ml of PBS, required at 2 μ g/ml, (adding 60 μ l of coat to 10.8 ml of PBS) and incubated overnight at 24°C. Next morning the plate was washed, blotted on a paper towel and blocked with TGF- β 1 block solution for approximately 1 hour. The TGF- β in the samples was activated with 1M HCL (50 μ l/250 μ l sample) for 10 minutes and neutralised with 1.2M NaOH. As foetal calf serum contains TGF- β , a control sample of culture medium was activated, neutralised and used in parallel with the samples. A standard curve was constructed using recombinant human TGF- β 1 in diluent with concentrations double diluted out from 2000 – 31.3 pg/ml. A blank sample of TGF- β 1 in diluent was included. After the 2 hours incubation period a biotinylated anti-human TGF- β 1 detection antibody was reconstituted with 1ml of PBS and 100 μ l/ well was added to the plate. It was used at a concentration at 300ng/ml, by adding 60 μ l to 10.8ml of diluent biotinylated detection antibody. After the incubation period of 2 hours, repeated washing and blotting, a HRP conjugate was added to the plate (100 μ l/well) at a dilution of 1:200 by adding 54 μ l of HRP to 10.8ml of diluent, and incubated at 24°C for 30 minutes. As substrate, OPD was used and the plate was incubated for further 30 minutes. The colour development was stopped with 3M sulphuric acid and plates were read at 490nm on an ELISA reader.

4.5.5. Interference of enteral diet with ELISA assay

Primary ELISA assays for IL1 β , IL1ra, IL10 and TGF- β 1 were performed in a random selection of enteral diet modifications to exclude any interference of the diet with the ELISA assay. These assays were performed in Waymouth medium, elemental diet (EAA), in olive oil (EOL), sunflower oil (ESU), safflower oil (ESA) and canola oil (ECA); diets were diluted at 1:10. Seven concentrations of cytokine standard were added to all the different ED compositions.

Plotting of ELISA for IL1 β , IL1ra, IL10 and TGF- β showed that overall, known added concentrations correlated well with the ELISA results by plotting all seven-concentration levels. Therefore increasing concentrations of cytokine standards added to all ED compositions resulted in an appropriate proportional increase in the ELISA concentrations of the cytokine mixed in ED composition. It proved that diet compositions did not interfere with the assays significantly. Correlations of added concentrations of standards to assayed cytokine concentrations are in Table 10.

Table 10 Correlation between graded standard concentrations of cytokines added to enteral diet composition and results of ELISA performed on these solutions

Assay conc. & correlation	EAA	EOL	ESU	ESA	ECA
IL1 β 250 pg, 125pg, 62.5pg, 31.3 pg, 15.6pg, 7.8 pg, 3.9 pg- correlation : r	0.99	0.99	0.99	0.98	0.99
IL1ra 2500 pg, 1250pg, 625pg, 313pg, 156pg, 78pg, 39pg correlation: r	0.99	0.99	0.99	0.99	0.99
IL10 2000 pg, 1000pg, 500pg, 250pg, 125pg, 62.5pg, 31.3pg, correlation: r	0.99	0.99	0.99	0.99	0.97
TGF- β 1 2000 pg, 1000pg, 500pg, 250pg, 125pg, 62.5pg, 31.3pg, correlation: r	0.99	0.99	0.99	0.99	0.99

4.5.5.1. Recovery of cytokines after incubation in enteral diet formulae

Cytokine recovery experiments were only carried out in EAA, as it was limited by availability of the diets. It is unlikely that cytokine recovery might be significant influenced by differences in amino acid compositions or fatty acid composition as the basic composition of these dietary modifications were all based on EAA (EO28). For diet details refer to Table 2 and Table 3. Elemental diet EAA was diluted in 1:10 dilution and 'spiked' with two known concentrations of cytokines for IL1 β , IL1ra, IL10 and TGF- β 1. This was similarly performed for medium. The 'unspiked' and 'spiked' diet and medium were incubated for four time points i.e. 0h, 3h, 6h and for 24h, each cytokine incubated for unspiked and two spiked concentrations. After each time point, culture fluid was collected, stored and frozen for assay.

The test was executed for two 'spiked' concentrations of 'low' and 'high'.

A low concentration was determined as IL1ra 100pg/ml; IL1 β of 10pg/ml; IL10 100pg/ml and TGF- β 1 of 100pg/ml. A high concentration was determined as IL1ra of 1000pg/ml; IL1 β 100pg/ml; IL10 1000pg/ml and TGF- β 1 1000pg/ml.

Results of recovery experiments show that IL1 β at low concentration was detectable at all time points to 100 % and at high concentration 100 % detectable at 0h, 3h and 6 hours but only 60 % at 24 hours (Figure 31A)

Recovery of IL1ra at low concentration showed that more than 50 % of cytokines was detected over entire 24 hours (69.3% at 24 hours), and at high concentration a steady decrease of detectable cytokines from 100 % at 0 h to 52 % after 24 h was seen (Figure 31B).

For IL10 at low concentration nearly 80% was detectable after 0 h and 40% after 24 h. At high concentration the detected amount of cytokines decreased to 40% after 24 h (Figure 31C).

Since IL1 β at low concentration was stable and 100% detectable at all time points, it is likely that IL1ra /IL1 β ratio in these experiments is conservative, as IL1ra was less stable over a period of time. IL10 was the least stable.

TGF- β 1 was stable and 100 % detectable at all time points (Figure 31D).

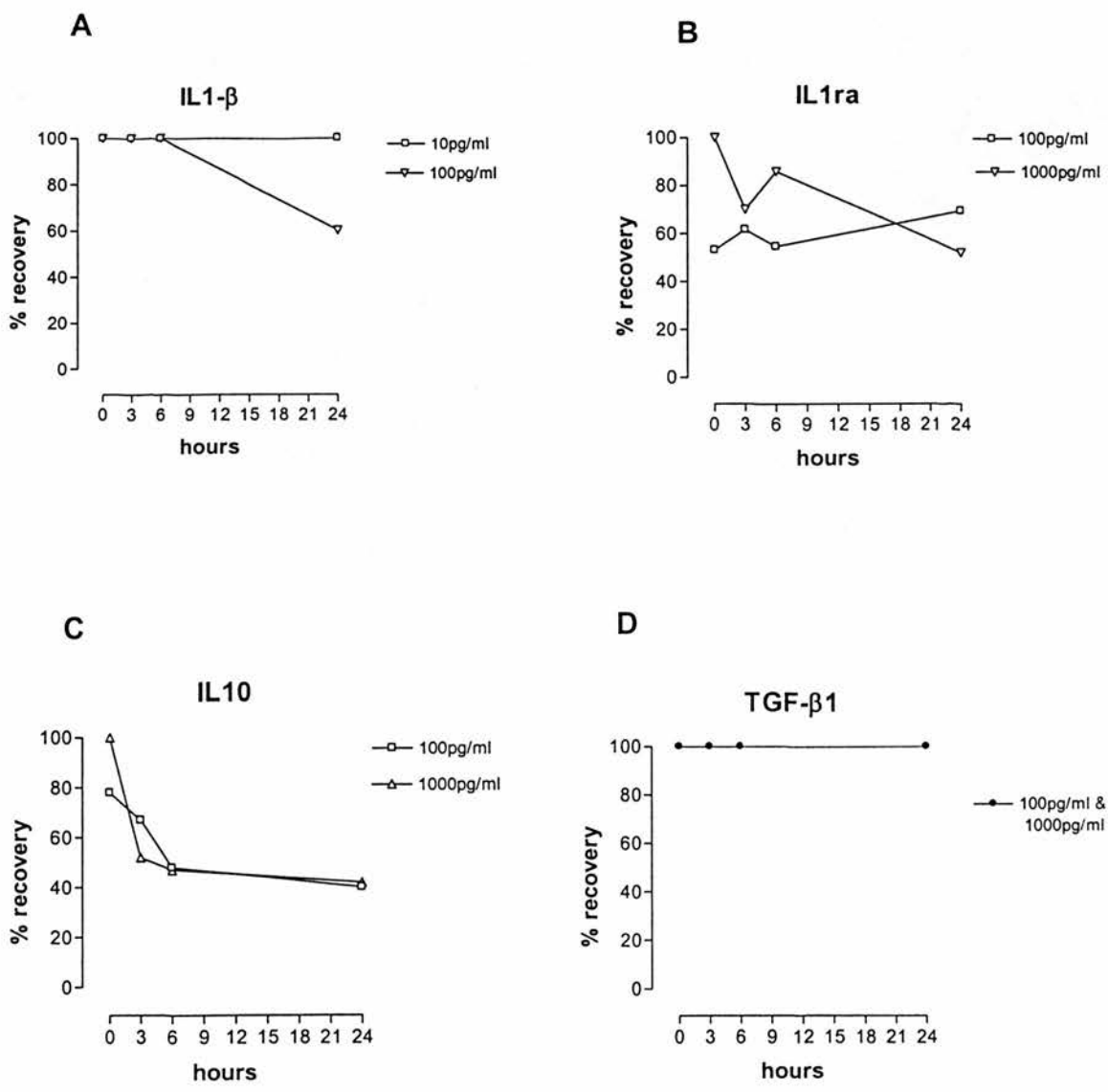


Figure 31 Graphs A to D showing % recovery of cytokines by ELISA up to 24-hours. The two different lines indicate two different initial spiking concentrations

4.6. Statistics

All the experiments were performed with a control, which was incubation of tissue from the same patient with medium control (MC). In addition, experiments were performed with tissue derived from control patients i.e. non-inflamed, non-IBD patients.

Differences of cytokine ratios of anti-inflammatory to pro-inflammatory cytokines after incubation with modified diets were all compared with MC unless otherwise specifically stated in text. Differences in TGF- β 1 expression as immunostaining area calculated as a percentage of total area of tissue in mm² was compared with MC staining expression if not otherwise stated in text. All data are expressed as mean and standard error of the mean (SEM) found as \pm in brackets. Graphically, the data are shown schematically as vertical bars with error bars plotting the mean and SEM.

All comparisons were statistically analysed against the MC using comparative statistics. The data on cytokine ratios after incubation with elemental diet, enteral diet –casein and enteral diet -whey were analysed using Student's t-test as the data was parametrically distributed. All other data on cytokine ratios were analysed using non-parametric Mann-Whitney U-test for comparison with MC. The data on TGF- β 1 expression was analysed using unstacked ANOVA estimation of variance. The differences were considered to be significant at $p \leq 0.05$ level. In general, the data from three dilutions of dietary formulae (1:20, 1:10, 1:5) were compared with MC and no correction for multiple testing was performed.

Correlation between cytokine ratios and TGF- β 1 expression was calculated using Pearson's correlation coefficient (r) and the significance of the correlation coefficient was calculated from the regression equation. Again, the results were considered to be significant at $p \leq 0.05$ level.

All statistical calculations were performed using the MINITAB (release 10).

SECTION II

Study Plan: Experiments and Subjects

Incubation of biopsy tissues with enteral diets presented in this thesis was performed on 117 patients. From these patients biopsies were obtained during endoscopy and incubated with diets modified in protein and fatty acid composition, assayed for cytokines and stained for BrdU to assess tissue viability. Only viable tissue was included in the final analysis. None of the IBD patients recruited in this study were on enteral nutritional therapy, prior to, or at the time of endoscopy.

In a smaller cohort of patients additional biopsies were immunohistochemically stained for TGF- β 1 and TGF- β 1 cytokine was assayed. Figure 32 describes experiment design with reference to patient diagnosis.

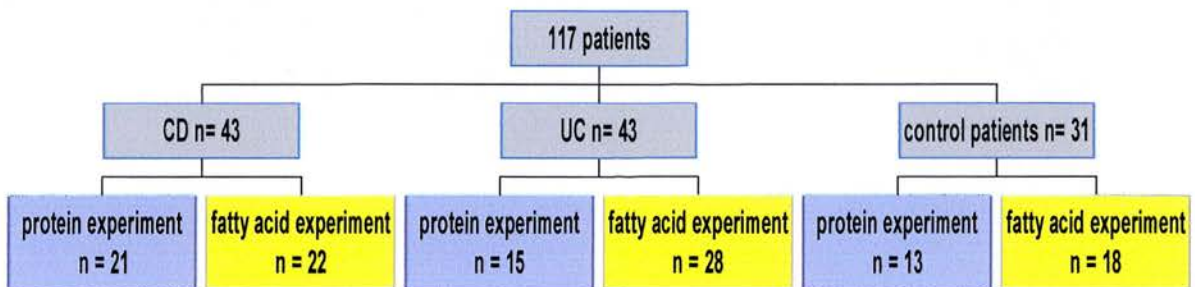


Figure 32 Patient design for experimental set up
The number of patients in different groups included for final analysis

4.7. Enteral diet protein modifications

Experiments for protein incubations were performed in 36 patients with IBD (21 CD, 15 UC) and 13 non-inflamed control patients. The incubation design for the diets is shown in Figure 33. Clinical characteristics and patient details for protein experiment is summarised in Table 11. A detailed separate description of patient information is included in the result sections for each of the protein incubations. Each of the result chapters for enteral diet modifications contains a brief summary of results obtained and a discussion. All the biopsies were handled and processed in the same manner as described in Chapter 4, section I and the method is therefore not repeated.

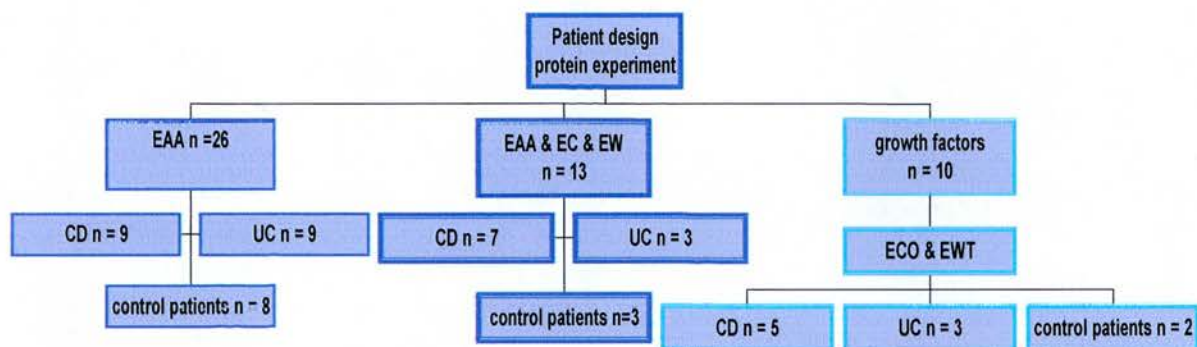


Figure 33 Patient design for protein experiment
Number of patients in different groups included in the study.

Table 11 Clinical characteristics of patients for protein incubations

Diagnosis	Disease location	Surgery	Age	Sex
CD 21	5 colorectal; 11 ileocolonic; 4 colonic; 1 rectosigmoid	2 Hemi-colectomy+ileostomy ; 3 terminal ileoectomy; 5 hemicolectomy	38.4 ± 3	10 F 11 M
UC 15	2 proctitis; 5 distal proctitis; 3 left-side proctitis; 4 colorectal ; 1 quiescent proctitis	No information	40.8 ± 3	6 F 9 M
Control patients 13	3 Post-infectios IBS; 6 diarrhoea; 2 IBS; 2 abdominal pain	-	49.4 ± 5	8 F 5 M

4.7.1. Biopsies for protein incubation

From these patients 191 biopsies were obtained and incubated with either EAA (1:20, 1:10, 1:5) or EAA & EC & EW (1:20, 1:10, 1:5), respectively and medium control. Further 70 biopsies were incubated with diets modified for ECO & EWT. Additionally, one biopsy for EAA in 1:10 dilution was used as a second control along with 10 biopsies obtained for medium control (MC) for the ECO and EWT incubations. Table 12 describes the number of incubations in IBD, control patients and medium control in protein experiments, which also shows the actual number of biopsies from which data could be obtained for final analysis.

Table 12 Biopsies in protein experiment

Incubation in	No. incubation / data obtained		No. incubation / data obtained
	EAA	EAA & EC & EW	ECO & EWT & EAA 1:10
IBD & control patients	49 / 39	105 / 94	70 / 50
CD	73 / 60		35 / 21
CD medium	13 / 13		5 / 3
UC	42 / 38		21 / 13
UC medium	15 / 7		3 / 1
Control patients	39 / 35		14 / 10
Control pat. medium	9 / 5		2 / 2
Total diet and medium	191 / 158		80 / 50

4.8. Enteral diet fatty acid modifications

68 patients (CD, UC and control patients) were recruited in order to obtain biopsies for the fatty acid experiments. From each patient 7 biopsies were obtained and incubated in pairs with two diets modified in fatty acid composition (dilutions 1:20, 1:10 and 1:5). This included one biopsy incubated with medium as medium control (MC). The study plan and performance of experiment is described in Chapter 4, section I. The number of

fatty acid experimental incubations in IBD and control patients is shown in Table 13.

Table 13 Patient design for fatty acid experiment

Diagnosis	EOL n 15	ESA n 15	ECA n 14	ESY n 14	EFI n 20	ESU n 20	COCF n 19	COC n 19
CD	6	4	4	6	4	4	8	8
UC	5	6	6	4	12	11	6	6
Control patients	4	5	4	4	4	5	5	5

Table 14 summaries the clinical characteristics of patients recruited for fatty acid experiments, which are also listed in the result section of fatty acid experiments, specifically itemized for each series of individual experiments.

Table 14 Clinical characteristics of patients for fatty acid experiments

Diagnosis	Disease location	Surgery	Age	Sex
CD 22	7 colorectal; 1 quiescent; 1 perianal; 1 colitis; 1 small bowel; 11 no information	1 (right hemicolectomy + ileocaecalresection); 1 (proctocolectomy+ileostomy); 1 (small bowel resection); 1 (right hemicolectomy); 1(colectomy+ileostomy); 1 (right hemicolectomy+small bowel resection)	45.4 ± 4	9 M 13 F
UC 28	5 quiescent; 6 pancolitis; 2 proctitis; 3 left site colitis; 2 distal colitis; 10 no information	1 temp.pancolectomy	52.6 ± 3	13 M 15 F
Control patients 18	8 IBS; 2 polyp removal; 8 diarrhoea;	-	52.8 ± 4	9 M 9 F

4.8.1. Biopsies for fatty acid incubation

From these 68 patients 473 biopsies were obtained and incubated for 24 hours as described in organ culture protocol in Chapter 4, section I. Table 15 explains the study design for the experiments in relation to number of biopsies taken for IBD, control patients and medium control, and the number included for final analysis.

Table 15 Biopsies in fatty acid experiments

Incubation in	No. of incubations / data obtained									
	MC	EOL	ESA	ECA	ESY	EFI	ESU	COCF	COC	total
IBD & Control patients	68 / 55	45 / 32	45 / 45	42 / 35	42 / 30	60 / 45	60 / 60	56 / 28	55 / 24	473 / 354
CD	22 / 19	18 / 15	12 / 12	12 / 11	18 / 15	12 / 12	12 / 12	24 / 10	24 / 8	154 / 114
UC	28 / 23	15 / 8	18 / 18	18 / 16	12 / 9	36 / 21	33 / 33	17 / 8	16 / 8	193 / 141
Control patients	18 / 16	12 / 9	15 / 15	12 / 8	12 / 6	12 / 12	15 / 15	15 / 10	15 / 8	126 / 99

Table 16 Table of the 117 patients in whom incubation experiments have been performed with different defined formulae diets

Patient Details				Medication			State of tissue		Diagnosis	Experiment
N	M	I	Age	5-ASA	AZA	Steroid	inflamed	not inflamed		
1.	x		46	x			x		CD	EAA
2.		x	40				x		UC	EAA
3.	x		36					x	Control	EAA
4.	x		41	x		x	x		UC	EAA
5.	x		22		x		x		UC	EAA
6.		x	16		x		x		CD	EAA
7.	x		40		x	x	x		CD	EAA
8.		x	30	x			x		CD	EAA
9.	x		64				x		CD	EAA
10.	x		37	x		x	x		CD	EAA
11.		x	39			x	x		UC	EAA
12.	x		45				x		UC	EAA
13.		x	33	nk	nk	nk	x		UC	EAA
14.	x		35	nk	nk	nk	x		UC	EAA
15.		x	51	nk	nk	nk	x		UC	EAA
16.		x	75					x	Control	EAA
17.	x		42					x	Control	EAA
18.	x		46	x		x	x		CD	EAA
19.		x	44					x	Control	EAA
20.		x	37					x	Control	EAA
21.	x		51				x		CD	EAA
22.	x		60					x	Control	EAA
23.		x	17				x		CD	EAA
24.	x		23					x	Control	EAA
25.		x	38				x		UC	EAA
26.		x	20					x	Control	EAA
27.	x		33		x		x		CD	EAA,EC, EW
28.		x	38				x		CD	EAA,EC, EW
29.	x		37	x			x		UC	EAA,EC, EW
30.		x	31	nk	nk	nk	x		CD	EAA,EC, EW
31.	x		47				x		CD	EAA,EC, EW
32.	x		38	x			x		CD	EAA,EC, EW
33.		x	68					x	Control	EAA,EC, EW
34.		x	19			x	x		CD	EAA,EC, EW
35.	x		51	x		x	x		CD	EAA,EC, EW
36.		x	37					x	Control	EAA,EC, EW
37.	x		24		x	x	x		UC	EAA,EC, EW
38.		x	43					x	Control	EAA,EC, EW
39.	x		48	x			x		UC	EAA, EC,EW
40.	x		60					x	Control	ECO, EWT
41.		x	53					x	Control	ECO, EWT
42.	x		69	x			x		UC	ECO, EWT
43.		x	52	nk	nk	nk	x		CD	ECO, EWT
44.	x		52	x		x	x		UC	ECO, EWT
45.		x	54				x		CD	ECO, EWT
46.		x	39				x		UC	ECO, EWT
47.		x	15	x			xx		CD	ECO, EWT
48.	x		42			x	x		CD	ECO, EWT
49.		x	49	x			x		CD	ECO, EWT

N	M	F	Age	5-ASA	AZA	Steroid	inflamed	not inflamed	Diagnosis	Experiment
50.	x		71				x		CD	ESA, ECA
51.		x	48				xx		CD	COCF, COC
52.	x		60			x	x		CD	COCF, COC
53.	x		48				x		CD	COCF, COC
54.		x	44				x		CD	EOL, ESY
55.		x	61				x		CD	EOL, ESY
56.		x	38			x	x		CD	COCF, COC
57.		x	34				x		CD	ESA, ECA
58.	x		26			x	x		CD	EOL, ESY
59.		x	39				x		CD	ESA, ECA
60.		x	38	x		x	x		CD	ESA, ECA
61.	x		18		x		x		CD	EFI, ESU
62.	x		19		x		xx		CD	EFI, ESU
63.	x		52		x	x	x		CD	EFI, ESU
64.		x	75			x	x		CD	EFI, ESU
65.		x	90				x		CD	EOL, ESY
66.		x	20				x		CD	COCF, COC
67.		x	60				x		CD	COCF, COC
68.		x	48			x	x		CD	EOL, ESY
69.		x	53	x		x	x		CD	COCF, COC
70.	x		19				x		CD	COCF, COC
71.	x		37			x	x		CD	EOL, ESY
72.		x	29					x	Control	EFI, ESU
73.		x	75					x	Control	COCF, COC
74.	x		64					x	Control	EFI, ESU
75.	x		73					x	Control	ESA, ECA
76.	x		77					x	Control	ESA, ECA
77.		x	24					x	Control	ESU, ECA
78.		x	58					x	Control	EFI, ESU
79.	x		39					x	Control	EFI, ESU
80.		x	18					x	Control	EFI, ESU
81.	x		68					x	Control	ESA, ECA
82.		x	46					x	Control	EOL, ESY
83.		x	58					x	Control	COCF, COC
84.		x	60					x	Control	COCF, COC
85.	x		59					x	Control	COCF, COC
86.	x		58					x	Control	EOL, ESY
87.	x		66					x	Control	EOL, ESY
88.	x		28					x	Control	EOL, ESY
89.		x	52					x	Control	COCF, COC
90.		x	29		x		x		UC	EFI, ESU
91.	x		63	x			x		UC	ESA, ECA
92.		x	75				x		UC	EFI, ESU
93.	x		56				x		UC	EFI, ESU
94.		x	64			x	x		UC	EFI, ESU
95.	x		20		x	x	x		UC	EFI, ESU
96.		x	46	x			x		UC	EFI, ESU
97.		x	69				x		UC	EFI, ESU
98.		x	51	x			xx		UC	EFI, ESU
99.		x	69				x		UC	EFI, ESU
100.	x		25	nk	nk	nk	x		UC	EFI, ESU
101.		x	36				xx		UC	COCF, COC
102.		x	53				x		UC	COCF, COC
103.	x		60				x		UC	EOL, ESY

N	M	F	Age	5-ASA	AZA	Steroid	Inflamed	not inflamed	Diagnosis	Experiment
104.	x		27				x		UC	EOL, ESY
105.	x		51				x		UC	EOL, ESY
106.	x		38				x		UC	EOL, ESY
107.	x		79				x		UC	ESA, ECA
108.		x	59	x			xx		UC	COCF, COC
109.	x		69				x		UC	COCF, COC
110.		x	46	x			x		UC	EFI, ESU
111.	x		53	x		x	x		UC	ESA, ECA
112.		x	48	nk	nk	nk	x		UC	COCF, COC
113.		x	55	x			x		UC	ESA, ECA
114.		x	73	x			x		UC	ESA, ECA
115.	x		37		x		x		UC	ESA, ECA
116.	x		69				x		UC	COCF, COC
117.		x	65				x		UC	EOL, ESY

(N: numbers; M; male; F: female; n.k.: nothing known; x: inflamed; xx: severe inflamed)

RESULTS

SECTION I

Manipulation of the Nitrogen Source of Enteral Diets

CHAPTER FIVE

5. Effects after incubation of inflamed and non-inflamed tissues with elemental diet (EAA), enteral diet- casein (EC) and enteral diet-whey (EW).

Introduction

Low nutritional intake is common in IBD, and may result in a lack of essential nutrients and malnutrition (Ferguson et al., 1998). Nutritional deficiencies are more common in CD than in UC. Nutritional therapy is not considered to be useful in inducing or maintaining remission in UC, although a milk-free diet has been reported to be more beneficial compared with a milk-containing diet which increased relapses (Wright R. and Truelove S.C., 1965). Nutritional therapy is more widely used and effective in CD, where it may improve disease activity; e.g. parenteral nutrition tends to improve nutrition and diminish inflammation, possibly by gut rest. However, treatment with a defined formula diet is cheaper, more effective and results in improvement of inflammatory symptoms (Teahon et al., 1990; Rigaud et al., 1991). The mechanism of action is unknown but possible explanations proposed include alteration of bacterial flora, exclusion of protein antigens, enterocytic immune response and a low fat content (Murch and Walker-Smith, 1998). Defined formula diets improve nutritional status and often induce remission of disease activity (Teahon et al., 1995). The efficacy of ED in inducing clinical remission was seen in 66% of CD patients after 4 weeks of treatment; ED allowed earlier clinical improvement and also a greater decrease in protein-losing enteropathy compared with polymeric diets (Rigaud et al., 1991). In adults an improvement in clinical features and long-term remission in uncomplicated CD could be achieved by ED (Teahon et al., 1990).

As discussed in Chapter 2, pro-inflammatory cytokines such as IL1 β , TNF α (Breese et al., 1994) or indirectly induced cytokines such as IL6, as well as anti-inflammatory cytokines (Reimund JM et al., 1996), such as IL1ra, are altered in IBD (Andus et al., 1997). A significantly raised production of IL1 β compared to controls was found in fresh and 24 hour cultured CD biopsy tissue (Ligumsky et al., 1990). A decreased ratio

of IL1ra/IL1 β seems to be more common in IBD compared with controls and suggests the hypothesis that an imbalance of IL1ra/IL1 β ratio may play an important role in IBD (Dionne et al., 1998; Casini-Raggi et al., 1995). IL10 has an anti-proliferative effect on human intestinal lamina propria T-cells, and also induces IL1ra secretion in peripheral monocytes and intestinal lamina propria mononuclear cells. In addition it is known to restore a decreased IL1ra/IL1 β in IBD to normal levels (Schreiber et al., 1995).

In vivo, a commercial polymeric enteral diet is reported to have a down-regulatory effect on pro-inflammatory cytokines (Fell J.M.E. et al., 2000). Data from our unit has also shown a decrease in IL1 β levels in gut lavage fluid after treatment with elemental diet (Ferguson et al., 1998). A crucial question is whether EAA is merely an exclusion diet or whether it possesses direct anti-inflammatory effect. If the mechanism of action of EAA was better understood, defined formula diets could be improved further to help reduce inflammation.

The project described in this Chapter investigates the effect of enteral diet on anti-inflammatory to inflammatory cytokine ratio after incubation with different enteral diet modifications.

Subjects and Methods

The anti-inflammatory to pro-inflammatory cytokine ratios for IL1ra / IL1- β and IL10 / IL1- β after enteral diet incubation using three different preparations; EO28 (EAA), EO-Casein (EC) and EO-Whey (EW) were obtained in 39 patients.

The patient groups were

CD (16), (10) M, (6) F, mean age (37 \pm 3) years.

UC (12), (7) M, (5) F, mean age (37 \pm 3) years.

Control patients (11), (4) M, (7) F, mean age (48 \pm 6) years.

From these patients a total of 191 biopsies were obtained and 158 biopsies were finally analysed for incubation with the different enteral diet formulae as follows:

CD: EAA (29), EC (16), EW (15), medium control (MC) (13).

UC: EAA (21), EC (9), EW (8), MC (7)

Control patients: EAA (21), EC (8), EW (6), MC (5)..

Site of biopsy taken were:

CD: (12) colon, (4) ileum

UC: (11) colon, (1) rectum

Control patients: (10) colon, (1) sigmoid

Time period from diagnosis to biopsies taken, were as follows:

1-3 years: (3) CD; (6) UC

4-6 years: (2) CD; (2) UC

> 6 years: (7) CD; (3) UC

Medications taken at time of taking of biopsies were:

CD: steroids (2), 5-ASA (4), AZA (1), steroids (2) and 5-ASA (3), AZA and steroids (2).

UC: steroid (1), 5-ASA (2), steroids and 5-ASA (2), steroids and AZA (1).

5.1. IL1ra / IL1β ratios after incubation with amino-acid based elemental diet (EAA) enteral diet containing casein (EC) and enteral diet containing whey (EW)

5.1.1. Crohn`s disease

Incubation of CD tissues with EAA increased the IL1ra / IL1β ratio in all three dilutions compared with MC, but the increase reached statistical significant only in 1:10 dilution compared with MC (Figure 34).

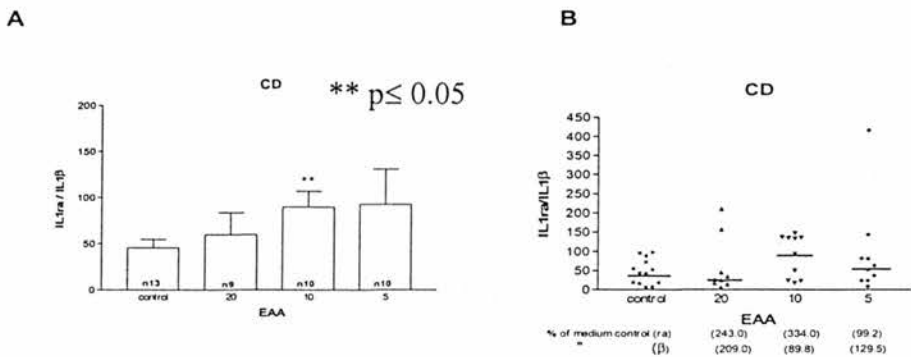


Figure 34 A+B (A) IL1ra / IL1β ratio after 24-h EAA incubation in CD. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β

Incubation of CD tissue with EC resulted in a significant increase of the IL1ra / IL1β ratio in all three dilutions ($p \leq 0.05$) compared with MC. The increase was not concentration dependent as the highest ratio was seen with 1:10 dilution (Figure 35).

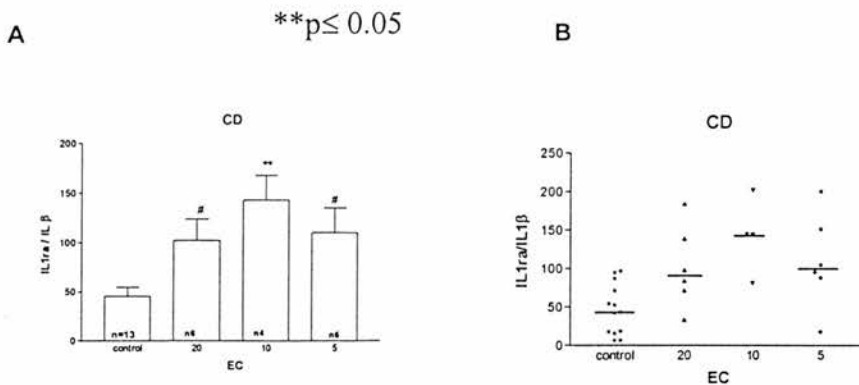


Figure 35 A+B (A) IL1ra / IL1β ratio after 24-h EC incubation in CD. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β

Incubation of CD tissue with EW increased the IL1ra / IL1 β ratio for 1:5, 1:10 and 1:20 but did not reach statistical significance. However, by combining the ratios for IL1ra / IL1 β in EW for all three dilutions of 1:5, 1:10 and 1:20 together, a significant increase compared with MC was demonstrated (Figure 36 and Table 17).

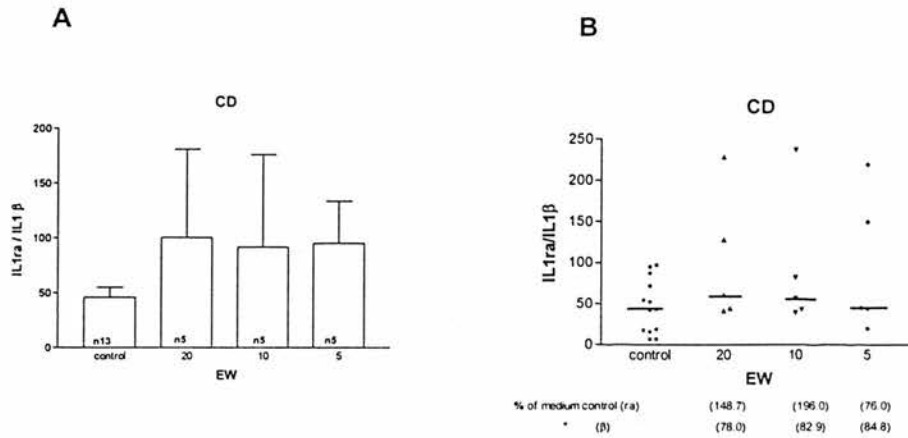


Figure 36 A+B (A) IL1ra / IL1 β ratio after 24-h EW incubation in CD. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

5.1.2. Ulcerative colitis

Incubation of UC tissues with EAA resulted in no significant change in the IL1ra / IL1 β ratio for any dilution (Figure 37). The incubations of UC tissue with EC and EW resulted in no significant change in the IL1ra / IL1 β ratio either. The MC ratio of IL1ra / IL1 β was similar to the MC ratio in CD (Figure 37, Figure 38).

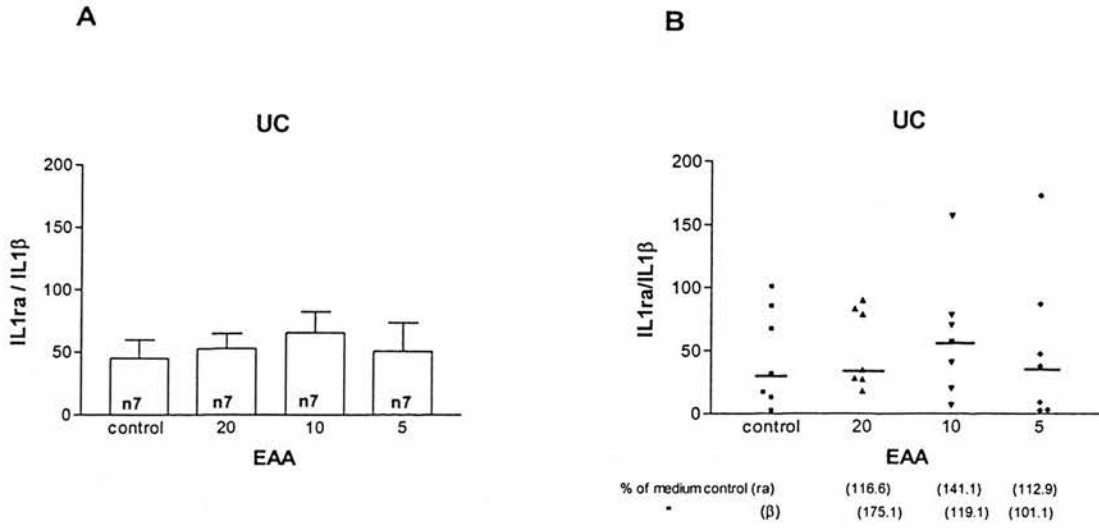


Figure 37 A+B (A) IL1ra / IL1 β ratio after 24-h EAA incubation in UC. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

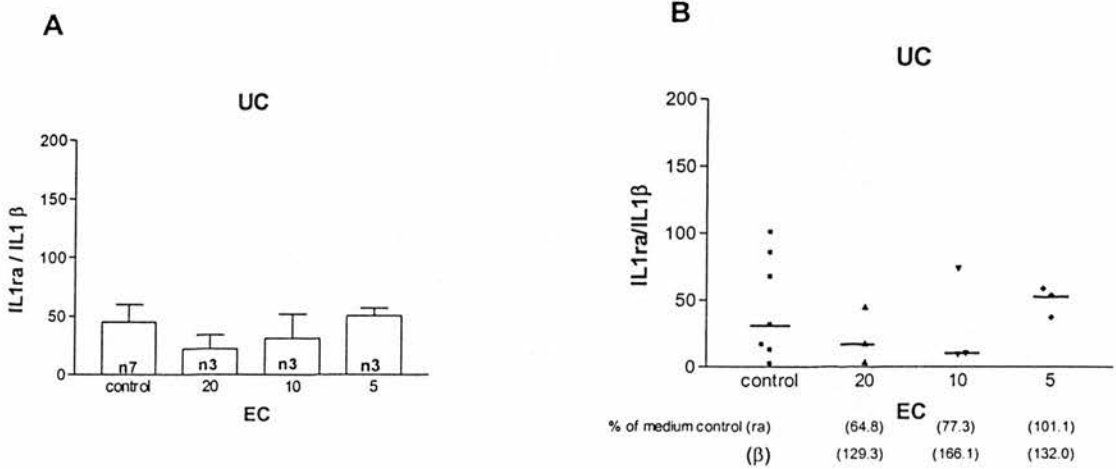


Figure 38 A+B (A) IL1ra / IL1 β ratio after 24-h EC incubation in UC. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

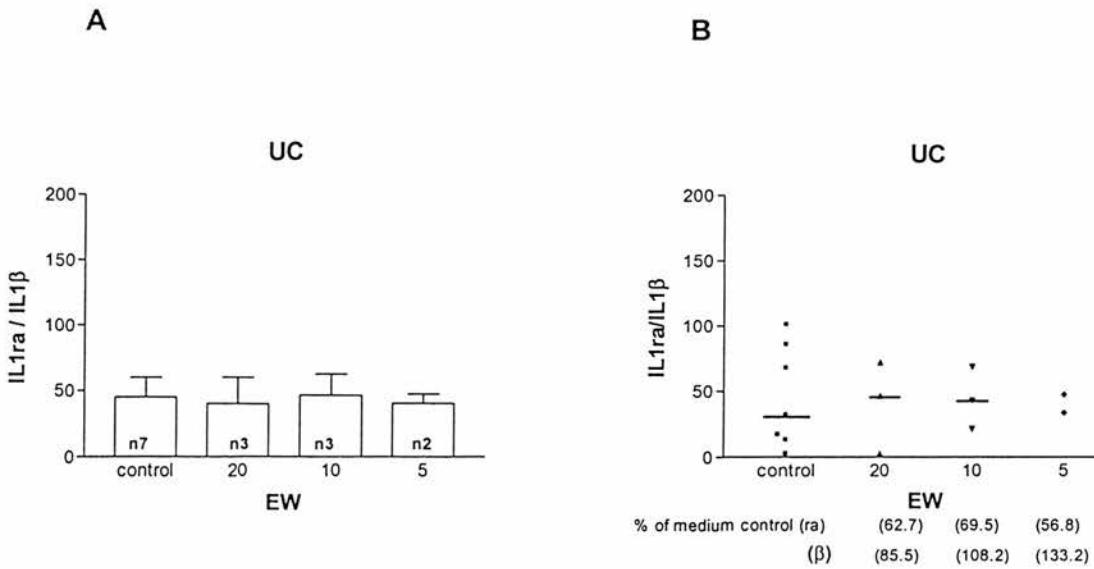


Figure 39 A+B (A) IL1ra / IL1 β ratio in EW after 24-h incubation in UC. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

5.1.3. Control patients

In patient control tissues incubated with EAA IL1ra / IL1 β ratio was higher than in the CD and UC specimens. A modest increase at 1:10 was noted vs. control, but this did not reach statistical significance. For the other dilutions of 1:5 and 1:20 there was no change in the IL1ra / IL1 β ratio (Figure 40)

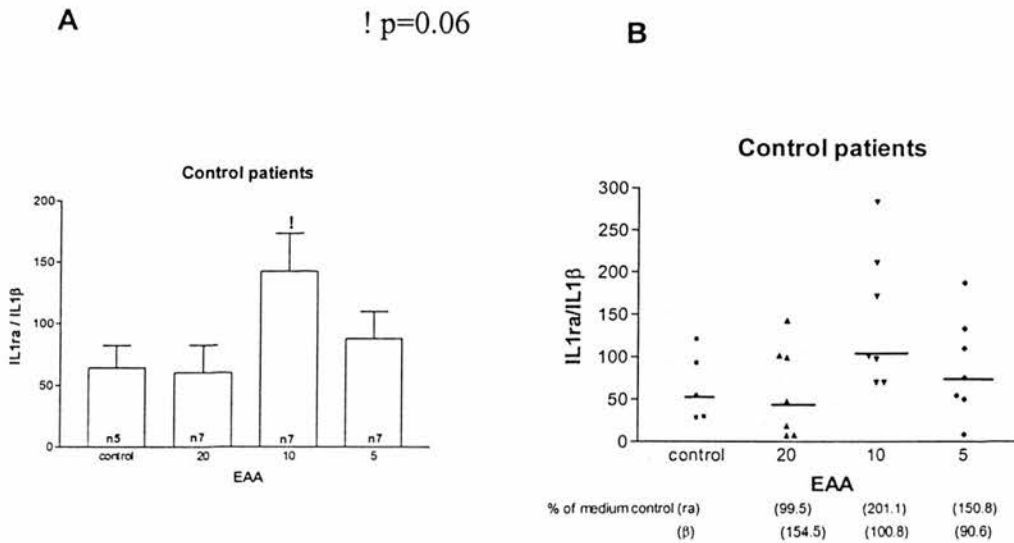


Figure 40 A+B (A) IL1ra /IL1 β ratio after 24-h EAA incubation Control patients. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

The incubation with EC resulted in a decrease in IL1ra / IL1 β for all three dilutions. This decrease was different from the experiments in CD affected tissue, where a consistent increase in the ratio was seen (). The incubation of patient control tissues with EW showed no significant change in the ratio of IL1ra/IL1 β for any dilutions compared with MC (Figure 41 and Table 17).

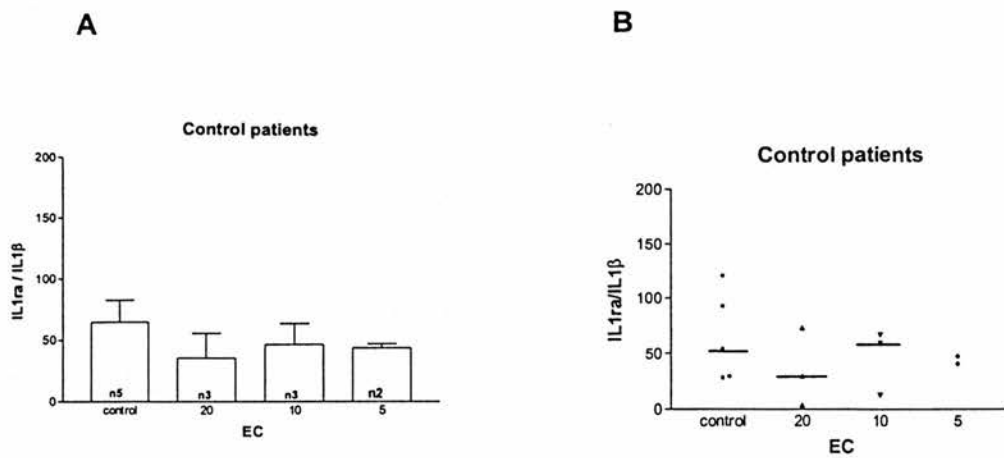


Figure 41 A+B (A) IL1ra / IL1 β ratio after 24-h EC incubation in Control patients. (B) IL1ra / IL1 β showing median and % of medium control of IL1ra + IL1 β

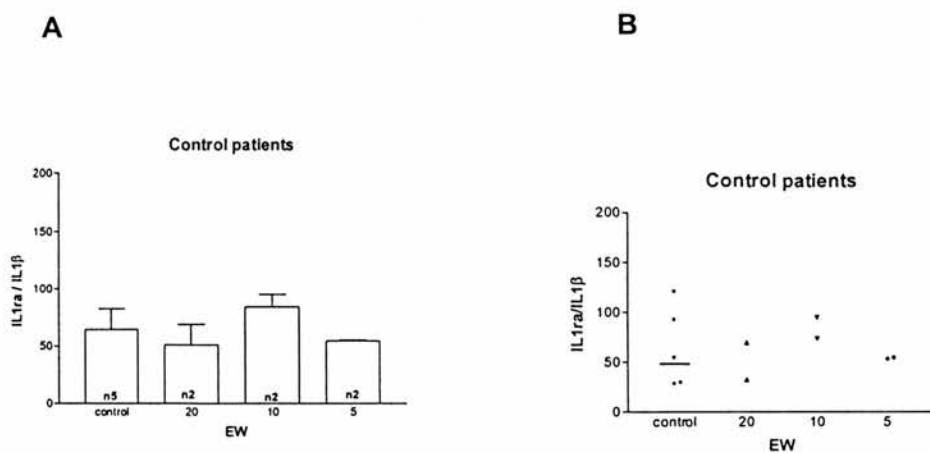


Figure 42 A+B (A) IL1ra / IL1 β ratio after 24-h EW incubation in Control patients. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

Table 17 IL1ra / IL1 β after 24h incubation with EAA, EC and EW

IL1ra / IL1 β	CD			UC			Control patients		
	20	10	5	20	10	5	20	10	5
EAA	59.7 ± 24.0	89.6** ± 17.0	93.0 ± 38.0	53.0 ± 12.0	65.8 ± 17.0	51.2 ± 23.0	60.6 ± 20.0	143.0! ± 31.0	88.2 ± 22.0
MC	45.7 (± 9.1)**			45.1 (± 15.0)			64.4 (± 18.0)!		
EC	101.8# ± 22.0	142.8** ± 24.6	109.7# ± 25.0	22.3 ± 12.0	31.0 ± 21.0	50.6 ± 6.6	35.4 ± 20.0	46.2 ± 17.0	43.7 ± 3.3
MC	45.7 (± 9.1)**#			45.1 (± 15.0)			64.4 (± 18.0)		
EW	100.4 ± 36.0	91.6 ± 37.0	95.2 ± 38.0	40.2 ± 20.0	46.6 ± 16.0	40.32 ± 6.9	51.2 ± 18.0	84.2 ± 11.0	45.3 ± 1.0
MC	45.7 (± 9.1)			45.1 (± 15.0)			64.4 (± 18.0)		

**p \leq 0.05 ; #p=0.05; ! p=0.06 vs. MC (mean \pm SEM)

5.2. IL10 / IL1 β ratio after incubation with EAA, EC and EW

5.2.1. Crohn`s disease

In CD tissue incubated with EAA there was no significant increase in IL10 / IL1 β ratio at any dilution vs. MC.

In CD tissue incubated with EC there appeared to be an increase in IL10 / IL1 β ratio at 1:20 and 1:5 dilutions. However, sample size was small and this did not reach statistical significance. For the 1:10 dilution no data were available.

EW incubation in CD tissue did not significantly change the IL10 / IL1 β ratio although a slight non-significant increases was noted at 1:10 (Table 18).

5.2.2. Ulcerative colitis

In UC tissue incubated with EAA, EC and EW formulae, no significant change in the ratio of IL10 / IL1 β was noted, but again the sample size was small (Table 18).

5.2.3. Control patients

In control patient tissue incubated with EAA, EC and EW there was no change in the IL10/ IL1 β ratio in all three enteral diet compositions (Table 18).

Table 18 IL10 / IL1 β ratio after 24 h incubation with EAA, EC and EW

IL10 / IL1 β	CD			UC			Control patients		
	20	10	5	20	10	5	20	10	5
EAA	28.3 ± 11.0	43.1 ± 23.0	18.9 ± 6.5	297.0 ± 199	96.0 ± 47.0	723.0 ± 579	58.2 ± 24.0	212.0 ± 135.0	136.0 ± 75.0
MC	21.4 (± 9.2)			5.12 (± 2.8)			84.1 (± 31.0)		
EC	75.4 ± 12.0	- \pm	48.12 ± 6.7	- \pm	- \pm	1.76 ± 2.08	- \pm	2.37 \pm	34.2 ± 26.9
MC	21.4 (± 9.2)			5.12 (± 2.8)			84.1 (± 31.0)		
EW	34.0 ± 18.0	165.0 ± 140	21.1 ± 7.0	8.62 ± 2.37	8.82 ± 1.7	2.93 ± 3.52	13.55 ± 9.41	13.12 \pm	80.15 ± 12.7
MC	21.4 (± 9.2)			5.12 (± 2.8)			84.1 (± 31.0)		

5.3. Discussion

In this study, changes in the IL1 α /IL1 β cytokine balance after incubation with elemental diet (EAA) and with comparable enteral formulae containing casein (EC) and whey (EW) could be demonstrated. These changes affected tissues differently, depending on whether the tissue was obtained from patients with CD, UC or control patients. It was shown that EAA does not interfere with the cytokine assays (Chapter 4). Recovery experiments showed that variable proportion of different cytokines were present after 24 hours of incubation. In practice however, cytokines were constantly being secreted and added to the supernatant over 24 hours, and therefore the net recovery is likely to be higher than the 24 hour recovery experiments. It can be considered that these results reflect the amount produced in tissues during incubation with EAA. There is no reason to believe that biopsies incubated with EAA would behave differently than those incubated with medium, as these results are consistent in patients. One biopsy from each individual patient was always used for medium control. No effect of medications could be detected on subgroup analysis, although the numbers are small and the possibility of a Type II error is quite large. This study was not designed to detect the effect of concurrent medications.

Several studies have shown that in both CD and UC, mucosal molar ratio of IL1ra/IL1 β ratio is lower in inflamed tissue compared with control tissue (Hyams et al., 1995; Casini-Raggi et al., 1995; Dionne et al., 1998). In this study a consistent increase in IL1ra/IL1 β ratio was seen in tissues affected by CD after incubation with enteral diet. This increase was concentration dependent with EAA, but less marked with EC and EW. EC at 1:10 dilution appeared to increase the ratio most markedly. In contrast, in UC and in control patient tissue, no statistically significant alteration in the IL1ra/IL1 β ratio was observed. This finding is consistent with the clinical observation that elemental diet is ineffective in UC. In both CD and UC the ratios of IL1ra/IL1 β in medium control alone without incubation in enteral diets were comparable, but lower than control patient tissue. This is consistent with previous findings.

The ratio of IL10/IL1 β showed no statistically significant rise in CD tissue after incubation with the formula diets, with EC again appearing to show a trend towards an increase. Little change in the IL10/IL1 β ratio was seen in UC or control patients. IL10 expression in UC and CD tissues might be variable (Akagi et al., 2000) but resolution of inflammation in IBD is expected to increase IL10 (Ishizuka K. et al., 2001) and therefore the IL10/IL1 β ratio; in the current study no such significant increase was observed, which might indicate that an increase in IL1ra is the preferential effect of enteral diets especially in CD. Also, since most of the IL10 is derived from lamina propria mononuclear cells (Gasche et al., 2000), the colonoscopic mucosal biopsies might have been weighted towards epithelial secretion of IL1ra, rather than lamina propria secretion of IL10. A further explanation could be the rather high loss of IL10 of approximately 60%/24hour in the cytokine recovery experiment in EAA at both concentrations of 1000pg/ml and 100pg/ml which might influence the IL10/ IL1 β ratio. Impaired production of IL10 in UC mucosa (Ishizuka K. et al., 2001) has been reported. As IL10 downregulates IL1 β (Schreiber et al., 1995) the low concentration of IL10 in UC might not downregulate IL1 β enough to increase the IL1ra / IL β ratio after incubation with enteral diets. Finally, as numbers are small, the possibility of a Type II error cannot be excluded.

These findings show that enteral diet has a direct anti-inflammatory effect on cytokine production on intestinal biopsy tissue from patients with CD. This direct anti-inflammatory effect is manifested by an increase in IL1ra/IL1 β ratio, but the effect on IL10/IL1 β is minimal. This would support *in vivo* studies, such as those of Fell *et al* (Fell J.M.E. et al., 2000) who investigated the effect of a polymeric diet, with casein as a protein source, on mucosal healing and expression of cytokine mRNA IL1 β , IFN γ and IL8 before and after 8 weeks of treatment. Their results also showed a decrease of IL1 β mRNA after treatment in ileum and colonic biopsies compared with controls. Ferguson *et al* also reported a decrease IL1 β in whole gut lavage fluid after treatment with elemental diet (Ferguson et al., 1998). The immunomodulatory effect of formula diets on CD was not restricted to EAA alone. EC and EW to a lesser extent also showed immunomodulatory properties. Casein is one of the major milk proteins and consists of four major protein fractions (α_s1 -casein, α_s2 casein, β casein and κ casein). Caseins are proteins known to have immune modulatory effects on cellular immune function. Casein, especially β casein and κ casein, has a range of stimulatory and immune-suppressive effects on mononuclear cell function. The carbohydrate-rich glycomacropeptide component of κ casein particularly acts as an active component (Wong et al., 1996; Cross and Gill, 1999). Kohyama *et al* (Kohyama et al., 1998) have reported the effect of a bovine α_{1s} casein in induction of CD8⁺ T-cells for IL10 production; they showed that a single specific amino acid substitution could induce a separate production of IL10 or IFN- γ from a CD8⁺ T-cell clone. Subtle variations in amino acid content or peptide sequence may influence the balance of Th1/Th2 responses. The immunomodulatory effect of casein was seen in the increased production of IL1ra in CD at all dilutions of EC, resulting in an increased IL1ra/IL1 β ratio. Whey originates from the production of cheese; it remains as a fluid phase after coagulation and extraction of the cheese product. The major whey proteins are β lactoglobulin and α lactalbumin. Some isolated proteins from whey have also been shown to have potential effects on cellular immune function (Cross and Gill, 1999). In our organ culture system we saw no significant differences in anti-/pro-inflammatory cytokine production with EW, though there was a trend towards an increased ratio of IL1ra/IL1 β in CD. It must

be stressed that the presented *in vitro* study is based by necessity on a very reductionist model. As such it does not parallel the *in vivo* situation of oral ingestion of EAA where EAA is almost completely absorbed in the proximal small intestine with very little reaching the colon. Also, the whole proteins used in the whey and casein-based formulae are partly digested *in vivo* so that a mixture of unabsorbed protein, peptides and amino acids are presented to the intestinal epithelium. These results must therefore be interpreted with caution. Differential degradation of the cytokines could affect the ratios and this might be particularly relevant for IL10/IL1 β ratio, which did not change significantly in this study. Finally, in this study colonic biopsies were studied, but small intestinal epithelium may behave differently. It is difficult however to consistently obtain inflamed intestinal biopsies from patients due to the limited accessibility of the small bowel.

The influence of enteral diet on the distal colonic mucosa is not known because most of the components will not reach the distal colon by oral feeding. These results have shown that in this *in vitro* model, when enteral diet has direct contact with the lamina propria it has a direct-acting immune-modulating influence on cells producing cytokines. The direct contact of EAA components might enhance a modulating effect on IL1 β mRNA expression, which is expressed in the lamina propria (Woywodt et al., 1999). This might open further considerations for EAA as an effective treatment after surgery e.g. ileal resection in IBD, to inject EAA into a stoma or, more unconventionally, infuse EAA as a high enema into the colon. The focus of this study was to detect any direct influence of enteral diets on the intestinal mucosa in the release of pro-and anti-inflammatory cytokines. The results demonstrated variation in ratios of pro-and anti-inflammatory cytokines released by IBD tissues incubated with EAA compared with non-inflamed non-IBD as controls. In conclusion, to our knowledge, this is the first *in vitro* study to show any direct anti-inflammatory response to EAA in intestinal biopsies of patients with IBD. The results confirm a positive direct anti-inflammatory effect of EAA on intestinal tissue affected by CD. This anti-inflammatory effect is preserved if casein and to a lesser extent, whey are substituted for amino acids in EAA. However the specific mechanism whereby EAA results in improvement in IBD is still unknown.

CHAPTER SIX

6. Effect of growth factors: TGF- β 1 expression after incubation with enteral diet-colostrum (ECO), enteral diet-whey extract enriched with TGF- β (EWT) and amino-acid based enteral diet (EAA)

Introduction

There has been a recent interest in the family of peptide-derived growth factors and their effects and mechanism of action in wound healing and re-epithelialisation (Brauchle et al., 1996; Dammeier et al., 1998). To this family belongs the epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF) and TGF- β . Transforming growth factor beta occurs in all mammalian tissue in three isoforms of TGF- β 1, TGF- β 2 and TGF- β 3. TGF- β 1 occurs in a predominant form in the gut. Each of these ligands interacts with specific, transmembrane serine/threonine kinase protein receptors. Produced by macrophages and lymphocytes, TGF- β 1 inhibits proliferation of B- and T-cells and has been found to promote intestinal restitution after mucosal injury and stimulate growth and differentiation of mesenchymal cells such as fibroblasts and dendritic cells (Lionetti et al., 1999). It has been shown in IBD that there is an increased expression of TGF- β in the mucosa of active disease (Babyatsky et al., 1996). TGF- β has been found to preserve, defend and maintain epithelial barrier function (Planchon et al., 1999; Planchon et al., 1994). An over-expression of TGF- β including their signalling receptors was found in CD (di Mola et al., 1999) along with a loss of intestinal signalling for TGF- β , which might contribute to the progression of IBD (Hahn et al., 2001). A disruption in TGF- β signal cascade (Monteleone et al., 2001) was recently documented. In immuno-histochemical studies and in-situ hybridisation the number of immune positive cells (T cells, neutrophils, monocytes and macrophages) in the lamina propria for TGF- β 1 increased both in CD and UC (Xian et al., 1999). The mRNA expression in this study showed that in IBD there was no evidence of a lack of epithelial growth factors, TGF- α and TGF- β peptides in the epithelium but suggested that a higher density of TGF- β positive cells could suppress the inflammatory response

in IBD. Avery *et al* (Avery et al., 1993) showed a different TGF- β staining pattern in normal compared to cancer tissue, with TGF- β expression in the crypts of normal but not cancer tissue.

Colostrum is known as the first milk produced after birth, rich in immunoglobulins, hormones and growth factors such as insulin-like growth factor, transforming growth factor α and β , cytokines and minerals. In calves it has been reported that after maximised colostrum feeding there is decreased apoptosis of the mucosal epithelial cells, a decreased villus growth after formula feeding compared to colostrum feeding and lower epithelial cell proliferation rate in the duodenum in formula feed compared to colostrum feed (Blaettler et al., 2001), suggesting that these bioactive molecules in colostrum might have more influence on intestinal growth than the supply of nutrients alone. A study of the ingestion of a TGF- β enriched diet showed a decrease of inflammatory markers and induction of remission and mucosal healing, as well as reduced mRNA levels of pro inflammatory cytokines and increased endogenous TGF- β (Donnet-Hughes, 2000). Whey protein is a by-product of cheese production, and proteins purified from whey have been shown to be potent modulators of immune function. The relevant proteins are lactoperoxidase, lactoferrin and milk growth factors (Cross and Gill, 1999). It was also shown that whey proteins could change immune competence when provided as dietary supplement (Woodward, 1998).

The following results document the uptake and expression of TGF- β 1 by immunostaining in intestinal tissue *in vitro* after 24 hours incubation. The tissue was incubated with (a) an enteral diet rich in colostrum bioactivity (ECO), containing TGF- β 2 with a TGF- β 1 content of 10-20% of TGF- β 2 (b) an enteral diet -whey enriched with TGF- β 2 (EWT) and (c) commercial amino acid based (EAA) enteral diet. The tissues were obtained from patients with CD, UC and control patients and compared with medium and control patients.

Subjects and Methods

TGF- β 1 staining expression in tissue after incubation with enteral diet enriched with

colostrum (ECO), whey extract enriched with TGF- β 2 (EWT) and EAA as a comparative diet (from 6 patient with CD, 2 patient in UC and 4 control patients described in the previous chapter). Therefore data were analysed in 21 patients after 24-h incubation. The method of TGF- β 1 immuno staining is described in chapter 4 section I.

Subjects

CD: (11); (5) M, (7) F, mean age (39 ± 4) years.

UC (4); (3) M, (1) F, mean age (50 ± 5) years.

Control patients: (6); (1) M, (5) F, mean age (50 ± 5) years.

Biopsies were obtained taken from the following anatomic areas:

CD: (5) colon, (2) ileocolonic, (1) ileum, (2) sigmoid, (1) recto-sigmoid area.

UC: (2) colon, (1) sigmoid, (1) rectum area

Control patients: (4) colon, (2) sigmoid area

Biopsies were generally taken from areas with inflamed appearance but not ulcerated.

Diagnosis was confirmed by histopathology. .

Time periods from initial diagnosis to biopsies were as follows:

1-3 years: 3 UC, 2 CD

4-6 years: 1 CD

> 6 years: 8 CD 1 UC

Medications at time of biopsy were as follows:

CD: steroids (4), 5-ASA (4)

UC: steroids and 5-ASA (2), 5-ASA (2)

Biopsy tissues were incubated with the different enteral diet formulae and 86 biopsies were analysed as follows:

CD: ECO (7), EWT (10), EAA (20), MC (8)

UC: ECO (5), EWT (6), EAA (7), MC (2)

Control patients: ECO (5), EWT (5), EAA (8), MC (3).

The method of quantitating the expression of TGF- β 1 is outlined in chapter 4. section I.

6.1. Expression of TGF- β 1 immunostaining after incubation with different enteral formulae.

6.1.1. Crohn`s disease

In CD after incubation with ECO a significant increase in percentage TGF- β 1 staining expression was seen at all three dilutions ($43.87 \pm .19$, $p < 0.001$; 27.35 ± 7.13 , $p < 0.001$; 29.4 ± 14.0 , $p < 0.001$ vs. 3.57 ± 0.6) for 1:20, 1:10, 1:5 vs. MC, respectively. This increase was also pronounced and significant vs. control by combining the three dilutions in colostrum (ECO-all) (32.94 ± 6.24 vs. 3.57 ± 0.6 ; $p < 0.001$), but was not significant for EAA 1:10 vs. ECO-all (17.27 ± 5.23 vs. 32.94 ± 6.24 , $p = 0.08$) (Figure 43).

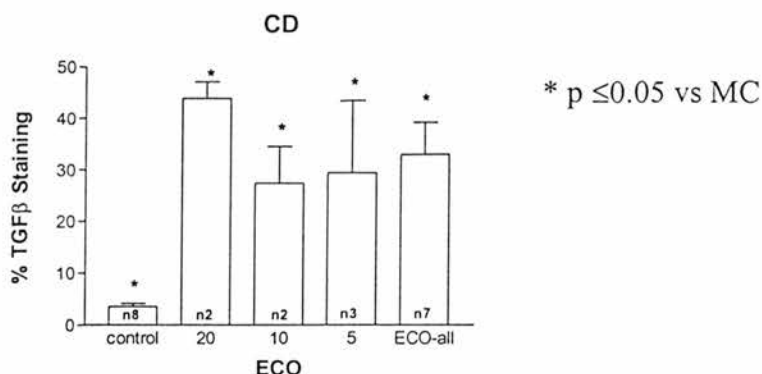


Figure 43 %TGF- β staining in CD after 24- h incubation with ECO

In CD after incubation with EWT a similar response was seen (32.87 ± 5.78 , $p < 0.001$; 28.1 ± 11.4 , $p = 0.01$; 33.1 ± 11.8 , $p < 0.001$ vs. 3.57 ± 0.6) for 1:20, 1:10, 1:5 vs. MC, respectively. A combination of all 3 dilutions of EWT was significant (31.05 ± 5.42 vs. 3.57 ± 0.6 , $p < 0.001$) vs. MC (Figure 44).

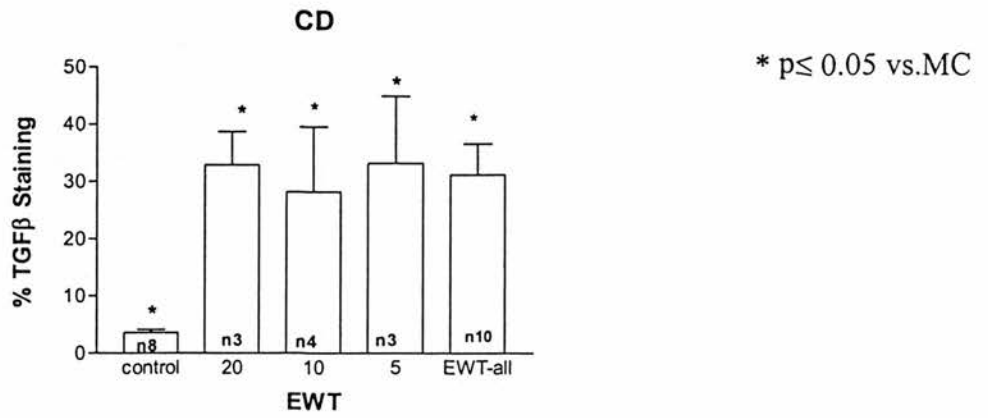


Figure 44 % TGF-β1 staining in CD after 24-h incubation with EWT

CD after incubation with EAA a significant increase in TGF-β1 staining expression was seen only at a dilution of 1:10 (17.72 ± 5.23 vs. 3.57 ± 0.6 , $p=0.03$). Combining all three dilution's EAA-all (1:5, 1:10, 1:20) was not significantly different vs. MC (Figure 45 and Table 19).

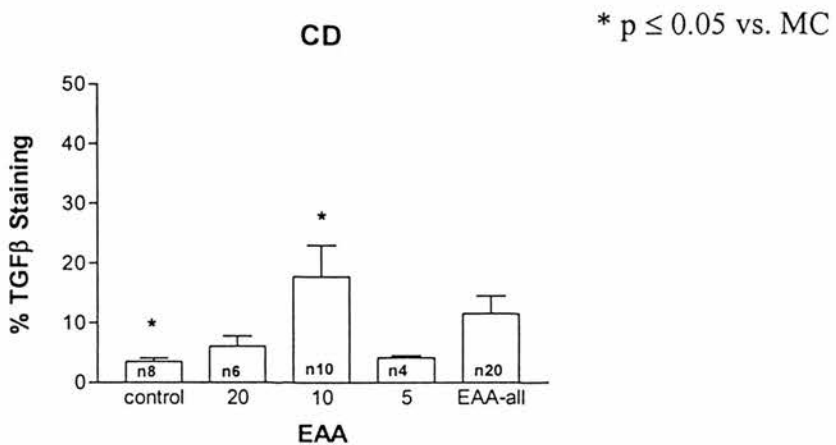


Figure 45 % TGF-β1 staining in CD after 24-h incubation with EAA

6.1.2. Ulcerative colitis

In UC neither ECO nor EWT resulted in an increase in TGF- β 1 expression compared with MC (Figure 46 and Figure 47). When combining all dilutions EAA-all was significant (8.43 ± 1.22 vs. 2.53 ± 1.65 ; $p < 0.05$) and EAA10 (diluted 1:10) was significant vs. MC ($p < 0.05$) in UC (Figure 48 and Table 19).

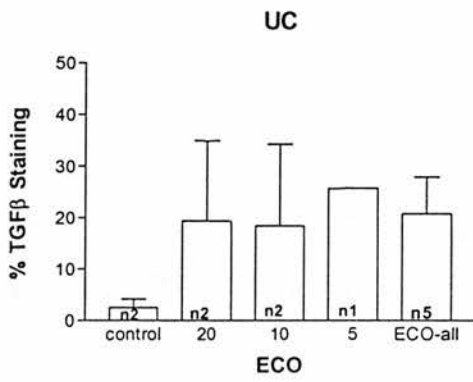


Figure 46 % TGF- β 1 staining in UC after 24-h incubation with ECO

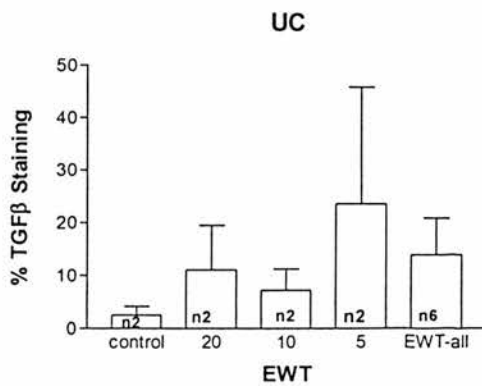


Figure 47 % TGF- β 1 staining in UC after 24-h incubation with EWT

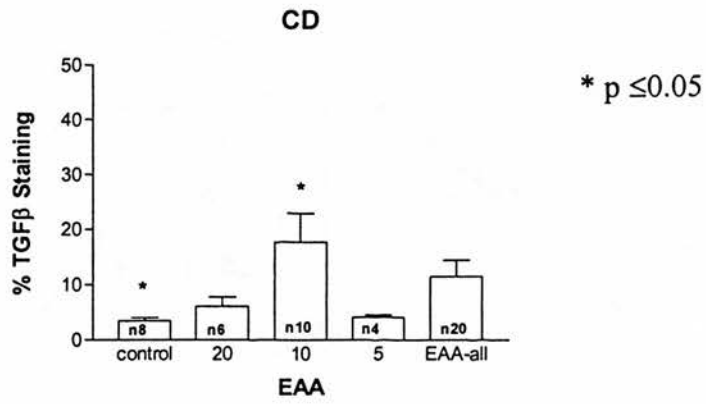


Figure 48 % TGF-β1 staining in UC after 24-h incubation with EAA

6.1.3. Control patients

In tissues from these patients, none of the enteral diet preparations resulted in significant increase of % TGF-β1 staining expression. Only for colostrum incubation (ECO) in dilution 1:20, was there a trend towards an increase, but this was not significant. (Figure 49, Figure 50, Figure 51 and Table 19).

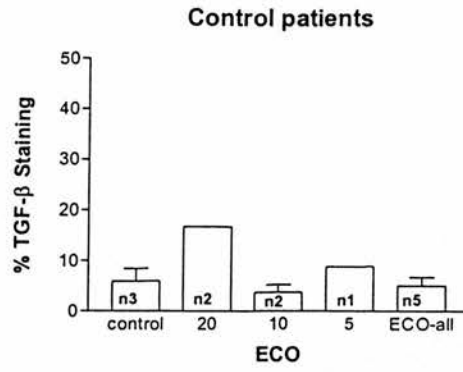


Figure 49 % TGF-β1 staining in Control patients after 24-h incubation with ECO

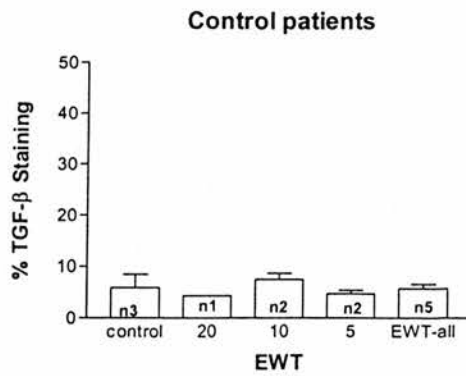


Figure 50 % TGF-β1 staining in Control patients after 24-h incubation with EWT

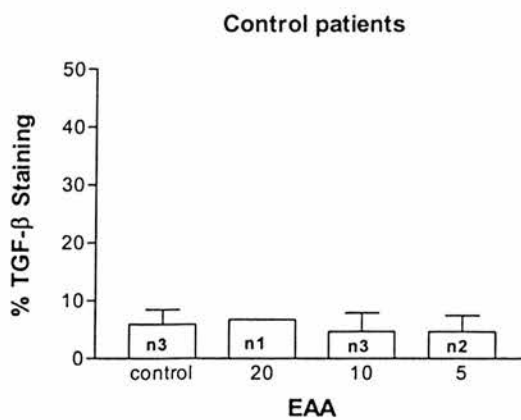


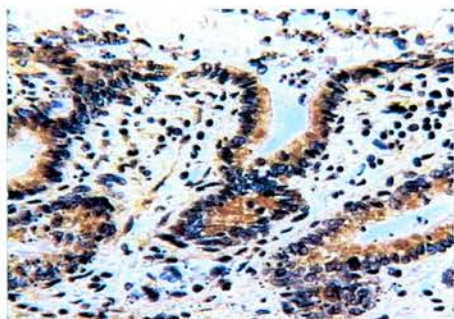
Figure 51 % TGF-β1 staining in Control patients after 24-h incubation with EAA

Table 19 Percentage of TGF-β1 staining expression after 24-h incubation with EAA, ECO and EWT

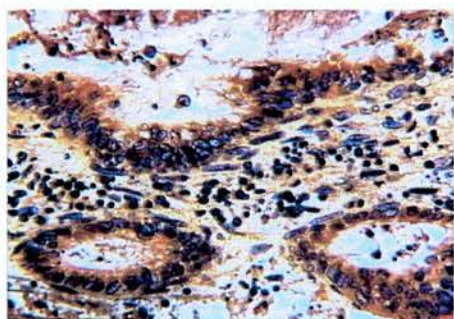
Diagnosis & Dilutions	EAA			ECO			EWT		
	20	10	5	20	10	5	20	10	5
CD	6.09 ±1.69	17.72* ±5.23	4.11 ±0.4	43.87* ±3.19	27.35* ±7.13	29.4* ±14.0	2.87* ±5.79	28.1* ±11.4	33.1* ±11.8
MC (1:20, 1:10, 1:5) EAA 1:10 all	3.57 (±0.59) 11.5 (±2.98) 17.72 (±5.23)*			3.57 (±0.59) 32.94 (± 6.24)* -			3.57 (±0.59) 31.05 (±5.42)* -		
UC	5.78 ±2.55	10.66* ±1.43	7.75 ±1.87	19.3 ±15.6	18.4 ±5.8	25.7 ±0	11.06 ±8.45	7.29 ±3.99	23.6 ±22.2
MC (1:20, 1:10, 1:5) EAA 1:10 all	2.53 (±1.65) 8.43 (±1.22)^ 10.66 (±1.43)			2.53 (±1.65) 20.24 (±7.15) -			2.53 (±1.65) 13.99 (±6.96) -		
Control patients	6.76 ±/	4.69 ±3.21	4.68 ±2.79	16.71 ±0	3.72 ±1.55	8.82 ±/	4.41 ±/	7.47 ±1.22	4.75 ±0.68
MC (1:20, 1:10, 1:5) EAA 1:10 all	5.94 (±2.55) 5.03 (± 1.64) 4.69 (±3.21)			5.94 (±2.55) 7.53 (±1.67) -			5.94 (±2.55) 5.77 (±0.83) -		

*p<0.05 vs. MC; ^p=0.05 vs. MC

A



B



C

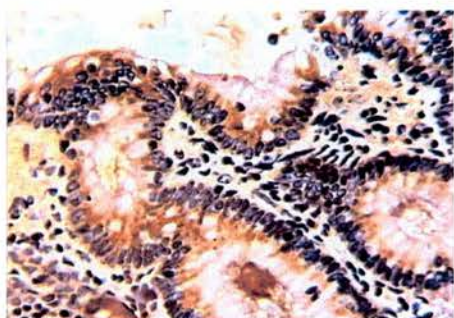
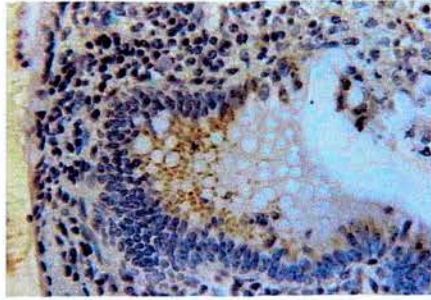


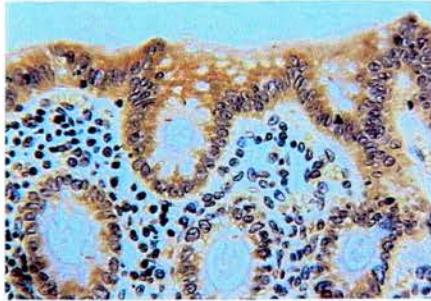
Figure 52 TGF- β 1 staining in CD

TGF- β 1 expression (brown staining) in CD. TGF- β 1 expression was found on surface epithelium and crypt epithelium, with differences in staining intensity after incubation with (A) elemental diet EAA; (B) ECO and (C) EWT. Magnification x 250.

A



B



C

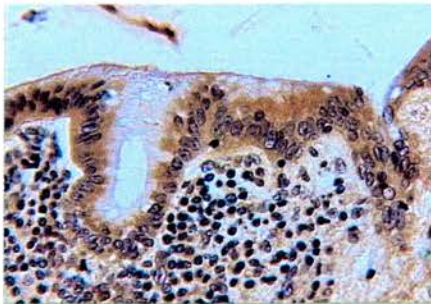


Figure 53 TGF- β 1 staining in UC

TGF- β 1 expression (brown staining) in UC. TGF- β 1 expression was found on surface epithelium and crypt epithelium with differences in staining intensity after incubation with (A) medium control (Magnification x400) ; (B) elemental diet EAA and (C) EWT. Magnification x 250.

6.2. Discussion: TGF- β 1 expression after incubation

This chapter describes the expression of TGF- β 1 in biopsy tissues after incubation with (a) enteral diet – colostrum (ECO), (b) enteral diet – whey enriched with TGF- β 2 (EWT) and (c) enteral diet (EAA) in organ culture in patients with CD, UC and control patients. Overall TGF- β 1 immunostaining was expressed in surface epithelia and crypt epithelium, and occasionally also in lamina propria in CD and UC. Staining area was different in CD, UC and control patients after incubation with modified enteral diets of ECO, EWT and EAA. TGF- β 1 expression in intestinal tissue sections was reported by Xian *et al*, where TGF- β 1 immunoreactivity was located in the surface epithelium and along the crypt epithelium in rectum or sigmoid colon mucosa of normal colon, CD and UC tissues. An increased number of TGF- β 1 positive cells in the lamina propria in active disease of CD and UC have also been reported (Xian et al., 1999). Enhanced expression of TGF- β 1 in IBD was reported in actively inflamed tissues of CD and UC. TGF- β mRNA was present in low or undetectable levels in normal mucosa, and in uninvolved mucosa of IBD patients, when assessed by Northern blot (Babyatsky et al., 1996).

In CD, incubation with ECO and EWT resulted in a significant increase in TGF- β 1 staining expression vs. medium control incubation. However, incubation with these enteral diet modifications in UC displayed no significant increase in TGF- β 1 staining vs. control. Incubation with EAA 1:10 showed an increase in TGF- β 1 staining expression in CD and a modest increase in UC vs. MC. This was not seen in the control patient group for EAA incubation. The control patient group tissue did not significantly alter TGF- β 1 expression when exposed to any of the ED modifications. Though these results were established in only a small group of patients with IBD, the results demonstrate that TGF- β 1 tissue expression can be manipulated by different enteral feeds rich in TGF- β . It also showed that this response can be disease specific and different in CD and UC. It also has shown that addition of TGF- β (in this case mainly

TGF- β 2) may lead to an upregulation of TGF- β , which was an upregulation of TGF- β 1 staining expression, as the major TGF- β isoform occurring in the intestinal mucosa. To further validate these findings it would have been very useful to perform in these groups in situ hybridisation to investigate differences in TGF- β mRNA expression in response to the dietary modifications after 24-h incubation. Unfortunately this was not performed due to lack of time and expertise in the laboratory, but could be an approach in a future study. Nevertheless, using this relatively simple study design it was possible to demonstrate significant differences in tissue TGF- β 1 response to different enteral diet modifications in CD. An increase in TGF- β expression may correspond to downregulation of inflammation in CD and provides the rationale for growth factor enrichment of enteral formulae. Such an approach has been tried *in vivo* by Fell *et al*, who fed a polymeric diet rich in casein and TGF- β 2 to children with CD. After 8 weeks of treatment 79% were in clinical remission. Decreases in IL1 β mRNA in colon and ileum, IFN- γ in ileum, and IL8 in colon were demonstrated after treatment. An increase of TGF- β 1 mRNA was found in ileum in endoscopic biopsies, but this was not found in the colon where no significant change in IFN- γ and TGF- β 1 occurred (Fell J.M.E. *et al.*, 2000). Recently other approaches, such as epidermal growth factor (EGF) enema have been tried in ulcerative proctitis with some success (Sinha *et al.*, 2001). Growth factors administered as colostrum (or enriched whey) may therefore have therapeutic value and peptides generated after partial digestion of these proteins may also be bioactive (Kuwata H *et al.*, 2001).

Further clinical trials with such enriched diets are therefore warranted especially in CD, compared with conventional elemental diet. Currently, no such clinical trial has been reported.

CHAPTER SEVEN

7. TGF- β 1 immunostaining expression after incubation with enteral diet - casein (EC) and enteral diet- whey (EW)

Introduction

Casein and whey are two of the major milk proteins. Casein consists of four fractions α_s1 -(alpha-S1)-casein, α_s2 -(alpha-S2)-casein, β -(beta) and κ -(kappa) casein. The carbohydrate rich glycomacropeptide of κ -casein is thought to have suppressive and immune stimulatory effects on mononuclear cell function and β -casein was found to have immune modulator activities such as promoting antibody synthesis and phagocytosis (Wong et al., 1996; Cross and Gill, 1999). The major whey proteins purified from whey are lactoperoxidase, lactoferrin and some milk growth factors, all of which have also been shown to have potent modulator effects on immune function (Cross and Gill, 1999). Whey, containing high levels of cysteine, may be superior to casein in supporting antibody response and whey proteins are more potent in supporting antibody-based resistance of adult mice to pneumococcal infections (Woodward, 1998). The immune modulator effects of milk proteins are still not clearly identified, but it seems that their functions become clearer and more evident in highly purified states obtained from the original parent product (Cross and Gill, 1999). In this study we used casein and whey proteins in modified enteral formulae, which were not highly purified.

Subjects and Methods

Detection of TGF- β 1 expression was performed as described in chapter 4 section I, in CD, UC and control patients after incubation with enteral diet formulae. Colonoscopic biopsies were incubated with enteral diets modified in protein content with casein (EC) and whey (EW) protein. The method of TGF- β 1 immunostaining is described in chapter 4 section I, and results were expressed as % area of TGF- β 1 immunostaining (mean \pm SEM) of total tissue area.

Subjects

CD: (5); (3) M, (2) F; mean age (35 ± 5) years

UC: (1) F, age (48) years.

Control patients: (2) F, mean age (50 ± 10) years.

Time periods from diagnosis to biopsies were as follows:

4-6 years: 2 CD, 1 UC

> 6 years: 3 CD

A total of 26 biopsies (for medium control these are identical to biopsies taken for TGF- β 1 staining for ECO and EWT, biopsy numbers are not included) were stained for TGF- β 1. The diagnosis was confirmed by histopathology. The biopsy sites were as follows:

CD: (4) colon, (1) ileum

UC: (1) colon

Control patients: (2) colon

Medications at time of biopsy:

CD: steroids and 5-ASA (1) , steroids and AZA (1), 5-ASA (1), 5AZA (1)

UC: 5-ASA (1)

Biopsies were incubated with enteral diet formulae as follows:

CD: EC (6), EW (8)

UC: EC (1), EW (3)

Control patients: EC (4), EW (4)

7.1. Percentage of TGF-β1 staining expression

7.1.1. Crohn`s disease

After incubation of CD affected tissues with EC a significant increase in % staining representing upregulation of expression of TGF-β1, was only seen at 1:5 dilution ($p < 0.05$). For 1:10 no data was available and dilution 1:20 showed no significant change between MC incubation and EC incubation. Combining all available dilutions used in incubations for EC there was no significant increase in % staining expression for TGF-β1 vs. MC. As the numbers were small a type II error is possible (Figure 54).

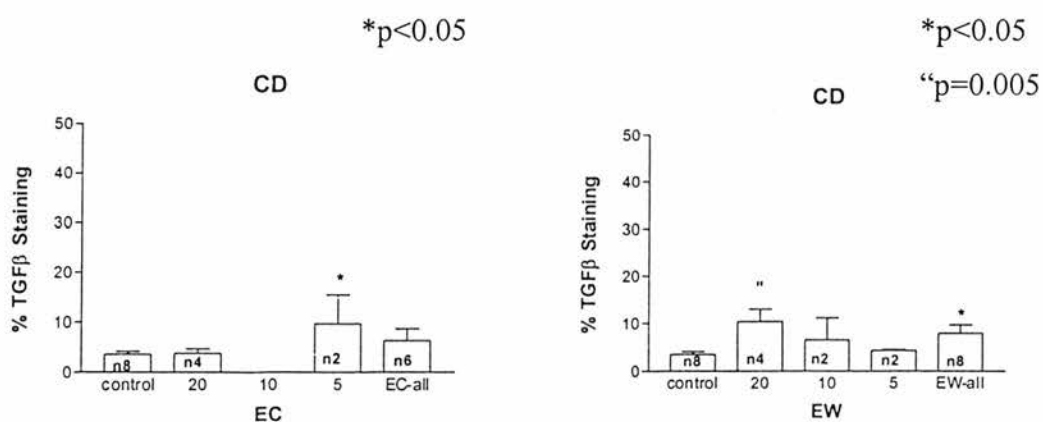


Figure 54 (left): % TGF-β1 staining in CD after 24-h incubation with EC

Figure 55 (right): % TGF-β1 staining in CD after 24-h incubation with EW

In contrast, in CD affected tissues after EW incubation a trend towards an increased % of TGF-β1 staining expression for 1:20, 1:10 and 1:5 was observed, which reached significance at 1:20 dilution ($p = 0.005$). After combining all data for EW, a significant % increase of TGF-β1 staining for EW vs. MC ($p < 0.05$) was noted (Figure 55). By comparing the TGF-β1 staining expression in CD for EC vs. EW at dilution 1:20, a significantly higher increase ($p < 0.05$) TGF-β1 expression was seen for EW compared

with EC (Figure 54 and Figure 55).

None of the modified enteral feed incubations of CD affected tissues resulted in significant difference in % TGF- β 1 staining expression when compared with EAA 1:10 (Table 20).

7.1.2. Ulcerative colitis

In UC, the number of incubations was small. EC incubation resulted in no significant increase in TGF- β 1 expression on immunohistochemistry at 1:20 dilution, but no data were available for 1:10 and 1:5 dilutions (Figure 56).

Incubation of UC tissue in EW resulted in a trend towards an increase at all three dilutions, but the numbers were low; when combining all three dilution's statistically significant increase in % TGF- β 1 expression vs. MC was demonstrated ($p < 0.01$) (Figure 57 and Table 20).

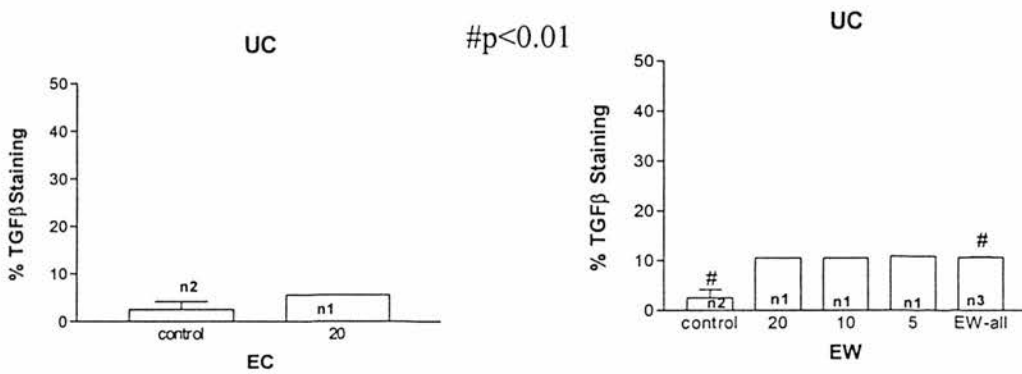


Figure 56 (left): % TGF- β 1 staining in UC after 24-h incubation with EC

Figure 57 (right): % TGF- β 1 staining in UC after 24-h incubation with EW

7.1.3. Control patients

Incubation of control patient tissue with EC and EW resulted in no significant increase in % staining expression at any dilution (no Figure shown, Table 20).

**Table 20 % TGF-β1 staining expression in EC and EW.
All results as mean (SEM)**

Diagnosis	EC			EW		
	20	10	5	20	10	5
CD	3.78 ±0.89	- ±/	9.69* ±5.8	10.45! ” ±2.55	6.55 ±4.69	4.41 ±0.21
MC (1:20, 1:10, 1:5)	3.57 (±0.59) * 6.30 (±2.39)			3.57 (±0.59) “ 7.97 (±1.77)		
UC	5.56 ±/	- ±/	- ±	10.50 ±/	10.53 ±/	10.9 ±/
MC (1:20, 1:10, 1:5)	2.53 (± 1.65) -			2.53 (±1.65) 10.64 (±0.129)#		
Control patients	6.78 ±/	14.41 ±0.12	18.92 ±/	14.04 ±/	5.88 ±/	7.99 ±4.34
MC (1:20, 1:10, 1:5)	5.94 (± 2.55) 13.63 (±2.52)			5.94 (±2.55) 8.97 (± 2.5)		

*p< 0.05; “p=0.005; #p<0.01 vs. MC respectively.

7.2. Discussion: TGF-β1 staining after EC and EW incubation

In this part of the study tissues obtained from patients with CD, UC and control patients were incubated with enteral diet-casein (EC) and enteral diet –whey (EW) and immunohistochemically stained for TGF-β1 expression in tissues. TGF-β1 is the main isoform occurring in the intestinal tract. As before, results were expressed as % TGF-β1 expression per unit area of the biopsies.

Incubation of CD tissues with EC resulted in a significant increase only for one dilution.

Combining the staining results for different dilutions for EC in CD did not reach any statistically significant increase. But the numbers of CD patients for EC were small and type II error is possible. TGF- β 1 staining after incubation of CD tissue with EW was increased in all three dilutions vs. MC, reaching a significant peak in 1:20. Combining the % staining areas for all three dilutions in CD tissue for EW incubation showed a significant increase vs. MC. EW produced a more impressive upregulation of TGF- β 1 expression compared with EC.

In UC there was no significant increase in % staining expression after EC incubation, but incubation with EW increased TGF- β 1 expression at all three dilutions vs. MC. Combining the three dilutions also showed significantly increased TGF- β 1 expression. For the control group there was no significant increase in TGF- β 1 expression.

Staining for TGF- β 1 in ED compositions not containing additional TGF- β , such as EC and EW, did not increase the TGF- β 1 expression as markedly as after incubation with ECO and EWT (chapter 6). Although it appears that incubation with EW did increase TGF- β 1 staining pattern more than incubation with EC, the numbers were small, and definite conclusions cannot be drawn. By combining all three dilutions the increase in % staining was greater in EW than in EC, even although the whey composition did not contain added TGF- β .

TGF- β expression is upregulated in inflamed tissue with IBD as reported by di Mola *et al* (di Mola et al., 1999). Addition of whey protein may have boosted tissue TGF- β 1 expression during culture period, and therefore resulted in greater detection of TGF- β 1 staining. The numbers in UC for EW were too small to draw meaningful conclusions. These results support the impression that although expression of the counter-inflammatory cytokine TGF- β 1 may be modestly increased after defined formulae diet incubation, added TGF- β or formulae containing growth factors can result in more marked expression of TGF- β 1 in inflamed tissue, especially in CD. Therefore, such enrichment of enteral diet formulae used for the treatment of CD deserves further *in vitro* studies. The fact that drinking water enriched with growth factor extract derived

from whey reduced ethane production and lipid peroxidation in the early phase of experimental colitis, suggests that some of these growth factors may reach the distal intestine and exert their action (Porter et al., 1998). Therefore, both local administration and oral administration are worthy of further exploration.

CHAPTER EIGHT

8. Summary: TGF- β 1 expression in IBD affected tissues after incubation with enteral diets

As presented in previous chapters, in this section of the project endoscopic tissue biopsies from patients with CD, UC and control patients were incubated with five different enteral diet modifications. Two of these were rich in TGF- β 2, as enteral diet–colostrum (ECO) and enteral diet whey enriched with TGF- β 2 (EWT). The remaining formulae were enteral diet amino acid based (EAA), enteral diet–casein (EC) and enteral diet-whey (EW), with no addition of TGF- β 2.

All biopsies were incubated for 24-hours in the relevant enteral diet formulae and TGF- β 1 immunostained area was quantified. In CD, bovine colostrum (ECO) and TGF- β enriched whey extract (EWT) incubations resulted in a significantly higher TGF- β 1 expression pattern compared to MC incubation alone. As both ECO and EWT composition were prepared or mixed with EAA, it seems likely that addition of TGF- β , present in colostrum and whey does significantly increase TGF- β 1 expression in the tissue. Xian *et al* (Xian et al., 1999) had reported an up regulation of TGF- β positive cells during active disease in IBD. The biopsies here were taken from actively diseased areas, but all were compared with medium control slide. This may suggest, that the increased TGF- β 1 staining pattern might be due to the addition of TGF- β in the diet, and therefore anti-inflammatory modulation of inflammatory response might be possible due to external addition of TGF- β . Fell *et al* reported a positive clinical response after treatment of paediatric CD patients with a polymeric enteral diet rich in TGF- β 2 (Fell J.M.E. et al., 2000). This was associated with down regulation of pro-inflammatory cytokine mRNA measured in the ileum and colon. Furthermore, an increase in TGF- β 1 mRNA expression in the terminal ileum was observed. In the study presented here, there was an increase in TGF- β 1 staining expression, which was significant in CD in both enteral diets enriched with TGF- β , compared with MC. This increased TGF- β 1 response was not remarkable in EAA, containing no TGF- β , even

though EAA directly altered the anti-inflammatory to pro-inflammatory cytokine ratio, as reported in chapter 5.

In contrast in UC TGF- β 1 expression after incubation with ECO and EWT was not significantly increased. Control patients incubations with ECO, EWT or EAA resulted in no increase in TGF- β 1 expression. As TGF- β acts as a multifunctional cytokine, involved in regulation of growth, differentiation and restoration of intestinal immunity and tolerance (Wahl, 1994), it seems reasonable that addition of TGF- β in enteral diets might contribute to the restitution and healing of the inflamed mucosa. Studies in CD patients have confirmed improvement in inflamed intestinal mucosa after feeding diets supplemented with TGF- β (Fell J.M.E. et al., 2000). Though TGF- β is normally expressed in the intestinal tract, differential production of TGF- β can be found in health in different parts of the intestine, but significant differences seem to occur only between the rectum and ascending colon (Kushiyama et al., 2000). The biopsies in this study were taken randomly, but preference was given for macroscopically inflamed areas, mainly from a sigmoid-colonic site. However, in a single set of incubation, all biopsies including that in control medium were obtained from the same site. It is not possible to determine how this site of biopsy influenced the staining expression. Differences between IBD and control patients in TGF- β 1 expression demonstrate in this study that it is likely that these differences in expression of TGF- β 1 were a result of inflamed mucosa and the different enteral formulae used in the study. The changes were most marked in CD but were insignificant in UC or controls with normal non-inflamed intestine.

Since the anti-inflammatory cytokine TGF- β is expressed in inflamed mucosa but does not restore the imbalance of inflammation, another explanation for its mechanism of action is offered. In a recent study, Hahn *et al* (Hahn et al., 2001) reported loss of TGF- β signal transduction as a cause of intestinal inflammation. Further recently Monteleone and colleagues (Monteleone et al., 2001) showed that despite TGF- β detection in CD, no immediate improvement occurred. In this study it was reported, that in CD a decreased phosphorylation of Smad 3 and an increase of Smad 7 exists. Smads are signal proteins acting as substrates for TGF- β receptors I (T β RI) and TGF- β receptors II (T β RII), which after binding with TGF- β initiate the downstream signal transduction

cascade for DNA transcription factors. Smad 7 acts as an antagonist for the signal cascade and Smad 3 together with Smad 2 and Smad 4 form the signal complex to activate transcription. Therefore a disruption of Smad 3 or an over expression of Smad 7 leads to inflammation. Monteleone *et al* reported that blockage of Smad 7 with a specific antisense oligonucleotide leads to restoration of the TGF- β signalling pathway. Furthermore, addition of a neutralising TGF- β 1 antibody to organ cultures decreased the Smad 7 antisense effect on cytokine expression and therefore modulated proinflammatory cytokine expression. It is possible that the results from the data presented may be explained by the external addition of TGF- β positively influencing the Smad signal cascade. However, the findings of Montelone *et al* may not be directly relevant to the results of the present study using a simple organ culture *in vitro* model. Modulation of pro-inflammatory cytokines by external addition of anti-inflammatory cytokines such as TGF- β as well as growth factors via diets may be a potential therapeutic advance and positively influence and restore imbalances in the signal cascade resulting in reduced inflammation.

On the other hand, the TGF- β 1 staining pattern for the enteral diet protein modifications, not containing any added TGF- β (i.e. EC and EW) displayed a different pattern, characterised by a lack of intensity in staining expression as compared with ECO and EWT. Incubation in CD with EC was followed by a significant increase in % staining expression only in one dilution vs. MC. TGF- β expression was greater after EW than EC incubation showing an increase, which was significant at a dilution of 1:20. Overall, by comparing the ED modifications of EC and EW, EW incubation displayed a more intense staining pattern compared with EC, suggesting a higher downregulatory effect on inflammation. The numbers are small and definite conclusions are difficult. However a protective effect of whey has been reported. Taylor *et al* investigated a (Taylor et al., 2001) mitogenic bovine whey extract (MBWE) for its protective activity against chemotherapy-induced damage in cultured mink lung cells. The results showed an attenuated cell death after addition of MBWE. It was concluded, that MBWE was cytoprotective against two chemotherapy drugs when added before and after drug exposure with a contribution from TGF- β to this protective activity, which was in this case TGF- β 2. Further in a study of Hakkak *et al* (Hakkak et al., 2001) the

influence of whey and casein diets on azoxymethane induced colon cancer in rats was investigated. Rats fed on the whey diet had a lower incidence of colon tumours than those fed on casein diet. Both studies showed a superior effect of whey protein on epithelial cells cultured *in vitro* and significantly more protective effect *in vivo* on intestinal tissue in rats. In the current study, although numbers were small, the results showed an overall increase in % TGF- β 1 staining expression after EW incubations, representing a symbiotic tissue protection of EW possibly mediated via TGF- β in the tissue. The reason for the lesser influence of casein in these experiments is difficult to explain. However, Sudlow *et al* (Sudlow and Wilde, 1994) found that TGF- β 1 suppressed the onset of lactation and production of beta-casein in mammary glands of pregnant mice. Their data suggest an inhibitory effect of TGF- β on casein secretion *in vivo*. Therefore it is also possible, that in the study presented, there was a reciprocal effect of addition of casein to the diet preventing the active tissue expression of TGF- β 1.

These results for TGF- β 1 staining in five different enteral diet modifications demonstrate that addition of TGF- β to the diet increases TGF- β 1 expression in CD tissues, whereas the effect is very limited in UC tissues. TGF- β 1 expression is significantly increased in CD vs. MC incubation. In diets where no TGF- β was added this effect of upregulation of TGF- β 1 expression was observed in CD tissue incubated with EW, but only modestly. Clearly, the exact mechanism by which TGF- β enrichment leads to TGF- β 1 expression in CD tissue is unclear and can merely be a matter of speculation. Smad signalling pathways may be relevant. As colostrum preparations have also been shown to be protective against NSAID induced gut damage (Playford *et al.*, 1999) such proteins may help preserve intestinal integrity and function (Playford *et al.*, 1993). Kuwata *et al* has shown for lactoferrin, that some of these milk-derived proteins are actually resistant and survive proteolytic degradation in the small intestine of adult rats (Kuwata H *et al.*, 2001). It seems that these specific protein compositions rich in growth factors, immunoglobulins, as well as other milk derived proteins may have important protective and anti-inflammatory effects, (Playford *et al.*, 2000), and these are being increasingly recognised. These findings offer an opportunity to design functional

foods with beneficial effects on specific forms of gut inflammation. However, clinical trials are required with these formulations, especially in CD, to determine the beneficial effects compared with elemental diet alone, which has been used for many years without much modification.

CHAPTER NINE

9. Cytokine response after incubation with enteral diet - colostrum (ECO) and enteral diet diet-whey enriched with TGF- β (EWT) in CD, UC and control patients tissues and correlation with TGF- β 1 immunostaining

Introduction

In IBD there is a well known imbalance between pro-and anti-inflammatory cytokines (Andus et al., 1997), (Rogler and Andus, 1998). Modulation of this cytokine pattern in favour of an increase in anti-inflammatory response by nutritional modulation has been demonstrated in several studies (Grimble, 1992), (Fell J.M.E. et al., 2000), (Fell J.M.E. et al., 1998), (Beattie et al., 1994). This chapter describes the cytokine response after incubation of IBD affected and control patients tissue with enteral diet–colostrum (ECO) and enteral diet –whey enriched with TGF- β (EWT).

Subjects and Methods

The patient cohort used in this part of the study was the same as in Chapter 6 and 7 where tissues were immunostained for TGF- β 1 expression. The cytokine results were matched with the data obtained for TGF- β 1 staining expression.

Subjects

These patients are the same as described for TGF- β 1 staining and are as follows:

CD: (11); (5) M, (7) F, mean age (39 ± 4) years.

UC: (4); (3) M, (1) F, mean age (50 ± 5) years.

Control patients: (6); (1) M, (5) F, mean age (50 ± 5) years.

Biopsy tissues were incubated with the different enteral formulae as follows:
 CD: ECO (7), EWT (10), EAA (20), MC (8)
 UC: ECO (5), EWT (6), EAA (7), MC (2)
 Control patients: ECO (5), EWT (5), EAA (6), MC (3).

9.1. IL1ra / IL1 β ratios after incubation with ECO and EWT

9.1.1. Crohn`s disease

There was no significant increase in the anti-inflammatory / pro-inflammatory cytokine ratio of IL1ra / IL1 β for either ECO or EWT incubation vs. MC (Figure 58 and Figure 59).

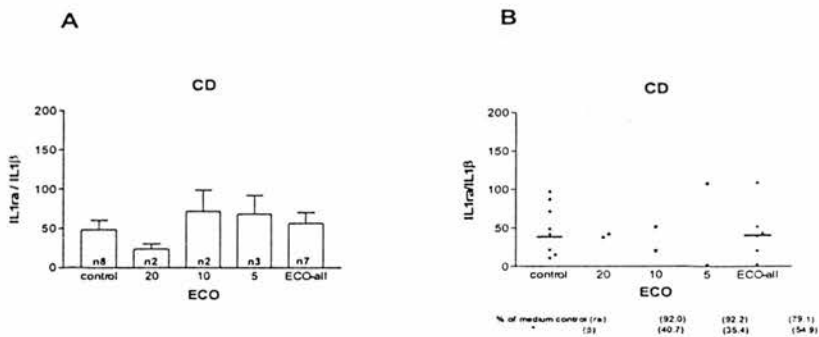


Figure 58 A+B (A) IL1ra/IL1 β ratio after incubation with ECO in CD. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

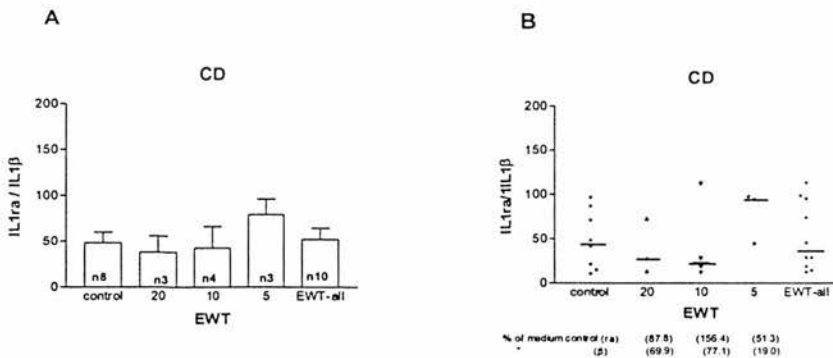


Figure 59 A+B (A) IL1ra /IL1 β ratio after incubation with EWT in CD. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

9.1.2. Ulcerative colitis

For UC there was no significant increase in IL1ra / IL1 β ratio for either ECO or EWT incubations (Figure 60, Figure 61 and Table 21).

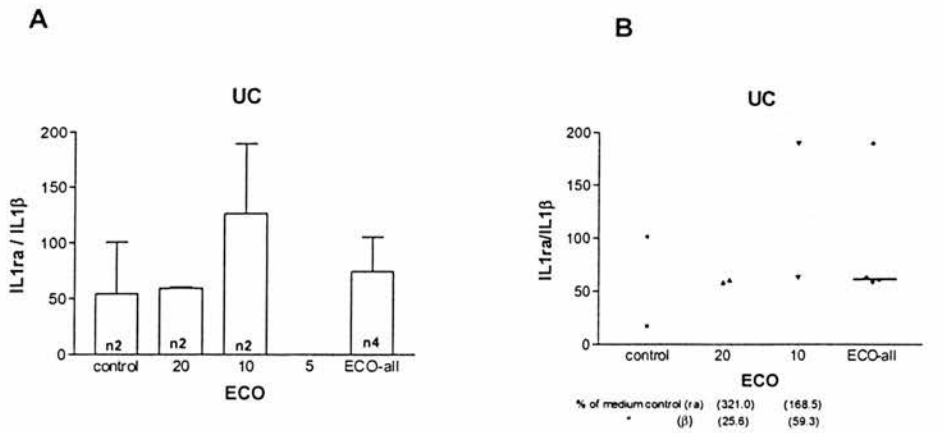


Figure 60 A+B (A) IL1ra /IL1 β ratio after incubation with ECO in UC. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

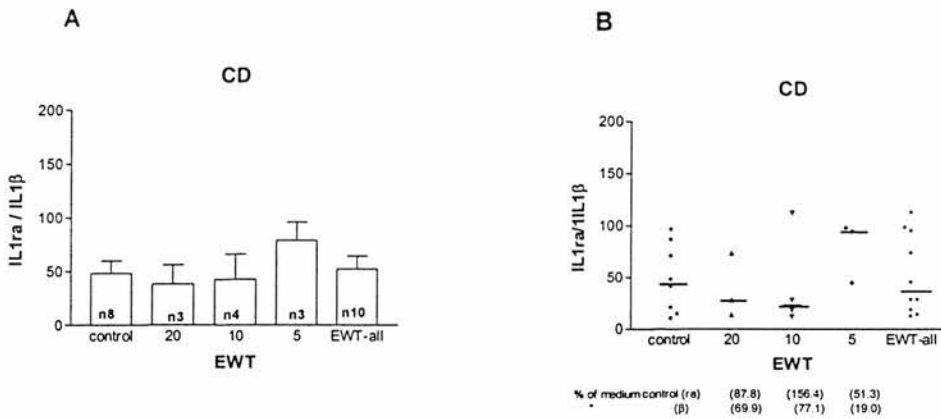


Figure 61 A+B (A) IL1ra /IL1 β ratio after incubation with EWT in UC. (B) IL1ra/ IL1 β showing median and % of medium control of IL1ra + IL1 β

9.1.3. Control patients

In these patients there was no significant increase in IL1ra / IL1 β ratio for incubation with ECO. Incubation with EWT resulted in a significant increase in the ratio at 1:5 dilution ($p < 0.05$) (Figure 62, Figure 63 and Table 21).

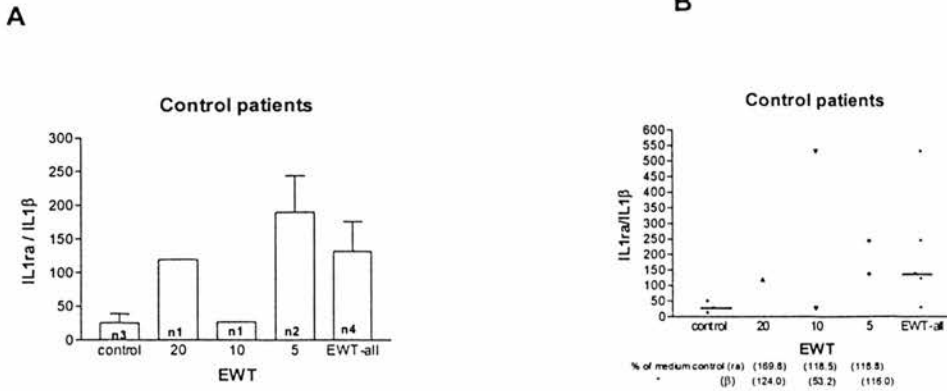


Figure 62 A+B (A) IL1ra /IL1 β ratio after incubation with ECO in Control patients. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

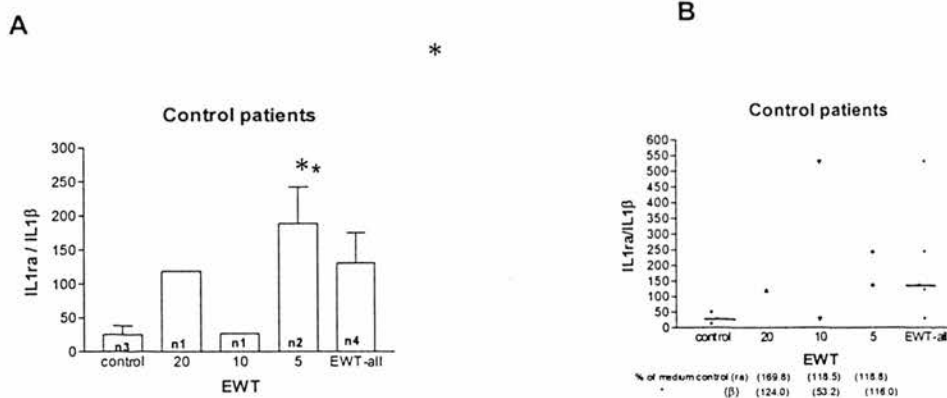


Figure 63 A+B (A) IL1ra / IL1 ratio after incubation with EWT in Control patients. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

Table 21 IL1ra / IL1 β ratios after 24-h incubation with ECO and EWT (data are as mean and SEM)

Diagnosis	ECO			EWT		
	20	10	5	20	10	5
CD	23.62 ± 6.74	71.5 ± 27.0	68.4 ± 23.5	38.3 ± 18.0	42.7 ± 23.5	79.2 ± 17.1
MC (1:20, 1:10, 1:5)	48.4 (± 11.7) 56.5 (± 13.7)			48.4 (± 11.7) 52.3 (± 12.2)		
UC	59.2 ± 1.16	126.0 ± 63.4	nd \pm	87.1 ± 20.3	70.1 ± 57.1	140.7 ± 14.9
MC (1:20, 1:10, 1:5)	54.3 (± 46.3) 74.3 (± 31.0)			54.3 (± 46.3) 99.2 (± 20.9)		
Control patients	64.6 ± 1.11	246.0 ± 100	106.3 \pm	119.2 \pm	26.77 \pm	189.7* ± 53.5
MC (1:20, 1:10, 1:5)	25.1 (± 13.6) 145 (± 52.5)			25.1 (± 13.6) 131.3 (± 44.3)		

* $p < 0.05$ vs. MC (medium control)

9.2. Interleukin 10 concentration in supernatant after incubation with ECO and EWT and TGF- β 1 concentration as % of medium control

The production of IL10 in the supernatant was expressed as IL10 concentration after incubation with enteral diet as a percentage of IL10 in medium control. This is due to the fact that the tissue was not processed for protein weight estimation and therefore an expression of IL10 per pg of protein weight was not available. As the tissue was taken with standard size of endoscopy forceps and by the same endoscopist, representing generally the same tissue size, this method was considered acceptable. Unlike the EAA, EC and EW experiments reported previously, this standardisation helped in analysis of results without tissue weighing. This tissue was required after organ culture for assessment of viability by BrdU uptake.

9.2.1. Crohn`s disease

In CD there was a no substantial increase in % IL10 compared with medium control after either ECO or EWT incubation (Figure 64 and Figure 65).

9.2.2. Ulcerative colitis

Similarly in UC, no substantial increase in % IL10 compared with medium control could be detected after either ECO or EWT incubation. In general, incubation with EWT resulted in more IL10 production than ECO (Figure 66)

9.2.3. Control patients

In control tissues incubated with ECO IL 10 production was more marked (Figure 64). Incubation with EWT resulted in some increase in 1:10 and 1:5 dilution (Figure 65).

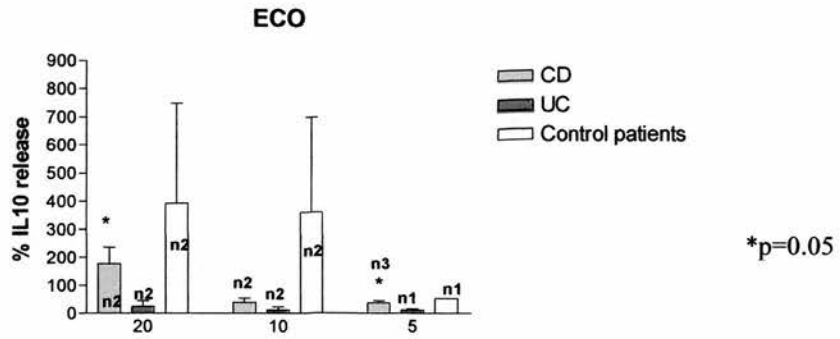


Figure 64 % IL10 after ECO in CD, UC and Control patients

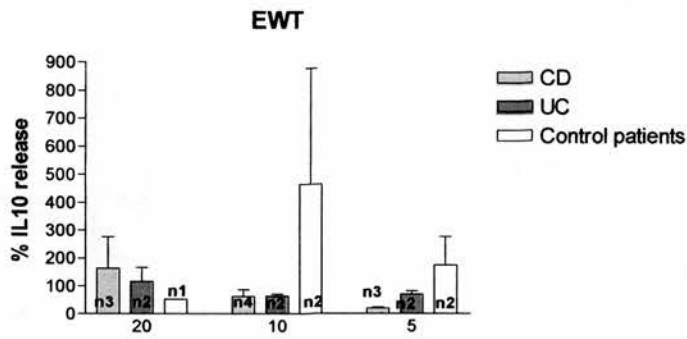


Figure 65 % IL10 after EWT in CD, UC and Control patients

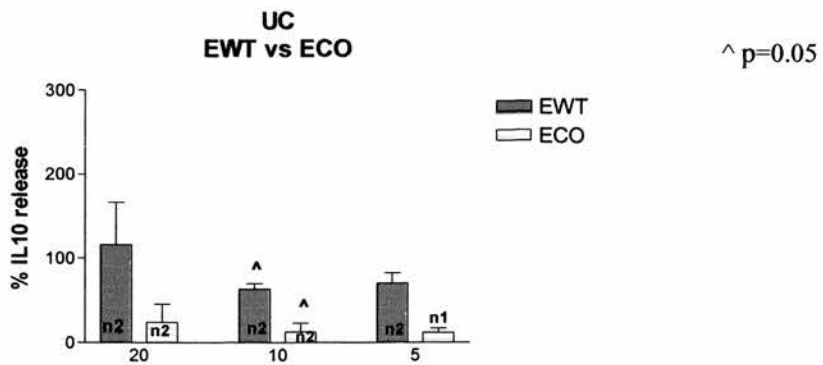


Figure 66 % IL10 in after ECO and EWT in UC

TGF- β concentrations are expressed as % of medium control (MC)

9.2.4. Crohn`s disease

In CD after ECO incubation % TGF- β 1 concentration showed the most marked increase at 1:20 dilution (Figure 67).

After EWT incubation, the increase in % TGF- β concentration was less (Figure 68) and after EAA incubation there was little increase in % TGF- β concentration (Figure 69 and Table 22) showing that TGF- β 1 release was ECO>EWT>EAA.

9.2.5. Ulcerative colitis

In UC after ECO incubation there was no or negligible detectable TGF- β 1 in the supernatant (Figure 67). After incubation with EWT, the increase was obvious, stepwise with the dilutions, but not significant (Figure 68). After EAA incubation with UC tissue, there was little increase in TGF- β 1 concentration (Figure 69 and Table 22) showing that TGF- β 1 release in UC was EWT>ECO>EAA.

9.2.6. Control patients

Tissues of control patients incubated with ECO (Figure 67), or EAA showed little increase in TGF- β 1 (Figure 69). After EWT incubation, an increase in TGF- β 1 was demonstrated at 1:5 dilution (Figure 68 and Table 22).

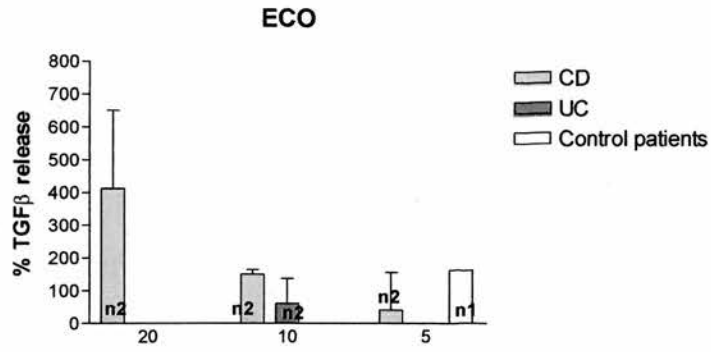


Figure 67 % TGF-β1 release after ECO incubation compared with medium control

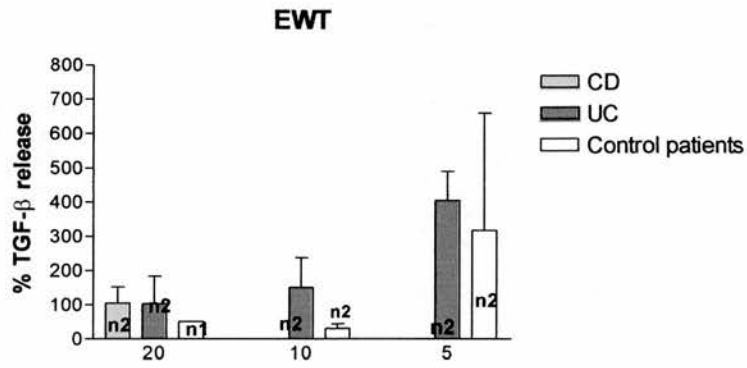


Figure 68 % TGF-β1 release after EWT incubation compared with medium control

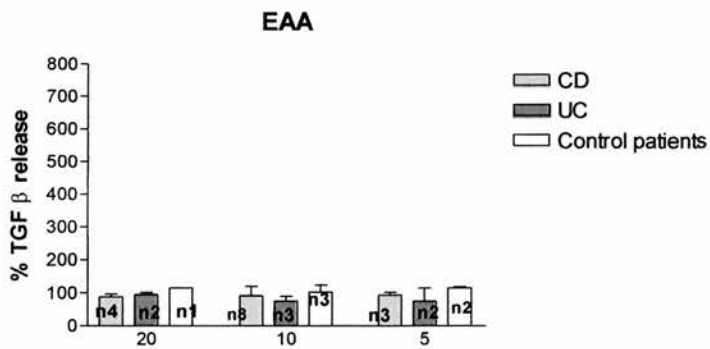


Figure 69 % TGF-β1 release after EAA incubation compared with medium control

Table 22 Percentage of TGF- β 1 release of control in CD, UC and control patients (% mean and SEM)

Diagnosis	EAA			ECO			EWT		
	20	10	5	20	10	5	20	10	5
CD	87.8 ± 9.51	91.0 ± 28.9	93.6 ± 8.6	412 ± 239	150.3 ± 14.3	40 ± 116	104.5 ± 47.6	nd -	nd -
UC	94.6 ± 6.9	75.7 ± 14.2	75.3 ± 39.6	nd -	61.1 ± 75.8	nd -	102.9 ± 80	151.2 ± 86.6	404.6 ± 84.5
Control patients	114.9 \pm	102.2 ± 21.5	115.7 ± 3.3	nd -	nd -	162.9 \pm	51.2 \pm	30.2 ± 14.0	317 ± 341

nd =not detected

9.3. Discussion of cytokine supernatant response assayed in the supernatant after incubation with ECO and EWT

These results summarise the cytokine response after 24-hour incubation with enteral diet colostrum (ECO) and enteral diet -whey enriched with TGF- β (EWT).

Incubation in ECO and EWT did not increase IL1 α / IL1 β ratio significantly. This was different from previous experiments where a significant increase in the ratio was seen after EAA. The lack of increase in IL1 α / IL1 β ratio after incubation with ECO and EWT was unexpected, as a downregulation of pro-inflammatory cytokines after *in-vivo* intake of polymeric diets containing TGF- β has been reported (Fell J.M.E. et al., 2000). A favourable response in TGF- β 1 expression after ECO and EWT incubation was reported in the previous chapter 6. As the present experiment was based on an *in vitro* model a possible influence on the IL1 α / IL1 β ratio in this experiment might be due to the addition of TGF- β . Wahl *et al* (Wahl et al., 1993) showed that TGF- β exerts its immunomodulatory effect on the TH1 mediated cytokine response of IL1 β in a sequential stepwise effect. It was shown that supernatant of TGF- β treated monocytes showed higher IL1 activity in supernatant within 24 hours, suggesting a differential regulation of IL1 β and IL1 α by TGF- β . Messenger RNA coding for IL1 β was

expressed earlier within 4 hours and showed higher expression after stimulation with TGF- β , while synthesis and secretion of IL1ra antagonist occurred later. This could explain the low or insignificant increase in IL1ra / IL1 β ratio after 24- hours incubation with ECO and EWT containing TGF- β in the current experiment. However, UC is known as being TH2 mediated and the IL1ra / IL1 β ratio was slightly higher in UC compared with CD (though not significant) but the increase in ratio was again seen by combining all three dilution for IL1ra / IL1 β ratio in UC.

Measurement of IL10 as a percentage of medium control in CD revealed a modest release of IL10 in supernatant after incubation with ECO and EWT, which could be either due to a low production of IL10 or due to an inhibitory effect on IL10 release triggered by TGF- β .

In UC there was a significant increase in IL10 production after incubation with EWT and to a lesser extent with ECO. Ishizuka *et al* (Ishizuka K. et al., 2001) has shown an increase in IL10 and TGF- β in culture supernatant of colonic biopsies from patients with UC in the resolving stage. Using EWT there was an increased release of % TGF- β in UC, but this was not found after incubation with ECO. IL10 release in UC was significantly different between diets containing TGF- β , showing a decreased or non-detectable release of IL10 in ECO. Ishizuka *et al* reported further a decreased IL1ra / IL1 β ratio in the active stage, and addition of IL10 to the culture fluid produced an inhibition of IL1 β . Contrary to this, after EAA incubation there was an increased release of IL10 but an insignificant increase of IL1ra /IL1 β ratio in UC.

Measurement of TGF- β 1 after incubation with ECO and EAA has methodological problems. This is due to the presence of foetal calf serum in the medium. It would have been useful using foetal calf serum as a blank in the assay. Unfortunately this has not been performed. Therefore there is no information how the foetal calf may have interfered with the assay. Therefore interpretation of these data should be with considerable caution. But as % TGF- β 1 release after incubation with EAA without added TGF- β was not different between the three disease groups, it is likely that variation of TGF- β 1

release after incubations with ECO in both CD and in UC is a reflection of the influence of these enteral diet compositions.

9.4. Correlation between TGF- β 1 expression and cytokine concentration after incubation with ECO and EWT

In the previous three chapters TGF- β 1 staining expression and cytokine response in tissues affected with CD, UC and control patients tissues after incubation with modified enteral diets of ECO and EWT have been discussed and data presented.

The correlations between cytokine ratio IL1ra / IL1 β vs. TGF- β 1 staining expression and between percentage IL10 of medium control vs. TGF- β 1 staining expression are shown in the next section. The objective was to determine if *in vitro* TGF- β 1 staining expression is correlated with IL1ra / IL1 β ratio and % IL10 of medium control in culture supernatant of CD, UC and control patients affected tissues *in vitro*.

The subjects were the same as described in chapter 6 for enteral diet colostrum (ECO) and TGF- β enriched whey extract (EWT) incubation immunostaining for TGF- β 1. For details refer to chapter 6.

9.4.1. Correlation between TGF- β 1 expression by immunostaining and IL1ra / IL1 β ratio

9.4.1.1. Crohn`s disease

TGF- β 1 expression and IL1ra / IL1 β ratio in CD after incubation with ECO were not correlated ($r=0.12$, $p=0.66$). The incubation with EWT resulted in no significant correlation of TGF- β 1 expression and IL1ra / IL1 β ratio, ($r = 0.24$, $p=0.33$) (Figure 70 and Figure 71).

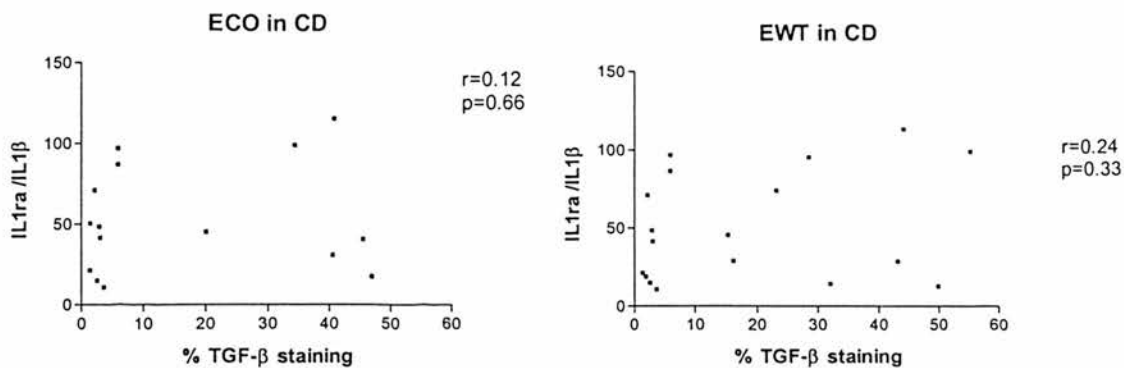


Figure 70 (left) % TGF-β1 staining vs. IL1ra /IL1β ratio after incubation with ECO in CD
 Figure 71 (right) % TGF-β1 staining expression vs. IL1ra /IL1β ratio after incubation with EWT in CD

9.4.1.2. Ulcerative colitis

Incubation with ECO and EWT in UC did not show any correlation between TGF-β1 staining expression and IL1ra / IL1β ratio ($r = -0.37$, $p = 0.42$ and $r = 0.42$; $p = 0.30$ respectively (Figure 72 and Figure 73).

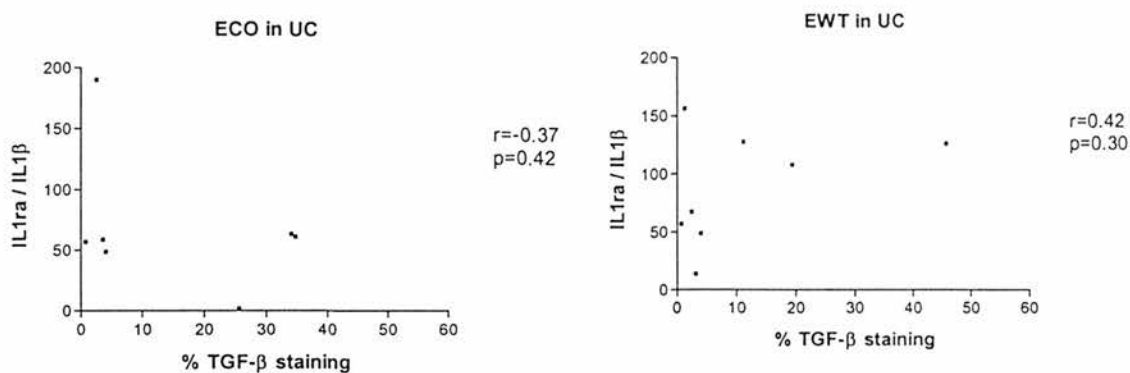


Figure 72 (left) % TGF-β1 staining vs. IL1ra /IL1β ratio after incubation with ECO in UC
 Figure 73 (right) % TGF-β1 staining vs. IL1ra / IL1β ratio after incubation with EWT in UC

9.4.1.3. Control patients

TGF- β 1 staining expression and IL1ra / IL1 β ratio in control patients showed no significant correlation ($r=-0.19$, $p=0.65$; $r=-0.20$, $p=0.67$, respectively) (Figure 74 and Figure 75).

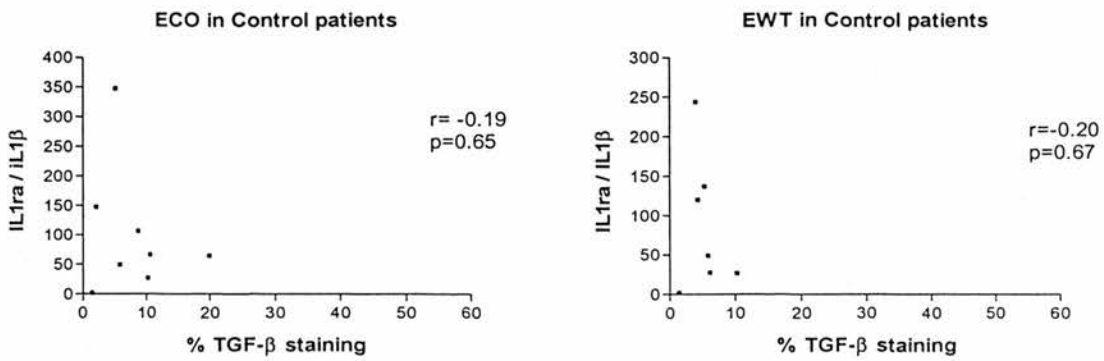


Figure 74 (left) % TGF- β 1 staining expression vs. IL1ra /IL1 β ratio after incubation with ECO in Control patients

Figure 75 (right) % TGF- β 1 staining expression vs. IL1ra / IL1 β ratio after incubation with EWT in Control patients

9.4.2. Correlation between TGF- β 1 expression by immunostaining and percentage IL10 of medium control

There was no correlation between TGF- β 1 staining expression and percentage IL10 of control in CD, UC and control patients.

9.4.3. Discussion

These results showed no correlation between percentage of TGF- β 1 staining expression and IL1ra / IL1 β ratio and between percentage of TGF- β 1 expression and percentage of IL10 of medium control after incubation with enteral diet colostrum (ECO) and enteral diet with TGF- β enriched whey extract (EWT) in CD, UC and control patients.

The data presented show that after incubation with ECO and EWT in CD no significant correlation between TGF- β 1 staining expression and IL1ra / IL1 β ratio could be found. Again in UC, no correlation was noted after incubation with ECO and EWT.

These results suggest that the effect of enteral diets in directly altering the anti-inflammatory to pro-inflammatory cytokine ratio is independent of the effect on TGF- β 1 expression. While elemental diet (EAA) has a direct anti-inflammatory effect by increasing the IL1ra / IL1 β ratio, the formulae enriched with growth factor peptides, such as ECO or EWT are more effective enhancing TGF- β 1 expression though these diets had no significant or major effect on altering the IL1ra / IL1 β ratio. Different dietary formulae may therefore have divergent effects on the intestinal mucosal inflammation. The group of Wahl *et al* reported a in TGF- β treated monocyte supernatant for IL1 β bioactivity revealed that supernatant generated at early time points (≤ 18 h) contained higher levels of detectable IL1 β activity than supernatant harvested later (≥ 24 h) (Wahl et al., 1993). This could also be a possible explanation for a) the non-significant increase in IL1ra / IL1 β ratio in ECO and EWT incubation vs. MC, and b) that in these data no correlation between IL1ra / IL1 β ratio and % TGF β 1 expression after 24-hours incubation occurred. It may be possible that IL1ra / IL1 β ratio might have significantly increased after a longer incubation period with these diets.

Immunomodulatory effect *in vivo* after consumption of enteral diets containing TGF- β is primarily mediated via upregulation of TGF- β with far less marked effect on the IL1ra / IL1 β ratio. However, many different factors and state of activation of the tissues determine the response and the assay conditions may not replicate the *in vivo* condition.

RESULTS

Section II

Manipulation of the Fatty Acids in Enteral Diets

CHAPTER TEN

10. Effects after incubation of inflamed and non-inflamed tissues with enteral diet- fish oil (EFI)

Introduction

The major source of ω -3 long chain polyunsaturated fatty acids in diet is from fish oil in the form of EPA and DHA. Fish oil in the form of encapsulated fish oil preparations has been used beneficially in several inflammatory conditions, including IBD. This is true especially in UC where it seems to have a better impact and modulation of inflammatory response than in CD (Ross, 1993; Lorenz-Meyer et al., 1996; Kim, 1996). The evidence however is conflicting (for details refer to chapter 3).

The following data present the results of organ culture incubation with enteral diet modified with fish oil (EFI) in IBD and control patients.

Subjects and Methods

Fifteen patients were included in this study. The data for the ELISA assay were analysed both for IL1 α and IL1 β anti-inflammatory / pro-inflammatory cytokine ratio and for percentage of IL1 β and IL1 α production in EFI incubation compared with medium control. The data are displayed as mean and standard error of the mean (\pm SEM). Enough data were not available for IL10 assays to perform any meaningful analysis.

Subjects

CD: (4), (1) F, (3) M, mean age (41 ± 14) years.

UC: (7), (6) F, (1) M, mean age (56 ± 6) years.

Control patients: (4), (3) F, (1) M, mean age (36 ± 9) years.

One biopsy was taken from each patient for medium control (MC) incubation. 45 biopsies (MC not included) were analysed, taken from the following anatomic areas:
CD: (2) sigmoid, (1) rectum, (1) ileocolonic anastomosis
UC: (5) sigmoid, (2) rectum
Control patients: (4) sigmoid
Biopsies were generally taken from inflamed looking areas, which were not ulcerated and diagnosis was confirmed by histopathology.

Biopsies were incubated with EFI:

CD: EFI (12), MC (4)

UC: EFI (21), MC (6)

Control patients: EFI (12), MC (4)

Time from diagnosis to biopsies taken were as follows:

1-3 years: 1 CD, 2 UC

4-6 years: 1 CD, 1 UC

>6 years: 2 CD, 4 UC

Medication at time of biopsy:

CD: AZA (2), steroids and AZA (1)

UC: steroids (1), AZA (2), 5-ASA (2)

10.1. IL1ra / IL1β ratio after EFI incubation

10.1.1. Ulcerative colitis

A significant increase was seen in the IL1ra / IL1β ratio in all three dilutions (1:20, 1:10 and 1:5; $p < 0.05$; $p = 0.05$ and $p < 0.02$, respectively) (Figure 76 and Table 23).

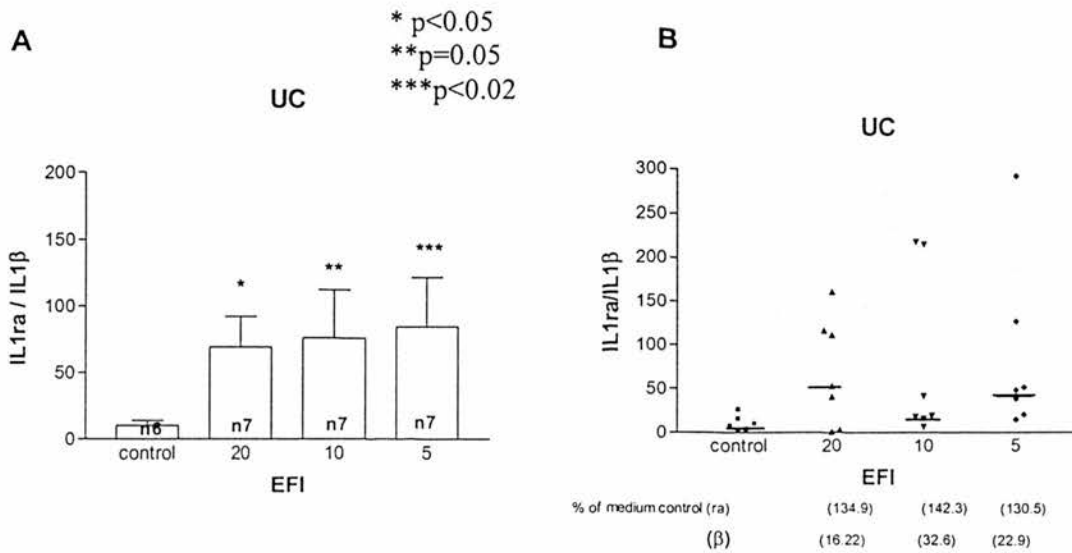
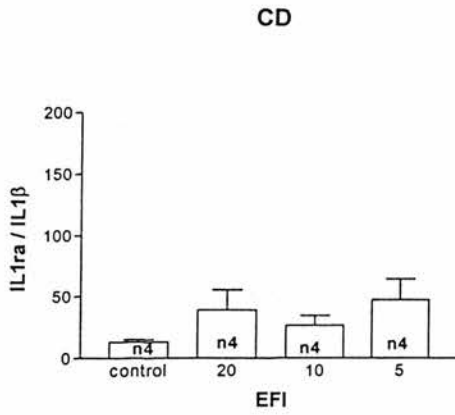


Figure 76 A+B (A) IL1ra / IL1β ratio in UC after 24-h incubation with EFI . (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1 β

10.1.2. Crohn`s disease

There was a slight increase in the IL1ra / IL1β ratio for 1:20, 1:10 and 1:5, but none of these reached statistical significance (Figure 77 and Table 23).

A



B

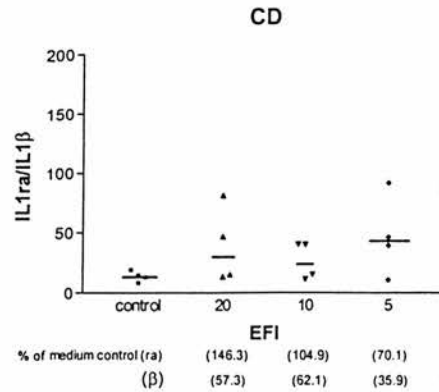
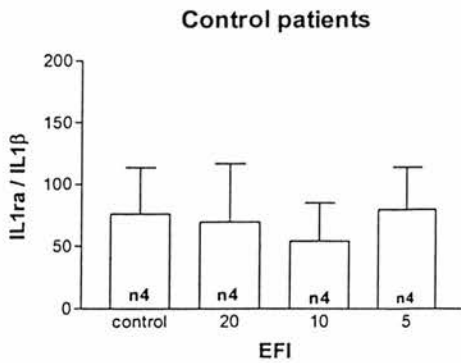


Figure 77 A+B (A) IL1ra / IL1β ratio in CD after 24-h incubation with EFI. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β

10.1.3. Control patients

The IL1ra / IL1β ratio did not increase after incubation with any of the dilutions. The ratio in MC was high (as expected in non-inflamed tissues) and this did not increase any further with EFI incubation (Figure 78 and Table 23).

A



B

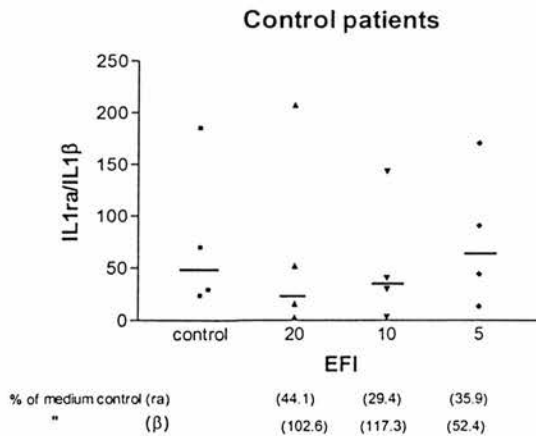


Figure 78 A+B (A) IL1ra / IL1β ratio in Control patients after 24-h incubation with EFI. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β

Table 23 IL1ra / IL1 β ratio in EFI

	EFI		
	20	10	5
CD	39.1 (\pm 16.1)	26.5 (\pm 7.8)	47.1 (\pm 16.8)
MC	13.0 (\pm 2.2.)		
UC	69.1* (\pm 32.2)	76.1** (\pm 36.4)	84.5*** (\pm 37.3)
MC	10.2 (\pm 3.7)		
control patient	69.3 (\pm 47.0)	54.1 (\pm 30.6)	79.4 (\pm 34.0)
MC	76.1 (\pm 37.3)		

* $p < 0.05$, ** $p = 0.05$, *** $p < 0.02$ vs. MC.

10.2. Changes in individual cytokine production after incubation with enteral diet-fish oil (EFI) as a percentage of incubation with medium control.

10.2.1. IL1 β

Percentage production of IL1 β compared with MC in UC was lower at all three dilutions compared with CD and this reached significance for 1:20 dilution ($p = 0.02$). The average production of IL1 β was significantly more suppressed in UC than in CD ($p < 0.05$).

In UC the decrease in production of IL1 β was also significant compared with control patients 1:20 ($p = 0.02$). In control patients there was little suppression of IL1 β except at the highest concentration of 1:5 (Figure 79 and Table 24).

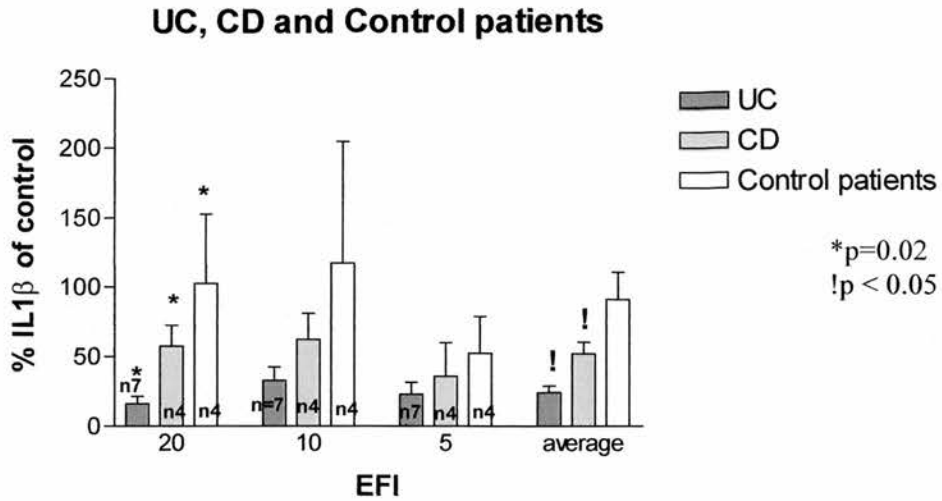


Figure 79 % IL1 β in UC, CD and Control patients after 24-h incubation with EFI

10.2.2. IL1ra

Production of IL1ra was increased at all three dilutions in CD compared with control patients, (in whom IL1ra decreased) reaching significance for 1:10 (p=0.03). The average increase in IL1ra in CD was significant vs. control patients (p=0.03).

In UC production of IL1ra was increased compared with control patients and the average production of IL1ra significantly increased (p<0.0001). Compared with CD, IL1ra production in UC was higher at 1:10 and 1:5 dilution, the average production of IL1ra was not significantly increased compared with MC. In control patients, IL1ra production decreased compared with MC, unlike CD and UC (Figure 80 and Table 24).

UC, CD and Control patients

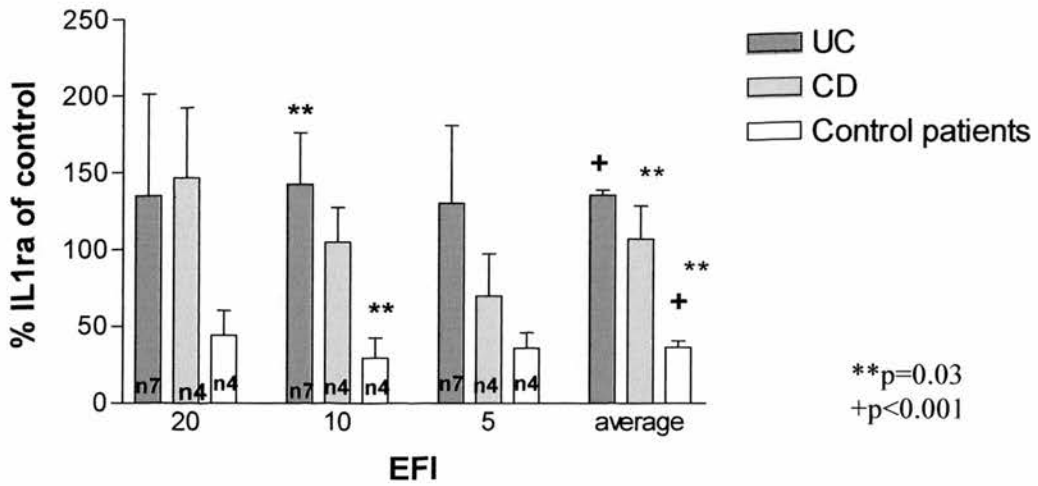


Figure 80 % IL1ra in UC, CD and Control patients after 24-h incubation

Table 24 Percentage of IL1 β and percentage of IL1ra production of MC in CD, UC and Control patients

Diagnosis	% IL1 β			% IL1ra		
	20	10	5	20	10	5
CD	57.3* (\pm 15.0)	62.1 (\pm 19.1)	35.9 (\pm 24.1)	146.3 (\pm 45.6)	104.9** (\pm 13.2)	70.1 (\pm 35.9)
average	51.8 (\pm 8.1)!			107.1 (\pm 22.0)**+		
UC	16.22* (\pm 5.2)	32.6 (\pm 9.8)	22.9 (\pm 8.7)	134.9 (\pm 66.1)	142.3 (\pm 33.2)	130.5 (\pm 50.3)
average	24.0 (\pm 4.8)!			135.9 (\pm 3.4)		
Control patients	102.6* (\pm 50.0)	117.3 (\pm 87.3)	52.4 (26.4)	44.1 (\pm 16.2)	29.4** (\pm 13.2)	35.9 (\pm 10.1)
average	90.8 (\pm 19.6)			36.5 (\pm 4.3) +**		

%IL1 β : *p=0.02 UC vs. CD & UC vs. Control patients (1:20)
!p<0.05 UC vs. CD (average)

% IL1ra: **p=0.03 CD vs. Control patients (1:10) & CD vs. Control patients (average)
+p<0.001 UC vs. Control patients & CD vs. Control patients (average)

10.3. Discussion

Fish oil preparations in the form of gelatin coated fish oil capsules have been used to treat UC in several studies. Hawthorne *et al* (Hawthorne et al., 1992), Aslan *et al* (Aslan and Triadafilopoulos, 1992), Stenson *et al* (Stenson et al., 1992) and Loeschke and his group (Loeschke et al., 1996) investigated (placebo controlled trials) the influence of ω -3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) derived from fish oil on prostaglandin and leukotriene synthesis in patients with UC. These studies illustrate that treatment with fish oil leads to a corticosteroid sparing effect, to a reduction in leukotriene B₄ (LTB₄) measured in colonic mucosa and rectal dialysate and to a suppressed synthesis of LTB₄ in peripheral blood neutrophils (Hawthorne et al., 1992; Aslan and Triadafilopoulos, 1992), (Stenson et al., 1992). In contrast to these studies in the current organ culture model, the direct influence of fish oil containing enteral diet on pro-and anti-inflammatory cytokine production *in vitro* was investigated. A significant increase in IL1ra / IL1 β ratio was found in UC compared with MC caused by an increased production of IL1ra and decreased production of IL1 β . This increase was not found in control patients and was less marked in CD patients. Average % IL1ra release in UC was higher than in CD and significantly higher compared with control patients. The percentage of IL1 β production of MC at all three dilutions showed a more marked decrease in UC compared with CD and control patients. This showed that enteral diets containing ω -3 fatty acids have a direct anti-inflammatory immunomodulatory impact *in vitro*, especially in UC in this model. It would appear that fish oil not only increases IL1ra, but also inhibits production of IL1 β . Such modulation of cytokine response by fish oil in UC patients might be due to modification of the fatty acid profile with increased ω -3 fatty acids. This supplies the substrate, which promotes production of prostaglandins and thromboxanes of series 3 and leukotrienes of series 5, with decreased pro-inflammatory properties. In a study by Mahida *et al* (Mahida et al., 1991) a similar organ culture model as the current study was used. Colonic biopsies of patients with UC were incubated with 5-aminosalicylic acid (5-ASA). Significantly higher IL1 β was detected in inflamed colonic biopsies compared to normal controls. After incubation with 5-ASA there was a significant dose

dependent decrease of IL1 β production as a percentage of control. They also showed a significant correlation of IL1 β and TXB₂ synthesis in culture medium of inflamed tissue compared with control. A significant decrease of LTB₄ was found in inflamed tissue treated with 5-ASA. 5-ASA can inhibit the synthesis of PGE₂, LTB₄ and their products of the 5-lipoxygenase pathway. It is likely that ω -3 fatty acids either affect the IL1 β synthesis by altering the eicosanoid synthesis, or as a direct influence of fatty acids on gene expression. The two major PUFA in fish oil are EPA and DHA, which are also major PUFAs incorporated into cell membrane. Eicosapentaenoic acid is the substrate for eicosanoid production via cyclooxygenase pathway for PG₃ and lipoxygenase pathway for LTB₅, both having less inflammatory properties. However, DHA is not a precursor for eicosanoid synthesis (Calder, 1998). Watanabe *et al* (Watanabe S. et al., 2000) showed that using LPS stimulated mice lymphocytes, where mice had been fed with beef tallow, DHA and EPA produced different IL1 β mRNA expression. Decreased production of IL1 β mRNA after EPA and DHA, was significant in DHA vs. tallow and slightly lower in EPA compared with DHA. Production of PGE₂ was significantly decreased in EPA and DHA, though DHA is not a precursor for eicosanoids (Calder, 1998). As IL1 β mRNA response differed between EPA and DHA, although the PGE₂ production was similar, it is possible that the balance of EPA/DHA in cellular lipids might be important in affecting intracellular signalling through modifications of specific gene transcription (Watanabe S. et al., 2000). Fatty acids in the cell are bound on fatty acid binding proteins (FABP), which exist in the cytosol and nucleus, in the form of fatty acid acyl-CoA (FA-CoA). As the nuclear receptors peroxisome proliferator-activated receptors (PPARs), occurring in three identified subtypes of α , β and γ , are regulated by fatty acids, it is likely that certain fatty acids or their metabolites may act as hormones in controlling specific transcription factors. For example PPAR α is required for fish oil mediated induction of mRNA_{AOX} (mRNA – Acyl-CoA oxidase). In addition, nuclear transcription factor NF κ B is required in genes involved in pro-inflammatory responses, such as COX2 and cytokines. The effect of ω -3 fatty acids might be attributed to the PPAR α activation and the regulation of NF κ B-mediated transcription (Jump and Clarke, 1999). As incubation with fish oil results in marked and divergent anti- and – pro-inflammatory cytokine response it is possible, that

ω -3 PUFA result in different cytokine production in various cell types (Watanabe S. et al., 2000). It may also reflect a different response in disease subtypes as IL1ra /IL1 β ratio was not increased in CD significantly after EFI incubation. Furthermore as EPA and DHA act differently in eicosanoid and IL1 β production, both of these ω -3 fatty acids may contribute to the anti-inflammatory effect of fish oil, possibly due to eicosanoid production by EPA and perhaps also through fatty acid modulated gene transcription through DHA. However, both of these pathways as a possible, maybe complementary anti-inflammatory response, need to be further researched.

Therefore, in summary, though EAA as well as modified protein enteral diets such as EC, preferentially downregulate inflammation in CD compared with UC, the situation was reversed in modified fatty acid enteral diet such as EFI. EFI preferentially downregulated inflammation in UC, and this was significantly more marked than in CD. This highlights the complexity of nutrient-inflamed gut interactions.

CHAPTER ELEVEN

11. Effects after incubation of inflamed and non-inflamed tissues with enteral diet sunflower oil (ESU) and enteral diet safflower oil (ESA)

Introduction

Sunflower oil and safflower oil are derived from plants generally known as being high in polyunsaturated fatty acid linoleic acid (18: 2- ω -6). The following experiments were set up to investigate the incubation with enteral diet modified in its fatty acid composition by sunflower oil (ESU) and safflower oil (ESA). The modified diets were specially prepared by SHS, UK. The rest of the composition of diet (EO28) was unchanged. This modified ESU is different from the usual commercial fatty acid profile of sunflower oil, i.e. rich in linoleic acid. This preparation of ESU contained monounsaturated fatty acid oleic acid (C18: 1), whereas safflower oil (ESA) contained as its major content both C18: 1 and C18: 2- ω -6 linoleic acid. The standard protocol as described in the previous chapters was followed.

Subjects and Methods

Biopsies from 34 patients were used to study enteral diets modified in fatty acid composition in respect of ESU and ESA. Some of the biopsies incubated with ESU were from the same patient as those incubated with EFI in previous chapter, as ESU was incubated in the experimental design along with EFI. For details refer to chapter 4 section I Figure 18. From each patient a biopsy was taken for MC incubation.

Subjects

CD: (8); (4) M, (4) F; mean age (43 ± 8) years.

UC: (17); (7) M, (10) F; mean age (52 ± 5) years.

Control patients: (9); (4) M, (5) F; mean age (50 ± 8) years.

From these patients biopsies were obtained, taken from the anatomical sites as follows:

CD: (3) sigmoid, (2) rectum, (1) colon (ascending/transverse), (1) ileocolonic anastomosis (1) ileum.

UC: (9) sigmoid, (4) rectum, (3) recto-sigmoid, (1) colon (ascending/transverse)

Control patients: (7) rectum, (1) colon (transverse), (1) colon (recto-sigmoid).

Biopsies were usually taken from areas appearing inflamed at endoscopy and diagnosis was confirmed by histopathology

Biopsies were incubated with the different enteral formulae and from 105 biopsies supernatants were analysed for:

CD: ESA (12), ESU (12)

UC: ESA (18), ESU (33)

Control patients: ESA (15), ESU (15) (from one patient biopsies were included in both diets)

Time from diagnosis to biopsies taken were:

<1 year: 3 CD, 3 UC

1-3 years: 1 CD

4-6 years: 1 CD, 2 UC

< 6 years: 12 UC, 3 CD

Medications at time of biopsies were:

CD: AZA (3); 5-ASA (1), steroids and 5-ASA (1)

UC: steroid (1), AZA (2), steroids and AZA (1); steroids and 5-ASA (1), 5-ASA (6)

11.1. IL1ra / IL1 β ratios after incubation with ESU and ESA

11.1.1. Crohn`s disease

In CD after incubation with ESU a significant increase in IL1ra / IL1 β ratios at all three dilutions of 1:20, 1:10 and 1:5 were observed ($p=0.03$, $p=0.01$ and $p=0.01$, respectively (Figure 81). After incubation of CD tissue with ESA no significant increase in the ratio of IL1ra / IL1 β compared with MC was observed (Figure 82 and Table 25).

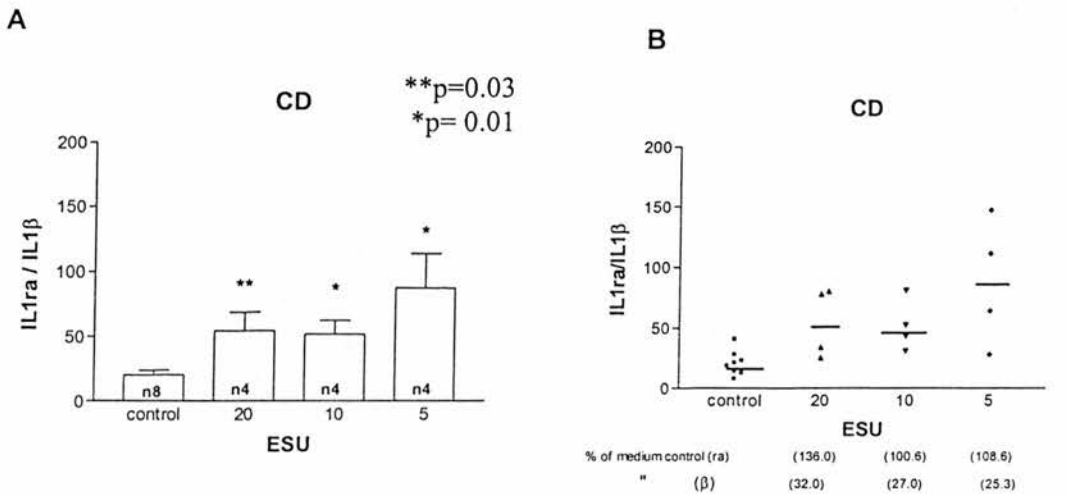


Figure 81 A+B (A) IL1ra / IL1 β ratio in CD after 24-h incubation with ESU. (B) IL1ra/IL1 β showing median and % of medium control IL1ra + IL1 β

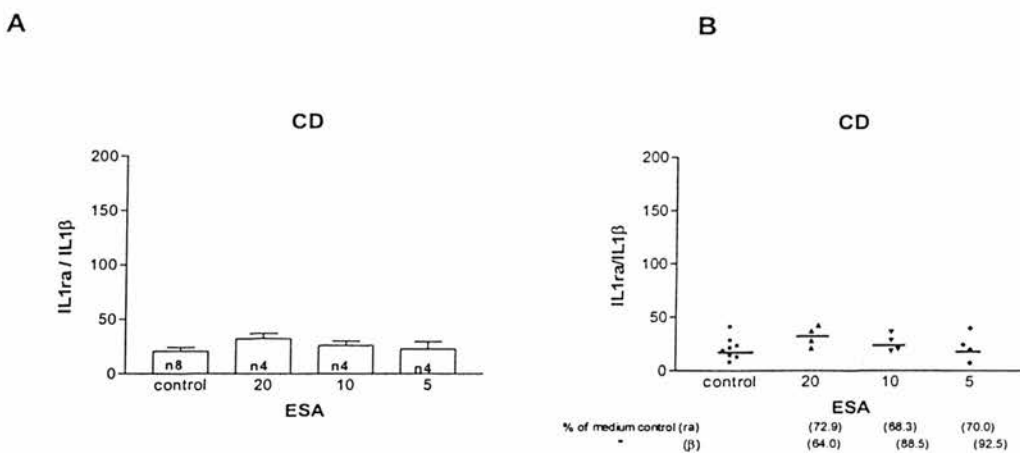


Figure 82 A+B (A) IL1ra / IL1 β ratio in CD after 24-h incubation with ESA. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra+IL1 β

11.1.2. Ulcerative colitis

In UC after incubation with ESU the IL1ra / IL1 β increased, but was only significant at 1:5 dilution (p=0.02) (Figure 83). In contrast, after UC tissue was incubated with ESA, there was no increase in the ratio IL1ra / IL1 β , in fact the ratio of ESA incubations was not different from MC (Figure 84 and Table 25).

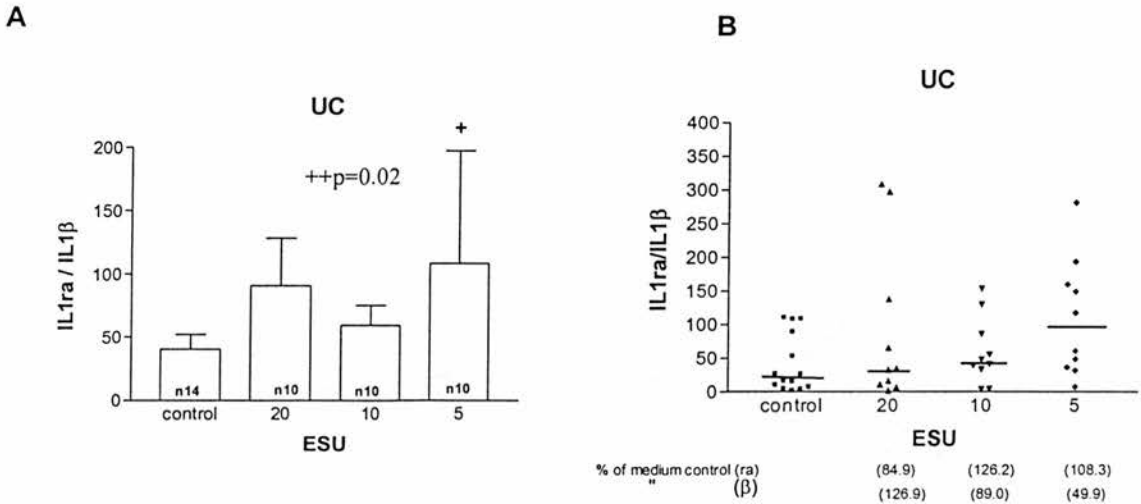


Figure 83 A+B (A) IL1ra / IL1 β ratio in UC after 24-h incubation with ESU. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

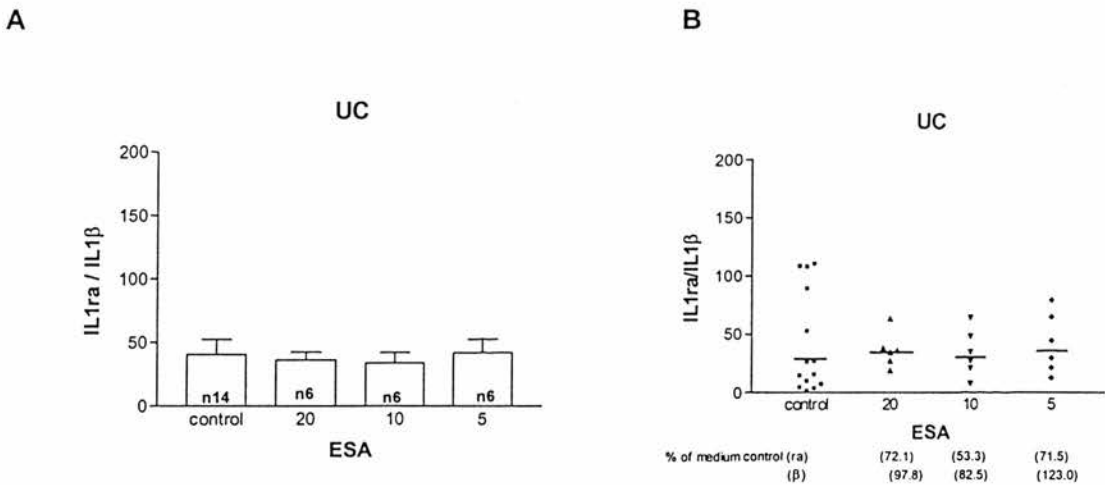


Figure 84 A+B (A) IL1ra / IL1 β ratio in UC after 24-h incubation with ESA. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

11.1.3. Control patients

After incubation in ESU, the IL1ra / IL1 β ratio increased at 1:5 dilution (p=0.05) (Figure 85). After incubation with ESA, there was no significant increase in the IL1ra / IL1 β ratio at any dilution (Figure 86 and Table 25).

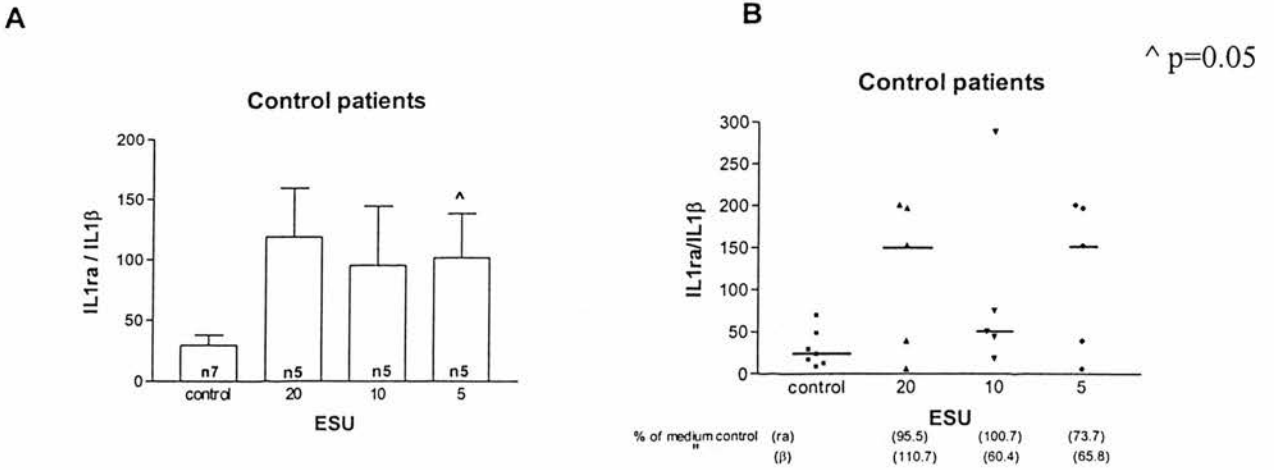


Figure 85 A+B (A) IL1ra / IL1 β ratio in Control patients after 24-h incubation with ESU. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

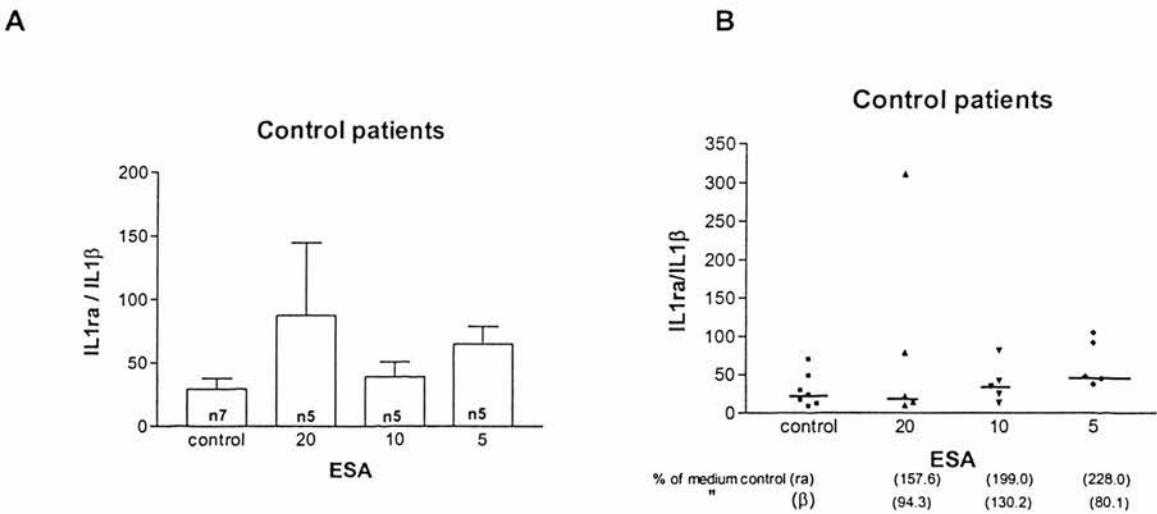


Figure 86 A+B (A) IL1ra / IL1 β in Control patients after 24 h incubation with ESA. (B) IL1ra/IL1 β showing median and % of medium control IL1ra + IL1 β

Table 25 IL1ra/IL1 β ratio for ESU and ESA incubation in CD, UC and Control patients

Diagnosis	ESU			ESA		
	20	10	5	20	10	5
CD	54.5** (\pm 14.4)	52.0* (\pm 10.7)	87.8* (\pm 26.2)	31.9 (\pm 4.8)	25.8 (\pm 4.1)	22.6 (\pm 6.7)
MC	20.5 (\pm 3.6)			20.5 (\pm 3.6)		
UC	91.1 (\pm 37.5)	59.4 (\pm 15.8)	108.7+ (\pm 27.8)	36.2 (\pm 6.1)	33.9 (\pm 8.2)	41.9 (\pm 10.6)
MC	40.5 (\pm 11.7)			40.5 (\pm 11.7)		
Control patients	119.2 (\pm 40.6)	95.7 (\pm 49.1)	102.0^ (\pm 36.6)	87.4 (\pm 57.5)	39.3 (\pm 11.6)	65.3 (\pm 13.8)
MC	29.5 (\pm 8.5)			29.5 (\pm 8.5)		

*p=0.03; **p=0.01; +p=0.02; ^p=0.05 vs. MC, respectively.

11.2. IL10/IL1 β ratios after ESU and ESA incubation

11.2.1. Crohn`s disease

In CD after ESU incubation, there was a small increase in IL10 / IL1 β ratio at all three dilutions, which was significantly higher at 1:20 vs. MC (p=0.05).

After ESA incubation of CD affected tissue there was no significant increase in the IL10 / IL1 β ratio compared with MC (Table 26).

11.2.2. Ulcerative colitis

Incubation in ESU resulted in a significant increase in the IL10/IL1 β ratio vs. MC (p<0.05) at 1:5. In ESA there was no change the IL10 / IL1 β (Table 26)

11.2.3. Control patients

In this group IL10 / IL1 β ratios were not significantly affected by incubation with ESU and ESA (Table 26).

Table 26 IL10/IL1 β ratio for ESU and ESA in CD, UC and Control patients

Diagnosis	ESU			ESA		
	20	10	5	20	10	5
CD	31.8 [^] (\pm 9.5)	35.8 (\pm 23.6)	214 (\pm 203)	18.9 (\pm 11.0)	15.6 (\pm 9.0)	11.6 (\pm 5.7)
MC	9.1 (\pm 2.2)			9.1 (\pm 2.2)		
UC	41.8 (\pm 14.0)	23.7 (\pm 6.6)	130.7* (\pm 95.9)	11.6 (\pm 2.8)	19.5 (\pm 7.0)	6.8 (\pm 2.8)
MC	26.8 (\pm 15.3)			26.8 (\pm 15.3)		
Control patients	24.0 (\pm 9.9)	26.3 (\pm 16.4)	84.5 (\pm 68.8)	41.3 (\pm 23.0)	6.3 (\pm 3.7)	9.8 (\pm 3.9)
MC	5.1 (\pm 1.5)			5.1 (\pm 1.5)		

[^]p=0.05 and *p < 0.05 vs. MC respectively

11.3. Changes in IL1 β after incubation of CD, UC and Control patients tissue with ESU and ESA, as percentage of medium control.

11.3.1. Crohn`s disease

IL1 β concentration in CD tissues incubated with ESU displayed a decrease of IL1 β as a percentage of MC at all three dilutions (Figure 87). The average concentration of IL1 β as a percentage of medium control was significantly lower in ESU compared with ESA ($p=0.004$) when all dilutions were considered together (Figure 88 and Table 27).

11.3.2. Ulcerative colitis

The average concentration of IL1 β as a percentage of MC was lower after ESU incubation (Figure 87) compared with % IL1 β concentration in ESA for UC tissues (Figure 88).

IL1 β concentration as percentage of MC was significantly lower after ESU incubation in CD when compared with UC ($p=0.05$) (Table 27).

11.3.3. Control patients

There was no significant change in percentage of IL1 β concentration compared with MC after incubation with ESU (Figure 87). This was also true for ESA incubation (Figure 88). After incubation with ESU, concentration of IL1 β as a percentage of MC was significantly lower in CD compared with control patients ($p=0.03$) (Table 27).

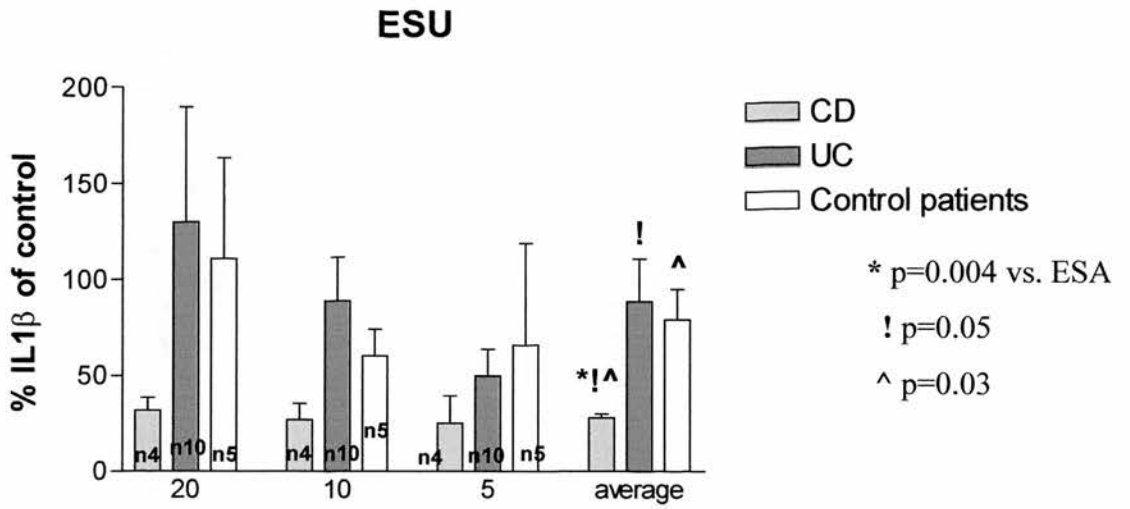


Figure 87 % IL1 β of control after incubation with ESU in CD, UC and Control patients

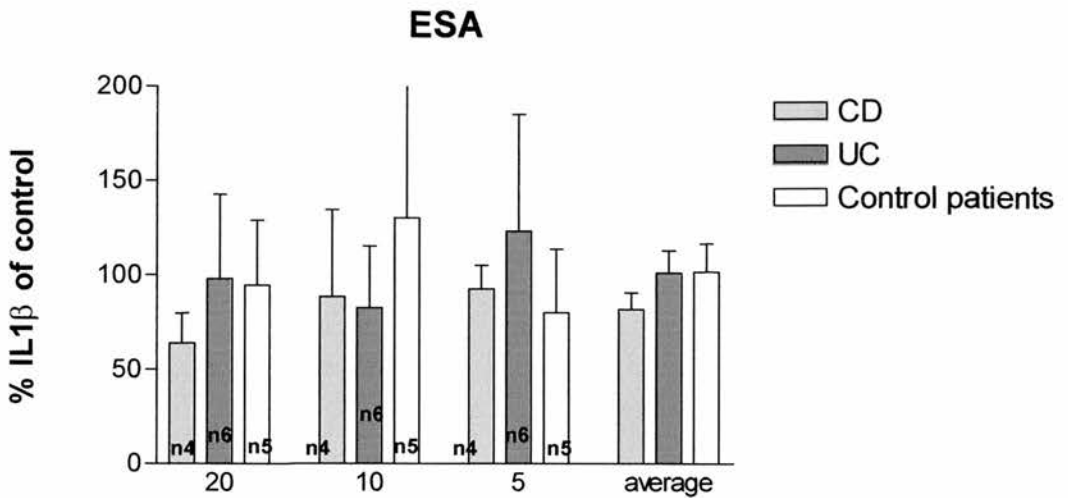


Figure 88 % IL1 β of control after incubation with ESA in CD, UC and Control patients

Table 27 Percentage of IL1 β of control for ESU and ESA in CD, UC and Control patients

Diagnosis	ESU			ESA		
	20	10	5	20	10	5
CD average	32.0 (± 6.6)	27.0 (± 8.6)	25.3 (± 14.3)	64.0 (± 15.8)	88.5 (± 46.1)	92.5 (± 12.5)
	28.1 (± 2.0)*! [^]			81.7 (± 8.9)*		
UC average	126.9 (± 59.8)	89.0 (± 22.7)	49.9 (± 13.9)	97.8 (± 44.8)	82.5 (± 32.7)	123.0 (± 62.3)
	88.6 (± 22.2) !			101.1 (± 11.8)		
Control patients average	110.7 (± 52.6)	60.4 (± 13.8)	65.8 (± 53.0)	94.3 (± 34.4)	130.2 (± 72.4)	80.1 (± 33.6)
	79.0 (± 15.9) [^]			101.5 (± 14.9)		

*p=0.004 ESU vs. ESA in CD (average)

! p= 0.05 CD vs. UC in ESU (average)

[^]p= 0.03 CD vs. Control patients in ESU (average).

11.4. Changes in IL1ra concentration of CD, UC and Control patients tissues as percentage of medium control after incubation with ESU and ESA

Changes in concentration of anti-inflammatory cytokine IL1ra were assessed after ESU and ESA incubation of CD, UC and control patient tissues.

11.4.1. Crohn`s disease

In CD, after ESU incubation, percentage of IL1ra concentration compared with MC was increased at all three dilutions (Figure 89), compared with ESA incubations and the average increase for ESU was significantly greater vs. average increase for ESA (p=0.01) (Figure 90 and Table 28).

11.4.2. Ulcerative colitis

This also showed an increase in IL1ra percentage compared with MC in all three dilutions of ESU compared with ESA. The average increase in UC after incubation in ESU (Figure 89) was significantly higher than the average increase after ESA incubation ($p < 0.05$) (Figure 90 and Table 28).

After ESU incubation, the percent increase in IL1ra compared with MC was similar in UC and CD.

11.4.3. Control patients

After ESU incubation the concentration of IL1ra showed a less marked increase as a percent of MC compared with CD and UC tissues (Figure 89).

The average concentration of IL1ra in the three dilutions after ESA incubation expressed as percentage of MC was significantly higher than after ESU incubation ($p < 0.01$). This was also significantly higher than CD and UC tissues ($p < 0.005$) (Figure 90 and Table 28).

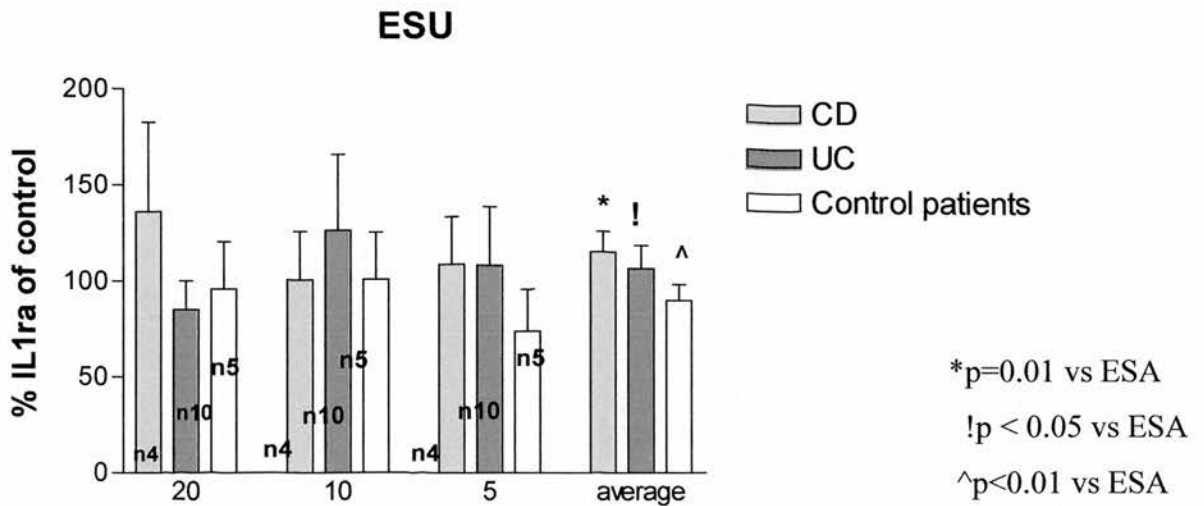


Figure 89 % IL1ra after incubation with ESU in CD, UC and Control patients

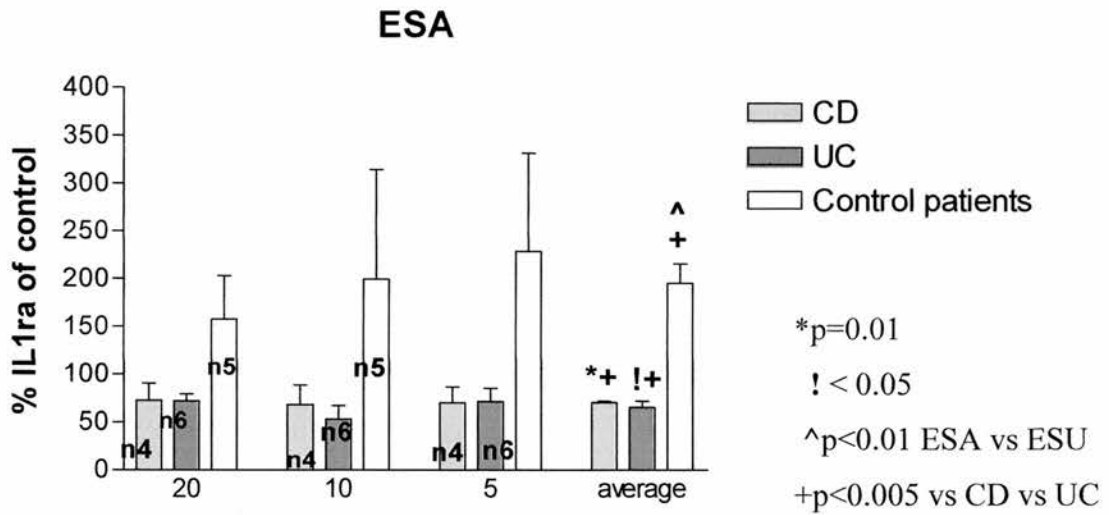


Figure 90 % of IL1ra after incubation with ESA in CD, UC and Control patients.

Table 28 Percentage of IL1ra of control in and ESA for CD, UC and Control patients

Diagnosis	ESU			ESA		
	20	10	5	20	10	5
CD	136.0 (±46.5)	100.6 (±25.2)	108.6 (±24.6)	72.9 (±17.5)	68.3 (±20.2)	70.0 (±15.5)
average	115.1 (±10.7)*			70.4 (±1.3)*+		
UC	84.9 (±15.0)	126.2 (±39.4)	108.3 (±30.2)	72.1 (±7.27)	53.3 (±13.9)	71.5 (±13.6)
average	106.5 (±12.0) !			65.6 (±6.2)!+		
Control patients	95.5 (±24.8)	100.7 (±24.50)	73.7 (±21.8)	157.6 (±45.2)	199 (±115)	228 (±103)
average	89.9 (±8.3)^			194.9 (±20.4)+^		

*p=0.01 in ESU vs. ESA (average)

! p<0.05 in ESU vs. ESA (average)

+P <0.005 in ESA Control patients vs. CD and vs. UC (average), respectively

^p<0.01 ESA vs ESU Control patients (average)

11.5. Discussion

Incubation of colonic biopsies with enteral diet modified in fatty acid composition with sunflower oil (ESU) and safflower oil (ESA) resulted in changes in the IL1ra / IL1 β ratio and in IL10/IL1 β ratio indicating an alteration in the balance between pro- and anti-inflammatory cytokines. In CD, incubation with ESU resulted in a significant increase in the anti-inflammatory / pro-inflammatory cytokine ratio of IL1ra and IL1 β . In UC after ESU incubation there was a significant increase in IL1ra / IL1 β ratio only at 1:5 dilution. Therefore in both CD and UC, incubation with ESU resulted in a favourable change in the anti-inflammatory direction. In CD patients there was a significant decrease in IL1 β as a result of ESU incubation, as well as an increase in IL1ra. Furthermore, incubation of CD tissue with ESU resulted in an increase in IL10 / IL1 β ratio, which was significant at 1:20. An increase in IL10 / IL1 β ratio was also significant at 1:5. Incubation with ESA did not increase IL10 / IL1 β ratio to the same extent as ESU incubation, either in CD or in UC. ESU suppressed IL1 β more effectively than ESA, especially in CD patients. IL10/ IL1 β ratio in CD and UC were unaffected by

ESA.

Overall, incubation with ESU demonstrated some evidence of a positive anti-inflammatory response, shown by a change in cytokine balance. This was especially true for CD patients, but also to some extent for UC. The beneficial effect on anti-inflammatory / pro-inflammatory cytokine production was more evident after incubation with ESU than ESA. This was characterised by an increase in IL1ra / IL1 β ratio and a decrease of IL1 β and increase of IL1ra. In addition in CD and UC the IL10 / IL1 β ratio increased after ESU incubation. The sunflower oil modification used in this study contained ~ 61.88 % oleic acid (18:1) and ~ 21.99 % stearic acid (18:0), compared with safflower oil 72.42 % (18:1), 2.19 % (18:0) and 14.4 % linoleic acid (18:2). This positive response of ESU could be possible due to the presence of stearic acid, which is higher in ESU (21.99%) compared with ESA (2.19%).

ESA did not achieve the same significant alteration of anti-and pro-inflammatory cytokine ratios. ESA contained linoleic acid, known as a plant derived essential fatty acid, which is a precursor of prostaglandin synthesis of ω -6 pathway for dihomo- γ -linolenic acid (20:3- ω -6) precursor for less inflammatory eicosanoid production, and arachidonic acid (20:4- ω -6), known for production of more inflammatory eicosanoids. These have been discussed in details in chapter 2. The positive effects of ESU can therefore be tentatively explained by the hypothesis suggesting that the composition of oleic acid and stearic acid together may have positive influenced the inflammatory response. Analogous to a high intake of linoleic acid causing elevated intragastric PGE₂ levels, (Sammon, 1999), it is possible that the presence of linoleic acid in ESA might have influenced PGE₂ synthesis in the present model, leading to an increase in IL1 β . Furthermore IL1ra / IL1 β ratio did not change after ESA incubation compared with MC in UC, which is known as Th2 lymphocyte modulated disease. Subtle differences in fatty acid composition of enteral formulae may therefore modulate inflammation differently, possibly via divergent pathways. However, these *in vitro* findings are preliminary and require further *in vitro* research investigating mRNA expression of immune response genes and expression microarray technique would be particularly suitable.

CHAPTER TWELVE

12. Effects after incubation of inflamed and non-inflamed tissue with enteral diet olive oil (EOL) and enteral diet canola oil (ECA)

Introduction

Olive oil has a high content of monounsaturated oleic acid (C18: 1) and is believed to have a beneficial anti-atherogenic effect. People resident in the Mediterranean are known to have a low incidence of coronary heart disease compared with Northern European countries. The effect is thought to be due to the combination of oleic acid and vegetables. Arteriosclerosis is mediated by eicosanoids due to platelet stimulation factor (PAF) and it is thought that high intake of oleic acid may direct eicosanoid production into pathways of low inflammatory potential and stimulate eicosanoids, and thereby prevent or have a protective effect against arteriosclerosis.

Canola oil, or rapeseed oil is known to have a relatively high content of essential linoleic acid (18:2 ω -6), which in turn is also believed to have a positive influence on eicosanoid synthesis.

The following experiments describe incubation of endoscopic biopsies from IBD and control patients with enteral diet modified in its fatty acid composition for olive oil (EOL) and canola oil (ECA). The aim was to establish whether the immunomodulatory effects of oleic acid and canola oil might also be reflected in downregulation of intestinal inflammation *in vitro* in IBD affected tissues.

Subjects and Methods

Endoscopic biopsy incubation in the above enteral diet fatty acid modifications was performed in 15 patients using EOL and in 14 patients using ECA. In each patient one biopsy was always used for MC incubation, which are not separately listed. As shown in the experimental design (chapter 4 section I, Figure 18) incubation for EOL was

performed with ESY, and therefore the data are analysed with ECA and MC results from ECA incubation, respectively.

Subjects

CD: (10); (7) F, (3) M, mean age (49 ± 6) years.

UC: (11); (4) F, (7) M, mean age (52 ± 6) years

Control patients: (8); (3) F, (5) M, mean age (55 ± 7) years.

A total of 87 biopsies were obtained, taken from the anatomic areas:

CD: (3) sigmoid, (1) rectum, (2) ascending/transverse colon, (4) ileum

UC: (7) sigmoid, (4) recto-sigmoid,

Control patients: (4) sigmoid, (3) ascending/transverse colon, (1) recto-sigmoid

Time from diagnosis to biopsy taken were:

< 1 year: 1 UC

1-3 years: 5 CD, 4 UC

4-6 years: 1 CD, 1 UC

> 6 years: 4 CD, 5 UC

Medications at time of biopsy were:

CD: steroid (3), steroid and 5-ASA (1)

UC: 5-ASA (4), steroids and 5-ASA (1), AZA (1)

87 Biopsies were incubated with EOL and ECA as follows:

CD: EOL (18), ECA (12)

UC: EOL (15), ECA (18)

Control patients: EOL (12), ECA (12) and 67 data were analysed for cytokines.

12.1. IL1ra / IL1 β ratios after incubation with EOL and ECA

12.1.1. Crohn`s disease

Tissues incubated with EOL did not show any increase in IL1ra / IL1 β ratio significantly (Figure 91). In tissues incubated with ECA the IL1ra / IL1 β ratio decreased (Table 29).

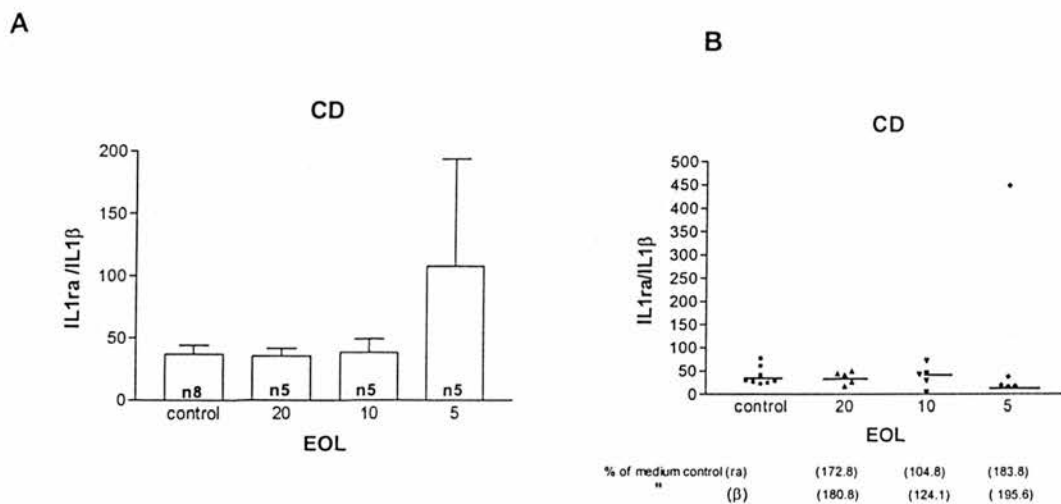


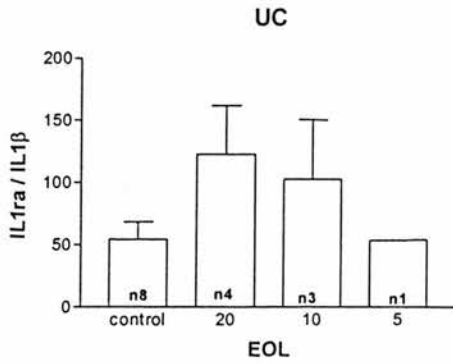
Figure 91 A+B (A) IL1ra / IL1 β ratio in CD after 24-h incubation with EOL. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

12.1.2. Ulcerative colitis

Tissues incubated with EOL showed a trend towards an increased IL1ra / IL1 β ratio but this was not significant vs. MC (Figure 92). A type II error cannot be excluded.

Incubation with ECA did not change the IL1ra / IL1 β ratios and these were lower than MC in CD (Table 29).

A



B

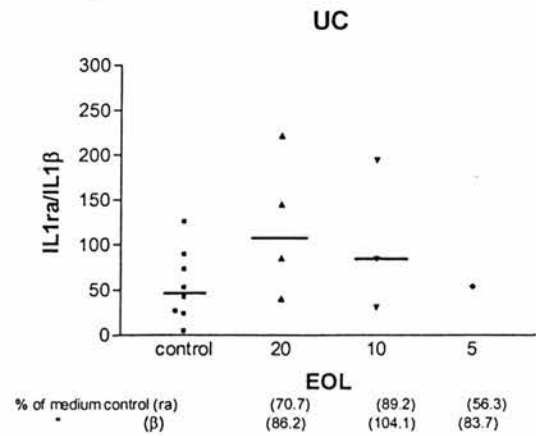
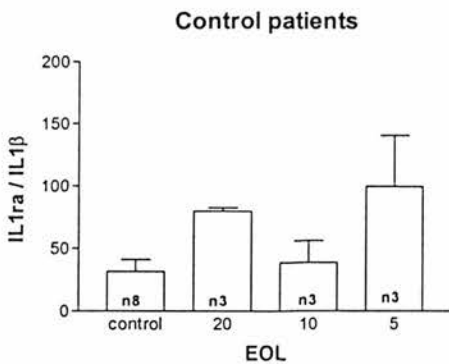


Figure 92 A+B (A) IL1ra / IL1β ratio in UC after 24-h incubation with EOL. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β

12.1.3. Control patients

The ratio increased modestly for 1:20 and 1:5 dilution of EOL (Figure 93). Incubation with ECA resulted in no significant alteration in the IL1ra / IL1β ratio at any of the three dilutions (Table 29).

A



B

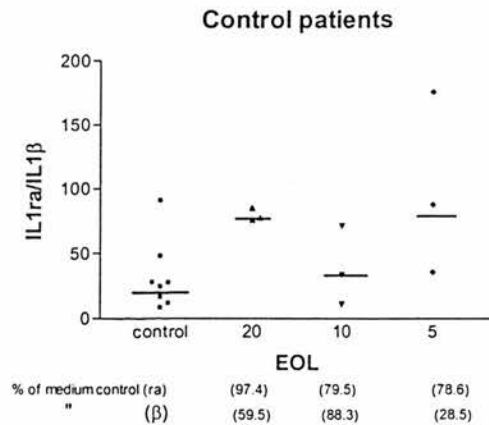


Figure 93 A+B (A) IL1ra / IL1β ratio in Control patients after 24-h incubation with EOL. (B) IL1ra/IL1b showing median and % of medium control of IL1ra + IL1b

Table 29 IL1ra / IL1 β ratio in EOL and ECA for CD, UC and Control patients

Diagnosis	EOL			ECA		
	20	10	5	20	10	5
CD	35.6 (± 6.1)	38.6 (± 11.0)	108.0 (± 85.5)	28.2 (± 6.7)	28.2 (± 4.1)	18.8 (± 1.8)
MC	37.1 (± 7.1)			37.1 (± 7.1)		
UC	122.9 (± 39.3)	102.9 (± 48.2)	53.9 (\pm)	37.3 (± 8.2)	43.0 (± 21.5)	24.3 (± 8.5)
MC	54.3 (± 14.0)			54.3 (± 14.0)		
Control patients	79.7 (± 2.9)	38.8 (± 17.6)	99.8 (± 40.8)	41.8 (± 15.1)	38.7 (± 14.8)	50.3 (± 14.4)
MC	31.7 (9.5)			31.7 (± 9.5)		

Estimation of IL10/IL1 β ratio in the supernatant after incubation with EOL and ECA did not demonstrate any appreciable anti-inflammatory effect. These data are therefore not presented in detail. In UC, after EOL incubation a significant increase in IL10 / IL1 β ratio (1:20) was observed vs. MC (118.3 ± 40.5 vs. 22.45 ± 9.39 ; $p < 0.02$).

12.2. Changes in IL1 β after incubation of inflamed and non-inflamed tissues in EOL and ECA as a percentage of medium control

After incubation with EOL, the concentration of IL1 β as a percentage of MC was significantly lower in UC compared with CD, and the average decrease in IL1 β was greater than in CD ($p < 0.03$). (Figure 94). The percentage decrease after ECA incubation was more marked than the actual increase after EOL incubation at 1:5 ($p = 0.05$). In UC, there was no significant change (Figure 95 and Table 30).

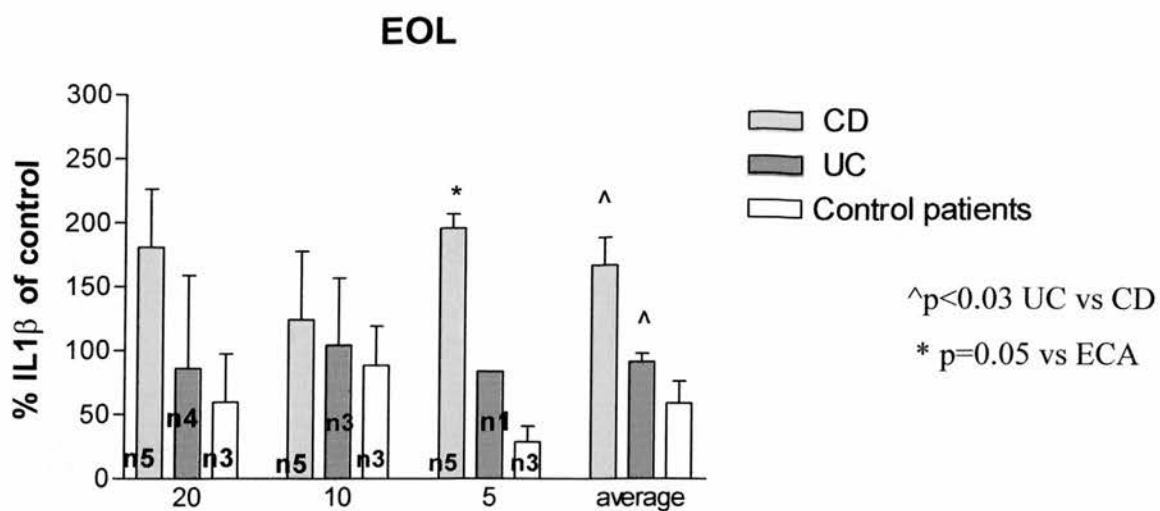


Figure 94 % IL1 β after incubation with EOL in CD, UC and Control patients

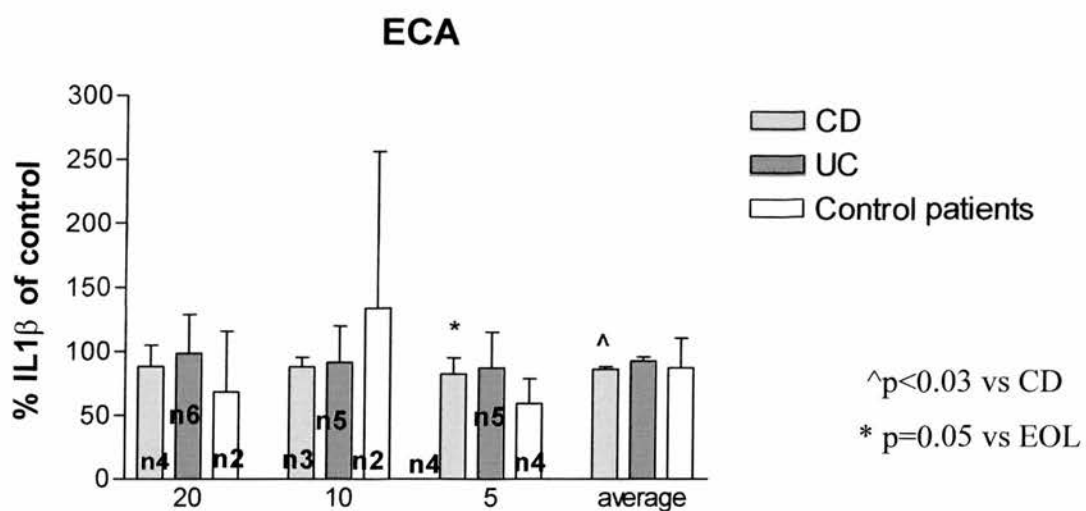


Figure 95 % IL1 β after incubation with ECA in CD, UC and Control patients

Table 30 Percentage of IL1 β of MC in CD, UC and Control patients after 24-h incubation with EOL and ECA

Diagnosis	EOL			ECA		
	20	10	5	20	10	5
CD average	180.8 (\pm 45.6)	124.1 (\pm 53.5)	195.6* (\pm 11.1)	88.3 (\pm 16.9)	88.1 (\pm 7.5)	82.3* (\pm 12.7)
	166.8 (\pm 21.8)^			86.2 (\pm 2.0)^		
UC average	86.2 (\pm 72.7)	104.1 (\pm 52.6)	83.7 (\pm)	98.5 (\pm 30.5)	91.6 (\pm 28.7)	87.0 (\pm 28.4)
	91.3 (\pm 6.4)^			92.4 (\pm 3.3)		
Control patients average	59.5 (\pm 37.9)	88.3 (\pm 30.9)	28.5 (\pm 12.4)	68.3 (\pm 47.7)	134.0 (\pm 122)	59.0 (\pm 19.7)
	58.8 (17.3)			87.1 (\pm 23.6)		

*p= 0.05 for EOL vs. ECA in CD (1:5)

^p <0.03 UC vs CD (EOL vs ECA, average)

12.3. Changes in IL1ra concentration in the supernatant after incubation in EOL and ECA, as a percentage of medium control

Incubation with EOL increased % IL1ra concentration significantly more in CD compared with UC (p<0.05) and with control patients (p<0.05). EOL did not change % IL1ra in UC () and ECA did not significantly change % IL1ra in CD or UC (Figure 96). Incubation with ECA in control patients tissues caused a significant increase in % IL1ra, which was significantly higher than in UC (p=0.01). Overall, ECA incubation increased % IL1ra concentration in IBD patients (CD + UC) compared with control patients (p=0.002) (Figure 97 and Table 31).

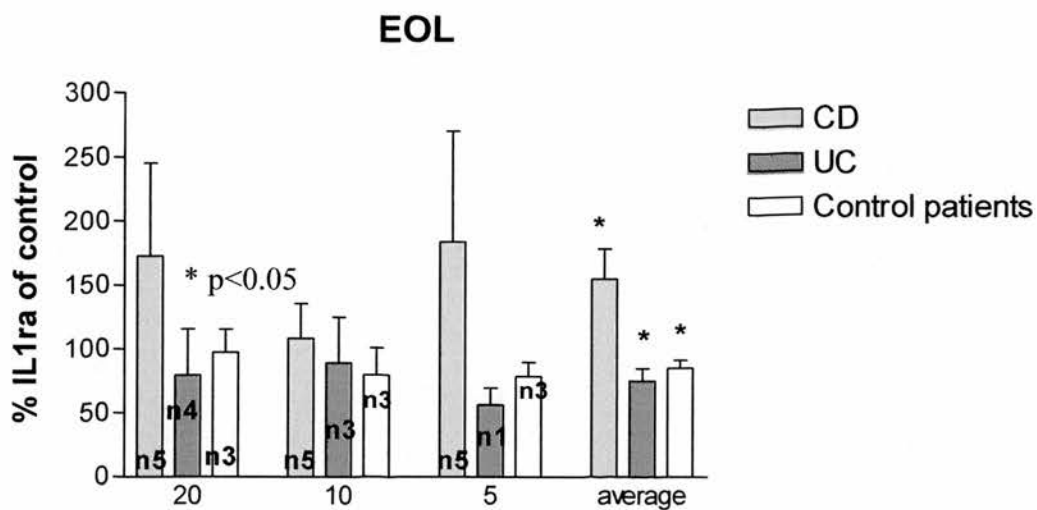


Figure 96 % IL1ra after incubation with EOL in CD, UC and Control patients

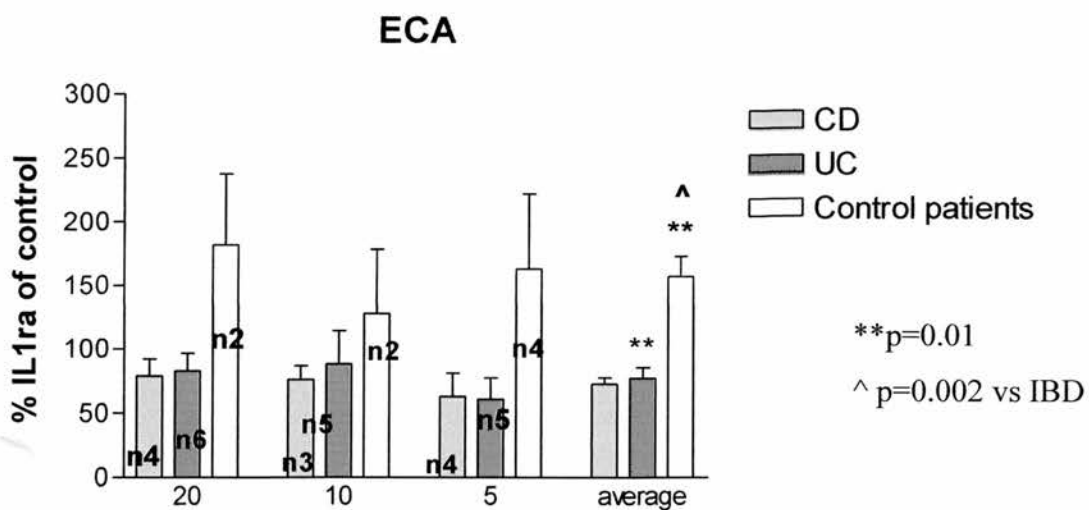


Figure 97 % IL1ra after incubation with ECA in CD, UC and Control patients

Table 31 Percentage of IL1ra of control in CD, UC and Control patients after 24- h incubation with EOL and ECA

Diagnosis	EOL			ECA		
	20	10	5	20	10	5
CD	172.8 (±72.5)	108.4 (±27.3)	183.8 (±86.3)	79.1 (±13.2)	76.3 (±10.9)	63.1 (±18.3)
average	155.0 (±23.5)*			72.8 (±4.9)^		
UC	79.7 (±35.9)	89.2 (±35.7)	56.3 (±13.3)	83.1 (±13.7)	88.3 (±26.0)	60.9 (±16.7)
average	75.1 (±9.8)*			77.4 (±8.4)**^		
Control patients	97.4 (±17.9)	79.5 (±21.4)	78.6 (±10.9)	181.8 (±55.8)	127.9 (±50.6)	163.2 (±58.7)
average	85.2 (±6.1)*			157.6 (±15.8)**^		

*p< 0.05 CD vs. UC and Control patients in EOL

**p=0.01 Control patients vs. UC in ECA (average)

^p = 0.002 Control patients vs. IBD patients in ECA

12.4. Discussion

In these experiments colonic biopsies from patients with CD, UC and control patients were incubated with enteral diet using olive oil (EOL) and canola oil (ECA), with the amino acid composition unchanged from EAA. Incubation of CD tissues with EOL resulted in no anti-inflammatory benefit as IL1ra / IL1β ratio did not change significantly. EOL incubation increased both IL1β and IL1ra as percentage of MC. Incubation with ECA in CD showed no change in IL1ra / IL1β ratio. In UC the IL1ra / IL1β ratio did not increase significantly after incubation with EOL compared MC. This is despite an increase in % IL1ra indexed against MC. Incubation with ECA in UC again did not increase IL1ra/ IL1β ratio.

These results demonstrate that oleic acid does not have a major effect on pro- and anti-inflammatory cytokine production in inflamed tissue *in vitro*, as might have been expected. Yaqoob *et al* reported that caution should be taken using oleic acid as placebo since it influences cytokine production in healthy humans (Yaqoob et al., 1998). This was shown in the present study in control patients where some modulation of cytokine

levels occurred.

These results indicate that dietary cytokine modulation is dependent on the nature and site of inflammation and is tissue specific. The increase in IL1 α / IL1 β ratio in UC and control patients may be an indirect reflection of the Mediterranean diet effect. Also in UC after EOL incubation a significant increase in IL10/IL1 β ratio in 1:20 vs. MC was noted. However it is unlikely that such oleic acid enriched diet will have a major impact in the therapy of IBD. Though IBD was previously thought to be commoner in Northern Europe compared to Southern Europe, these differences are narrower on more recent data (Shivananda S et al., 1996)

CHAPTER THIRTEEN

13. Effects of incubating inflamed and non-inflamed tissues with enteral diet fractionated coconut oil (COCF) and enteral diet coconut oil (COC)

Introduction

Saturated short and medium chain (C6 – C12) and saturated long chain fatty acids (C14 – C24) are components of the so-called 'Western diets'. Saturated fatty acids have the abilities to increase risk of coronary heart disease, arteriosclerosis and increased inflammatory mediator production.

A commonly employed processing method to purify fatty acids and remove 'undesirable components' is the so-called fractionation of the oil, which is a three step process of 1) 'Folding', where the oil is re-distilled to remove some but not all of the terpenes in the oil. 2) 'Terpeneless' where the oil is re-distilled and all the terpenes are removed and the 3) 'Rectification' where a part of the oil has been removed by either re-distilling or heating or chilling the oil, which allows parts of the oil to separate and be removed.

Coconut oil and its fatty acid profile are known to be high in short chain saturated fatty acids. The following experiments describe the impact of coconut oil and fractionated coconut oil incubation on cytokine pattern in CD and UC and control patient tissue after 24-h incubation, with modified enteral diets, using an enteral diet with modified fatty acid composition.

Subjects and Methods

The analysis of enteral diet incubation modified for coconut oil fractionated (COCF) and enteral diet modified for unfractionated coconut oil (COC) included only 11 patients out of 19. In some patients the assay for IL1 β was suboptimal and therefore no data could be analysed though other cytokine results were available. From each patient,

endoscopic biopsies were incubated with COCF and COC with a matched biopsy for MC.

Subjects

CD: (4); (2) F, (2) M; mean age (49 ± 5) years.

UC: (3); (3) F; mean age (46 ± 5) years.

Control patients: (4); (4) F; mean age (61 ± 5) years.

From these patients 58 biopsies were obtained for diet and MC incubation, taken from the anatomic areas:

CD: (4) colon

UC: (1) rectum, (2) colon

Control patients: (4) colon

Time period from diagnosis to taking biopsies:

> 1 year: 2 CD

1-3 years: 1 UC

4-6 years: 2 CD

< 6 years: 2 UC

Medications at the time of biopsy were:

CD: steroids (2)

UC: 5-ASA (2)

Fifty-two biopsy tissues were analysed, incubated with the diet formulae for COCF and COC

CD: COCF (10), COC (8)

UC: COCF (8), COC (8)

Control patients: COCF (10), COC (8)

13.1. IL1ra / IL1 β ratios after incubation with COCF and COC

13.1.1. Crohn`s disease

Incubation with COCF or with COC resulted in no significant increase in IL1ra / IL1 β ratio in any of the three dilutions. With COCF, a trend towards a stepwise increase in the ratio was observed with increasing concentration (Figure 98 and Table 32).

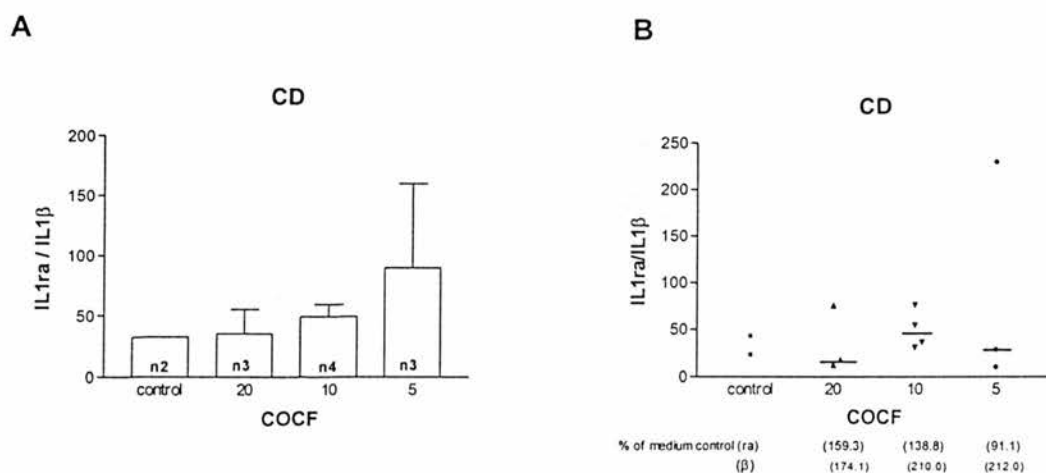


Figure 98 A+B (A) IL1ra/IL1 β ratio in CD after 24-h incubation with COCF. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

13.1.2. Ulcerative colitis

A similar concentration dependent increase in IL1ra / IL1 β ratio occurred in UC tissues incubated with COCF, but none reached statistical significance. Incubation with COC did not result in any significant increase in ratio (Figure 99 and Table 32)

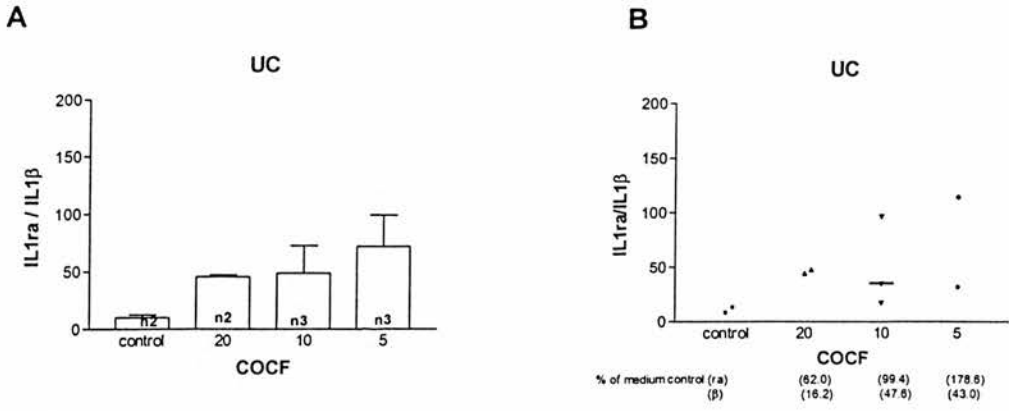


Figure 99 A+B (A) IL1ra / IL1β ratio in UC after 24-h incubation with COCF. (B) IL1ra/IL1β showing median and % of medium control of IL1ra + IL1β

13.1.3. Control patients

Neither COCF nor COC incubation resulted in any significant alteration in the IL1ra / IL1β ratio (Figure 100 and Table 32),

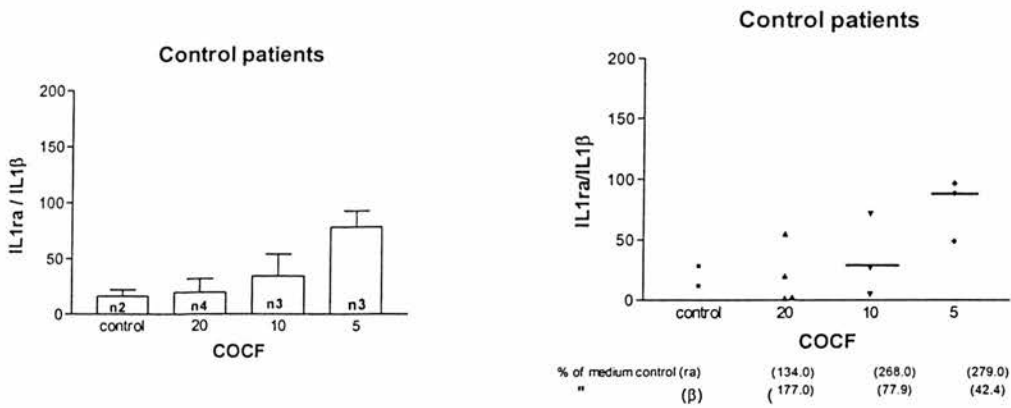


Figure 100 A+B (A) IL1ra / IL1β ratio in Control patients after 24-h incubation with COCF. (B) IL1ra / IL1β showing median and % of medium control of IL1ra + IL1β

Investigation of the percentage production of IL1 β and IL1ra of MC and the ratio of IL10 / IL1ra after incubation with coconut oils showed no significant changes and these results have therefore not been presented.

Table 32 IL1ra / IL1 β ratio for COCF and COC in CD, UC and Control patients

Diagnosis	COCF			COC		
	20	10	5	20	10	5
CD	35.0 (\pm 20.0)	49.2 (\pm 10.2)	89.8 (\pm 70.7)	42.2 (\pm 17.9)	54.6 (\pm 10.3)	40.8 (\pm 20.1)
MC	32.21 (\pm 9.9)			32.21 (\pm 9.9)		
UC	45.9 (\pm 1.7)	49.1 (\pm 24.0)	72.8 (\pm 27.7)	43.5 (\pm 23.3)	34.8 (\pm 4.2)	36.2 (\pm 3.9)
MC	10.1 (\pm 2.5)			10.1 (\pm 2.5)		
Control patients	19.8 (\pm 12.5)	34.5 (\pm 19.6)	78.2 (\pm 14.7)	96.2 (20.7)	50.5 (8.6)	79.6 (\pm 59.5)
MC	16.4 (\pm 5.6)			16.4 (\pm 5.6)		

13.2. Discussion

Incubation of colonoscopic biopsies with enteral diet modified in its fatty acid composition with fractionated coconut oil and coconut oil overall did not show any major changes in the cytokine response in either IBD affected tissues or in control patients. Only the incubations with COCF resulted in a trend towards a step-wise increase in the IL1ra / IL1 β ratio in both CD and UC. The numbers were particularly small in this series of experiments and therefore the possibility of Type II error is large.

By fractionation it is possible to remove unwanted components of fat or to enhance or improve wanted triacylglycerides in the fat. Recently in palm oil there was a demand to fractionate and isolate some of the fat components from the oil and this has been the trend in the food industry as a result of a health-conscious public.

Though the numbers were small, the increases after COCF incubation in IL1ra / IL1 β ratio in CD and UC may be interesting. It is possible that as a result of fractionation, short and medium chain fatty acids in the modification of coconut oil used, such as caproic acid (C6), caprylic acid (C8), capric acid (C10) and lauric acid (C12) have become more accessible to be used as fatty acid substrate by colonic tissue cells *in vitro*. Short chain fatty acids such as butyrate (C4) can influence proliferation and differentiation of various cell types, including colonocytes, and may trigger rapid apoptosis. There is evidence that butyrate has the capacity to modulate survival and death of colonocytes, (Fitch and Fleming, 1999). Short chain fatty acids serve as essential metabolic substrate for colonocytes. The use of such short chain fatty acids is reviewed in chapter 3. Unlike enteral diets modified with fish oil however, immunomodulatory effect of unfractionated coconut oil is minimal. It is unlikely that this will have application to improve gut inflammation in IBD.

CHAPTER FOURTEEN

14. Effects after incubation of inflamed and non-inflamed tissues with enteral diet soya oil (ESY)

Introduction

Soya oil is derived from soybean, but generally soybean is more known for its derived products such as soy sauce and tofu. The soya bean has recently attracted attention for its nutritional properties, containing protein, vitamins, minerals and vegetal hormones.

Subjects and Methods

Colonoscopy biopsies from IBD and control patients were incubated with enteral diet modified in its fatty acid composition with soya oil (ESY). Biopsy incubations were performed in the experiment design in pairs of two different fatty acid modifications in the same patient (for details refer to chapter 4 section I, Figure 18). For soya oil this was done in combination with olive oil. Therefore were MC data obtained in ECA incubation was combined with the MC data in ESY for CD, UC and control patients.

Subjects

CD: (6); (4) F, (2) M; mean age (61 ± 9) years.

UC: (4); (2) F, (2) M; mean age (45 ± 8) years.

Control patients: (2); (1) F, (1) M; mean age ($52 \pm 6.$) years.

From these 12 (out of 14) patients a total of 48 biopsies were obtained, taken from the anatomic sites:

CD: (1) sigmoid, (1) recto-sigmoid, (1) ascending/transverse colon, (3) ileum (1 anastomosis, 2 ileostomy),

UC: (3) sigmoid, (1) recto-sigmoid

Control patients: (1) sigmoid, (1) ascending/transverse colon. .

Time period from diagnosis to biopsies were:

< 1 year: 2 CD

1-3 years: 1 CD, 2 UC

4-6 years: 1 CD, 1 UC

> 6 years: 2 CD, 1 UC

Medications at time of biopsy taken were:

CD: steroids (3)

UC: 5-ASA (1).

36 biopsies were incubated with ESY and 30 biopsies were analysed for:

CD: ESY (15)

UC: ESY (9)

Control patients: ESY (6)

14.1. IL1ra / IL1 β ratios after incubation with ESY

14.1.1. Crohn`s disease

IL1ra / IL1 β ratio showed a small increase at 1:5 and 1:20, which reached significance for 1:5 ($p < 0.05$) (Figure 101 and Table 33).

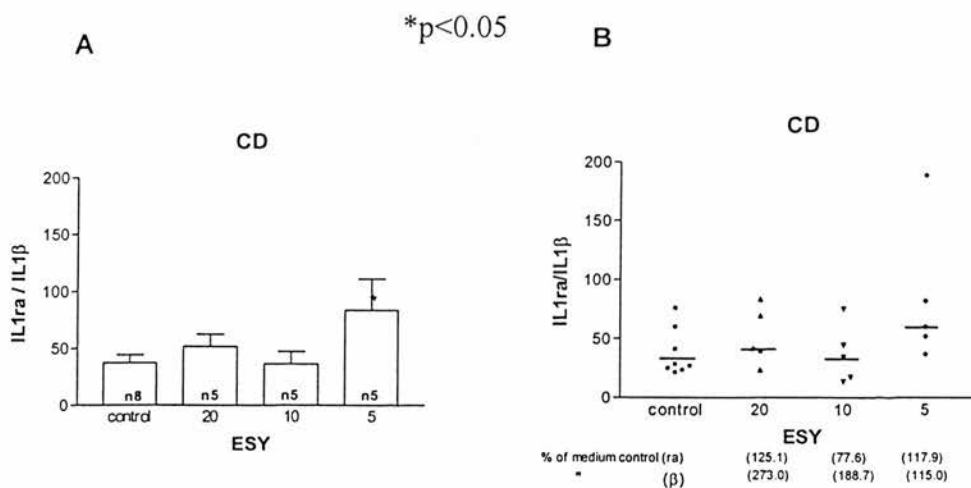


Figure 101 A+B (A) IL1ra / IL1 β ratio in CD after 24-h incubation with ESY. (B) IL1ra / IL1 β showing median and % of medium control of IL1ra + IL1 β

14.1.2. Ulcerative colitis

After ESY incubation an increase in the IL1ra / IL1 β ratio was noted, which was significant in 1:10 dilution (Figure 102 and Table 33).

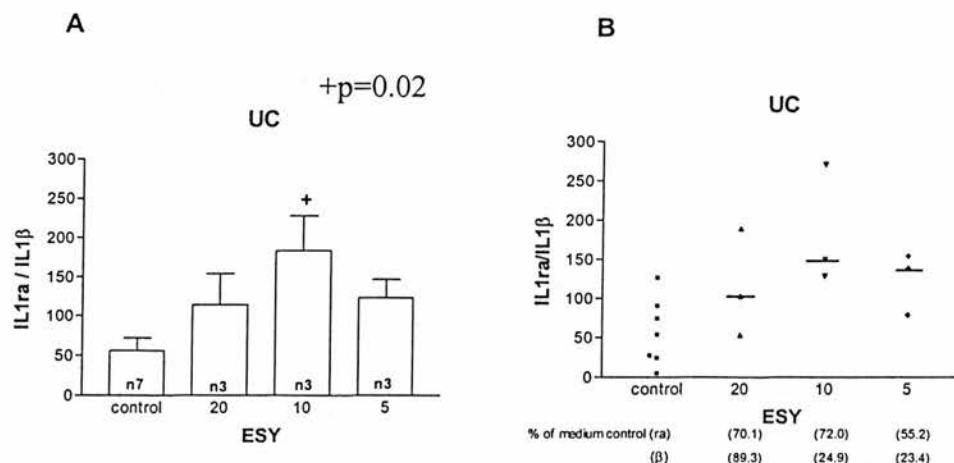


Figure 102 A+B (A) IL1ra / IL1 β ratio after 24-h incubation with ESY in UC. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

14.1.3. Control patients

In these patients, an increase in the IL1ra / IL1 β ratio for 1:10 and 1:5 vs. MC was shown but this did not reach significance (Figure 103 and Table 33).

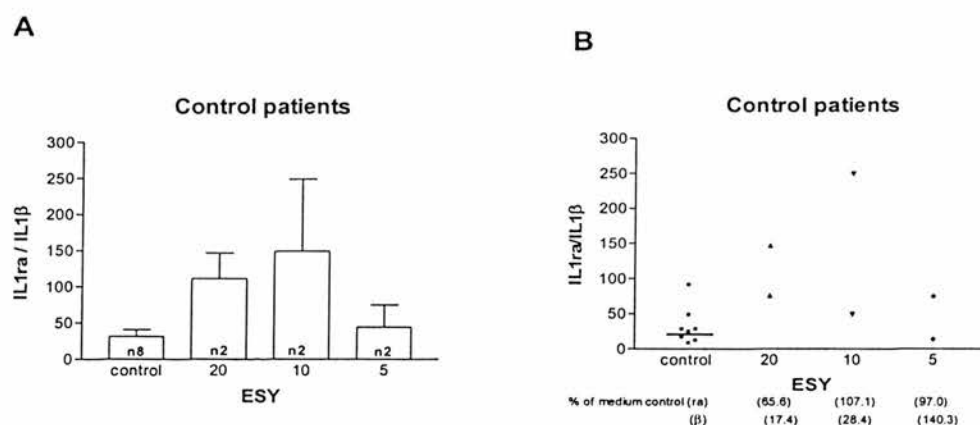


Figure 103 A+B (A) IL1ra / IL1 β ratio in Control patients after 24-h incubation with ESY. (B) IL1ra/IL1 β showing median and % of medium control of IL1ra + IL1 β

Table 33 IL1ra / IL1 β ratio for ESY in CD, UC and Control patients

Diagnosis	ESY		
	20	10	5
CD	51.7 (\pm 10.9)	36.7 (\pm 11.1)	83.9 * (\pm 27.1)
MC	37.1 (\pm 7.1)		
UC	114.0 (\pm 39.9)	182.3 + (\pm 44.6)	123.2 (\pm 23.1)
MC	56.1 (\pm 16.0)		
Control patients	111.6 (\pm 35.4)	149.0 (\pm 100)	44.4 (\pm 30.5)
MC	31.7 (\pm 9.5)		

*p = 0.02 vs. MC.

+p<0.05 vs. MC

14.2. Discussion

Incubation of tissues with enteral diet modified in its fatty acid composition by soya oil (ESY) had only a small influence on the cytokine balance.

Incubation with ESY in CD resulted in a significant increase in IL1 α / IL1 β ratio vs. MC at 1:5 dilution. IL1 α /IL1 β ratio increase was very modest in CD compared with UC.

In contrast, in UC after incubation with ESY an increase in anti-inflammatory / pro-inflammatory cytokine ratios was seen at all three dilutions. This increase was significant for 1:10 ($p=0.02$).

In control patients IL1 α / IL1 β ratio was increased after incubation with ESY similar to those in UC, but was not sufficient for statistical significance.

These patterns of IL1 α / IL1 β cytokine response for CD and UC were similar to those seen after incubation with enteral diet EOL, where also no effective change was seen in the ratio in CD. The fatty acid profile in ESY consist of ~ 23.41% stearic acid (C18: 0), a high content ~ 50.67% oleic acid (C18: 1) and to a lesser extent ~ 7.17% linoleic acid (C18: 2). Similar fatty acid profile is seen in EOL for (C18: 1) ~ 67.4% and (C18: 2) ~ 11.23%, though (C18: 0) was lower in EOL ~ 1.91%. Details of fatty acid profiles are presented in chapter 4 section I Table 6.

However the fatty acid profile in ESU (chapter 11) was (C18:0) ~ 21.99 %, (C18:1) ~ 61.18%, which is much closer to the fatty acid composition seen in ESY, except for the content of linoleic acid (C18:2) ~ 7.17%. However, it does not show the same positive anti-inflammatory effect in CD for ESU incubation, which was significant in all three dilutions.

These results are difficult to verify but may indicate that 1) a combination of representative amounts of C18:1 and C18:2 can initiate fatty acid modulated cytokine response via cyclooxygenase and lipoxygenase pathways which can produce disease specific response as in the case of UC. 2) The major difference in fatty acid profile in ESY and ESU was the presence of (C18:2) ~ 7.17 % in ESY. In CD, for ESY the increase in IL1 α / IL1 β was very modest and significant only in one dilution, but was more consistently increased in ESU not containing C18:2. It may support again the hypothesis of a specific fatty acid modulated and disease selective inflammatory response in IBD. Though, these results are obtained in a simple model, these do show differences in inflammatory cytokine response *in vitro*, and needs to be further clarified by in-situ hybridisation for mRNA expression.

CHAPTER FIFTEEN

Summary and Discussion

15. Principal observations

This thesis describes in an *in vitro* study the influence of different enteral diet modifications, modified in amino acid and fatty acid composition, on cytokine response. These have been investigated after a 24-hour incubation period of biopsy tissue in an organ culture chamber. The endoscopic biopsies were obtained from patients with IBD and non-inflamed control patients. Inflammatory cytokine IL1 β and anti-inflammatory cytokine IL1ra and IL10 were measured in culture supernatant. The expression of TGF- β 1 after tissue incubation with some enteral diet modifications was also studied. This enabled the project to give some data on potential beneficial effects in immunological terms of defined formulae diets in IBD. This may form the basis of clinical studies in future. Individual sets of results have been discussed along with each chapter, but this final chapter gives an overall overview.

15.1. Intestinal biopsy cytokine responses after incubation with elemental diet and enteral diets modified in their amino acid composition

It was found that selected enteral diets can have an anti-inflammatory effect *in vitro*, which was characterised by an increase in anti-inflammatory / pro-inflammatory cytokine ratio of IL1ra and IL1 β and to a lesser extent in some cases by an increase in IL10 / IL1 β ratio.

Tissues affected with IBD and control patient tissues were incubated with enteral diets modified in their nitrogen source by casein (EC), whey (EW) as well as commercial amino acid based enteral diet (EAA). Additionally in some patients biopsies were incubated with enteral diet modified in amino acid content by colostrum protein (ECO) and whey extract enriched with TGF- β mixed into EAA (EWT).

Incubation of biopsies from tissues affected with CD resulted in an increase in IL1ra / IL1 β ratio compared with MC. These increases were significant, but not dose dependent for EC incubation at all three enteral diet dilutions of 1:5, 1:10 and 1:20 vs. MC. The increase vs. MC after incubation with enteral diet-whey (EW) was not significant. Furthermore after incubation with EAA an increase in the ratio of IL1ra / IL1 β was observed, reaching significance at 1:10 dilution. Similar increases at some dilutions for the ratio of IL10 / IL1 β after incubation with EC, EW and EAA were observed in CD, but none of these were significant and the sample size small.

Incubation with EC, EW and EAA in CD in these experiments have been shown to increase the IL1ra / IL1 β ratio depending on the nitrogen source. Although the commercial amino acid based enteral diet EAA raised anti-inflammatory / pro-inflammatory cytokine ratio, a similar anti-inflammatory effect was also seen after incubation with EC. Firm conclusion cannot be drawn from such *in vitro* studies, but the direct anti-inflammatory effect of enteral diets is not abolished by replacing amino acid based formulae with whole protein based formulae such as casein and whey. Incubation with milk-product based ECO and EWT in CD did not significantly influence IL1ra / IL1 β ratio.

In contrast, in UC biopsies after incubation with enteral diet formulae EC, EW and EAA, there were no increases in the ratio of IL1ra / IL1 β compared with MC. Nor was there any change in the IL10 / IL1 β ratio. No significant change in IL1ra / IL1 β ratio was observed after incubation with ECO and EWT, however the numbers were small. These *in vitro* studies are consistent with clinical experience. In CD, elemental diet and polymeric diets are effective in reducing inflammation, but such diets do not result in beneficial effects in UC.

In contrast with CD, the control patients showed a trend towards a decrease in IL1ra / IL1 β ratio compared with MC. Incubation with EAA and EW resulted in a non-significant change in the ratio of IL1ra / IL1 β and in the ratio of IL10 / IL1 β . Similarly in control tissue, there was no significant change in the IL1ra / IL1 β ratio after ECO and EWT incubation.

15.2. Cytokine response after incubation with enteral diet modified in fatty acid composition

In further incubation experiments, endoscopic biopsies from patients with CD, UC and control patients were incubated for 24-h with enteral diets, which were modified in their fatty acid composition by fish oil (EFI), sunflower oil (ESU), safflower oil (ESA), olive oil (EOL), canola oil (ECA), fractionated coconut oil (COCF), coconut oil (COC) and soya oil (ESY). The compositions are given in chapter 4 section I.

In CD, incubation with EFI resulted in no significant increase in the IL1ra / IL1 β ratio. In contrast, after incubation with ESU there was a significant increase in IL1ra / IL1 β ratio at all three dilutions vs. MC. After incubation with ESA a very modest increase in the IL1ra / IL1 β ratio was observed, which did not differ markedly from the MC ratio and did not reach statistical significance.

In CD after incubation with COCF there was a tendency towards an increase in the IL1ra / IL1 β ratio vs. MC, which was similar after incubation with COC in dilution 1:5, but overall, incubation with COC did not change IL1ra / IL1 β ratio in a beneficial direction. After incubation with EOL, there was no significant increase in the IL1ra / IL1 β ratio. After incubation with ECA, there was no increase in the IL1ra / IL1 β ratio vs. MC. After incubation with ESY, there was an increase in the IL1ra / IL1 β ratio for two dilutions, reaching significance in one of those.

It would appear that tissues affected with CD respond differently in *in vitro* cytokine expression after incubation with enteral diets, which differ in their fatty acid composition. An enteral diet high in polyunsaturated fatty acids preferably those of ω -3 origins, as in fish oil has no impact on anti-inflammatory cytokine production in CD *in vitro*. After incubation with diets modified in their fatty acid composition a positive response indicated by an increase in IL1 α / IL1 β ratio vs. MC, occurred only in ESU and to a lesser extent after incubation with ESY and EOL. It can be speculated that the balance of long-chain fatty acids, in this case of C18:0 and C18:1 as in ESU and ESY may give the appropriate substrate 'starting package' into eicosanoid synthesis and/or into fatty acid induced gene transcription of a reduced anti-inflammatory response. These changes of IL1 α / IL1 β ratio were not observed after incubation with EOL in CD. The impact on IL1 α / IL1 β ratio after ESA was not significant. ESA in the present composition has a similar fatty acid profile to EOL, and it is possible that such profiles have no impact on cytokine modulation in CD.

ECA, which showed no effect on cytokine ratio IL1 α / IL1 β *in vitro* is composed of long-chain fatty acids, where the balance is C18:0 > C18:1. The slight (but not statistically significant) increase in the ratio of IL1 α / IL1 β vs. MC for COCF but not COC could be a response to the purification of COCF and the presence of only short- and medium- chain fatty acids with their metabolic influences. The increase in the IL1 α / IL1 β ratio for COCF is also seen in UC.

IL10 / IL1 β ratios in CD after incubation with ESU increased at all three dilutions reaching significance at 1:20 dilution. For ESA the increase in IL10 / IL1 β ratio was not significant.

In UC, after incubation with EFI there was a significant increase in IL1 α / IL1 β ratio after incubation at all three dilutions. This effect of EFI was different from incubation with CD tissue. Incubation with ESU also resulted in an increase in IL1 α / IL1 β ratio, reaching significance at one dilution. After incubation with ESA there was no increase in the IL1 α / IL1 β ratio. Further after incubation with COCF a trend towards an

increase in the IL1ra / IL1 β ratio was noted, but this was not significant. Incubation with COC resulted in no significant increase in IL1ra /IL1 β ratio. After biopsy incubation with EOL there was a trend towards an increase in the ratio. Incubation with ECA showed a trend towards a decrease in the ratio, similar to those observed in CD, and no evidence of any beneficial change.

After incubations with ESY at all three dilutions, there was an increase in the ratio of IL1ra / IL1 β which was significant at 1:10 dilution. *In vitro* IL1ra / IL1 β cytokine response after incubation of UC biopsy tissue with modified enteral diets does show an improvement in some cases. This suggests that clinical trials with appropriate enteral diets for UC may prove beneficial. A major difference was seen in the increase in IL1ra / IL1 β ratio in UC after incubation with EFI, which was not observed in CD. This suggests a disease specific *in vitro* immune modulator response to fatty acid profile of ω -3 fatty acids. After incubation with ESU an increase in IL1ra / IL1 β cytokine ratio was observed both in UC and CD.

Incubation of UC tissue with ESA showed no change in IL1ra / IL1 β ratio.

The change in IL1ra / IL1 β in UC after COCF incubation was similar to that in CD, with a trend towards a step like increase in the ratio. However MC ratio in UC was lower than that in CD, and showed a more effective increase in ratio in UC than in CD. It might be possible that as UC is always anatomically sited in the rectal and distal colonic area, a different colonic bacterial environment has adapted to more effective metabolism of short chain fatty acids compared with CD. This response was not seen after incubation with COC, composed of a mixture of short chain and medium chain fatty acids.

After incubation with EOL, there was an increase in IL1ra / IL1 β ratio, which was more marked in UC than in CD. The EOL composition contained some long-chain saturated and unsaturated fatty acids of C22 docosanoic and docosaenoic origin. These can be further metabolised becoming more unsaturated (C24) and β -oxidised (C22:6), and therefore having immune modulator impact on cytokine synthesis similar to those with EFI for UC. Again, this effect of an increase in IL1ra / IL1 β ratio in UC for EOL

incubation was not observed in CD. This suggests again that fatty acids can have immunological and disease-selective properties on the inflammatory processes. The non-response in UC for ECA was similar to CD, as the fatty acid composition was not conducive to any immune regulatory response. Enteral diet ESY incubation of UC tissue had a greater impact on an increase in IL1ra / IL1 β ratio than in CD.

Data on IL10 / IL1 β ratio in UC after incubation with ESU displayed a significant increase at 1:5 dilution, while there was no change after ESA incubations.

In control patients, incubation with EFI resulted in no differences in the ratio of IL1ra / IL1 β . In ESU there was an increase at all dilutions reaching significance at 1:5. After incubation in ESA there was a trend to increase the ratio at 1:20 and 1:5.

Incubation with COCF displayed a similar step like increase in the IL1ra / IL1 β ratio towards the higher concentration of 1:5. The ratio after incubation with COC was not changed. For EOL there was a trend towards an increase in two dilutions.

In contrast to the incubations for ECA in CD and UC there was a modest increase in the ratio in control patients. Incubation with ESY in control patients showed no change in IL1ra / IL1 β ratio.

Incubation of control tissue with enteral diets modified in their fatty acid composition has been shown to have some immune modulator effect. This was noticeable after incubation with ESA and ECA showing an increase in the anti-inflammatory / pro-inflammatory ratios, which was not observed for CD and UC. Handling of the tissues prior to organ culture might have induced some inflammatory changes in the tissues though in general the IL1ra / IL1 β ratio was higher in control tissue than in inflamed tissue. Generally, immunomodulatory effects of enteral diet modifications were most marked *in vitro* selectively on inflamed tissue rather than control tissue.

In vivo, any dietary effect on inflammation would be modified by the intestinal flora. This was not replicated in the *in vitro* experiments by addition of lipopolysaccharides (LPS) to the culture media, as this would adversely affect tissue viability. Addition of LPS often augments the pro-inflammatory cytokine response *in vitro*.

15.3. TGF- β 1 tissue expression by immunostaining after incubation with enteral diet modifications

In further experiments, TGF- β 1 immunostaining expression as percentage of total tissue area of endoscopic biopsies after 24-h incubation with enteral diet rich in colostrum (ECO) and enteral diet mixed with TGF- β enriched whey extract (EWT) were analysed. The TGF- β isoform of these diets was TGF- β 2. TGF- β 1 expression was compared with percentage of TGF- β 1 after incubation in MC. In a smaller group of patients TGF- β 1 immunohistochemical staining was also performed in tissues incubated with EC and EW.

In CD, after incubation with ECO, TGF- β 1 immunohistochemical staining was significantly increased as a percentage of TGF- β 1 tissue expression. After incubation with EWT, there was a similar significant increase in staining expression as in ECO. Incubation with amino acid based enteral diet EAA led to an increase in TGF- β 1 expression, reaching significance at 1:10 dilution. After EC incubations, there was a significant increase in %TGF- β 1 expression at 1:5 dilution. However, incubation with EW displayed an increase in % TGF- β 1 expression, being significant at 1:20. Combining the data obtained for EW incubation for all dilutions there was a significant increase vs. MC. Again, comparing TGF- β 1 staining expression for EC with EW showed a significant increase for EW. The data obtained for incubations in CD displayed a superior effect for EW in enhancing TGF- β 1 staining expression compared with EC.

In UC, incubation with ECO and incubation with EWT did not result in any increase in % TGF- β 1 staining expression. In UC, for incubation with EAA an increase in staining expression was detected; being significant at 1:10 and being significant by combining all three dilutions of EAA together, similar to CD. For EC incubation there were no increase in TGF- β 1 staining. For EW a significant increase in TGF- β 1 expression was

detected, when all three dilutions were combined.

In control patients, incubation with ECO, EWT and EAA resulted in no significant increase in % TGF- β 1 staining expression. Data obtained for EC and EW were small, and no significant increase in TGF- β 1 expression was detectable.

15.4. Summary

15.4.1. Incubation with enteral diets modified in protein composition – cytokine ratios

Endoscopic biopsies incubated with amino acid based enteral diet **EAA** in patients with CD, UC and non-inflamed controls resulted in a significant increase only in CD.

- Direct anti-inflammatory modulation of cytokine ratio by EAA in CD.

Incubation with **EC** resulted in a significant increase in IL1ra / IL1 β ratio in CD but not in UC and control patients. Similar response was noted for IL10 / IL1 β ratio.

- Direct anti-inflammatory modulation of cytokine ratio by EC in CD.

Incubation with **EW** increased IL1ra / IL1 β ratio in CD, but no change in the ratio was noted in UC and control patients. Similar increase for IL10 / IL1 β ratio was also seen in CD.

- Some evidence of direct anti-inflammatory modulation of cytokine ratio by EW in CD.

Incubation with **ECO** did not increase IL1ra / IL1 β ratio in CD, UC or control patients.

Incubation with **EWT** did not increase IL1ra / IL1 β ratio in CD, UC or control patients.

- No effect of TGF- β enriched enteral diets on anti / pro-inflammatory cytokine ratio of IL1ra / IL1 β *in vitro*.

15.4.2. Incubation with enteral diets modified in protein composition – TGF- β 1 expression

Incubation with **ECO** and immune histochemical staining for TGF- β 1 showed a significant increase of TGF- β 1 expression in CD.

- Anti-inflammatory TGF- β 1 increased in expression after ECO incubation in CD.

Incubation with **EWT** resulted in increased TGF- β 1 expression in CD, but not in UC and control patients.

Incubation with **EAA** resulted in a significant increase in staining expression in CD and UC in a single dilution.

- Some response in CD and UC after EAA incubation with upregulation of TGF- β 1 expression.

Incubation with **EC** resulted in some upregulation of TGF- β 1 expression in CD, but not in UC or control patients

- Incubation with EC results in some upregulation of TGF- β 1 expression in CD.

Incubation with **EW** resulted in upregulation of TGF- β 1 expression in a single dilution in CD and all dilutions combined in UC.

- Incubation with EW may result in upregulation of TGF- β 1 expression.

Overall, the most marked upregulation of TGF- β 1 expression was seen after incubation of CD affected tissues with TGF- β enriched diets ECO and EWT.

15.4.3. Incubation with enteral diets modified in fatty acid composition- cytokine ratios

Incubation with **EFI** was shown to have no anti-inflammatory influence in biopsy tissue affected by CD and biopsy tissue from control patients. However, EFI significantly increased IL1ra / IL1 β ratio in UC.

- Incubation with fish oil appears to have a direct anti-inflammatory effect in only UC affected tissue.

In vitro incubation with enteral diet modified in its fatty acid composition have shown that incubation with **ESU** resulted in an increase in anti-inflammatory / pro-inflammatory cytokine ratio both IL1ra / IL1 β and IL10 / IL1 β in CD, UC and control patients tissue. This was most prominent in tissues affected by CD.

- Direct anti-inflammatory effect of ESU on CD and UC affected tissues as well as controls.

Incubation with **ESA** did not seem to influence IL1ra / IL1 β ratio in CD and UC.

- No effect of ESA incubation on CD and UC affected tissues.

Fractionated coconut oil **COCF** was shown in CD and UC patients to positively influence IL1ra / IL1 β ratio, seen as an increase in the ratio. Although these increases are relatively modest, incubation with COCF displayed this trend in all patient groups and stepwise in increasing dilutions.

- A trend toward a direct anti-inflammatory effect in CD and UC.

In contrast incubation with **COC** in CD, UC and control patients showed no consistent increase in IL1ra / IL1 β ratio.

Incubation with enteral diet oleic acid **EOL** resulted in an increase in IL1ra / IL1 β ratio in a single dilution in CD and in three dilutions in UC.

- Some anti-inflammatory effect in UC and less in CD of EOL incubation.

Incubation with **ECA** did not result in any significant change in the cytokine profile.

There was no anti-inflammatory response from incubation of tissues with ECA.

Finally incubation with **ESY** increased the ratio in CD and UC, reaching significance in CD at one dilution and in UC at two dilutions.

- Incubation with ESY has some anti-inflammatory effect in UC and CD.

Table 34 A summary of IL1ra / IL1 β response after fatty acid incubations

Diagnosis	EFI	ESU	ESA	ECA	EOL	COCF	COC	ESY
CD	-	***	-	-	-	-	-	*
UC	***	*	-	-	-	-	-	*

*** significance in 3 dilutions vs. MC

** significance in 2 dilutions vs. MC

* significance in 1 dilution vs. MC

15.5. Comparison of our findings with other published data

Enteral diets, elemental or polymeric have been widely investigated in clinical trials, although there is still debate about the relative merits of polymeric and elemental diets. The effect of enteral diets in respect of downregulation of inflammatory mediator production, decrease in clinical activity and histological improvement of intestinal mucosa have been documented (Mansfield et al., 1995) (Rigaud et al., 1991) (Beattie et al., 1994) (Fell J.M.E. et al., 2000) and are discussed in the literature review.

Few studies have been performed which define the cytokine response in *in vivo* or *in vitro* studies after manipulation of enteral diets with casein or whey extracts. Studies in IBD have been conducted in comparison with steroid treatment or during remission phase after commercial enteral diet. The following discussion describes the immunological response to casein and whey, although reports of diet modification with casein and or whey have been reported only in polymeric diets enriched with casein and TGF- β .

Milk derived casein and whey proteins, such as β - and κ -casein of the former and lactoperoxidase and lactoferrin of the latter and milk growth factors are known to have immuno-modulator functions. The positive bioactivity is increased with the degree of purification (Cross and Gill, 1999). Beta-casein is the predominant fraction in human milk and is present in bovine milk in only about 25-30% of the total casein. A study by the group of Cross *et al* (Cross and Gill, 1999) investigated immunomodulatory effect of purified whey extract on lymphocyte proliferation *in vitro* after stimulation with Con A, PHA and LPS. The results demonstrated different degrees of suppression with the lowest (38%) in PHA induced proliferation. Cytokines IFN- γ and IL4 were measured in culture supernatant after stimulation with Con A, resulting in suppression of IFN- γ and IL4 secretion. Suppression of activated CD25 T-cells after stimulation, and suppression of CD4 and CD8 T-cell phenotypes in presence of purified whey protein was observed. A study by Porter *et al* (Porter et al., 1998) investigated the effect of a growth factor extract derived from bovine whey in orally induced colitis in rats. The effect was

measured by the ethane breath test, as lipid peroxidation of PUFA induced by hydroxyl (OH[•]) and superoxide (O₂^{•-}) radicals are possible mechanism of bowel damage in UC. The data suggest that whey-derived growth factor is as effective as drug therapy in suppressing lipid peroxidation in the phase before the chronic phase of disease process. *In vitro* and *in vivo* immune response of bovine β-casein have shown a significant and powerful effect on superoxide production after stimulation, a dose dependent potentiating effect on T lymphocyte proliferation response after stimulation by Con A, and an enhancing effect of β-casein on LPS induced B lymphocyte proliferation. It has been shown, that bovine β-casein had stimulatory effects on proliferative responses of T- and B- cells after mitogenic stimulation, which was not demonstrated for κ-casein. As IFN-γ production from Con A stimulated lymphocytes was not significant it is possible that β-casein is stimulating lymphocyte function by specific pathways (Wong et al., 1996). The effective immune modulation of bovine whey proteins has been reported as being superior to those of casein and other animal protein in supporting antibody response. Additionally its been documented that whey protein concentrate is superior to casein in supporting antibody based resistance to pneumococcal infection in young adult mice. The effectiveness of whey proteins in maximising cell-mediated adaptive immune competence has also become apparent (Woodward, 1998). The study of Kuwata *et al* has shown that lactoferrin, a bioactive constituent of whey is resistant and survives proteolytic degradation in the small intestine of adult rats (Kuwata H et al., 2001), which proves that such active proteins are still active after partial digestion in the gut.

The biological properties of transforming growth factor family with different TGF-β isoforms have recently received increased attention. An interest has been shown in the role of TGF-β in IBD and its positive mucosa repairing and inflammation downregulatory properties. It has been recently reported that an interruption in TGF-β signal cascade might be involved in IBD (Hahn et al., 2001) (Monteleone et al., 2001). TGF-β are bioactive growth factors present in human and bovine milk (Saito et al., 1993) (Playford et al., 2000) and have been shown to remain biologically active after manufacturing processes in milk derived products depending on the milk protein source and processing conditions (Donnet-Hughes, 2000). Effects of diet induced cytokine

modulation after stimulation with IL1 β *in vitro* in cell lines has been recently shown with a decrease in TNF- α , IL6 and IL8 secretion by addition of breast milk and enteral polymeric diet Modulen®. A decrease in clinical inflammatory parameters occurred after one week of dietary treatment (Croft et al., 2001)

To date few studies have been performed to investigate modulation of inflammatory response by addition of TGF- β . The use of polymeric diets in CD containing TGF- β 2 showed that such a diet was effective in inducing remission in paediatric patients with small bowel CD (Beattie et al., 1994) and CD (Fell J.M.E. et al., 1998). This was associated with remission, mucosal healing and normalisation of ESR and CRP levels along with an increase in serum albumin levels (Beattie et al., 1994). Further, there was a reduction in mRNA levels for IL1 β , IL8 and IFN- γ and an increase in endogenous TGF- β (Fell J.M.E. et al., 1998). Studies have been carried out with diets containing TGF- β isoforms, such as TGF- β 2 (Fell J.M.E. et al., 2000), demonstrating an increase in ileal TGF- β 1 mRNA, suggesting that presence of specific TGF- β isoforms or balance of these might be independent of actual TGF- β 1 expression.

Expression of TGF- β 1 positive cells was documented by several research groups showing in paraffin sections positive staining of surface epithelial cells of normal colonic mucosa, which became weaker in cells more deeply situated within the crypts (Avery et al., 1993). In normal intestinal biopsies TGF- β protein expression was found in the epithelium and prominent in villus tips of small intestinal biopsies (Lionetti et al., 1999). Di Mola *et al* reported a weak immunoreactivity for TGF- β 1 in normal intestinal tissue samples and an intense TGF- β 1 expression accompanied by an intense immunoreactivity for TGF- β receptors T β R-1_{ALK5} and T β R-II in CD tissue. These were localised in macrophages, lymphocytes and fibroblasts of lamina propria and in remaining epithelial cells. The highest intensity of signals was located in cells close to the luminal surface epithelium (di Mola et al., 1999). Further expression of TGF- β 1 in IBD tissue was documented by Xian *et al* (Xian et al., 1999) and Babyatsky *et al* (Babyatsky et al., 1996).

Several clinical trials have investigated the effect of fish oil, rich in ω -3 fatty acids on inflammatory response, prostaglandin release, and incorporation of EPA into mucous membrane and cytokine response. These studies were generally conducted in UC as overall it has been shown that fish oils are more effective in UC than in CD. Studies in CD have shown that ω -3 fatty acids do not extend the remission period in CD (Lorenz-Meyer et al., 1996), although others have found some effect of fish oil in CD (Kim, 1996). In general, trials using ω -3 fatty acid preparations are more often conducted in UC patients, suggesting a greater benefit in anti-inflammatory response or remission period. Ingestion of fish oil capsules in patients with UC led to clinical improvement and reduction in colonic LTB₄ levels. Reduction of LTB₄ concentrations in colonic mucosa after treatment with fish oil was seen in UC but also in placebo group receiving corn oil. A slight improvement in histological scoring was noted, which was worse in the placebo group (Aslan and Triadafilopoulos, 1992). Further in a study by Hawthorne *et al* (Hawthorne et al., 1992) patients with UC were given 20ml fish oil and as placebo olive oil, over a period of one year. The results showed that despite the taste of fish oil, long-term compliance could be achieved, as this is often an argument against fish oil treatment. Furthermore, it led to a sustained increase in membrane levels of EPA and enhanced synthesis of LTB₅ and a reduction of LTB₄. Long-term treatment with fish oil led to a corticosteroid sparing effect, though it did not increase remission time. In a multicentre randomised trial it has been found that after 4 months of dietary treatment with fish oil capsules there was a reduction in rectal dialysate levels of LTB₄ and improvement in histological appearance (Stenson et al., 1992). Improvement of disease activity in UC, seen in reduction of clinical and sigmoidoscopic scores, was again found after 6 months of treatment with fish oil capsules. Serum levels of LTB₄ were significantly reduced, and undetectable in some patients after the treatment period (Almallah et al., 2000). In rats, the effect of ω -3 fatty acids by enema in trinitrobenzene (TNB) induced colitis was investigated (Yuceyar et al., 1999). The results postulated that a diet rich in ω -3 fatty acids had an anti-inflammatory effect on TNB induced colonic lesions. Use of ω -3 fatty acid containing enemas had a suppressant effect on LTB₄ and LTC₄ levels in the chronic stage, but did not heal the colonic lesions. A similar experiment by Grisham *et al* (Grisham et al., 1996) investigated the effect of orally ingested sulphasalazine (SAZ), fish oil diet or oligosaccharide rich diet, which

can attenuate inflammatory response in chronically induced colitis in rats. The results revealed that the diets used had similar efficacy in producing an anti-inflammatory response as SAZ in colitis. Less strictures and adhesions were found after TNBS induced colitis in rats fed with ω -3 rich diet (FO) compared with rats fed on olive oil (OO) and phospholipid rich (PL) diet; occurrence of stenosis and ulcers were highest in phospholipid group. PGE₂ levels in colonic mucosa were lower in FO and OO group and LTB₄ was higher in PL group (Nieto et al., 1998). A recent study by Dichi *et al* (Dichi et al., 2000) compared the use of ω -3 fatty acid with SAZ in UC, suggesting that though ω -3 fatty acid resulted in an improvement in UC, overall, the treatment with SAZ was superior to treatment with fish oil alone in treatment of mild to moderate UC.

The modulating influence of short-chain fatty acids (SCFA) on inflammation is of interest since acetate, propionate and butyrate are produced in the caecum and colon by fermentation of unabsorbed carbohydrates (Fitch and Fleming, 1999). Substrate concentration prior to fermentation influences the rate of fermentation and influences the luminal concentration of SCFA. The transport of SCFA across mucosa in the large bowel is concentration dependent (Ruppin H.Bar-Meir et al., 1980). UC may be considered as an energy deficient state in the colonic epithelium due to a block in uptake or oxidation of SCFA by colonocytes, or due to a high luminal concentration of hydrogen sulphide produced by sulphate-reducing bacteria (Roediger et al., 1993). Impaired butyrate oxidation was found to be more marked in the descending colon than in the ascending colon. A deficiency of SCFA in UC has not been shown to exist as reduced, normal or increased levels of SCFA have been found in stool samples of UC patients (Treem et al., 1996) (Roediger et al., 1982). The concept of treatment using SCFA in UC is based on the fact that high luminal SCFA concentrations may override the postulated metabolic defect of colonic mucosa in oxidising SCFA (Kim, 1998). Treatment with SCFA in colitis administered as rectal instillation was performed by Schauber and colleagues showing no significant difference in histological scoring, mucosal inflammation and symptoms compared with placebo control after 3 weeks of twice daily application in enemas containing SCFA (Schauber J et al., 2000). In contrast, Scheppach *et al* showed a marked effect on crypt cell proliferation in colonic biopsies of UC patients than on inflammation after irrigation with SCFA (Scheppach et

al., 1997). Further, Breuer *et al* reported beneficial effect with twice daily rectal irrigation of SCFA in UC (Breuer et al., 1991). Steinhart *et al* showed similar improvement after nightly treatment with butyrate enemas in UC patients. His group reported response in 6/10 patients, with complete response in 4/6, with a fall in mean disease activity score in all patients from 8.0 ± 2.4 to 4.3 ± 4.1 at end of follow up period (Steinhart et al., 1994).

Wallace *et al* showed that different fatty acids can stimulate or modulate cytokine production differently (Wallace et al., 1999b) in a lymphocyte proliferation-assay in rats after being fed with diets rich in coconut oil, olive oil, safflower oil or fish oil. Lymphocyte proliferation and IL2 production were decreased in diets rich in unsaturated fatty acids (olive -, safflower - and fish oil) compared with coconut-oil diet. Further, IFN- γ production was reduced by safflower or fish oil compared with coconut or olive oil rich diets. Compared with saturated fatty acids, polyunsaturated fatty acids decrease Th1 lymphocyte responses but no marked influence was seen in Th2 lymphocyte response. Polyunsaturated fatty acids have the ability to modulate cytokine response, seen in a decrease or inhibition of IL1 β , IL2 or TNF- α , (Meydani and Dinarello, 1993). PUFA of ω -3 origins have a positive immune-modulator effect on pro-inflammatory cytokines, (Grimble and Tappia, 1998). A study by the group of Wallace and colleagues (Wallace et al., 1999a) investigated the effect of LPS stimulated macrophages in mice fed diets containing coconut oil, safflower oil or fish oil and a low fat diet. Macrophages from fish oil fed group showed a significantly decreased production of IL1 β and TNF- α compared with other diets. Production of IL10 was the lowest in the coconut-oil group and diets rich in PUFA significantly increased IL10 production by resident macrophages. It has been shown that cytokine production by macrophages is influenced by dietary fat, but is dependent on the activation-state of the macrophages.

In this study, the enteral diet preparations modified in nitrogen content for casein (EC) and whey (EW) proteins have produced different responses. The casein preparation was not purified or separated in β -casein or κ -casein content, as this might have influenced the T and B cell reaction response. The study of Wong *et al* (Wong et al., 1996)

reported that β -casein had effects on both T and B cells following mitogenic stimulation. Similarly whey protein components, lactoferrin and lactoperoxidase have been found to have greater immune modulator properties after purification (Cross and Gill, 1999). Therefore the results presented have to be interpreted as an immune modulator response of both caseinates of β and κ for the EC incubations. Similarly for the interpretation of the data for whey composition (EW), it is likely that lactoferrin, lactoperoxidase and bovine milk derived growth factors, may all be exerting immunomodulatory effects, but this requires to be further investigated.

Overall it has been shown that incubation with enteral diets containing nitrogen modifications resulted in little anti-inflammatory response in UC *in vitro*. This is consistent with the fact that UC patients do not respond to treatment with elemental or polymeric diets. UC is generally located in the colon and rectum and usually *in vivo* uptake of nutrients do not occur via this site. Although 'the route' of administration in this *in vitro* model does not represent an *in vivo* situation, it was shown that commercial enteral formulae specifically modified in fatty acid composition can have a positive immunomodulatory influence on tissues affected by UC. The data suggested that using an *in vitro* model, enteral formulae specifically modulated in fatty acid composition might have an anti-inflammatory effect on tissues affected with UC. This is also confirmed by other studies in UC and treatment with ω -3 fatty acids *in vivo*. The positive effect in UC tissue in response to fatty acid modulation and the non-response to nitrogen source modulation is difficult to explain with current knowledge. It could be postulated that specific changes in mucin layer (Strugala et al., 2001) of UC biopsies may interfere with colonic protein fermentation, and colonocyte demand specific SCFA preferably butyrate (Jorgensen and Mortensen, 2000), which can be supplied purely by fatty acids either through β -oxidation or fermentation. This could partly contribute to the non-response to modified protein diets and the response to modified fatty acid diets in UC. It would also underline the disease and diet specific anti-inflammatory response, which is very relevant to dietary therapy of IBD. These differences in *in vitro* modulation by enteral diets again highlight immunological differences between UC and CD.

TGF- β 1 in tissues incubated with ECO, EWT, EC, EW and EAA was expressed differently in CD, UC and control patients. Positive staining for TGF- β 1 was found in all tissues on surface and crypt epithelium, but responded differently after enteral diets in different disease groups. TGF- β 1 expression was found in lamina propria of CD and UC tissues incubated with ECO and EWT, which was not seen in control tissues. Although the numbers studied were small, significant differences in TGF- β 1 expression as a percentage of TGF- β 1 / mm² of tissue was found. Significant increase of TGF- β 1 tissue expression was found in CD after incubation with ECO and EWT. For EC and EW incubations of CD an increase in TGF- β 1 was less consistently seen. In UC no significant increase in staining was seen for ECO and EWT. In control patients, TGF- β 1 expression did not increase after incubation with ECO, EWT, EC and EW. Expression of TGF- β 1 stained positive cells in IBD and control patients have been reported in frozen and paraffin sections (Xian et al., 1999). In normal recto-sigmoid colon TGF- β 1 immune reactive cells were located uniformly along surface and crypt epithelium and in a few cells scattered in the lamina propria. In active CD and UC the epithelial staining remained unchanged but an increased in lamina propria expression in inflamed tissues was seen.

Although the experiments presented in this thesis for TGF- β 1 immunostaining measured % staining of tissue area, the results obtained are similar to those of Xian *et al* (Xian et al., 1999). Though intense TGF- β 1 expression was noted in CD and UC, their response to specific enteral incubations were different. As reported, immune cell marker detection showed TGF-1 positive immune cells in active CD in lamina propria, including T cells, neutrophils, monocytes or macrophages. B-lymphocytes were rarely found, and similar results were found in UC (Xian et al., 1999). Babyatsky *et al* reported enhanced expression of TGF- β 1 in IBD, present in actively inflamed mucosa of CD and UC compared with controls (Babyatsky et al., 1996). Detection of TGF- β 1 mRNA by Northern blot was greater in IBD than in control tissue. Increased expression of TGF- β 1 was reported for CD and UC (Xian et al., 1999; Babyatsky et al., 1996) which compares well with our results after incubation with ED modifications. This study found no significant differences in UC, as found in CD for ECO, EWT, EAA and

similar but much weaker increase after EC and EW incubations in UC. It is possible that although TGF- β 1 is increased or detectable, TGF- α response is a more common and possibly distinct feature in UC (Babyatsky et al., 1996), where TGF- α and TGF- α mRNA were found to be upregulated in long-term inactive UC (Xian et al., 1999). Grip *et al* has shown that the number of TGF- α containing cells in the colonic mucosa is increased in both, during active inflammation and in clinical remission of UC. TGF- α a member of epidermal growth factor family, showed enhanced expression in UC, suggesting again to be a selective feature associated with UC, which was not found in patients with CD (Grip et al., 2000). In the current study, TGF- α expression was not investigated in the incubated tissues.

Incubation with EC and EW resulted in slight differences in staining expression, and also some differences were found between the IBD groups. Not only did CD respond better after nitrogen/TGF- β enriched diet modification, but whey modified diets increased TGF- β 1 staining expression, and EW increased IL1ra / IL1 β ratio in CD (Meister et al., 2002a).

The mechanism by which these nitrogen source and TGF- β modified enteral diets actually act in downregulating inflammatory cytokines (Fell J.M.E. et al., 2000; Fell J.M.E. et al., 1998; Beattie et al., 1994), in increasing immune positive cells stained for TGF- β 1 (Meister et al., 2002b) or in influencing TGF- β signal cascade (Monteleone et al., 2001) is unknown. A number of different molecules are likely to be involved in triggering an immunosuppressive effect and further specific research is needed.

Biopsies incubated with EFI, as a major source of ω -3 fatty acids, have shown a significant shift towards an increased anti-inflammatory to pro-inflammatory ratio only in UC. In current literature, the immunomodulatory properties of fish oil have been investigated and a response in prostaglandin profile seen as a decrease in LTB₄ or increased synthesis of LTB₅ (Aslan and Triadafilopoulos, 1992); (Hillier et al., 1991), but changes in IL1 β mRNA expression after LPS stimulation in mouse spleen lymphocytes have also been reported (Watanabe S. et al., 2000). The actual increase in IL1ra / IL1 β ratio may be interpreted as a response to eicosanoid modulated cytokine

production through G-proteins and/or fatty acid modulated gene transcription via transcription factors. The most significant component of fish oil having the greatest impact on immune modulator properties is unknown. However, several studies have found that DHA might be superior to EPA in immunomodulatory activity (Lochsen et al., 1999) (Watanabe S. et al., 2000), indicating that the effects of fish oil might be due to a fatty acid induced gene transcription. Eicosanoids are derivatives of dihomo- γ -linolenic acid (DLGA, 20:3 ω -6), arachidonic acid (AA C20:4 ω -6) and eicosapentaenoic acid (EPA C20:5 ω -3) (Calder, 1998) and not docosahexaenoic acid (DHA C22:6 ω -3). The non-response of EFI incubations in CD is reflected also in the current literature where treatment with encapsulated fish oil fatty acids are mainly used.

However, a positive anti-inflammatory response after biopsy incubations with ESU was found in all three groups, especially in CD. The hypothesis is that a 'starting package' in respect of specific C18:0 < C18:1 fatty acid composition of the enteral diet, similar to that found in ESY, might have influenced response, presenting a steady supply for delta 9 desaturase enzyme converting stearic acid into oleic acid. Increased incorporation of oleic acid into mucosa after 12-weeks dietary supply with olive oil in patients with IBD has been demonstrated (Hillier et al., 1991) accompanied by a decrease in C18:0. No significant change in prostaglandin and thromboxane synthesis in olive oil group was observed after treatment period. An improvement in endoscopic appearance was noted in six out of eight patients taking olive oil. However, this study took place over a period of 12 weeks and improvement was not immediate. Incorporation of oleic acid into colonic mucosa after fish oil intake was not increased. These data suggest that each tissue and cell type may have an individual pattern of fatty acid incorporation, which might be independent of the original presence and availability of fatty acids. These would also highlight the positive effect of fish oil supplementation in UC in the study presented, and the relative lack of effect of fish oil in CD. However, increased incorporation of oleic acid was present in PBMC, after consuming a diet high in oleic acid (Yaqoob et al., 1998). A slight decrease in natural killer cell activity after 2 months of experimental period and a decrease in adhesion molecule ICAM-1, which is thought to play a crucial role in growth of mononuclear cells in arteriosclerotic plaque, were noted. These data again confirm that the effect of oleic acid on plasma membrane

phospholipids should not be ignored, and caution should be taken when using oleic acid as placebo control group in such studies. The actual modulation of action due to oleic acid is not yet known.

Incubation with EOL showed no response in CD tissue and a small but insignificant increase in UC and control tissue for IL1 α / IL1 β ratio. Linoleic acid (C18:2) concentration was higher in EOL compared with ESY, for > ESY (7.17%) for > EOL (11.23%) and absent in ESU. However it is possible that the presence of linoleic acid in EOL has masked the effects of oleic acid (C18:1), as found in ESU and ESY incubation, leading to an increase in IL1 α / IL1 β ratio. It cannot be concluded that the positive effects of oleic acid in decreasing platelet aggregation (Burri et al., 1991) and lowering incidence of coronary heart diseases (Yaqoob et al., 1998) known as the Mediterranean diet effect, will necessarily translate into a beneficial effect in IBD.

In the present study there were no positive anti-inflammatory response after incubation with safflower oil (ESA) in CD and UC. This was similar after incubation with canola oil (ECA), leading to an actual decrease in IL1 α / IL1 β ratio in CD and UC. Again, it is difficult to reconcile these results and provide explanations based on these simple experiments. The variability of experimental conditions and assays may account for some of these differences.

Increases in the ratio of IL1 α / IL1 β after incubation with COCF may be explained by the presence of SCFA and MCFA serving as a fast substrate for intracellular FA- β oxidation commencing in metabolism of SCFA, butyrate, propionate and acetate, where especially butyrate serves as energy substrate for colonocytes. A recent study by Segain *et al* (Segain et al., 2000) have found that butyrate decreased TNF levels in a dose dependent manner in inflamed and non-inflamed biopsies. This was also seen for IL6 but less consistent for IL1 β . The group observed a decrease in cytokine mRNA in biopsies and isolated LPMC after treatment with butyrate, and a decrease in TNF- α , TNF- β , IL1 β and IL6 mRNA expression in PBMC after stimulation with LPS, due to a butyrate induced inhibition of LPS stimulated mRNA expression. Butyrate inhibited NF κ B p65 translocation from cytoplasm to nucleus, which was induced by LPS in

PBMC, and similar observations were made in cultured CD biopsies. This was associated with an increase in I κ B levels. Overall these studies show that butyrate has a downregulatory impact on inflammatory response through inhibition of NF κ B. This data might explain the slight increase in the IL1 α / IL1 β ratio in incubations with COCF which maybe due to a production of butyrate from SCFA. This may lead to a modest inhibitory effect on NF κ B induced IL1 β mRNA expression, leading to a decreased IL1 β and therefore to an increase in IL1 α / IL1 β ratio. However, we did not investigate *in vitro* production of butyrate in this simplified model, and therefore can only propose this hypothesis which may be confirmed in future studies. The same effect, i.e. an increase in the IL1 α /IL1 β ratio was not seen in COC with a fatty acid composition ranging from C6 to C18. Jeppesen *et al* investigated absorption of saturated fatty acids (C8-C20) and long-chain unsaturated (C16:1, C18:1, C18:2) fatty acids in patients with small bowel resection with or without a preserved colon, showing that fatty acids of C16-C18 were completely malabsorbed (98-100%) in three patients with a preserved full colon and ileocecal valve and in one patient with half a colon in continuity. It indicated that LCFA with a length of 16 or more carbon atoms are not absorbed in the colon. Better absorption was achieved for C8:0 to C14:0 in a chain length dependent pattern. In patients with functional colon, absorption of C8:0 was almost complete (more than 90%) at any level of C16 absorption (Jeppesen and Mortensen, 1998). The fatty acid profile in the coconut oil formulae for COCF and COC for C8:0 FA (55.39%) and (6.69%) respectively, could have favoured colonic uptake and metabolism of C:8 in COCF.

15.5.1. Strengths of the study

The experiments described in this thesis were performed with endoscopic tissue biopsies and incubated with specific modified enteral diets in a modular incubator chamber. Using tissue has some advantages. Although it is an *in vitro* model, it is more representative of an intact metabolic system and responses to any external addition. It has been shown that the incubated tissue is viable over a period of 24-h in culture

medium, as well as in enteral diet medium mixtures. Viable tissue for protein experiments (medium not included) in CD: 75%, UC: 81.5%, control patients: 85%; for fatty acid experiment (medium not included) in CD: 72.1%, UC: 73.3%, control patients: 77%. Total viable biopsy analysed (dietary and medium incubation) for cytokine assay and TGF- β 1 staining was 75.5% of biopsies. The 'loss' of 24.5 % of biopsies (which were excluded in the final analysis) consisted of biopsies that had not been viable over the culture period (no BrdU uptake), loss of biopsies during tissue processing cycle, failure of cytokine assay or insufficient tissue for staining (mucus rich, no tissue structure). The results showed that anti-inflammatory effect in culture supernatants manifested as cytokine modulation *in vitro* may be assayed. The design of these experiments was relatively simple but labour intensive. The results are reproducible and are similar to current literature on cytokine response *in vitro* in e.g. cell lines, lymphocytes or macrophages after stimulation with single fatty acids or in *in vivo* cytokine modulation or cytokine mRNA response after enteral feeding in patients with IBD. These anti-inflammatory effects due to dietary modulation have been further found to be similar to other studies in IBD after enteral feeding of polymeric diets, expression of cytokine mRNA in endoscopic biopsies (Fell J.M.E. et al., 2000) (Fell J.M.E. et al., 1998), and reduced mucosal inflammation and improvement of disease activity (Beattie et al., 1994).

Intestinal cytokine release has been further investigated in a variety of experiments. A similar organ culture model has been used by Mahida *et al*, where his group measured IL1 β cytokine response (homogenate and medium) and as % of medium control in endoscopic biopsies in patients with IBD, incubated with 5-ASA (Mahida et al., 1991). *In vivo* measurement of intestinal mucosa released cytokines and eicosanoids was performed in patients with UC by Carty *et al* using filter paper (Carty et al., 2000) and leukotriene concentration was measured in colonic dialysate in patients with UC after oral treatment with MK-591 (Roberts et al., 1997). Incubation of Caco2 cells grown in serum-free medium supplemented with whole protein diets was recently performed by Croft *et al*, establishing an anti-inflammatory effect of diets on intestinal epithelial cells (Croft et al., 2001).

It is not clear how handling of the tissue in the experiments presented in this thesis might have affected the measured cytokine response. All the diet formulation supplied by SHS were modified on the basis formulation of EO28. Therefore the emulsifiers used were the same in all formulae used in this experiment. We did not specified investigate the effect of the emulsifiers in these experiments. The difference between different diets is unlikely to be explained by any different emulsifier composition in the formulae.

However, the medium incubated anti-inflammatory / pro-inflammatory cytokine (IL1 α / IL1 β) ratio in experiments has been generally lower compared with dietary modulated groups in CD and UC. Therefore it is likely that the anti-inflammatory response seen as an increase in the ratio must be due to different dietary modulation. Further studies in this area of nutritional modulation in IBD should be approached with some more defined laboratory tools to avoid weaknesses of our study as discussed below. Using simple laboratory methods, these data have shown that enteral formulae have a direct anti-inflammatory effect on cytokine response *in vitro*.

To my knowledge, this is the first study where endoscopic tissue biopsies obtained from patients with IBD and non-inflamed control patients were incubated with enteral diet modifications. The use of biopsy tissue has the advantage of representing a microenvironment, which mirrors the actual intestinal mucosa of the subject under investigation. With this method it was proven, that enteral diets have a direct anti-inflammatory effect in patients with IBD.

15.5.2. Weaknesses of the study

Biopsies in this study were obtained from endoscopy. Although incubation of tissue has its advantages, the manipulation of the tissue by endoscopy forceps can lead to an increased cytokine release by traumatising the epithelial surface. Using cell cultures could have prevented this, but primary epithelial cell cultures are less robust and difficult to maintain. Transformed cell lines may not model IBD mucosa. The subjects

included in this study had diagnosed IBD, in different states of inflammatory activity, but an actual estimation of the clinical disease activity was not performed. This was not done because estimation by CDAI would involve diary card and laboratory blood parameters and most patients generally came as outpatients for routine endoscopy, staying in the day bed area. Characterisation of disease activity could therefore only be obtained by macroscopic and histological appearance of the intestinal mucosa. Medication used for IBD prior to biopsies has been recorded though the influence of medication in combination with the enteral diets was not analysed, as the numbers were small. In all the reviewed trials involving fatty acid modulation in patients with IBD, the patients have continued with their usual medication. Due to processing of tissue for BrdU uptake an estimate of protein weight was not possible, as the tissue as a whole would no longer have been available for immunostaining. Weighing of biopsies after endoscopy was initially performed but as the biopsies were small and the tissue kept moisturised in culture medium to prevent desiccation, measurements of biopsy weight were considered inaccurate and this was therefore discontinued. The cytokines IL1 β , IL1ra, IL10 and, to some extent TGF- β , have been measured in the culture supernatant and ratio of changes of anti-and pro-inflammatory cytokines determined. Using cytokine ratios is a reasonable method, since an imbalance of pro-inflammatory and anti-inflammatory cytokine ratio in IBD is known. This also avoids using tissue weights. It would have been useful also to measure other inflammatory cytokines such as IL6 and IL2 but this was not possible due to lack of available culture fluid, which was used up in the assays done. As thirteen different diets were investigated, in CD, UC and control patients, the actual data set of each patient group per diet was low. It also has to be considered that some data were excluded due insufficient BrdU uptake confirming no viable tissue during incubation period, due to failure of assay, leakage and loss of pressure during culture process, loss of tissue during tissue processing cycle or spoiling of supernatant. Finally, as in most such experimental designs, it is a reductionist model, which does not mirror the actual *in vivo* situation and therefore has to be interpreted with caution.

15.5.3. Proposals for further work

It is difficult to draw any unifying conclusion in respect of cytokine response after incubation in these diets modified in protein and fatty acid composition and many questions remain open. The principal results were obtained only for IL1 α and IL1 β and to a lesser extent in IL10, as these cytokines have shown to have a major impact in IBD and imbalance is well established in IBD. It was shown in these experiments that simple dietary modulation can have a major influence on these well known cytokine profiles. Enteral diet components may have a direct effect on intestinal mucosal cytokine modulation and a positive anti-inflammatory effect can result from such modulation. However, investigating other cytokines involved in IBD such as IL2, IL6 and IL8 and their possible change due to dietary manipulation could be an interesting approach in further studies. An encapsulated 'wrapping' could be considered for specific fatty acids, as some of these are unpalatable. Delivery systems to directly apply ω -3 fatty acid to mucosa may have clinical applications. Fatty acid enema can have some benefits especially in UC and such topical effects bypassing diets could be utilised. It would be difficult for orally taken enteral diets to reach the colon undigested, or ready for fermentation as in the case of SCFA. Further clinical trials are required.

At the current stage it would be useful to launch further work in this area studying the direct immunomodulatory effects of nutrient components. Using tissue does reflect a more intact metabolism, despite injuries due to biopsy forceps. As biopsy tissues are easy to obtain during routine endoscopy, it gives some advantages over cell cultures, especially primary cultures. This organ culture model has been shown to keep tissue viable over a period of 24-h. With appropriate facilities, it is possible to refine the work further by in situ hybridisation for cytokine mRNA measurements. Through measuring prostaglandin changes after dietary exposure its relationship to cytokine production can be clarified. Clinical trials to study a more defined *in vitro*, as proposed, and *in vivo* response after two to three weeks of oral enteral diet with modifications in patients with IBD could be interesting. A close monitoring of treatment response prior to and after

treatment with paired *in vitro* incubation of biopsies could be tried. This would highlight the actual onset of diet induced anti-inflammatory responses.

This project has opened up the possibility of clinical trials using some of the enteral diet modifications used for incubations of UC and CD affected tissues.

Appendix

MATERIAL SUMMARY

Waymouth medium MB705\1 (Flow-labs 12-522-54)

L-Ascorbic acid (Sigma A 4544)

Ferrous sulphate (Sigma F 8633)

Penicillin & Streptomycin (Flow labs 16-700-49)

Gentamycin (Flow-labs 16-760-45)

L-Glutamine (Gibco 43-8030)

L-Glutamine (Gibco-8030)

Universal container (Greiner bio-one, UK)

Modular Incubator chamber (ICN Biomedicals #6153000)

Culture dishes (Becton&Dickinson, Falcon # 35303, USA)

Metal triangular grids mesh (United wire, swg36, 0.190mm #50(304L), Edinburgh, UK).

◆

Enteral diet modifications (SHS, Liverpool, UK)

Distilled water (Baxter Health Care, Pharmacy WGH #F7124 Edinburgh, UK)

◆

Industrial Methylated Spirit (IMS 99) (Rothburn Chemicals Limited, UK)

Carnoy's fixative (30% Chloroform, 10% Acetic acid, 60% Industrial Methylated Spirit)

Microtome Leica 2045 Multicut, Nussloch, Germany

Paraffin wax (BDH Lab Supplies, # 361079E, UK).

◆

Hydrogen peroxide BP 6% (Hilcross Pharmaceuticals #HYD 355C, UK)

Concentrated Hydrochloride acid (BDH analar # 10125, UK)

3,3'-Diaminobenzidine (Sigma D-5637, UK)

Harris Haematoxylin (Sigma HHS-16, UK)

Rabbit serum (SAPU # S030-220, UK)

Monoclonal anti-Bromodeoxyuridine (Becton&Dickinson #7580, UK)

Biotinylated rabbit anti mouse immunoglobulines (Dako # E0413, UK)
StreptABCcomplex/peroxidase kit (Dako #K377-B, UK)
Trizma base analar (#10315, UK)
Sodium chloride (Baxter health care Pharmacy WGH # F7124 Edinburgh, UK)
HistoClear (Rothburn Chemicals Limited, UK)
Xylene (Rothburn Chemicals Limited, UK)
Pap pen (Dako)
DPX (Mounting medium, #1,522, Raymond A. Lamp, London, UK).
Cover slips (Microslides, #K Germany)
Slides (Microslides LSL, Limited Rochdale, UK)
Light microscope (Leitz Wetzlar, Germany)

◆

Normal Sheep serum (# Z5050020, Diagnostics Scotland, Edinburgh, UK)
TGF- β 1 rabbit polyclonal (Santa Cruz Biotechnology #sc-146, Germany)
Sheep Anti-rabbit IgG biotinylated (Chemicon International #AQ301B, UK)
Copper sulphate (BDH Chemicals Ltd, #72850, UK)
Leica-Video-Image Analyser (Q500MC, Leica Cambridge, UK)

◆

ELISA plates (Corning Costar, # 9018, UK)
Human IL1 β capture (#MAB601, R&D, UK)
Human IL1 β protein (#201-LB-005, R&D, UK)
Human IL1 β detection (#BAF201, R&D, UK)
Human IL1ra capture (#MAB280, R&D, UK)
Human IL1ra protein (#280-RA-010, R&D, UK)
Human IL1ra detection (#BAF280, R&D, UK)
Human IL10 capture (# MAB217, R&D, UK)
Human IL10 protein (# 217-IL-005, R&D, UK)
Human IL10 biotinylated detection (# BAF217, R&D, UK)

◆

Human TGF- β 1 DuoSet (#DY240, R&D, UK) containing:
TGF- β 1 capture (#840116)
TGF- β 1 protein (#840118)

TGF- β 1 biotinylated detection (# 840117)

Streptavidin – HRP conjugate (#890803)

Streptavidin-horse-radish peroxidase conjugate (HRP) (Zymed Laboratories, # 43-4323, UK)

◆

Phosphate buffer saline tablets (PBS) (Sigma, # P-4417, UK)

Tween 20 (Sigma # P-1379, UK)

Bovine Serum albumin (Sigma, # A-7906, UK)

Sucrose (Sigma, # S-9378, UK)

O-phenylene-edamine-dichloride -Fast-OPD tablets- (Sigma, # P-9187, UK)

Tetra-methylbenzidine substrate –TMB - (# DY999, R&D, UK)

HEPES, (Sigma # H-9136, UK)

3 N Sulphuric acid

Elisa Reader (# 992-0001-99, Dynex-Immunoassay Technologies, Guernsey Ltd, UK)

Bibliography

- Actis, G.C., Volpes, R. and Rizzetto, M. Oral microemulsion cyclosporin to reduce steroids rapidly in chronic active ulcerative colitis. *European Journal of Gastroenterology & Hepatology* 11:905-908, 1999.
- Adler, D.J. and Korelitz, B.I. The therapeutic efficacy of 6-mercaptopurine in refractory ulcerative colitis. *American Journal of Gastroenterology* 85:717-722, 1990.
- Ahmad, T; Armuzzi A; Bunce M; Mulcahy-Hawes, K; Marshall SE; Orchard TR; Grawshaw, J; Large, O; De Silva A; Cook JT; Barnado M; Cullen S; Welsh KI; Jewell DP; The Molecular Classification of the Clinical Manifestations of Crohn`s Disease/ *Gastroenterology* 122: 854-866, 2002
- Akagi, S., Hiyama, E., Imamura, Y., Takesue, Y., Matsuura, Y. and Yokoyama, T. Interleukin-10 expression in intestine of Crohn`s disease. *International Journal of Molecular Medicine* 5:389-395, 2000.
- Alexander, J.W. Immunonutrition: the role of omega-3 fatty acids. *Nutrition* 14:627-633, 1998.
- Almallah, Y.Z., El-Tahir, A., Heys, S.D., Richardson, S. and Eremin, O. Distal proctocolitis and n-3 polyunsaturated fatty acids:the mechanism(s) of natural cytotoxicity inhibition. *European Journal of Clinical Investigation* 30:58-65, 2000.
- Andus, T., Daig, R., Vogl, D., et al. Imbalance of the interleukin 1 system in colonic mucosa--association with intestinal inflammation and interleukin 1 receptor antagonist [corrected] genotype 2 *Gut* 41:651-657, 1997.
- Ang, Y.S., Mahmud, N., White, B., et al. Randomized comparison of unfractionated heparin with corticosteroids in severe active inflammatory bowel disease. *Alimentary Pharmacology & Therapeutics* 14:1015-1022, 2000.
- Ardizzone, S. and Porro, G.B. A practical guide to the management of distal ulcerative colitis.*Drugs* 55:519-542, 1998.
- Arlander, E., Ost, A., Stahlberg, D. and Lofberg, R. Ropivacaine gel in active distal ulcerative colitis and proctitis -- a pharmacokinetic and exploratory clinical study. *Alimentary Pharmacology & Therapeutics* 10:73-81, 1996.
- Armitage, E., Drummond, H., Ghosh, S. and Ferguson, A. Incidence of juvenile-onset Crohn's disease in Scotland *Lancet* 353:1496-1497, 1999.
- Armitage, E., Drummond, H., Wilson D. and Ghosh, S. Increasing incidence of both juvenile-onset Crohn`s disease and ulcerative colitis in Scotland. *European Journal of Gastroenterology & Hepatology* 13:1439-1447, 2001.

- Arora, S., Katkov, W., Cooley, J., et al. Methotrexate in Crohn's disease: results of a randomized, double-blind, placebo-controlled trial. *Hepato-Gastroenterology* 46:1724-1729, 1999.
- Aslan, A. and Triadafilopoulos, G. Fish Oil Fatty Acid Supplementation in Active Ulcerative Colitis: A Double-Blind, Placebo-Controlled, Cross-over Study. *The American Journal of Gastroenterology* 87:432-437, 1992.
- Austin, S.C. and Funk, C.D. Insight into prostaglandin, leukotriene, and other eicosanoid functions using mice with targeted gene disruptions. *Prostaglandins & Other Lipid Mediators* 58:231-252, 1999.
- Autrup, H., Barrett, L.A., Jackson, F.E., et al. Explant culture of human colon. *Gastroenterology* 74:1248-1257, 1978.
- Avery, A., Paraskeva, C., Hall, P., Flanders, K.C., Sporn, M. and Moorghen, M. TGF-beta expression in the human colon: differential immunostaining along crypt epithelium. *British Journal of Cancer* 68:137-139, 1993.
- Awad, J.A., Roberts, L.J., Burk, R.F. and Morrow, J.D. Isoprostanes - Prostaglandin-like compounds formed in vivo independently of cyclooxygenase. *Gastroenterology Clinics of North America* 25:409-427, 1996.
- Babyatsky, M.W., Rossiter, G. and Podolsky, D.K. Expression of transforming growth factors alpha and beta in colonic mucosa in inflammatory bowel disease. *Gastroenterology* 110:975-984, 1996.
- Ballinger, A.B., Camacho-Huebner, C. and Croft, N.M. Growth Failure and intestinal inflammation. *QJM* 94:121-125, 2001.
- Baron, T.H., Truss, C.D. and Elson, C.O. Low-dose oral methotrexate in refractory inflammatory bowel disease. *Digestive Diseases & Sciences* 38:1851-1856, 1993.
- Bauditz, J., Haemling, J., Ortner, M., Lochs, H., Raedler, A. and Schreiber, S. Treatment with tumour necrosis factor inhibitor oxpentifylline does not improve corticosteroid dependent chronic active Crohn's disease. *Gut* 40:470-474, 1997.
- Beattie, R.M., Schiffrin, E.J., Donnet-Hughes, A., et al. Polymeric nutrition as the primary therapy in children with small bowel Crohn's disease. *Alimentary Pharmacology & Therapeutics* 8:609-615, 1994.
- Belch, J.J. and Hill, A. Evening primrose oil and borage oil in rheumatologic conditions. *American Journal of Clinical Nutrition* 71:352S-356S, 2000.
- Bellona G, Turietti A, Artusio E, et al. Tumor-Associated Transforming Growth Factor- β and Interleukin-10 Contribute to a Systemic Th2 Immune Phenotype in Pancreatic Carcinoma Patients. *American Journal of Pathology* 155:537-543, 1999.

- Belluzzi, A., Brignola, C., Campieri, M., Pera, A., Boschi, S. and Miglioli, M. Effect of an enteric-coated fish-oil preparation on relapses in Crohn's disease. *New England Journal of Medicine* 334:1557-1560, 1996.
- Bernstein, C.N., Papineau, N., Zajackowski, J., Rawsthorne, P., Okrusko, G. and Blanchard, J.F. Direct hospital costs for patients with inflammatory bowel disease in a Canadian tertiary care university hospital. *American Journal of Gastroenterology* 95:677-683, 2000.
- Best, W.R., Bechtel, J.M., Singleton, J.W. and Kern, F., Jr. Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. *Gastroenterology* 70:439-444, 1976.
- Bhan, A.K., Mizoguchi, E. and Mizoguchi, A. New models of chronic intestinal inflammation. *Current Opinion in Gastroenterology* 10:633-638, 1994.
- Birch EE, Garfield S, Uauy R and Birch DG. A randomized controlled trial of early dietary supply of longchain polyunsaturated fatty acids and mental development in term infants. *Developmental Medicine & Child Neurology* 42:174-181, 2000.
- Bjorck, S., Dahlstrom, A., Johansson, L. and Ahlman, H. Treatment of the mucosa with local anaesthetics in ulcerative colitis. *Agents & Actions Spec No:C60-C72*1992.
- Bjorkman, D.J. Aspirin and NSAIDs: Mechanism of Action. *Clinical Perspectives in Gastroenterology* May/June:134-140, 1999.
- Blaettler, U., Hammon, H.M., Morel, C., et al. Feeding Colostrum, its Composition and Feeding Duration Variably Modify Proliferation and Morphology of the Intestine and Digestive Enzyme Activities of Neonatal Calves. *Journal of Nutrition* 4:1256-1263, 2001.
- Blomqvist, P. and Ekblom, A. Inflammatory bowel diseases: health care and costs in Sweden in 1994. *Scandinavian Journal of Gastroenterology* 32:1134-1139, 1997.
- Boidreau, M.D., Chanmugam, P.S., Hart, S.B., Lee, S.H. and Hwang, D.H. Lack of dose response by dietary n-3 fatty acids at a constant ratio of n-3 to n-6 fatty acids in suppressing eicosanoid biosynthesis from arachidonic acid. *American Journal of Clinical Nutrition* 54:111-117, 1991.
- Bonapace, C.R. and Mays, D.A. The effect of mesalamine and nicotine in the treatment of inflammatory bowel disease. *Annals of Pharmacotherapy* 31:907-913, 1997.
- Boot, A.M., Bouquet, J., Krenning, E.P. and de Muinck, K. Bone mineral density and nutritional status in children with chronic inflammatory bowel disease. *Gut* 42:188-194, 1998.
- Borgaonkar, M., MacIntosh, D., Fardy, J. and Simms, L. Anti-tuberculous therapy for maintaining remission of Crohn's disease. *Cochrane Database of Systematic*

Reviews [computer file] CD0002992000.

- Bousvaros, A., Kirschner, B.S., Werlin, S.L., et al. Oral tacrolimus treatment of severe colitis in children. *Journal of Pediatrics* 137:794-799, 2000.
- Brandtzaeg, P. Development and basic mechanisms of human gut immunity. *Nutrition Reviews* 56:S5-18, 1998.
- Brauchle, M., Madlener, M., Wagner, A.D., et al. Keratinocyte growth factor is highly overexpressed in inflammatory bowel disease. *American Journal of Pathology* 149:521-529, 1996.
- Breese, E.J., Michie, C.A., Nicholls, S.W., et al. Tumor necrosis factor alpha-producing cells in the intestinal mucosa of children with inflammatory bowel disease. *Gastroenterology* 106:1455-1466, 1994.
- Breuer, R., Buto SK, Christ ML, et al. Rectal irrigation with short-chain fatty acids for distal ulcerative colitis. Preliminary report. *Digestive Disease and Science* 36:185-7, 1991.
- Brynskov, J., Freund, L., Rasmussen, S.N., et al. A placebo-controlled, double-blind, randomized trial of cyclosporine therapy in active chronic Crohn's disease. [see comments]. *New England Journal of Medicine* 321:845-850, 1989.
- Burke, D.A., Axon, A.T., Clayden, S.A., Dixon, M.F., Johnston, D. and Lacey, R.W. The efficacy of tobramycin in the treatment of ulcerative colitis. *Alimentary Pharmacology & Therapeutics* 4:123-129, 1990.
- Burri, B.J., Dougherty, R.M., Kelley, D.S. and Iacono, J.M. Platelet aggregation in humans is affected by replacement of dietary linoleic acid with oleic acid. *American Journal of Clinical Nutrition* 54:359-362, 1991.
- Burton, J.L. Dietary fatty acids and inflammatory skin disease. *Lancet* 1:27-31, 1989.
- Calder, P.C. Dietary fatty acids and the immune system. *Nutrition Reviews* 56:S70-S83, 1998.
- Camma, C., Giunta, M., Rosselli, M. and Cottone, M. Mesalamine in the maintenance treatment of Crohn's disease: a meta-analysis adjusted for confounding variables. *Gastroenterology* 113:1465-1473, 1997.
- Campieri, M., Ferguson, A., Doe W, Persson T and Nilsson, L.G. Oral budesonide is as effective as oral prednisolone in active Crohn's disease. The Global Budesonide Study Group. *Gut* 41:209-14, 1997.
- Candy, S., Wright, J., Gerber, M., Adams, G., Gerig, M. and Goodman, R. A controlled double blind study of azathioprine in the management of Crohn's disease. *Gut* 37:674-678, 1995.
- Canva-Delcambre, V., Jacquot, S., Robinet, E., et al. Treatment of severe Crohn's

- disease with anti-CD4 monoclonal antibody. *Alimentary Pharmacology & Therapeutics* 10:721-727, 1996.
- Caprilli, R., Carratu, R. and Babbini, M. Double-blind comparison of the effectiveness of azathioprine and sulfasalazine in idiopathic proctocolitis. Preliminary report. *American Journal of Digestive Diseases* 20:115-120, 1975.
- Carbonnel, F., Boruchowicz, A., Duclos, B., et al. Intravenous cyclosporine in attacks of ulcerative colitis: short-term and long-term responses. *Digestive Diseases & Sciences* 41:2471-2476, 1996.
- Carty, E., Brabander, M.D., Feakin, R.M. and Rampton, D.S. Measurement of in vivo rectal mucosal cytokine and eicosanoid production in ulcerative colitis using filter paper. *Gut* 46:492-2000.
- Carty, E., Rampton, D.S., Schneider, H., Rutgeerts, P. and Wright, J.P. Lack of efficacy of ridogrel, a thromboxane synthase inhibitor, in a placebo-controlled, double-blind, multi-centre clinical trial in active Crohn's disease. *Alimentary Pharmacology & Therapeutics* 15:1323-1329, 2001.
- Casini-Raggi, V., Kam, L., Chong, Y.J., Fiocchi, Pizarro, T.T. and Cominelli, F. Mucosal imbalance of IL-1 and IL-1 receptor antagonist in inflammatory bowel disease. A novel mechanism of chronic intestinal inflammation. *Journal of Immunology* 154:2434-2440, 1995.
- Casson, D.H., Eltumi, M., Tomlin, S., Walker-Smith, J.A. and Murch, S.H. Topical tacrolimus may be effective in the treatment of oral and perineal Crohn's disease. *Gut* 47:436-440, 2000.
- Cellier, C., De Beenhouwer, H., Berger, A., et al. Mycobacterium paratuberculosis and Mycobacterium avium subsp. silvaticum DNA cannot be detected by PCR in Crohn's disease tissue. *Gastroenterologie Clinique et Biologique* 22:675-678, 1998.
- Cezard, J.P. and Messing B. Enteral Nutrition in inflammatory bowel disease: is there a special role for elemental diet? *Clinical Nutrition* 12:S75-S81 1993.
- Chapman, R.W., Selby, W.S. and Jewell, D.P. Controlled trial of intravenous metronidazole as an adjunct to corticosteroids in severe ulcerative colitis. *Gut* 27:1210-1212, 1986.
- Chey, W.Y. Infliximab for patients with refractory ulcerative colitis. *Inflammatory Bowel Diseases* 7 Suppl 1:S30-S33 2001.
- Chiba, M., Fukushima, T., Horie, Y., Iizuka, M. and Masamune, O. No Mycobacterium paratuberculosis detected in intestinal tissue, including Peyer's patches and lymph follicles, of Crohn's disease. *Journal of Gastroenterology* 33:482-487, 1998.
- Chong, R.Y., Hanauer, S.B. and Cohen, R.D. Efficacy of parenteral methotrexate in

- refractory Crohn's disease. *Alimentary Pharmacology & Therapeutics* 15:35-44, 2001.
- Cohen, R.D., Larson, L.R., Roth, J.M., Becker, R.V. and Mummert, L.L. The cost of hospitalization in Crohn's disease. *American Journal of Gastroenterology* 95:524-530, 2000.
- Cohen, R.D., Woseth, D.M., Thisted, R.A. and Hanauer, S.B. A meta-analysis and overview of the literature on treatment options for left-sided ulcerative colitis and ulcerative proctitis. *American Journal of Gastroenterology* 95:1263-1276, 2000.
- Colombel, J.F., Rutgeerts, P., Malchow, H., et al. Interleukin 10 (Tenovil) in the prevention of postoperative recurrence of Crohn's disease. *Gut* 49:42-46, 2001.
- Conference Report. Omega -3 fatty acids and health. *Nutrition Bulletin* 25:81-84, 2000.
- Corfield, A.P., Myerscough, N., Longman, R., Sylvester, P., Arul, S. and Pignatelli, M. Mucins and mucosal protection in the gastrointestinal tract: new prospects for mucins in the pathology of gastrointestinal disease. *Gut* 47:589-594, 2000.
- Cortot, A., Colombel, J.F., Rutgeerts, P., et al. Switch from systemic steroids to budesonide in steroid dependent patients with inactive Crohn's disease. *Gut* 48:186-190, 2001.
- Crawford, M.A. Commentary on the workshop statement. Essentiality of and recommended dietary intakes for Omega-6 and Omega-3 fatty acids. *Prostaglandins Leukotrienes & Essential Fatty Acids* 63:131-134, 2000.
- Croft, N.M., Kelly, E.J., Bannerjee, K., Dryhurst, K. and Sanderson, I. Diets can modulate the inflammatory response in vivo and in vitro. *Gut* 48:A3-004, 2001
- Cross, M.L. and Gill, H.S. Modulation of immune function by a modified bovine whey protein concentrate. *Immunology & Cell Biology* 77:345-350, 1999.
- Crowther, J.R. Basic principles of ELISA. In: *ELISA Theory and Practice* edited by Human Press Inc. Totowa, New Jersey: 1995, p. 35-217.
- D'Haens, G., Lemmens, L., Geboes, K., et al. Intravenous cyclosporine versus intravenous corticosteroids as single therapy for severe attacks of ulcerative colitis. *Gastroenterology* 120:1323-1329, 2001.
- Dammeier, J., Brauchle, M., Falk, W., Grotendorst, G.R. and Werner. Connective tissue growth factor: a novel regulator of mucosal repair and fibrosis in inflammatory bowel disease? *International Journal of Biochemistry & Cell Biology* 30:909-922, 1998.
- Danielsson, A., Hellers, G., Lyrenas, E., et al. A controlled randomized trial of budesonide versus prednisolone retention enemas in active distal ulcerative colitis. *Scandinavian Journal of Gastroenterology* 22:987-992, 1987.

- De Caterina, R., Bernini, W., Carluccio, M.A., Liao, J.K. and Libby, P. Structural requirements for inhibition of cytokine-induced endothelial activation by unsaturated fatty acids. *Journal of Lipid Research* 39:1062-1070, 1998.
- De Caterina, R., Liao, J.K. and Libby, P. Fatty acid modulation of endothelial activation. *American Journal of Clinical Nutrition* 71:213S-223S, 2000.
- De Caterina, R., Spiecker, M., Solaini, G., et al. The inhibition of endothelial activation by unsaturated fatty acids. *Lipids* 34 Suppl:S191-S194, 1999.
- de Deckere, E.A., Korver, O., Verschuren, P.M. and Katan, M.B. Health aspects of fish and n-3 polyunsaturated fatty acids from plant and marine origin. *European Journal of Clinical Nutrition* 52:749-753, 1998.
- Dear KL, Compston, J.E. and Hunter, J.O. Treatment for Crohn's disease that minimise steroid dosis are associated with a reduced risk of osteoporosis. *Clinical Nutrition* 20:541-6, 2001.
- Defries, E.A. and Franks, L.M. An organ culture method for adult colon from germfree and conventional mice: effects of donor age and carcinogen treatment on epithelial mitotic activity. *Journal of the National Cancer Institute* 58:1323-1328, 1977.
- Deitch, E.A., Xu, D., Naruhn, M.B., Deitch, D.C., Lu, Q. and Marino, A.A. Elemental Diet and IV-TPN-Induced Bacterial Translocation is Associated with Loss of Intestinal Mucosal Barrier Function against Bacteria. *Annals of Surgery* 221:299-307, 1995.
- di Mola, F.F., Friess, H., Scheuren, A., et al. Transforming growth factor-betas and their signaling receptors are coexpressed in Crohn's disease. *Annals of Surgery* 229:67-75, 1999.
- Dichi, I., Frenhane, P., Dichi, J.B., et al. Comparison of omega-3 fatty acids and sulfasalazine in ulcerative colitis. *Nutrition* 16:87-90, 2000.
- Dickinson, R.J., O'Connor, H.J., Pinder, I., Hamilton, I., Johnston, D. and Axon, A.T. Double blind controlled trial of oral vancomycin as adjunctive treatment in acute exacerbations of idiopathic colitis. *Gut* 26:1380-1384, 1985.
- Dionne, S., D'Agata, I.D., Hiscott, J., Vanounou, T. and Seidman, E.G. Colonic explant production of IL-1 and its receptor antagonist is imbalanced in inflammatory bowel disease (IBD). *Clinical & Experimental Immunology* 112:435-442, 1998.
- Donnet-Hughes, A. Bioactive molecules in milk and their role in health and disease: The role of transforming growth factor-[beta]. *Immunology & Cell Biology* 78:74-79, 2000.
- Draft recommendations on the essentiality of and recommended dietary intake for omega-6 and omega-3 fatty acids. 63(3), 117. 2000. Conference Proceeding

- Egan LJ, Tremaine, W.J., Mays DC, Lipsky, J.J. and Sandborn, W.J. Clinical outcome and pharmacokinetics after addition of low-dose cyclosporine to methotrexate: a case study of five patients with treatment-resistant inflammatory bowel disease. *Inflammatory Bowel Diseases* 6:286-9, 2000.
- Ehrenpreis, E.D., Kane, S.V., Cohen, L.B., Cohen, R.D. and Hanauer, S.B. Thalidomide therapy for patients with refractory Crohn's disease: an open-label trial. *Gastroenterology* 117:1271-1277, 1999.
- Elion GB. The comparative metabolism of imuran and 6-mercaptopurine in man. *Proceedings of the American Association of Cancer Research* 10:211969.
- Elion, G.B. The purine path to chemotherapy. *Science* 244:41-47, 1989.
- Esteve-Comas, M., Nunez, M.C., Fernandez-Banares, F., et al. Abnormal plasma polyunsaturated fatty acid pattern in non-active inflammatory bowel disease. *Gut* 34:1370-1373, 1993.
- Evans, R.C., Clarke, L., Heath, P., Stephens, S., Morris, A.I. and Rhodes, J.M. Treatment of ulcerative colitis with an engineered human anti-TNF α antibody CDP571. *Alimentary Pharmacology & Therapeutics* 11:1031-1035, 1997.
- Ewe, K., Bottger, T., Buhr, H.J., Ecker, K.W. and Otto, H.F. Low-dose budesonide treatment for prevention of postoperative recurrence of Crohn's disease: a multicentre randomized placebo-controlled trial. German Budesonide Study Group. *European Journal of Gastroenterology & Hepatology* 11:277-282, 1999.
- Ewe, K., Press, A.G., Singe, C.C., et al. Azathioprine combined with prednisolone or monotherapy with prednisolone in active Crohn's disease. *Gastroenterology* 105:367-372, 1993.
- Faubion, W.A.J., Loftus, E.V.J., Harmsen, W.S., Zinsmeister, A.R. and Sandborn, W.J. The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study. *Gastroenterology* 121:255-260, 2001.
- Feagan, B.G., McDonald, J.W., Rochon, J., et al. Low-dose cyclosporine for the treatment of Crohn's disease. The Canadian Crohn's Relapse Prevention Trial Investigators. *New England Journal of Medicine* 330:1846-1851, 1994.
- Feagan, B.G., Rochon, J., Fedorak, R.N., et al. Methotrexate for the treatment of Crohn's disease. The North American Crohn's Study Group Investigators. *New England Journal of Medicine* 332:292-297, 1995.
- Feagan, B.G., Vreeland, M.G., Larson, L.R. and Bala, M.V. Annual cost of care for Crohn's disease: a payor perspective. *American Journal of Gastroenterology* 95:1955-1960, 2000.
- Fedorak, R.N., Gangl, A., Elson, C.O., et al. Recombinant human interleukin 10 in the

- treatment of patients with mild to moderately active Crohn's disease. The Interleukin 10 Inflammatory Bowel Disease Cooperative Study Group. *Gastroenterology* 119:1473-1482, 2000.
- Feeney M., Clegg A., Winwood P. and Snook J. Measles Vaccination and Inflammatory bowel disease. *Lancet* 350:764-766, 1997.
- Fell J.M.E., Hollis A., Paintin M., Arnaud-Battandier F., MacDonald T.T and Walker-Smith, J.A. Normalisation of mucosal cytokine mRNA in association with clinical improvement in children with Crohn's disease treated with polymeric diet. *Journal of Pediatric Gastroenterology & Nutrition* 26:544/A341998
- Fell J.M.E., Paintin M., Arnaud-Battandier F., et al. Mucosal healing and a fall in mucosal pro-inflammatory cytokine mRNA induced by a specific oral polymeric diet in paediatric Crohn's disease. *Alimentary Pharmacology & Therapeutics* 14:281-289, 2000.
- Ferguson, A., Campieri, M., Doe, W., Persson, T. and Nygard, G. Oral budesonide as maintenance therapy in Crohn's disease--results of a 12-month study. Global Budesonide Study Group. *Alimentary Pharmacology & Therapeutics* 12:175-183, 1998.
- Ferguson, A., Glen, M. and Ghosh, S. Crohn's disease: nutrition and nutritional therapy. *Baillieres Clinical Gastroenterology* 12:93-114, 1998.
- Fiocchi, C., Binion, D.G. and Katz, J.A. Cytokine production in the human gastrointestinal tract during inflammation. *Current Opinion in Gastroenterology* 2:639-644, 1994.
- Fitch, M.D. and Fleming, S.E. Metabolism of short-chain fatty acids by rat colonic mucosa in vivo. *American Journal of Physiology* 277:G31-G401999.
- Frank, S., Madlener M and Werners S. Transforming Growth Factors β 1, β 2 and β 3 and their receptors are differentially regulated during normal and impaired wound healing. *The Journal of Biological Chemistry* 271:10188-10193, 1996.
- French, M.A., Parrott, A.M., Kielo, E.S., et al. Polyunsaturated fat in the diet may improve intestinal function in patients with Crohn's disease. *Biochimica et Biophysica Acta* 1360:262-270, 1997.
- Friedman, S., Marion, J.F., Scherl, E., Rubin, P.H. and Present, D.H. Intravenous cyclosporine in refractory pyoderma gangrenosum complicating inflammatory bowel disease. *Inflammatory Bowel Diseases* 7:1-7, 2001.
- Galli, C. Commentary on the workshop statement. Essentiality of and recommended dietary intake of Omega-6 and Omega-3 fatty acids. *Prostaglandins Leukotrienes & Essential Fatty Acids* 63:127-129, 2000.
- Garavito, R.M. and DeWitt, D.L. The cyclooxygenase isoforms: structural insights into

- the conversion of arachidonic acid to prostaglandins. *Biochimica et Biophysica Acta* 1441:278-287, 1999.
- Gasche, C., Bakos, S., Dejaco, C., Tillinger, W., Zakeri, S. and Reinisch, W. IL-10 secretion and sensitivity in normal human intestine and inflammatory bowel disease. *Journal of Clinical Immunology* 20:362-370, 2000.
- Geerling, B.J., Badart-Smook, A., van Deursen, C., et al. Nutritional supplementation with N-3 fatty acids and antioxidants in patients with Crohn's disease in remission: effects on antioxidant status and fatty acid profile. *Inflammatory Bowel Diseases* 6:77-84, 2000.
- Geerling, B.J., Dagnelie, P.C., Badart-Smook, A., Russel, M.G., Stockbrugger, R.W. and Brummer, R.J. Diet as a risk factor for the development of ulcerative colitis. *American Journal of Gastroenterology* 95:1008-1013, 2000.
- George, J., Present, D.H., Pou, R., Bodian, C. and Rubin, P.H. The long-term outcome of ulcerative colitis treated with 6-mercaptopurine. *American Journal of Gastroenterology* 91:1711-1714, 1996.
- Ghosh, S., Armitage, E., Wilson D. and Afzal, M.A. Detection of persistent measles virus infection in Crohn's disease: current status of experimental work. *Gut* 48:748-752 Leading article, 2001.
- Ghosh, S. and Meister, D. Osteopenia in inflammatory bowel disease. *Research and Clinical Forum* 22:159-170, 2000.
- Ghosh, S., Meister, D., Cowen, S., Hannan, W.J. and Ferguson, A. Body composition at the bedside. *European Journal of Gastroenterology & Hepatology* 9:783-788, 1997.
- Ghosh, S., Shand, A. and Ferguson, A. Ulcerative colitis. *BMJ* 320:1119-1123, 2000.
- Giaffer, M.H., Cann P and Holdsworth, C.D. Long-term effects of elemental and exclusion diets for Crohn's disease. *Alimentary Pharmacology & Therapeutics* 5:115-125, 1990.
- Gionchetti, P., Rizzello, F., Venturi, A., et al. Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. *Gastroenterology* 119:305-309, 2000b.
- Gionchetti, P., Venturi, A., Rizzello, F., et al. Retrograde colonic spread of a new mesalazine rectal enema in patients with distal ulcerative colitis. *Alimentary Pharmacology & Therapeutics* 11:679-684, 1997a.
- Goenka, M.K., Kochhar, R., Tandia, B. and Mehta, S.K. Chloroquine for mild to moderately active ulcerative colitis: comparison with sulfasalazine. *American Journal of Gastroenterology* 91:917-921, 1996.
- Gonzalez-Huix, F., Fernandez-Banares, F., Esteve-Comas, M., et al. Enteral versus

- parenteral nutrition as adjunct therapy in acute ulcerative colitis. *American Journal of Gastroenterology* 88:227-232, 1993.
- Gonzalez Z.A. Practical Aspects of Nutritional Modulation of the Cytokines Response. *Nutrition in Clinical Practice* 15:45-47, 2000.
- Gorard, D.A., Hunt, J.B., Payne-James, J.J., et al. Initial response and subsequent course of Crohn's disease treated with elemental diet or prednisolone. *Gut* 34:1198-1202, 1993.
- Gordon, F.H., Lai, C.W., Hamilton, M.I., et al. A randomized placebo-controlled trial of a humanized monoclonal antibody to alpha4 integrin in active Crohn's disease. *Gastroenterology* 121:268-274, 2001.
- Graham Robinson. Immunohistochemistry. In: *Theory and Practice of Histological Techniques*, edited by John D. Bancroft and Alan Stevens. 1982, p. 406-426.
- Gratzner, H.G. Monoclonal Antibody to 5-Bromo- and 5-Iododeoxyuridine: A New Reagent for Detection of DNA Replication. *Science* 218:475-1982.
- Green, E.P., Tizard, M.L., Moss, M.T., et al. Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. *Nucleic Acids Research* 17:9063-9073, 1989.
- Green, J.A., Edwards, R.E. and Manson, M.M. Immunohistochemical detection of bromodeoxyuridine-labeled nuclei for in vivo cell kinetic studies. *Methods in Molecular Biology* 80:251-254, 1998.
- Green, J.R., Gibson, J.A., Kerr, G.D., et al. Maintenance of remission of ulcerative colitis: a comparison between balsalazide 3 g daily and mesalazine 1.2 g daily over 12 months. ABACUS Investigator group. *Alimentary Pharmacology & Therapeutics* 12:1207-1216, 1998.
- Green, J.T., Thomas, G.A., Rhodes, J., et al. Pharmacokinetics of nicotine carbomer enemas: a new treatment modality for ulcerative colitis. *Clinical Pharmacology & Therapeutics* 61:340-348, 1997.
- Greenberg, G.R., Feagan, B.G., Martin, F., et al. Oral budesonide as maintenance treatment for Crohn's disease: a placebo-controlled, dose-ranging study. Canadian Inflammatory Bowel Disease Study Group. *Gastroenterology* 110:45-51, 1996.
- Gregory L, Eastwood MD, Jerry S and Trier MD. Organ Culture of Human Rectal Mucosa. *Gastroenterology* 64:375-382, 1973.
- Griffiths, A.M. Inflammatory bowel disease. *Nutrition* 14:788-791, 1998.
- Griffiths, A.M., Ohlsson, A., Sherman, P.M. and Sutherland, L.R. Meta-analysis of enteral nutrition as a primary treatment of active Crohn's disease. *Gastroenterology* 108:1056-1067, 1995.

- Grimble, G.K. and Tappia, P.S. Modulation of pro-inflammatory cytokine biology by unsaturated fatty acids. *Zeitschrift für Ernährungswissenschaft* 37:57-65, 1998.
- Grimble, R.F. Dietary manipulation of the inflammatory response. *Proceedings of the Nutrition Society* 51:285-294, 1992.
- Grip, O., Malm, J., Veress, B., Bjartell, A., Lindgren, S. and Egesten, A. Increased presence of cells containing transforming growth factor alpha (TGF- α) in ulcerative colitis, both during active inflammation and in remission. *European Journal of Gastroenterology & Hepatology* 12:761-766, 2000.
- Grisham, M.B., Demichele, S.J., Garleb, K.A. and Specian, R.D. Sulfasalazine or enteral diets containing fish oil or oligosaccharides attenuate chronic colitis in rats. *Inflammatory Bowel Diseases* 2:178-188, 1996.
- Gross, V., Andus, T., Caesar, I., et al. Oral pH-modified release budesonide versus 6-methylprednisolone in active Crohn's disease. German/Austrian Budesonide Study Group. *European Journal of Gastroenterology & Hepatology* 8:905-909, 1996.
- Gui, G.P., Thomas, P.R., Tizard, M.L., Lake, J., Sanderson, J.D. and Hermon-Taylor, J. Two-year-outcomes analysis of Crohn's disease treated with rifabutin and macrolide antibiotics. *Journal of Antimicrobial Chemotherapy* 39:393-400, 1997.
- Guslandi, M. and Tittobello, A. Pilot trial of nicotine patches as an alternative to corticosteroids in ulcerative colitis. *Journal of Gastroenterology* 31:627-629, 1996.
- Guslandi, M. and Tittobello, A. Outcome of ulcerative colitis after treatment with transdermal nicotine. *European Journal of Gastroenterology & Hepatology* 10:513-515, 1998.
- Hahn, K.-P., Im, Y.-H., Parks, T.W., et al. Loss of transforming growth factor β signalling in the intestine contributes to tissue injury in inflammatory bowel disease. *Gut* 49:190-198, 2001.
- Hakkak, R., Korourian, S.R.M. and Badger, T. Dietary whey protein protects against azoxymethane-induced colon tumors in male rats. *Cancer Epidemiology, Biomarkers & Prevention* 10:555-558, 2001.
- Handy, L.M., Ghosh, S. and Ferguson, A. Investigation of neutrophils in the gut lumen by assay of granulocyte elastase in whole-gut lavage fluid. *Scandinavian Journal of Gastroenterology* 31:700-705, 1996.
- Harvey R.F. and Bradshaw J.M. A simple index of Crohn's disease activity. *Lancet* 1:514-1980.
- Hawkey, C.J., Dube, L.M., Rountree, L.V., Linnen, P.J. and Lancaster, J.F. A trial of zileuton versus mesalazine or placebo in the maintenance of remission of

- ulcerative colitis. The European Zileuton Study Group For Ulcerative Colitis. *Gastroenterology* 112:718-724, 1997.
- Hawthorne, A.B., Daneshmend, T.K., Hawkey, C.J., et al. Treatment of ulcerative colitis with fish oil supplementation: a prospective 12 month randomised controlled trial. *Gut* 33:922-928, 1992.
- Hawthorne, A.B., Logan, R.F., Hawkey, C.J., et al. Randomised controlled trial of azathioprine withdrawal in ulcerative colitis. *BMJ* 305:20-22, 1992.
- Hay, J.W. and Hay, A.R. Inflammatory bowel disease: costs-of-illness. *Journal of Clinical Gastroenterology* 14:309-317, 1992.
- Hellers, G., Cortot, A., Jewell, D., et al. Oral budesonide for prevention of postsurgical recurrence in Crohn's disease. The IOIBD Budesonide Study Group. *Gastroenterology* 116:294-300, 1999.
- Herfarth, H.H. and Sartor, R.B. Cytokine regulation of experimental intestinal inflammation. *Current Opinion in Gastroenterology* 10:625-623, 1994.
- Heuschkel, R.B. Enteral nutrition in children with Crohn's disease. *Journal of Pediatric Gastroenterology & Nutrition* 31:575-2000.
- Hillier, K., Jewell, R., Dorrell, L. and Smith, C.L. Incorporation of fatty acids from fish oil and olive oil into colonic mucosal lipids and effects upon eicosanoid synthesis in inflammatory bowel disease. *Gut* 32:1151-1155, 1991.
- Hodges, G.M. and Melcher, A.H. Chemically-defined medium for growth and differentiation of mixed epithelial and connective tissues in organ culture. *In Vitro* 12:450-459, 1976.
- Hoffenberg, E.J., Fidanza, S. and Sauaia, A. Serologic testing for inflammatory bowel disease. *Journal of Pediatrics* 134:447-452, 1999.
- Hunter, J.O. Nutritional factors in inflammatory bowel disease. *European Journal of Gastroenterology & Hepatology* 10:235-237, 1998.
- Hyams, J.S., Fitzgerald, J.E., Wyzga, N., et al. Relationship of interleukin-1 receptor antagonist to mucosal inflammation in inflammatory bowel disease. *Journal of Pediatric Gastroenterology and Nutrition* 21:419-425, 1995.
- Ierardi, E., Principi, M., Francavilla, R., et al. Oral tacrolimus long-term therapy in patients with Crohn's disease and steroid resistance. *Alimentary Pharmacology & Therapeutics* 15:371-377, 2001.
- Innis, SM. The role of Dietary n-6 and n-3 fatty acids in the Developing brain. *Development in Neuroscience* 22:474-480, 2000.
- Irvine, E.J., Feagan BG, Rochon, J., et al. Quality of life: a valid and reliable measure of therapeutic efficacy in the treatment of inflammatory bowel disease. *Canada*

- Crohn's Disease Relapse Prevention Trial Study Group. *Gastroenterology* 106:287-96, 1994.
- Irvine, E.J., Greenberg, G.R., Feagan BG, et al. Quality of life rapidly improves with budesonide therapy for active Crohn's disease. Canadian Inflammatory Bowel Disease Study group. *Inflammatory Bowel Diseases* 6:181-7, 2000.
- Ishizuka K., Sugimura, K., Homma T., et al. Influence of Interleukin-10 on the Interleukin-1 Receptor Antagonist/Interleukin-1 β Ratio in the Colonic Mucosa of Ulcerative Colitis. *Digestion* 63:22-27, 2001.
- Jeppesen, P.B. and Mortensen, P.B. The influence of a preserved colon on the absorption of medium chain fat in patients with small bowel resection. *Gut* 43:478-483, 1998.
- Jewell, D.P. and Truelove, S.C. Azathioprine in ulcerative colitis: final report on controlled therapeutic trial. *BMJ* 4:627-630, 1974.
- Jorgensen, J. and Mortensen, P.B. Utilization of Short-chain Fatty acids by colonic mucosal tissue strips. *Scandinavian Journal of Gastroenterology* 35:659-666, 2000.
- Jump, D.B. and Clarke, S.D. Regulation of gene expression by dietary fat. *Annual Review of Nutrition* 19:63-90, 1999.
- Kamm, M.A. *Inflammatory Bowel Disease*, London:1996. pp. 1-57.
- Keller, R., Stoll, R., Foerster, E.C., Gutsche, N. and Domschke, W. Oral budesonide therapy for steroid-dependent ulcerative colitis: a pilot trial. *Alimentary Pharmacology & Therapeutics* 11:1047-1052, 1997.
- Kim, Y.I. Can fish oil maintain Crohn's disease in remission? *Nutrition Reviews* 54:248-252, 1996.
- Kim, Y.I. Short-chain fatty acids in ulcerative colitis. *Nutrition Reviews* 56:17-24, 1998.
- Kirk, A.P. and Lennard-Jones, J.E. Controlled trial of azathioprine in chronic ulcerative colitis. *British Medical Journal Clinical Research Ed* . 284:1291-1292, 1982.
- Klein, M., Binder, H.J., Mitchell, M., Aaronson, R. and Spiro, H. Treatment of Crohn's disease with azathioprine: a controlled evaluation. *Gastroenterology* 66:916-922, 1974.
- Ko, T.C., Beauchamp, R.D., Townsend, C.M.J., Thompson, E.A. and Thompson, J.C. Transforming growth factor-beta inhibits rat intestinal cell growth by regulating cell cycle specific gene expression. *American Journal of Surgery* 167:14-19, 1994.
- Kohyama, M., Kakehi, M., Totsuka, M., Hachimura, S., Hisatsune, T. and Kaminogawa, S. Selective induction of CD8+ T cell functions by single

- substituted analogs of an antigenic peptide: distinct signals for IL-10 production. *FEBS Letters* 423:138-142, 1998.
- Kono, T., Nomura, M., Kasai, S. and Kohgo, Y. Effect of ecabet sodium enema on mildly to moderately active ulcerative proctosigmoiditis: an open-label study. *American Journal of Gastroenterology* 96:793-797, 2001.
- Koolman J and Roehm KH. Lipide. In: *Taschenatlas der Biochemie*, Georg Thime Verlag, 1994, p. 42-46.
- Kozarek, R.A., Patterson, D.J., Gelfand, M.D., Botoman, V.A., Ball, T.J. and Wilske, K.R. Methotrexate induces clinical and histologic remission in patients with refractory inflammatory bowel disease. *Annals of Internal Medicine* 110:353-356, 1989.
- Krause, W. and DuBois, R.N. Eicosanoids and the large intestine. *Prostaglandins & Other Lipid Mediators* 61:145-161, 2000.
- Kremer, J.M. n-3 fatty acid supplements in rheumatoid arthritis. *American Journal of Clinical Nutrition* 71:349S-351S, 2000.
- Kruis, W., Schutz, E., Fric, P., Fixa, B., Judmaier, G. and Stolte, M. Double-blind comparison of an oral *Escherichia coli* preparation and mesalazine in maintaining remission of ulcerative colitis. *Alimentary Pharmacology & Therapeutics* 11:853-858, 1997.
- Kugathasan, S., Werlin, S.L., Martinez, A., Rivera, M.T., Heikenen, J.B. and Binion, D.G. Prolonged duration of response to infliximab in early but not late pediatric Crohn's disease. *American Journal of Gastroenterology* 95:3189-3194, 2000.
- Kulmacz, R.J. Cellular regulation of prostaglandin H synthase catalysis. *FEBS Letters* 430:154-157, 1998.
- Kushiya, Y., Fukuda, R., Suetsugu, H., et al. Site-dependent production of transforming growth factor beta1 in colonic mucosa: Its possible role in tumorigenesis of the colon. *Journal of Laboratory & Clinical Medicine* 136:201-208, 2000.
- Kuwata H, Yamauchi K, Teraguchi S, et al. Functional fragments of ingested lactoferrin are resistant to proteolytic degradation in the gastrointestinal tract of adult rats. *Journal of Nutrition* 131:2121-7, 2001.
- Latterie, M., Angeloni, T., Silveri, N., Manna, R., Gasbarrini, G. and Navarra, P. Pharmacokinetics of cyclosporin microemulsion in patients with inflammatory bowel disease. *Clinical Pharmacokinetics* 40:473-483, 2001.
- Lemann, M., Zenjari, T., Bouhnik, Y., et al. Methotrexate in Crohn's disease: long-term efficacy and toxicity. *American Journal of Gastroenterology* 95:1730-1734, 2000.

- Lennard, L. The clinical pharmacology of 6-mercaptopurine. *European Journal of Clinical Pharmacology* 43:329-339, 1992.
- Lennard, L. Clinical implications of thiopurine methyltransferase--optimization of drug dosage and potential drug interactions. *Therapeutic Drug Monitoring* 20:527-531, 1998.
- Lichtiger, S. Cyclosporine therapy in inflammatory bowel disease: open-label experience. *Mount Sinai Journal of Medicine* 57:315-319, 1990.
- Lichtiger, S. and Present, D.H. Preliminary report: cyclosporin in treatment of severe active ulcerative colitis. *Lancet* 336:16-19, 1990.
- Lichtiger, S., Present, D.H., Kornbluth, A., et al. Cyclosporine in severe ulcerative colitis refractory to steroid therapy. *New England Journal of Medicine* 330:1841-1845, 1994.
- Ligumsky, M., Simon, P.L., Karmeli, F. and Rachmilewitz, D. Role of interleukin 1 in inflammatory bowel disease--enhanced production during active disease. *Gut* 31:686-689, 1990.
- Lionetti, P., Pazzaglia, A., Moriondo, M., et al. Differing patterns of transforming growth factor-beta expression in normal intestinal mucosa and in active celiac disease. *Journal of Pediatric Gastroenterology & Nutrition* 29:308-313, 1999.
- Lochs, H., Mayer, M., Fleig, W.E., et al. Prophylaxis of postoperative relapse in Crohn's disease with mesalamine: European Cooperative Crohn's Disease Study VI. *Gastroenterology* 118:264-273, 2000.
- Lochs, H., Steinhardt, H.J., Klaus-Wentz B., et al. Comparison of Enteral Nutrition and Drug Treatment in Active Crohn's Disease. *Gastroenterology* 101:881-888, 1991.
- Lochsen, T., Ormstad, H., Braud, H., Brodal, B., Christiansen, E.N. and Osmundsen, H. Effects of fish oil and n-3 fatty acids on the regulation of delta9-fatty acid desaturase mRNA and -activity in rat liver. *Lipids* 34 Suppl:S221-S222, 1999.
- Loeschke, K., Ueberschaer, B., Pietsch, A., et al. n-3 fatty acids only delay early relapse of ulcerative colitis in remission. *Digestive Diseases & Sciences* 41:2087-2094, 1996.
- Lofberg, R., Danielsson, A., Suhr, O., et al. Oral budesonide versus prednisolone in patients with active extensive and left-sided ulcerative colitis. *Gastroenterology* 110:1713-1718, 1996.
- Lofberg, R., Ostergaard, T.O., Langholz, E., et al. Budesonide versus prednisolone retention enemas in active distal ulcerative colitis. *Alimentary Pharmacology & Therapeutics* 8:623-629, 1994.
- Lofberg, R., Rutgeerts, P., Malchow, H., et al. Budesonide prolongs time to relapse in

- ileal and ileocaecal Crohn's disease. A placebo controlled one year study. *Gut* 39:82-86, 1996.
- Lorenz-Meyer, H., Bauer, P., Nicolay, C., et al. Omega-3 fatty acids and low carbohydrate diet for maintenance of remission in Crohn's disease. A randomized controlled multicenter trial. Study Group Members (German Crohn's Disease Study Group). *Scandinavian Journal of Gastroenterology* 31:778-785, 1996.
- Lorenz, R., Weber, P.C., Szimnau, P., Heldwein, W., Strasser, T. and Loeschke, K. Supplementation with n-3 fatty acids from fish oil in chronic inflammatory bowel disease--a randomized, placebo-controlled, double-blind cross-over trial. *Journal of Internal Medicine Supplement*. 225:225-232, 1989.
- Loser, C., Eisel, A., Harms, D. and Folsch, U.R. Dietary polyamines are essential luminal growth factors for small intestinal and colonic mucosal growth and development. *Gut* 44:12-16, 1999.
- Louvet, B., Buisine, M.P., Desreumaux, P., et al. Transdermal nicotine decreases mucosal IL-8 expression but has no effect on mucin gene expression in ulcerative colitis. *Inflammatory Bowel Diseases* 5:174-181, 1999.
- Lowry, P.W., Weaver, A.L., Tremaine, W.J. and Sandborn, W.J. Combination therapy with oral tacrolimus (FK506) and azathioprine or 6-mercaptopurine for treatment-refractory Crohn's disease perianal fistulae. *Inflammatory Bowel Diseases* 5:239-245, 1999.
- Mack, D.R., Young, R., Kaufman, S.S., Ramey, L. and Vanderhoof, J.A. Methotrexate in patients with Crohn's disease after 6-mercaptopurine. *Journal of Pediatrics* 132:830-835, 1998.
- Madsen, S.M., Schlichting, P., Davidsen, B., et al. An open-labeled, randomized study comparing systemic interferon-alpha-2A and prednisolone enemas in the treatment of left-sided ulcerative colitis. *American Journal of Gastroenterology* 96:1807-1815, 2001.
- Mahadevan, U., Tremaine, W.J., Johnson, T., et al. Intravenous azathioprine in severe ulcerative colitis: a pilot study. *American Journal of Gastroenterology* 95:3463-3468, 2000.
- Mahida, Y.R., Lamming, C.E., Gallagher, A., Hawthorne, A.B. and Hawkey, C.J. 5-Aminosalicylic acid is a potent inhibitor of interleukin 1 beta production in organ culture of colonic biopsy specimens from patients with inflammatory bowel disease. *Gut* 32:50-54, 1991.
- Malchow H, Ewe, K., Brandes JW, et al. European Cooperative Crohn's Disease Study (ECCDS): results of drug treatment. *Gastroenterology* 86:249-66, 1984.
- Male David, Cooke Anne, Owen Michael, Trowsdale John and Champion Brian. Cytokines that modulate inflammation. In: *Advanced Immunology*, 1996,

- Mansfield, J.C., Giaffer, M.H. and Holdsworth, C.D. Controlled trial of oligopeptide versus amino acid diet in treatment of active Crohn's disease. *Gut* 36:60-66, 1995.
- Mantzaris, G.J., Hatzis, A., Kontogiannis, P. and Triadaphyllou, G. Intravenous tobramycin and metronidazole as an adjunct to corticosteroids in acute, severe ulcerative colitis. *American Journal of Gastroenterology* 89:43-46, 1994.
- Mantzaris, G.J., Petraki, K., Archavlis, E., et al. A prospective randomized controlled trial of intravenous ciprofloxacin as an adjunct to corticosteroids in acute, severe, ulcerative colitis. *Scandinavian Journal of Gastroenterology* 36:971-974, 2001.
- Markowitz, J., Grancher, K., Kohn, N., Lesser, M. and Daum, F. A multicenter trial of 6-mercaptopurine and prednisone in children with newly diagnosed Crohn's disease. *Gastroenterology* 119:895-902, 2000.
- Marnett, L.J. Cyclooxygenase mechanisms. *Current Opinion in Chemical Biology* 4:545-552, 2000.
- Marshall, J.K. and Irvine, E.J. Rectal corticosteroids versus alternative treatments in ulcerative colitis: a meta-analysis. *Gut* 40:775-781, 1997.
- Mayer, L. Mucosal immunity and gastrointestinal antigen processing. *Journal of Pediatric Gastroenterology & Nutrition* 30 Suppl: S4-12, 2000.
- McAlindon, M.E., Hawkey, C.J. and Mahida, Y.R. Expression of interleukin 1 beta and interleukin 1 beta converting enzyme by intestinal macrophages in health and inflammatory bowel disease. *Gut* 42:214-219, 1998.
- McCartney, S. and Ballinger, A. Growth failure in inflammatory bowel disease. *Nutrition* 15:169-171, 1999.
- McClane, S.J. and Rombeau, J.L. Cytokines and inflammatory bowel disease: *Journal of Parenteral & Enteral Nutrition* 23:S20-S24, 1999.
- McCoy, K.L. Programmed B and T cell development. *Nutrition Reviews* 56:S19-S26, 1998.
- McGee, D.W., Beagley, K.W., Aicher, W.K. and McGhee, J.R. Transforming growth factor-beta enhances interleukin-6 secretion by intestinal epithelial cells. *Immunology* 77:7-12, 1992.
- McIntosh GH, Royle PJ, Le Leu RK, et al. Whey proteins as Functional Food ingredients? *International Dairy Journal* 8:434, 1998.
- McMurray, D.N., Jolly, C.A. and Chapkin, R.S. Effects of Dietary n-3 Fatty Acids on T Cell Activation and T Cell Receptor-Mediated Signalling in a Murine Model. *The Journal of Infectious Diseases* 182:103-107, 2000.

- Meister, D., Bode, J. and Ghosh, S. TGF- β 1 expression is upregulated in Crohn's disease (CD) but not in ulcerative colitis (UC) mucosa after incubation with elemental diet-whey enriched with TGF- β (EWT) and elemental diet - colostrum (ECO). *Gut* 53:2002b.
- Meister, D., Bode, J., Shand, A. and Ghosh, S. Anti-inflammatory effect of enteral diet components on Crohn's disease affected tissue in-vitro. *Digestive and Liver Disease* 34: 430-8, 2002a
- Messori, A., Brignola, C., Trallori, G., et al. Effectiveness of 5-aminosalicylic acid for maintaining remission in patients with Crohn's disease: a meta-analysis. *American Journal of Gastroenterology* 89:692-698, 1994.
- Meydani, S.N. and Dinarello, C.A. Influence of dietary fatty acids on cytokine production and its clinical implications. *Nutrition in Clinical Practice* 8:65-72, 1993.
- Miehlsler, W., Reinisch, W., Moser, G., Gangl, A. and Vogelsang, H. Is mycophenolate mofetil an effective alternative in azathioprine-intolerant patients with chronic active Crohn's disease? *American Journal of Gastroenterology* 96:782-787, 2001.
- Monteleone, G., Kumerova, A., Croft, N.M., McKenzie, C., Steer, H.W. and MacDonald T.T. Blocking Smad 7 restores TGF- β 1 signaling in chronic inflammatory disease. *The Journal of Clinical Investigation* 108:601-609, 2001.
- Moum, B., Ekbo, A., Vatn, M.H., et al. Clinical course during the 1st year after diagnosis in ulcerative colitis and Crohn's disease. Results of a large, prospective population-based study in southeastern Norway, 1990-93. *Scandinavian Journal of Gastroenterology* 32:1005-1012, 1997.
- Mueller, S.D. *Ernaehrung bei chronischen entzuendlichen Darmerkrankungen*, Freiburg, Germany:2001. Ed.19 pp. 3-58.
- Munkholm, P., Langholz, E., Davidsen, M. and Binder, V. Frequency of glucocorticoid resistance and dependency in Crohn's disease. *Gut* 35:360-362, 1994.
- Murch, S.H. and Walker-Smith, J.A. Nutrition in inflammatory bowel disease. *Baillieres Clinical Gastroenterology* 12:719-738, 1998.
- Myrvik, Q.N. Immunology and Nutrition. In: *Modern Nutrition in health and disease*, edited by Shils, M.E., Olson, J.A. and Shike, M. Philadelphia, Baltimore, Hong Kong, London, Munich, Sydney, Tokio: Lea & Febiger, 1994, p. 623-656.
- Nagura, H., Ohtani, H., Sasano, H. and Matsumoto, T. The Immuno-Inflammatory Mechanism for Tissue Injury in Inflammatory Bowel Disease and Helicobacter pylori-infected Chronic Active Gastritis. *Digestion* 63:12-21, 2001.
- Nakamura, M., Saito, H., Kasanuki, J., Tamura, Y. and Yoshida, S. Cytokine production in patients with inflammatory bowel disease. *Gut* 33:933-937, 1992.

- Neurath, M.F., Wanitschke, R., Peters, M., Krummenauer, F., Meyer, z.B.K. and Schlaak, J.F. Randomised trial of mycophenolate mofetil versus azathioprine for treatment of chronic active Crohn's disease. *Gut* 44:625-628, 1999.
- Nicoletti, C. Unsolved mysteries of intestinal M cells. *Gut* 47:735-739, 2000.
- Nielsen, O.H., Ahnfelt-Ronne, I. and Elmgreen, J. Abnormal metabolism of arachidonic acid in chronic inflammatory bowel disease: enhanced release of leucotriene B4 from activated neutrophils. *Gut* 28:181-185, 1987.
- Nieto, N., Fernandez, M.I., Torres, M.I., Rios, A., Suarez, M.D. and Gil, A. Dietary monounsaturated n-3 and n-6 long-chain polyunsaturated fatty acids affect cellular antioxidant defense system in rats with experimental ulcerative colitis induced by trinitrobenzene sulfonic acid. *Digestive Diseases & Sciences* 43:2676-2687, 1998.
- Nikolaus, S., Raedler, A., Kuhbacker, T., Sfikas, N., Folsch, U.R. and Schreiber, S. Mechanisms in failure of infliximab for Crohn's disease. *Lancet* 356:1475-1479, 2000.
- O'Donoghue, D.P., Dawson, A.M., Powell-Tuck, J., Bown, R.L. and Lennard-Jones, J.E. Double-blind withdrawal trial of azathioprine as maintenance treatment for Crohn's disease. *Lancet* 2:955-957, 1978.
- Ortiz, V., Nos, P., Aguilera, V., Berenguer, J. and Ponce, J. Cyclosporin in an oral microemulsion as the initial treatment of corticoid-refractory ulcerative colitis. *Gastroenterologia y Hepatologia* 23:120-122, 2000.
- Pacheco, S., Hillier, K. and Smith, C. Increased arachidonic acid levels in phospholipids of human colonic mucosa in inflammatory bowel disease. *Clinical Science* 74:361-364, 1987.
- Panes, J., Esteve, M., Cabre, E., et al. Comparison of heparin and steroids in the treatment of moderate and severe ulcerative colitis. *Gastroenterology* 119:903-908, 2000.
- Papa, A., Danese, S., Gasbarrini, A. and Gasbarrini, G. Review article: potential therapeutic applications and mechanisms of action of heparin in inflammatory bowel disease. *Alimentary Pharmacology & Therapeutics* 14:1403-1409, 2000.
- Papadakis, K.A. and Targan, S.R. Role of cytokines in the pathogenesis of inflammatory bowel disease. *Annual Review of Medicine* 51:289-298, 2000.
- Papi, C., Luchetti, R., Gili, L., Montanti, S., Koch, M. and Capurso, L. Budesonide in the treatment of Crohn's disease: a meta-analysis. *Alimentary Pharmacology & Therapeutics* 14:1419-1428, 2000.
- Patz, J., Jacobsohn, W.Z., Gottschalk-Sabag, S., Zeides, S. and Braverman, D.Z. Treatment of refractory distal ulcerative colitis with short chain fatty acid enemas. *American Journal of Gastroenterology* 91:731-734, 1996.

- Pearson, D.C., May, G.R., Fick, G. and Sutherland, L.R. Azathioprine for maintaining remission of Crohn's disease. *Cochrane Database of Systematic Reviews* [computer file] CD0000672000.
- Pearson, M., Teahon, K., Levi, A.J. and Bjarnason, I. Food intolerance and Crohn's disease; *Gut* 34:783-787, 1993.
- Pinna-Pintor, M., Arese, P., Bona, R., et al. Severe steroid-unresponsive ulcerative colitis: outcomes of restorative proctocolectomy in patients undergoing cyclosporin treatment. *Diseases of the Colon & Rectum* 43:609-613, 2000.
- Planchon, S., Fiocchi, C., Takafuji, V. and Roche, J.K. Transforming growth factor-beta1 preserves epithelial barrier function: identification of receptors, biochemical intermediates, and cytokine antagonists. *Journal of Cellular Physiology* 181:55-66, 1999.
- Planchon, S.M., Martins, C.A., Guerrant, R.L. and Roche, J.K. Regulation of intestinal epithelial barrier function by TGF-beta 1. Evidence for its role in abrogating the effect of a T cell cytokine. *Journal of Immunology* 153:5730-5739, 1994.
- Playford, R.J. Peptides and gastrointestinal mucosal integrity. *Gut* 36:1995.
- Playford, R.J., Floyd, D.N., Macdonald, C.E., et al. Bovine colostrum is a health food supplement which prevents NSAID induced gut damage. *Gut* 44:653-658, 1999.
- Playford, R.J., Macdonald, C.E. and Johnson, W. Colostrum and milk-derived peptide growth factors for the treatment of gastrointestinal disorders. *American Journal of Clinical Nutrition* 72:5-14, 2000.
- Playford, R.J., Woodmann, A.C., Clark, P., et al. Effects of luminal growth factor preservation on intestinal growth. *Lancet* 341:843-848, 1993.
- Pompeia, C., Lopes, L.R., Miyasaka, C.K., Procopio, J., Sannomiya, P. and Curi, R. Effect of fatty acids on leukocyte function. *Brazilian Journal of Medical & Biological Research* 33:1255-1268, 2000.
- Porter, S.N., Howarth, G.S. and Butler, R.N. An orally administered growth factor extract derived from bovine whey suppresses breath ethane in colitic rats. *Scandinavian Journal of Gastroenterology* 33:967-974, 1998.
- Potten CS, Keller M, Roberts SA, Rew DA and Wilson GD. Measurement of in vivo proliferation in human colorectal mucosa using bromodeoxyuridine. *Gut* 33:71-78, 1992.
- Potten, C.S., Kellett, M., Rew, D.A., Roberts and SA. Proliferation in human gastrointestinal epithelium using bromodeoxyuridine in vivo: data for different sites, proximity to a tumour, and polyposis colitis. *Gut* 33:524-529, 1992.
- Present, D.H., Korelitz, B.I., Wisch, N., Glass, J.L., Sachar, D.B. and Pasternack, B.S. Treatment of Crohn's disease with 6-mercaptopurine. A long-term, randomized,

- double-blind study. *New England Journal of Medicine* 302:981-987, 1980.
- Present, D.H., Rutgeerts, P., Targan, S., et al. Infliximab for the treatment of fistulas in patients with Crohn's disease. *New England Journal of Medicine* 340:1398-1405, 1999.
- Pritchett, C.J., Senior, P.V., Sunter, J.P., et al. Cell proliferation in human colorectal mucosa in organ culture: the early adaptive changes. *Journal of Anatomy* 141:171-179, 1985.
- Pullan, R.D., Rhodes, J., Ganesh, S., et al. Transdermal nicotine for active ulcerative colitis. *New England Journal of Medicine* 330:811-815, 1994.
- Rampton D.S. and Shanahan, F. *Inflammatory Bowel Disease*, Fine Print, Oxford UK, 2000. pp. 5-99.
- Raouf, A.H., Hildrey, V., Daniel, J., et al. Enteral feeding as sole treatment for Crohn's disease: controlled trial of whole protein v amino acid based feed and a case study of dietary challenge. *Gut* 32:702-707, 1991.
- Reimund JM, Wittersheim C, Dumont S, et al. Increased production of tumor necrosis factor-alpha interleukin-1beta, and interleukin-6 by morphologically normal intestinal biopsies from patients with Crohn's disease. *Gut* 39:684-689, 1996.
- Rhodes, J., Bainton, D., Beck, P. and Campbell, H. Controlled trial of azathioprine in Crohn's disease. *Lancet* 2:1273-1276, 1971.
- Ricart E, Panaccione R, Loftus, E.V., Tremaine, W.J. and Sandborn, W.J. Infliximab for Crohn` disease in clinical practice at the Mayo Clinic: the first 100 patients. *American Journal of Gastroenterology* 96:122-9, 2001.
- Ricart, E., Panaccione, R., Loftus, E.V., Tremaine, W.J. and Sandborn, W.J. Successful management of Crohn's disease of the ileoanal pouch with infliximab. *Gastroenterology* 117:429-432, 1999.
- Rigaud, D., Cosnes, J., Le Quintrec, Y., Rene, E., Gendre, J.P. and Mignon, M. Controlled trial comparing two types of enteral nutrition in treatment of active Crohn's disease: elemental versus polymeric diet. *Gut* 32:1492-1497, 1991.
- Roberts, W.G., Simon, T.J., Berlin, R.G., et al. Leukotrienes in ulcerative colitis: results of a multicenter trial of a leukotriene biosynthesis inhibitor, MK-591. *Gastroenterology* 112:725-732, 1997.
- Roe, D.J., Alberts, D.S., Wargovich, M.J., et al. Reproducibility of the measurement of colonic proliferation using bromodeoxyuridine across laboratories. *Cancer Epidemiology, Biomarkers & Prevention* 5:349-353, 1996.
- Roediger, W.E., Duncan A, Kapaniris O and Millard S. Sulphide impairment of substrate oxidation in rat colonocytes: a biochemical basis for ulcerative colitis? *Clinical Science (London)* 85:623-7, 1993.

- Roediger, W.E., Heyworth M, Willoughby P, Piris J, Moore A and Truelove S.C. Luminal ions and short chain fatty acids as markers of functional activity of the mucosa in ulcerative colitis. *Journal of Clinical Pathology* 35:323-6, 1982.
- Rogler, G. and Andus, T. Cytokines in inflammatory bowel disease. *World Journal of Surgery* 22:382-389, 1998.
- Rose, D.P. and Connolly, J.M. Omega-3 fatty acids as cancer chemopreventive agents. *Pharmacology & Therapeutics* 83:217-244, 1999.
- Rosenberg, J.L., Levin, B., Wall, A.J. and Kirsner, J.B. A controlled trial of azathioprine in Crohn's disease. *American Journal of Digestive Diseases* 20:721-726, 1975.
- Rosenberg, J.L., Wall, A.J., Levin, B., Binder, H.J. and Kirsner, J.B. A controlled trial of azathioprine in the management of chronic ulcerative colitis. *Gastroenterology* 69:96-99, 1975.
- Ross, E. The role of marine fish oils in the treatment of ulcerative colitis. *Nutrition Reviews* 51:47-49, 1993.
- Royuela M, D.M.P.B.F.S.-C.M.F.B. and Paniagua R. Transforming growth factor beta 1 and its receptor types I and II. Comparison in human normal prostate, benign prostatic hyperplasia, and prostatic carcinoma. *Growth Factors* 16:101-110, 1998.
- Ruppin H.Bar-Meir, Soergel, K., Wood, C. and Schmitt, M. Absorption of short-chain fatty acids by the colon. *Gastroenterology* 78:1500-7, 1980.
- Rutgeerts, P., D'Haens, G., Targan, S., et al. Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (infliximab) to maintain remission in Crohn's disease. *Gastroenterology* 117:761-769, 1999.
- Rutgeerts, P., Lofberg, R., Malchow H, et al. A comparison of budesonide with prednisolone for active Crohn's disease. *New England Journal of Medicine* 331:842-5, 1994.
- Saito, S., Yoshida, M., Ichijo, S. and Tsujii, T. Transforming growth factor-beta (TGF- β) in human milk. *Clinical & Experimental Immunology* 94:220-224, 1993.
- Sammon, A.M. Dietary linoleic acid, immune inhibition and disease. *Postgraduate Medical Journal* 75:129-132, 1999.
- Sandborn, W.J. Preliminary report on the use of oral tacrolimus (FK506) in the treatment of complicated proximal small bowel and fistulizing Crohn's disease. *American Journal of Gastroenterology* 92:876-879, 1997.
- Sandborn, W.J., Feagan, B.G., Hanauer, S.B., et al. An engineered human antibody to TNF (CDP571) for active Crohn's disease: a randomized double-blind placebo-controlled trial. *Gastroenterology* 120:1330-1338, 2001.

- Sandborn, W.J., Tremaine, W.J., Leighton, J.A., et al. Nicotine tartrate liquid enemas for mildly to moderately active left-sided ulcerative colitis unresponsive to first-line therapy: a pilot study. *Alimentary Pharmacology & Therapeutics* 11:663-671, 1997.
- Sandborn, W.J., Tremaine, W.J., Offord, K.P., et al. Transdermal nicotine for mildly to moderately active ulcerative colitis. A randomized, double-blind, placebo-controlled trial. *Annals of Internal Medicine* 126:364-371, 1997.
- Sandborn, W.J., Tremaine, W.J., Schroeder, K.W., et al. A placebo-controlled trial of cyclosporine enemas for mildly to moderately active left-sided ulcerative colitis. *Gastroenterology* 106:1429-1435, 1994.
- Sandborn, W.J., Tremaine, W.J., Schroeder, K.W., Steiner, B.L., Batts, K.P. and Lawson, G.M. Cyclosporine enemas for treatment-resistant, mildly to moderately active, left-sided ulcerative colitis. *American Journal of Gastroenterology* 88:640-645, 1993.
- Sandborn, W.J., Tremaine, W.J., Wolf, D.C., et al. Lack of effect of intravenous administration on time to respond to azathioprine for steroid-treated Crohn's disease. North American Azathioprine Study Group. *Gastroenterology* 117:527-535, 1999.
- Sands, B.E., Bank, S., Sninsky, C.A., et al. Preliminary evaluation of safety and activity of recombinant human interleukin 11 in patients with active Crohn's disease. *Gastroenterology* 117:58-64, 1999.
- Sands, B.E. and Podolsky, D.K. New life in a sleeper: thalidomide and Crohn's disease. *Gastroenterology* 117:1485-1488, 1999.
- Sands, B.E., Tremaine, W.J., Sandborn, W.J., et al. Infliximab in the treatment of severe, steroid-refractory ulcerative colitis: a pilot study. *Inflammatory Bowel Diseases* 7:83-88, 2001.
- Santos, J., Baudet, S., Casellas, F., Guarner, L., Vilaseca, J. and Malagelada, J.R. Efficacy of intravenous cyclosporine for steroid refractory attacks of ulcerative colitis. *Journal of Clinical Gastroenterology* 20:285-289, 1995.
- Saverymattu, S.H. Clinical remission in Crohn's disease--assessment using faecal 111In granulocyte excretion. *Digestion* 33:74-79, 1986.
- Schauber J, Bark T, Jaramillo E, Katouli M, Sandstedt B and Svenberg T. Local short-chain fatty acids supplementatin without beneficial effect on inflammation in excluded rectum. *Scandinavian Journal of Gastroenterology* 35:184-9, 2000.
- Scheppach, W. Treatment of distal ulcerative colitis with short-chain fatty acid enemas. A placebo-controlled trial. German-Austrian SCFA Study Group. *Digestive Diseases & Sciences* 41:2254-2259, 1996.
- Scheppach, W., Muller JG, Boxberger F, et al. Histological changes in the colonic

- mucosa following irrigation with short-chain fatty acids. *European Journal of Gastroenterology & Hepatology* 9:163-8, 1997.
- Schreiber, S., Fedorak, R.N., Nielsen, O.H., et al. Safety and efficacy of recombinant human interleukin 10 in chronic active Crohn's disease. Crohn's Disease IL-10 Cooperative Study Group. *Gastroenterology* 119:1461-1472, 2000.
- Schreiber, S., Heinig, T., Thiele, H.G. and Raedler, A. Immunoregulatory role of interleukin 10 in patients with inflammatory bowel disease. *Gastroenterology* 108:1434-1444, 1995.
- Schreiber, S., Nikolaus, S., Malchow, H., et al. Absence of efficacy of subcutaneous antisense ICAM-1 treatment of chronic active Crohn's disease. *Gastroenterology* 120:1339-1346, 2001.
- Segain, J.P., Raingeard, d.l.B., Bourreille, A., et al. Butyrate inhibits inflammatory responses through NF κ B inhibition: implications for Crohn's Disease. *Gut* 47:397-403, 2000.
- Senior, P.V., Pritchett, C.J., Sunter, J.P., Appleton, D.R., Watson and AJ. Crypt regeneration in adult human colonic mucosa during prolonged organ culture. *Journal of Anatomy* 134:459-469, 1982.
- Seo, M., Okada, M., Yao, T., Furukawa, H. and Matake, H. The role of total parenteral nutrition in the management of patients with acute attacks of inflammatory bowel disease. *Journal of Clinical Gastroenterology* 29:270-275, 1999.
- Serizawa, H., Miura, S., Tashiro, H., et al. Alteration of mucosal immunity after long-term ingestion of an elemental diet in rats. *Journal of Parenteral & Enteral Nutrition* 18:141-147, 1994.
- Serrano, MS., Schmidt-Sommerfeld, E., Kilbaugh, TJ., Brown, RF., Udall, J. and Mannick, EE. Use of infliximab in pediatric patients with inflammatory bowel disease. *Annals of Pharmacotherapy* 35:823-828, 2001.
- Shah, N. Effects of milk derived bioactivity: an overview. *British Journal of Nutrition* 84: S3-S. 10, 2000.
- Shike, M. Enteral Feeding. In: *Modern Nutrition in health and disease*, edited by Shils, M.E., Olson, J.A. and Shike, M. Malvern, PA: Lea & Febiger, 1994, p. 1417-1427.
- Shivananda S, Lennard-Jones J, Logan R, et al. Incidence of inflammatory bowel disease across Europe: is there a difference between north and south ? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD). *Gut* 39:690-7, 1996.
- Silbernagel A and Despopoulos A. Endokrines System und Hormone. In: *Taschenatlas der Physiologie*, Georg Thieme Verlag, Stuttgart, 1991, p. 242-244.

- Simms, L and Steinhart, A.H. Budesonide for maintenance of remission in Crohn's disease. *Cochrane Database Systemic Reviews* 1:2001.
- Simopoulos, A.P. Commentary on the workshop statement. Essentiality of and recommended dietary intakes for Omega-6 and Omega-3 fatty acids. *Prostaglandins Leukotrienes & Essential Fatty Acids* 63:123-124, 2000.
- Simopoulos, A.P., Leaf, A. and Salem, N., Jr. Workshop statement on the essentiality of and recommended dietary intakes for Omega-6 and Omega-3 fatty acids. *Prostaglandins Leukotrienes & Essential Fatty Acids* 63:119-121, 2000.
- Sinha, A., Nightingale, J., West, K., Berlangue-Acosta J and Playford, R.J. Can left-sided ulcerative colitis be treated with epidermal growth factor enemas? *Gut* 1:A1-2001
- Slonim, A.E., Bulone, L., Damore, M.B., Goldberg, T., Wingertzahn, M.A. and McKinley, M.J. A preliminary study of growth hormone therapy for Crohn's disease. *New England Journal of Medicine* 342:1633-1637, 2000.
- Steijns JM and van Hooijdonk ACM. Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. *British Journal of Nutrition* 84:11-17, 2000.
- Steinhart, A.H., Brzezinski, A. and Baker, J.P. Treatment of refractory ulcerative colitis proctosigmoiditis with butyrate enemas. *American Journal of Gastroenterology* 89:179-83, 1994.
- Steinhart, A.H., Ewe, K., Griffiths, A.M., Modigliani, R. and Thomsen, O.O. Corticosteroids for maintaining remission of Crohn's disease. *Cochrane Database of Systematic Reviews [computer file]* CD0003012000.
- Steinhart, A.H., Hemphill, D. and Greenberg, G.R. Sulfasalazine and mesalazine for the maintenance therapy of Crohn's disease: a meta-analysis. *American Journal of Gastroenterology* 89:2116-2124, 1994.
- Steinhart, A.H., Hiruki, T., Brzezinski, A. and Baker, J.P. Treatment of left-sided ulcerative colitis with butyrate enemas: a controlled trial. *Alimentary Pharmacology & Therapeutics* 10:729-736, 1996.
- Stenson, W.F., Cort, D., Rodgers, J., et al. Dietary supplementation with fish oil in ulcerative colitis. *Annals of Internal Medicine* 116:609-614, 1992.
- Strugala, V., Allen, A., Dettmar, P.W., Hainsworth P.J., Hudson, M. and Pearson, J. Thickness and continuity of human colonic mucosal layer is decreased in active UC but remains normal in quiescent UC. *Gut* 48:A832001.
- Sudlow, A. and Wilde, C.B.R. Transforming growth factor - beta 1 inhibits casein secretion from differentiating mammary-gland explants but not from lactating mammary cells. *Biochemistry Journal* 1:333-336, 1994.

- Sugihara, H., Hattori, T. and Fukuda, M. Immunohistochemical detection of bromodeoxyuridine in formalin-fixed tissues. *Histochemistry* 85:193-195, 1986.
- Summers, R.W., Switz, D.M., Sessions, J.T., et al. National Cooperative Crohn's Disease Study: results of drug treatment. *Gastroenterology* 77:847-869, 1979.
- Sutherland, L.R., May, G.R. and Shaffer, E.A. Sulfasalazine revisited: a meta-analysis of 5-aminosalicylic acid in the treatment of ulcerative colitis. *Annals of Internal Medicine* 118:540-549, 1993.
- Targan, S.R., Hanauer, S.B., Van Deventer, S.J., et al. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *New England Journal of Medicine* 337:1029-1035, 1997.
- Taylor, V., Goddard, C. and Read, L. A milk growth factor extract reduces chemotherapeutic drug toxicity in epithelial cells in vitro. *In Vitro Cell Development Biology in Animals* 37:310-318, 2001.
- Teahon, K., Bjarnason, I., Pearson, M. and Levi, A.J. Ten years' experience with an elemental diet in the management of Crohn's disease. *Gut* 31:1133-1137, 1990.
- Teahon, K., Pearson, M., Smith, T. and Bjarnason, I. Alterations in nutritional status and disease activity during treatment of Crohn's disease with elemental diet. *Scandinavian Journal of Gastroenterology* 30:54-60, 1995.
- Teahon, K., Smethurst, P., Pearson, M., Levi, A.J. and Bjarnason, I. The effect of elemental diet on intestinal permeability and inflammation in Crohn's disease. *Gastroenterology* 101:84-89, 1991.
- Thomas, G.A., Rhodes, J., Mani, V., et al. Transdermal nicotine as maintenance therapy for ulcerative colitis. *New England Journal of Medicine* 332:988-992, 1995.
- Thomas, G.A., Rhodes, J., Ragunath, K., et al. Transdermal nicotine compared with oral prednisolone therapy for active ulcerative colitis. *European Journal of Gastroenterology & Hepatology* 8:769-776, 1996.
- Thomas, G.A., Swift, G.L., Green, J.T., et al. Controlled trial of antituberculous chemotherapy in Crohn's disease: a five year follow up study. *Gut* 42:497-500, 1998.
- Thomsen, O.O., Cortot, A., Jewell, D., et al. A comparison of budesonide and mesalamine for active Crohn's disease. International Budesonide-Mesalamine Study Group. [see comments]. *New England Journal of Medicine* 339:370-374, 1998.
- Tidd DM and Paterson, A. Distinction between inhibition of purine nucleotide synthesis and the delayed cytotoxic reaction of 6-mercaptopurine. *Cancer Research* 34:733-7, 1974.

- Timmer, A., Breuer-Katschinski, B. and Goebell, H. Time trends in the incidence and disease location of Crohn's disease 1980-1995: a prospective analysis in an urban population in Germany. *Inflammatory Bowel Diseases* 5:79-84, 1999.
- Trallori, G. and Messori, A. Drug treatments for maintaining remission in Crohn's disease. A lifetime cost-utility analysis. *Pharmacoeconomics* 11:444-453, 1997.
- Treem, W.R., Ahsan N, Kastoff G and Hyams, J.S. Fecal short-chain fatty acids in patients with diarrhea-predominant irritable bowel syndrome: in vitro studies of carbohydrate Fecal short-chain fatty acids in patients with diarrhea-predominant irritable bowel syndrome: in vitro studies of fermentation. *Journal of Pediatric Gastroenterology & Nutrition* 23:280-6, 1996.
- van Beers-Schreurs, H.M., Nabuurs, M.J., Vellenga, L., Wensing, T. and Breukink, H.J. Role of the large intestine in the pathogenesis of diarrhea in weaned pigs. *American Journal of Veterinary Research* 59:696-703, 1998.
- van Dullemen, H.M., Van Deventer, S.J., Hommes, D.W., et al. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). *Gastroenterology* 109:129-135, 1995.
- van Hees, P.A., van, E., PH, van Lier, H.J. and van Tongeren, J.H. An index of inflammatory activity in patients with Crohn's disease. *Gut* 21:279-286, 1980.
- Vasiliauskas, E.A., Kam, L.Y., Abreu-Martin, M.T., et al. An open-label pilot study of low-dose thalidomide in chronically active, steroid-dependent Crohn's disease. *Gastroenterology* 117:1278-1287, 1999.
- Vasiliauskas, E.A., Kam, L.Y., Karp, L.C., Gaiennie, J., Yang, H. and Targan, S.R. Marker antibody expression stratifies Crohn's disease into immunologically homogeneous subgroups with distinct clinical characteristics. *Gut* 47:487-496, 2000.
- Venturi, A., Gionchetti, P., Rizzello, F., et al. Impact on the composition of the faecal flora by a new probiotic preparation: preliminary data on maintenance treatment of patients with ulcerative colitis. *Alimentary Pharmacology & Therapeutics* 13:1103-1108, 1999.
- Verma, S., Brown, S., Kirkwood, B. and Gaffer, M.H. Polymeric versus elemental diet as primary treatment in active Crohn's disease: a randomized, double-blind trial. *American Journal of Gastroenterology* 95:735-739, 2000.
- Verma, S., Kirkwood, B., Brown, S. and Gaffer, M.H. Oral nutritional supplementation is effective in the maintenance of remission in Crohn's disease. *Digestive & Liver Disease* 32:769-774, 2000.
- Vernia, P., Marcheggiano, A., Caprilli, R., et al. Short-chain fatty acid topical treatment in distal ulcerative colitis. *Alimentary Pharmacology & Therapeutics* 9:309-313, 1995.

- Wahl, S.M. Transforming growth factor beta: the good, the bad, and the ugly. *Journal of Experimental Medicine* 180:1587-1590, 1994.
- Wahl, S.M., Costa, G.L., Corcoran, M., Wahl, L.M. and Berger, A.E. Transforming growth factor-beta mediates IL-1-dependent induction of IL-1 receptor antagonist. *Journal of Immunology* 150:3553-3560, 1993.
- Wallace, F.A., Miles, E.A. and Calder, P.C. The effect of dietary fat on cytokine production by murine macrophages in different activation states. *Lipids* 34 Suppl:S1451999a.
- Wallace, F.A., Yaqoob, P., Miles, E.A. and Calder, P.C. Dietary fat influences the production of Th1- but not Th2-derived cytokines. *Lipids* 34 Suppl:S1411999b.
- Walmsley RS, Ayres RCS, Pounder RE and Allan RN. A simple clinical colitis activity index. *Gut* 43:29-32, 1998.
- Watanabe S., Katagiri K., Onozaki K., et al. Dietary docosahexaenoic acid but not eicosapentaenoic acid suppresses lipopolysaccharide-induced interleukin-1beta mRNA induction in mouse spleen leukocytes. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 62:147-152, 2000.
- Weinstein, T.A., Sciubba, J.J. and Levine, J. Thalidomide for the treatment of oral aphthous ulcers in Crohn's disease. *Journal of Pediatric Gastroenterology & Nutrition* 28:214-216, 1999.
- Whitney, E.N., Cataldo, C.B. and Rolfes, S.R. Fat and fatty acids. In: *Understanding Normal and Clinical Nutrition*, Thompson Publishing Group, 1998, p. 146-150.
- Willoughby, J.M., Beckett, J., Kumar, P.J. and Dawson, A.M. Controlled trial of azathioprine in Crohn's disease. *Lancet* 2:944-947, 1971.
- Wilschanski, M., Sherman, P., Pencharz, P., Davis, L., Corey, M. and Griffiths, A. Supplementary enteral nutrition maintains remission in paediatric Crohn's disease. *Gut* 38:543-548, 1996.
- Wilson, R.G., Smith, A.N. and Bird, C.C. Immunohistochemical detection of abnormal cell proliferation in colonic mucosa of subjects with polyps. *Journal of Clinical Pathology* 43:744-747, 1990.
- Wong, C.W., Seow, H.F., Liu, A.H., Husband, A.J., Smithers, G.W. and Watson, D.L. Modulation of immune responses by bovine beta-casein. *Immunology & Cell Biology* 74:323-329, 1996.
- Woodward, B. Protein, calories, and immune defenses. *Nutrition Reviews* 56:S84-S921998.
- Woywodt, A., Ludwig, D., Neustock, P., et al. Mucosal cytokine expression, cellular markers and adhesion molecules in inflammatory bowel disease. *European Journal of Gastroenterology & Hepatology* 11:267-276, 1999.

- Wright R. and Truelove S.C. A controlled Therapeutic Trial of Various Diets in Ulcerative Colitis. *British Medical Journal* 2:138-141, 1965.
- Wright, J.P., Winter, T.A., Candy, S. and Marks, I.S. Sucralfate and methylprednisolone enemas in active ulcerative colitis: a prospective, single-blind study. *Digestive Diseases & Sciences* 44:1899-1901, 1999.
- Wu, G. Intestinal mucosal amino acid catabolism. *Journal of Nutrition* 128:1249-1252, 1998.
- Wynford-Thomas, D. and Williams, E.D. Use of bromodeoxyuridine for cell kinetic studies in intact animals. *Cell & Tissue Kinetics* 19:179-182, 1986.
- Xian, C.J., Xu, X., Mardell, C.E., et al. Site-specific changes in transforming growth factor-alpha and -beta1 expression in colonic mucosa of adolescents with inflammatory bowel disease. *Scandinavian Journal of Gastroenterology* 34:591-600, 1999.
- Xu, D., Lu, Q. and Deitch, E.A. Elemental diet-induced bacterial translocation associated with systemic and intestinal immune suppression. *Journal of Parenteral & Enteral Nutrition* 22:37-41, 1998.
- Yang, V.W. Eicosanoids and inflammatory bowel disease. *Gastroenterology Clinics of North America* 25:317-332, 1996.
- Yaqoob, P., Knapper, J.A., Webb, D.H., Williams, C.M., Newsholme, E.A. and Calder, P.C. Effect of olive oil on immune function in middle-aged men. *American Journal of Clinical Nutrition* 67:129-135, 1998.
- Yuceyar, H., Ozutemiz, O., Huseyinov, A., et al. Is administration of n-3 fatty acids by mucosal enema protective against trinitrobenzene-induced colitis in rats? *Prostaglandins Leukotrienes & Essential Fatty Acids* 61:339-345, 1999.
- Ziboh, V.A., Miller, C.C. and Cho, Y. Metabolism of polyunsaturated fatty acids by skin epidermal enzymes: generation of antiinflammatory and antiproliferative metabolites. *American Journal of Clinical Nutrition* 71:361S-366S, 2000.

Published papers

Anti-inflammatory effects of enteral diet components on Crohn's disease-affected tissues in vitro

D. Meister
J. Bode
A. Shand
S. Ghosh

Background. The mechanism of action of elemental diet in Crohn's disease treatment, is unknown. Alteration of bacterial flora, low antigenicity, low fat content and improvement of nutritional status are postulated to play a role in the anti-inflammatory effect of elemental diet.

Aim. To determine whether elemental diet or its modifications has a direct anti-inflammatory effect on colonic tissue biopsies in vitro.

Patients and methods. Colonic or ileal biopsies from 39 patients with inflammatory bowel disease and control patients were incubated for 24 hours with enteral diets in which nitrogen sources were amino acids as in elemental diet, casein or whey. Tissues were incubated with elemental diet, casein or whey, at dilutions of 1:5, 1:10 or 1:20 in Waymouth's complete medium; a medium control was also included. Tissue viability was assessed by bromodeoxyuridine uptake. Interleukin-1 β , interleukin-1 receptor antagonist and interleukin-10 concentrations in supernatants were measured by immunoassay (enzyme-linked immunosorbent assay).

Results. Incubation of tissues from Crohn's disease with elemental diet resulted in an increase in the ratio of interleukin-1 receptor antagonist/interleukin-1 β vs control statistically significant at 1:10 (89.6 \pm 17 vs 45.7 \pm 9.1, $p < 0.05$). Incubation of Crohn's tissue with casein resulted in a significant increase of interleukin-1 receptor antagonist/interleukin-1 β ratio at dilutions 1:20, 1:10 and 1:5 (101.8 \pm 22.0, $p = 0.05$; 142.8 \pm 24.6, $p < 0.05$; 109.7 \pm 25.0, $p = 0.05$). In ulcerative colitis tissue and non-inflamed non-inflammatory bowel disease control tissue, no significant increase in interleukin 1 receptor antagonist/interleukin-1 β ratio was seen after incubation with elemental diet, casein and whey.

Conclusion. Elemental diet incubation increases anti-inflammatory:pro-inflammatory cytokine ratio in Crohn's disease and this anti-inflammatory effect is not specifically due to amino acid composition, as diets containing casein have similar anti-inflammatory effects.

From

Gastrointestinal Unit, Department of Medical Sciences, University of Edinburgh, Western General Hospital, Edinburgh, UK.

Address for correspondence

Dr. S. Ghosh, Imperial College School of Medicine, Hammersmith Hospital, Du Cane Road, London W12, 0NN, UK.
E-mail: s.ghosh@ic.ac.uk

Acknowledgements

Authors are grateful to S. Giffen of SHS International Ltd (SHS) Liverpool, UK for supplying specially prepared formula diets; to M. Aldhous for help with ELISA assay and reviewing paper; the endoscopy team and K. Kingstone for measuring osmolality.

Submitted September 4, 2001.

Accepted after revision January 7, 2002.

Part of study was presented at British Society of Gastroenterology Annual Conference, Birmingham 2000 (Gut 2000;46(Suppl 11):W42).

Digest Liver Dis 2002;34:430-8

Key words. Crohn's disease; cytokine interleukin-1 receptor antagonist/interleukin-1 β ratio; elemental diet; organ culture.

Introduction

Low nutritional intake is common in inflammatory bowel disease (IBD), and may result in a lack of essential nutrients and malnutrition¹. Nutritional therapy is not considered to be useful in inducing or maintaining remission in ulcerative colitis (UC), although a milk-free diet has been reported to be superior compared with a milk-containing diet which increased relapses². Nutritional therapy is more widely used and effective in Crohn's disease (CD), where it may improve disease activity³⁻⁵. The mechanism of

action is unknown but possible explanations, which have been proposed, include: alteration of bacterial flora, exclusion of protein antigens, enterocytic immune response and a low fat content^{5,6}.

Elemental diet (EAA) is a liquid chemical nutrition source where nitrogen is derived from free amino acids, the carbohydrate source is derived from simple digestible carbohydrates, and the fat source consists of triglycerides; vitamins, minerals and trace elements are added to provide a complete nutritional formula. EAA is a particularly popular treatment for paediatric CD¹⁷. In children with CD, supplemental feeding with EAA has been shown to maintain remission of disease activity, which suggests that EAA may not be merely an exclusion diet⁸. In adults, an improvement in clinical features and long-term remission in uncomplicated CD could be achieved by EAA³. Also in adult quiescent CD patients, supplemental elemental diet maintained remission, making it unlikely that elemental diet worked as an exclusion diet⁹.

Pro-inflammatory cytokines such as interleukin- (IL-) 1 β , tumour necrosis factor alpha (TNF- α)¹⁰ or indirectly induced cytokines such as IL-6, as well as anti-inflammatory cytokines¹¹, such as IL-1 receptor antagonist (IL-1ra), are altered in IBD¹². A significantly raised production of IL-1 β compared to controls was found in fresh and 24-hour cultured CD biopsy tissue¹³. A decreased ratio of IL-1ra/IL-1 β seems to be more common in IBD compared with controls and suggest the hypothesis that an imbalance in the IL-1ra/IL-1 β ratio may play an important role in IBD^{14,15}. IL-10 has an anti-proliferative effect on human intestinal lamina propria T-cells, and also induces IL-1ra secretion in peripheral monocytes and intestinal lamina propria mononuclear cells. It is also known to restore a decreased IL-1ra/IL-1 β in IBD to normal levels¹⁶.

In vivo, a commercial polymeric enteral diet is reported to have a down-regulatory effect on pro-inflammatory cytokines¹⁷. Data from our unit also showed a decrease in IL-1 β levels in gut lavage fluid after treatment with elemental diet¹. A crucial question is whether EAA is an exclusion diet or whether it possesses a direct anti-inflammatory effect. If the mechanism of action of EAA were better understood, defined formula diets may be improved further to help reduce inflammation.

The aim of this study was to determine whether EAA has a direct anti-inflammatory effect in vitro, using an organ culture model. The culture of colonic tissue has been described previously¹⁸⁻²⁰. With organ culture, it is possible to maintain the structure of the tissue, which is normally accompanied by a minimum outgrowth of the tissue and preserves the histological architecture for further investigations.

Patients and methods

Patients

Colonoscopic biopsy samples were collected from 39 patients, during 1998 and 1999. The patients required colonoscopy for medical reasons and consent was given prior to endoscopy. Patients with CD [n=16, mean age: 37 years (range 16-64), biopsy site: colon n=12, ileum n=4] and UC patients [n=12, mean age: 38 years, (range 22-55), biopsy site: colon]. In CD and UC, biopsies were taken from macroscopically affected areas and in control patients from the rectosigmoid region. The non-inflamed non-IBD control group [n=11, mean age: 48 years (range 20-78), biopsy site: rectosigmoid] was composed of patients with diagnoses such as irritable bowel syndrome, chronic constipation and incidental vascular malformations who had gastrointestinal symptoms but macroscopically normal intestine. When biopsies from a patient were taken for ED incubation, additional biopsies from the same patient were used for media control incubation. At time of biopsies, 11 out of 16 CD patients were on medications (2 = prednisolone, 4 = 5-ASA, 1 = azathioprine, 2 = prednisolone + 5-ASA, 2 = azathioprine and steroids); similarly in UC, 6 out of 12 patients were on medications (1 = prednisolone, 2 = 5-ASA, 2 = prednisolone + 5-ASA, 1 = prednisolone + azathioprine). The diagnoses were confirmed by characteristic radiological, endoscopic and histological features.

Elemental diet

EAA (E028 Extra) and preparations of comparable diets but with casein (EC) and whey (EW) as nitrogen sources were supplied by SHS International (Liverpool, UK). The nutritional profiles of the diets are shown in Tables I and II. The osmolality of the diluted elemental diets with distilled water and with Waymouth's medium (osmolality in 1:7.5 diluted with media to 1:5) were as follows: EAA 296 mosm/kg; EC 268 mosm/kg; EW 268 mosm/kg.

Organ culture

The tissue was cultured in Waymouth's medium MB752\1 (Flow-labs 12-52-54) which was modified and supplemented with 10% heat inactivated foetal calf serum (Ako Tech), L-Glutamine 200 mM (Gibco 43-8030), 300 μ g/ml Ascorbic acid (Sigma A 4544), 0.45 μ g/ml ferrous sulphate (Sigma F 8633), 50 mg/ml gentamicin (Flow-Labs 16-760-45) and 5000 IU/ml Penicillin and 5000 μ g/ml Streptomycin (Flow-labs 16-700-49). Bromodeoxy-Uridine (BrdU) (Sigma B5002) was added to each formula diet dilution, to give a final concentration of 200 μ M BrdU²¹.

A transport container to which the medium had already

Table I. Composition of formula diets.

Ingredient	Per 100 g powder EAA
Energy kcal	443
Carbohydrate g	60
Vitamin A μ g	330
Vitamin D μ g	1.9
Vitamin E mg	8.3
Vitamin C mg	28.3
Vitamin K μ g	25
Thiamine mg	0.6
Riboflavin mg	0.6
Niacin mg	4.2
Vitamin B ₆ mg	0.8
Vitamin B ₁₂	1.8
Biotin μ g	58.3
Pantothenic mg	2
Choline mg	91.6
Inositol mg	9.2
Sodium mg	305
Potassium mg	466
Chloride mg	333
Calcium mg	187.5
Phosphorus mg	200
Magnesium mg	81.6
Iron mg	4.2
Copper mg	0.4
Zinc mg	4.2
Iodine μ g	33.3
Manganese mg	0.6
Molybdenum μ g	33.3
Selenium μ g	15
Chromium μ g	15
Fat g	17.65
MCT%	35
LCT%	65

MCT: medium chain triglycerides; LCT: long chain triglycerides.

been added was weighed on a digital balance (Oertling, Smethwick, UK). The container was pre-gassed in the incubator chamber (ICN Pharmaceuticals, Ltd., UK) with 95% O₂ and 5% CO₂. Time between pre-gassing of the transport containers until tissue collection was between 60 and 150 minutes. The tissue was transported on ice in a plastic bag to prevent moisturising of the containers and re-weighted to calculate biopsy weight. The biopsies were carefully placed on a sterile triangular metal grid with luminal tissue surface face up, over a central well of an organ culture dish. Culture fluid medium or diluted elemental diet (1.5 ml), was added to the culture dish. The dishes were incubated at 37°C for 24 hours in a sealed chamber containing 95% O₂ and 5% CO₂ at a pressure of two atmospheres. After incubation, the tissue was placed in Carnoy's fixative (60% absolute alcohol,

Table II. Amino acid profiles of diets.

g/100g Amino acid	EAA	EC	EW
Protein Equivalent	12.63	13.57	12.13
Nitrogen	2.31	2.12	1.9
L-alanine	0.51	0.45	0.69
L-arginine	1.16	0.52	0.33
S-aspartic acid	1.00	1.05	1.49
L-cystine	0.07	0.07	0.27
L-glutamic acid	0.00	3.22	2.40
Glycine	0.87	0.26	0.24
L-histidine	0.63	0.40	0.23
L-isoleucine	0.86	0.76	0.87
L-leucine	1.48	1.40	1.43
L-lysine	1.10	1.10	1.25
L-methionine	0.50	0.42	0.25
L-proline	1.01	1.48	0.87
L-phenylalanine	1.22	0.70	0.39
L-serine	0.62	0.84	0.76
L-threonine	0.72	0.60	1.03
L-tryptophan	0.29	0.17	0.23
L-tyrosine	0.25	0.78	0.38
L-valine	0.94	0.97	0.80
L-carnitine	0.025	0.00	0.00
Taurine	0.047	0.00	0.00
L-glutamine	1.72	0.00	0.00

30% chloroform, 10% acetic acid) for approximately 16 hours and then transferred into industrial methylated spirits (IMS99) for further histological processing. The culture supernatants were immediately frozen at -70°C prior to immunoassay.

Assessment of tissue viability

BrdU is a thymidine analogue and labels cells which are in S-phase of the cell cycle²². BrdU uptake was assessed by immunostaining as described elsewhere^{21, 22}. BrdU stained sections in each experiment were systematically reviewed for crypt architecture, epithelial cell shape, and nuclear orientation, oedema, cell debris in the lamina propria and crypt necrosis. Viability was assessed by the morphologically intact structure of the tissue and intact epithelial integrity. Retention of normal morphology of the tissue and incorporation of BrdU in DNA of dividing cells are indices of the survival of explant tissues²¹. As it was not a histological study for colonic tissue proliferation, the number of BrdU labelled cells were not quantified, as in²³. Tissue was considered as viable in respect of the above-mentioned tissue appearance and the presence of BrdU labelled cells in horizontal and U-shaped crypts. To avoid double estimation of labelled cells, one tissue cut out of three cuts was used for assessing BrdU uptake. Tissue explants which did not fulfil these criteria, were excluded from further analysis

ELISAs for determination of IL-10, IL-1 β and IL-1RA in supernatant

IL-10, IL-1ra and IL-1 β were measured in culture supernatants using matched antibody pairs obtained from R&D Systems Briefly, ELISA plates (Corning Costar #9018) were coated with capture antibody (IL-10 MAB217, IL-1ra MAB280, IL-1 β MAB601, R&D Systems) at concentrations of 4, 9 and 3.5 μ g/ml, respectively, in PBS (Sigma, UK, 100 μ l/well). After overnight incubation, and washing, plates were blocked (300 μ l/well) with PBS containing 1% BSA (Sigma), 5% sucrose, 0.05% NaN₃ (BDH, UK) for 2 hours. Supernatant samples were added to plates (100 μ l/well) in duplicates. Standard curves were constructed in culture medium, using recombinant IL-10 (217-IL-005, R&D Systems) at concentrations double diluted out from 2000 – 31.3 pg/ml; for IL-1ra (280-RA-010, R&D Systems) at concentrations from 2500–39 pg/ml; for IL-1 β (201-LB-005, R&D Systems) at concentrations from 250–3.9 pg/ml. After 2 hours' incubation at 24°C biotinylated detection antibodies were used: for IL-10 (BAF217, R&D Systems) at a concentration of 420 ng/ml; for IL-1ra (BAF280, R&D Systems) at 60 ng/ml; for IL-1 β (BAF201, R&D Systems) at 90 ng/ml. Plates were incubated at 24°C for 2 hours. A streptavidin-horse-radish peroxidase conjugate (Zymed Laboratories, UK) at a dilution of 1:20,000 was added and plates were incubated at 24°C for 1 hour. TMB substrate (R&D Systems) was used for IL-10 and IL-1ra. OPD substrate was used for IL-1 β . After 30 minutes' incubation, the colour development was stopped and plates were read at 450 nm for IL-10 and IL-1ra, and at 490 nm for IL-1 β .

ELISA interference with elemental diet

Recovery was performed by incubating an EAA dilution of 1:10 either plain or "spiked" with a known amount of cytokine for 0, 3, 6 and 24 hours and afterwards immediately frozen to investigate whether EAA cross-reacts with cytokines. Standard curves were made up in EAA 1:5 dilution for IL-1 β , IL-1ra and IL-10 and compared with curves obtained with medium.

Statistical analysis

Results were analysed statistically in Minitab 10. Statistics used were mean and standard error of the mean (SEM). Differences between formula diet dilutions were calculated using Student t-test for unpaired values. Results are expressed as mean \pm SEM and significance was denoted by $p \leq 0.05$.

Results

A total of 158 organ culture experiments were included in the final analysis from 39 patients (16 CD, 12 UC

and 11 non-inflamed non-IBD controls). Measurement of cytokines for IL-1ra, IL-1 β and IL-10 were performed using the same supernatant from incubations. Specimens in which BrdU uptake was unsatisfactory were excluded. We did not find any specific differences between colonic and ileal biopsies in our experiments on cytokine balance. Ileal and colonic biopsies in CD are, therefore, considered together in this study.

In all the experiments included in the final analysis, the tissues had normal mucosal architecture after organ culture for 24 hours. The epithelial lining of the mucosa remained intact, with normal colonic crypt architecture. BrdU uptake could be detected in labelled cells in the crypt.

Recovery experiments of EAA spiked with cytokines showed that for IL-1ra at a low concentration (100 pg/ml), more than 50% of cytokine was detected over the entire 24-hour incubation period. At a high concentration of IL-1ra (1000 pg/ml), a steady decrease of detectable cytokine from 100% at 0 hours to 52% after 24 hours was seen. For IL-1 β at low concentration (10 pg/ml) 100% was detectable at all time points. At high concentration IL-1 β (100 pg/ml), 100% was detected at 0, 3 and 6 hours but only 60% at 24 hours. Since in the experiments, the IL-1 β concentrations corresponded to the low concentrations in the spiking experiments and were stable, it is likely that the ratio of IL-1ra/IL-1 β reported in this study is conservative. For IL-10 at low concentration (100 pg/ml), nearly 80% was detectable after 0 hour of incubation, and 40% after 24 hours. At high concentration (1000 pg/ml), the amount of cytokine detected decreased to 40% at 24 hours of incubation.

ELISA standard curves for EAA and medium for IL-1 β , for IL-1ra and for IL-10, were performed. These curves did not show any significant shift in standard curves between EAA and medium curves, and confirms no interference between EAA and the cytokine assays. An accurate estimation of the biopsy weight was problematic, as endoscopic biopsies were small. The degree of hydration considerably affected measurement of such small weights. Also the between measurement variation of the tissue weight was large, even using a sensitive digital balance. An alternative option of indexing results against protein or DNA content was not possible as the tissue was processed for BrdU uptake. Therefore, we have not presented the data as cytokine concentration per mg tissue weight, as these results will be greatly affected by even small inaccuracies of tissue weight measurement. Instead, results are presented as anti-inflammatory/pro-inflammatory cytokine ratios (IL-1ra/IL-1 β and IL-10/IL-1 β) which are independent of tissue weights. IL-1 β and IL-1ra are competing cytokines for the same membrane receptor and, therefore, an increase in the IL-1ra/IL-1 β ratio

gives an estimate for an anti-inflammatory effect in response to EAA.

No differences in cytokine production or anti-inflammatory to pro-inflammatory ratio could be detected when the biopsies were subgrouped according to medications. However, from each set of biopsies from an individual patient, a medium control group was always set up for comparison.

Analysis of cytokine data per mg tissue weight shows that in CD the predominant change after addition of EAA to media was a reduction in IL-1 β concentration in the supernatant at 24 hours. With medium alone the mean IL-1 β concentration was 2.96 pg/mg of tissue (± 1.59). With 1:20, 1:10 and 1:5 dilutions of EAA, the mean IL-1 β concentration was 3.64 pg/mg (± 2.06), 0.22 pg/mg (± 0.07) and 0.39 pg/mg (± 0.09), respectively. With 1:20, 1:10 and 1:5 dilutions of EC, the mean IL-1 β concentration was 1.09 pg/mg of tissue (± 0.97), 0.36 pg/mg (± 0.30) and 0.46 pg/mg (± 0.19), respectively. Finally with 1:20, 1:10 and 1:5 dilutions of EW, the mean IL-1 β concentration was 0.73 pg/mg (± 0.36), 0.48 pg/mg (± 0.2) and 1.85 pg/mg (± 0.94), respectively. However, since the reproducibility of weight of biopsies on repeated measurements was poor, we have avoided presenting the data as pg/mg tissue weight and, instead, used ratios of IL-1ra/IL-1 β and IL-10/IL-1 β , which cancels weight and, therefore, is independent of tissue weight.

IL-1ra/IL-1 β ratio after elemental diet incubation

Crohn's disease Figure 1 shows the IL-1ra/IL-1 β ratio after incubation of biopsies from patients with CD. Compared with medium control (45.7 \pm 9.1), incubation of biopsies with EAA increased the ratio at 1:20, 1:10 and 1:5 dilutions (59.7 \pm 24.0; 89.6 \pm 17.0; 93.0 \pm 38), although the result reached statistical significance ($p < 0.05$) only at 1:10 dilution compared with medium control alone (Fig. 1a).

An increase in the IL-1ra/IL-1 β ratio was shown with EC incubation compared with medium alone (control) in dilutions 1:20 (101.8 \pm 22.0; $p = 0.05$), 1:10 (142.8 \pm 24.6; $p < 0.05$) and in 1:5 (109.7 \pm 25.0; $p = 0.05$), respectively (Fig. 1b). The increase was not dose-dependent as the highest ratio was seen with 1:10 concentration of IL-1ra/IL-1 β .

The ratio of IL-1ra/IL-1 β after EW incubation increased at 1:20, 1:10 and 1:5 concentrations but the differences seen in all 3 dilutions compared with control did not reach statistical significance. The mean ratios (SEM) were 100.4 \pm 36.0; 91.6 \pm 37.0; 95.2 \pm 38.0 for 1:20, 1:10 and 1:5, respectively, compared with the control ratio (45.7 \pm 9.1) (Fig. 1c).

However, combining the IL-1ra/IL-1 β ratio for all dilutions from 1:5 to 1:20 in EW together (control $n = 13$ mean 45.7 \pm 9.1 vs combined $n = 15$ mean 95.7 \pm 20) re-

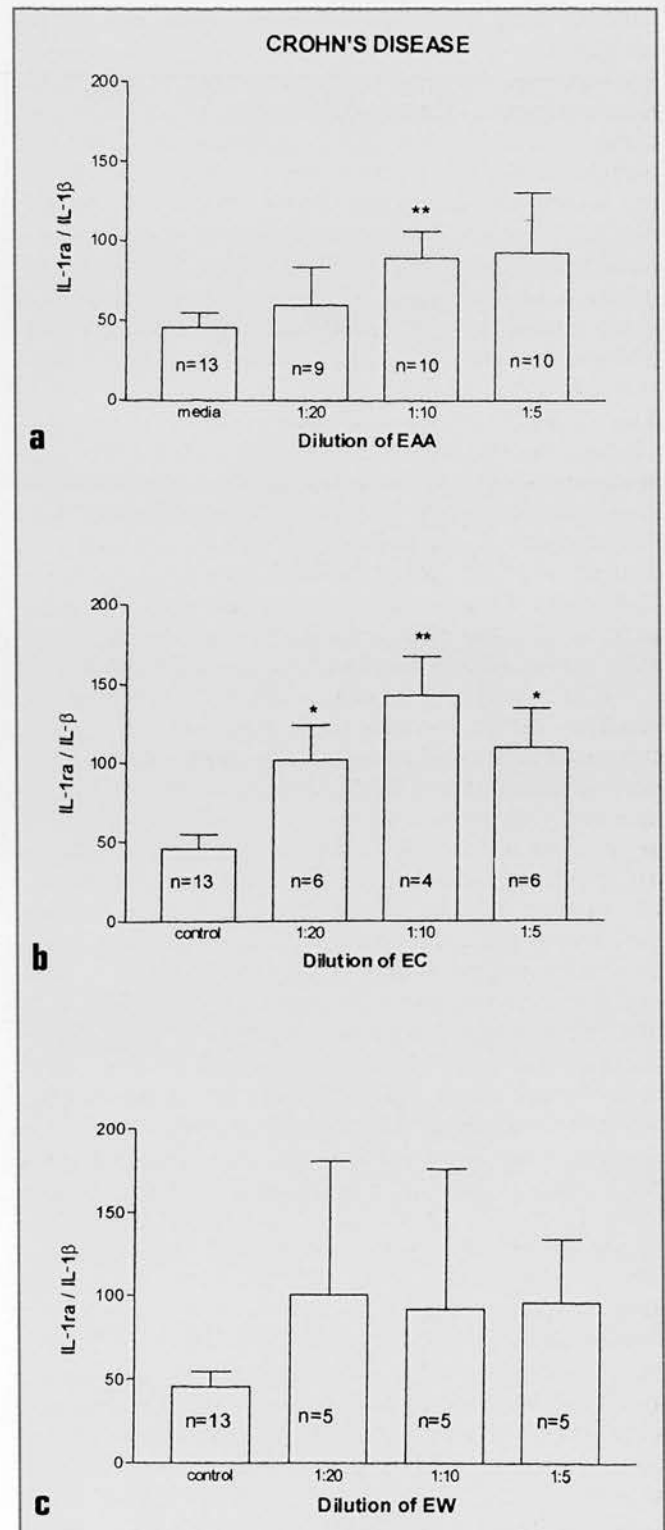


Fig. 1. IL-1ra/IL-1 β ratios in supernatant after 24 hours' incubation in Crohn's disease. a) IL-1ra / IL-1 β ratio after incubation with EAA in Crohn's disease. ** $p < 0.05$; b) IL-1ra/IL-1 β ratio after incubation with EC in Crohn's disease. ** $p < 0.05$, * $p = 0.05$; c) IL-1ra/IL-1 β ratio after incubation with EW in Crohn's disease.

sulted in a significant increase in the ratio ($p < 0.05$) after incubation with EW.

Ulcerative colitis Figure 2 shows the IL-1ra/IL-1 β ratio after incubation of biopsies from patients with UC. Incubation with EAA resulted in no significant changes in the IL-1ra/IL-1 β ratio. A slight increase in the mean ratio at 1:10 EAA compared with control (65.8 ± 17.0 vs 45.1 ± 15.0) was seen, but was not statistically significant. The mean ratios with 1:20 and 1:5 dilutions of EAA were 53.0 ± 12.0 and 51.2 ± 23.0 , respectively (Fig. 2a). Similarly, incubation with EC and EW resulted in no changes in the IL-1ra/IL-1 β ratio (Fig. 2b, c).

The IL-1ra/IL-1 β ratio in the control UC specimen (45.1 ± 15.0) was similar to the ratio in the control CD specimen (45.7 ± 9.1) without the addition of formula diet. **Non-inflamed non-IBD** Figure 3 shows the IL-1ra/IL-1 β ratio after incubation of biopsies from non-inflamed non-IBD patients. The ratio in the non-inflamed non-IBD specimen was higher than that in the previously noted inflamed specimens. Incubation with EAA produced an increase in the IL-1ra/IL-1 β ratio at 1:10 dilution vs control (143 ± 31 ; 64.4 ± 18) that nearly reached statistical significance ($p = 0.06$). At other dilutions of EAA, no significant change in the ratio was seen (60.6 ± 20.0 and 88.2 ± 22.0) for 1:20 and 1:5, respectively (Fig. 3a).

After incubation with EC, a decrease in the IL-1ra/IL-1 β ratio at 1:20, 1:10 and 1:5 was observed (35.4 ± 20.0 ; 46.2 ± 17.0 ; 43.7 ± 3.3 , respectively) compared with control (64.4 ± 18.0), respectively, but this was not significant for 1:20 and 1:10 (Fig. 3b). This was different from the experiments conducted with CD affected tissues, where a consistent increase in the ratio was seen.

In EW-incubated tissue, no change in the ratio of IL-1ra/IL-1 β was detected between the control (64.4 ± 18.0) and 1:20, 1:10 and 1:5 dilutions (51.2 ± 18.0 ; 84.2 ± 11.0 ; 54.3 ± 1.0 , respectively) (Fig. 3c).

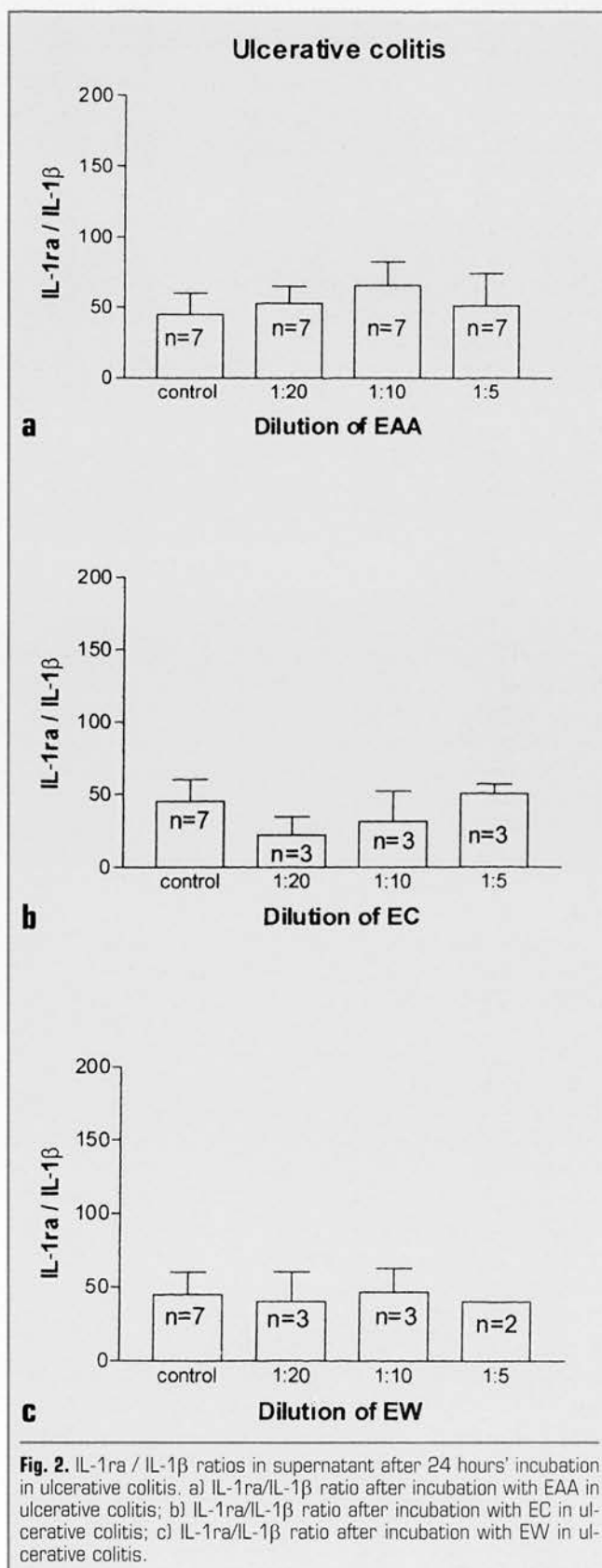
IL-10/IL-1 β ratio after elemental diet incubation

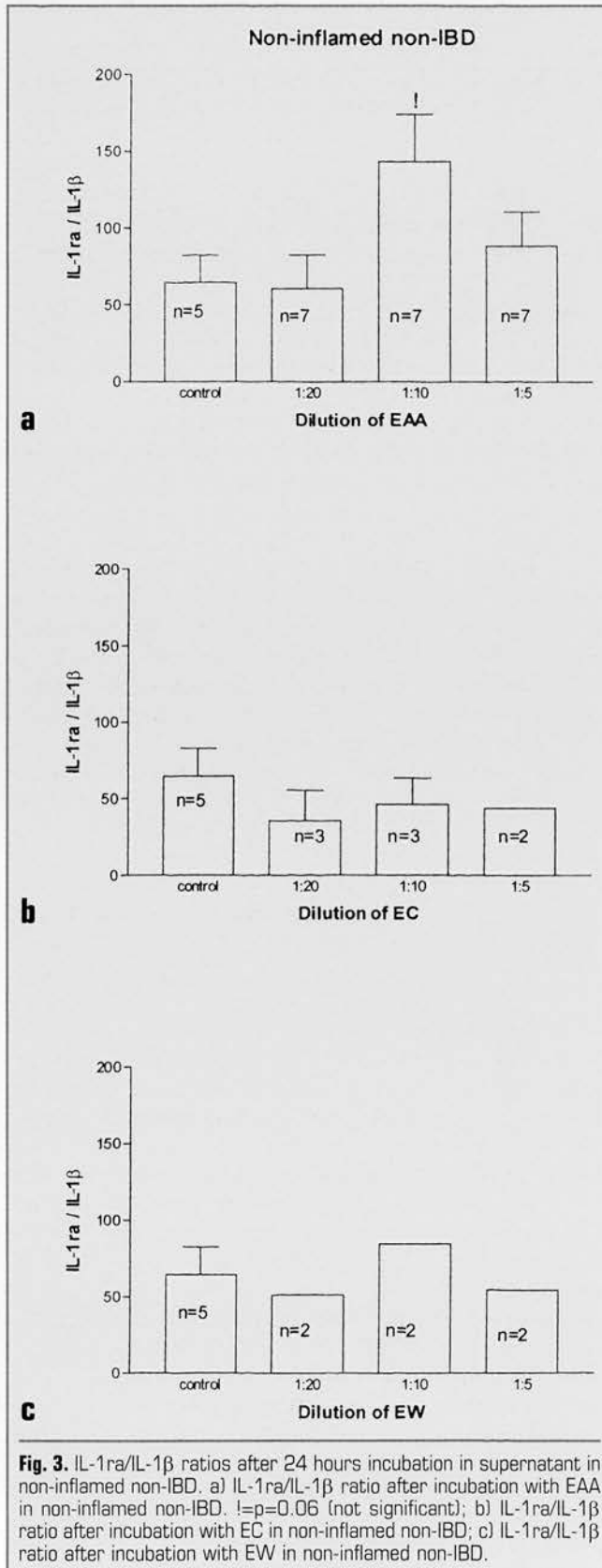
The IL-10/IL-1 β ratio in CD after incubation with EAA showed a slight increase in IL-10/IL-1 β ratio in 1:10 dilution vs control (43.1 ± 23 vs 21.4 ± 9.2) but this was not statistically significant (data not shown).

In EC, an increase in the ratio of IL-10/IL-1 β at 1:20 (75.4 ± 12) and at 1:5 (48.12 ± 6.7) occurred, but the sample size was small and the results did not reach statistical significance. For the 1:10 dilution, no data were available (data not shown).

In EW incubation, an increase in mean at 1:10 dilution compared with control (165 ± 140 vs 21.4 ± 9.2) occurred, however this did not reach statistical significance (data not shown).

In UC patients and in non-inflamed non-IBD controls,





no changes in IL-10/IL-1 β ratios were observed after incubation with EAA, EC or EW (data not shown).

Discussion

In this study, we have shown changes in the IL-1ra/IL-1 β cytokine balance after incubation with EAA and with comparable enteral formulas containing EC and EW. These changes affected tissues differently, depending on whether the tissue was obtained from patients with CD, UC or non-inflamed non-IBD. We showed that EAA does not interfere with the cytokine assays. Recovery experiments showed that variable proportions of different cytokines were present after 24 hours of incubation. In practice, however, cytokines were constantly being secreted and added to the supernatant over 24 hours, and, therefore, the net recovery is likely to be higher than the 24-hour recovery experiments. Therefore, as the ratio increases we consider that our results are a reflection of the amount produced in tissues during incubation with EAA. We have no reason to believe that biopsies incubated with EAA would act differently from those incubated with medium, as these results are consistent in our patients. One biopsy from each individual patient was always used for medium control. No effect of medications could be detected on subgroup analysis, though the numbers are small and the possibility of a type II error quite large. This study was not designed to detect the effect of concurrent medications.

Several studies have shown that both in CD and UC, the mucosal molar ratio of the IL-1ra/IL-1 β ratio is lower in inflamed tissue compared with control tissue^{14 15 24}. In this study, in tissues affected by CD, a consistent increase in the IL-1ra/IL-1 β ratio was seen after incubation with enteral diet. This was dose dependent with EAA, but the dose-dependency was less marked with EC and EW. EC at 1:10 dilution appeared to increase the ratio most markedly. In contrast, in UC and in non-inflamed non-IBD tissue, no statistically significant alteration in the IL-1ra/IL-1 β ratio was observed. This finding is consistent with the well-known clinical observation that EAA is ineffective in UC. Both in CD and in UC the ratios of IL-1ra/IL-1 β in media control alone without incubation in enteral diets were comparable, but lower than non-inflamed non-IBD control tissue.

The ratio of IL-10/IL-1 β showed no statistically significant rise in CD after incubation with the formula diets, with EC again appearing to show a trend towards an increase. Little change in the IL-10/IL-1 β ratio was seen in UC or non-inflamed non-IBD patients. IL-10 expression in UC and CD tissues might be variable²⁵ but resolution of inflammation in IBD is expected to increase IL-10²⁶ and, therefore, the IL-10/IL-1 β ratios;

in our study, no such significant increase was observed. This might indicate that an increase in IL-1ra is the preferential effect of enteral diets especially in CD. Also since most of the IL-10 is derived from lamina propria mononuclear cells²⁷, the colonoscopic mucosal biopsies might have been weighted towards epithelial secretion of IL-1ra, rather than lamina propria secretion of IL-10. A further explanation could be the rather high loss of IL-10 of approximately 60%/24 hour in the cytokine recovery experiment in EAA at both concentrations of 1000 pg/ml and 100 pg/ml which might influence the IL-10/IL-1 β ratio. Impaired production of IL-10 in UC mucosa²⁶ has been reported. As IL-10 downregulates IL-1 β ¹⁶ the low concentration of IL-10 in UC might not sufficiently downregulate IL-1 β and increase the IL-1ra/IL-1 β ratio after incubation with enteral diets.

Our findings show that enteral diet has a direct anti-inflammatory effect on cytokine production on intestinal biopsy tissue from patients with CD. This direct anti-inflammatory effect is manifested by an increase in the IL-1ra/IL-1 β ratio, but the effect on IL-10/IL-1 β is minimal. This would support *in vivo* studies, such as that by Fell et al.¹⁷ who investigated the effect of a polymeric diet, with casein as a protein source, on mucosal healing and expression of cytokine mRNA IL-1 β , interferon gamma (IFN- γ) and IL-8 before and after 8 weeks of treatment. Their results also showed a decrease of IL-1 β mRNA after treatment in ileum and colonic biopsies compared with controls. Likewise, Ferguson et al. also reported a decrease in IL-1 β in whole gut lavage fluid after treatment with elemental diet¹.

The immunomodulatory effect of formula diets on CD was not restricted to EAA alone. EC and EW also showed immunomodulatory properties, and this was particularly observed with EC. EC is one of the major milk proteins and consists of four major protein fractions (α_1 -casein, α_2 -casein, β casein and κ casein). ECs are proteins known to have immune modulator effects on cellular immune function. EC, especially β casein and κ casein, have a range of stimulatory and immunosuppressive effects on mononuclear cell function. The carbohydrate-rich glycomacropptide component of κ casein particularly acts as an active component^{28,29}. Kohyama et al.³⁰ have reported the effect of a bovine α_1 -casein in induction of CD8⁺ T-cells for IL-10 production; they showed that a single specific amino acid substitution could induce a separate production of IL-10 or IFN γ from a CD8⁺ T-cell clone. Subtle variations in amino acid content or peptide sequence may influence the balance of Th1/Th2 responses. The immunomodulatory effect of casein was seen in the increased production of IL-1ra in CD at all dilutions of EC, resulting in an increased IL-1ra/IL-1 β ratio.

Whey originates from the production of cheese; it remains as a fluid phase after coagulation and extraction of the cheese product. The major whey proteins are β lactoglobulin and α lactalbumin. Some isolated proteins from whey have also been shown to have potential effects on cellular immune function²⁹. In our organ culture system, we saw no significant differences in anti-/pro-inflammatory cytokine production with EW, though there was a trend towards an increased ratio of IL-1ra/IL-1 β in CD.

It must be stressed that our *in vitro* study is based, by necessity, on a very reductionist model. As such, it does not parallel the *in vivo* situation of oral ingestion of EAA where EAA is almost completely absorbed in the proximal small intestine with very little arriving in unchanged form in the colon. Also, the whole proteins used in the whey and casein-based formulas would have been partly digested *in vivo* so that a mixture of unabsorbed protein, peptides and amino acids would be presented to the colonocytes. These results must, therefore, be interpreted with caution. Differential degradation of the cytokines could affect the ratios and this might be particularly relevant for the IL-10/IL-1 β ratio, which did not change significantly in this study. We do not know the influence of enteral diet on the distal colonic mucosa because most of the components will not reach the distal colon by oral feeding. But we have shown in this *in vitro* model that when enteral diet has direct contact with the lamina propria, it has a direct-acting immune-modulating influence on cells producing cytokines. The direct contact of EAA components might enhance a modulating effect on IL-1 β mRNA expression, which is present in the lamina propria³¹. This might open further considerations for EAA as an effective treatment after surgery e.g. ileal resection in IBD, to inject EAA into a stoma or, more unconventionally, infuse EAA as a high enema into the colon.

The focus of this study was to detect any direct influence of elemental diets on the intestinal mucosa in the release of pro- and anti-inflammatory cytokines. We have demonstrated variation between ratios of pro- and anti-inflammatory cytokines released by IBD tissues incubated with EAA compared with non-inflamed non-IBD as controls.

In conclusion, to our knowledge, this is the first *in vitro* study to show any direct anti-inflammatory response to EAA in intestinal biopsies of patients with IBD. The results confirm a positive direct anti-inflammatory effect of EAA on intestinal tissue affected by CD. This anti-inflammatory effect is preserved if casein and to a lesser extent, whey are substituted for amino acids in EAA. However, the specific mechanism by which EAA leads to improvement in IBD is still unknown. Future work should include assessment of other key

cytokines such as TNF- α , IL-6, IL-12 and IFN- γ as well as quantitative reverse transcription-polymerase chain reaction of cytokine mRNA after incubation.

List of abbreviations

BrdU: bromodeoxyuridine; CD: Crohn's disease; EAA: elemental diet; EC: casein; ELISA: enzyme-linked immunosorbent assay; EW: whey; IBD: inflammatory bowel disease; IFN- γ : interferon-gamma; IL-1ra: interleukin-1 receptor antagonist; IL: interleukin; SEM: standard error of the mean; TMB: TNF- α : tumour necrosis factor alpha; UC: ulcerative colitis.

References

- Ferguson A, Glen M, Ghosh S. Crohn's disease: nutrition and nutritional therapy. *Baillieres Clin Gastroenterol* 1998;12:93-114.
- Wright R, Truelove SC. A controlled therapeutic trial of various diets in ulcerative colitis. *Br Med J* 1965;2:138-41.
- Teahon K, Bjarnason I, Pearson M, Levi AJ. Ten years' experience with an elemental diet in the management of Crohn's disease. *Gut* 1990;31:1133-7.
- Rigaud D, Cosnes J, Le Quintec Y, Rene E, Gendre JP, Mignon M. Controlled trial comparing two types of enteral nutrition in treatment of active Crohn's disease: elemental versus polymeric diet. *Gut* 1991;32:1492-7.
- Teahon K, Pearson M, Smith T, Bjarnason I. Alterations in nutritional status and disease activity during treatment of Crohn's disease with elemental diet. *Scand J Gastroenterol* 1995;30:54-60.
- Murch SH, Walker-Smith JA. Nutrition in inflammatory bowel disease. *Baillieres Clin Gastroenterol* 1998;12:719-38.
- Heuschkel RB, Walker-Smith JA. Enteral nutrition in inflammatory bowel disease of childhood. *J Parent Enteral Nutr* 1999;23:S29-32.
- Wilschanski M, Sherman P, Pencharz P, Davis L, Corey M, Griffiths A. Supplementary enteral nutrition maintains remission in paediatric Crohn's disease. *Gut* 1996;38:543-8.
- Verma S, Kirkwood B, Brown S, Gjafer MH. Oral nutritional supplementation is effective in the maintenance of remission in Crohn's disease. *Digest Liver Dis* 2000;32:769-74.
- Breese EJ, Michie CA, Nicholls SW, Murch SH, Williams CB, Domizio P, et al. Tumor necrosis factor alpha-producing cells in the intestinal mucosa of children with inflammatory bowel disease. *Gastroenterology* 1994;106:1455-66.
- Reimund JM, Wittersheim C, Dumont S, Muller CD, Kenney JS, Baumann R, et al. Increased production of tumor necrosis factor-alpha interleukin-1beta, and interleukin-6 by morphologically normal intestinal biopsies from patients with Crohn's disease. *Gut* 1996;39:684-9.
- Andus T, Daig R, Vogl D, Aschenbrenner E, Lock G, Hollerbach S, et al. Imbalance of the interleukin 1 system in colonic mucosa — association with intestinal inflammation and interleukin 1 receptor antagonist [corrected] genotype 2 [published erratum appears in *Gut* 1998;42:450]. *Gut* 1997;41:651-7.
- Ligumsky M, Simon PL, Karmeli F, Rachmilewitz D. Role of interleukin 1 in inflammatory bowel disease-enhanced production during active disease. *Gut* 1990;31:686-9.
- Dionne S, D'Agata ID, Hiscott J, Vanounou T, Seidman EG. Colonic explant production of IL-1 and its receptor antagonist is imbalanced in inflammatory bowel disease (IBD). *Clin Exper Immunol* 1998;112:435-42.
- Casini-Raggi V, Kam L, Chong YJ, Flocchi, Pizarro TT, Cominelli F. Mucosal imbalance of IL-1 and IL-1 receptor antagonist in inflammatory bowel disease. A novel mechanism of chronic intestinal inflammation. *J Immunol* 1995;154:2434-40.
- Schreiber S, Heinig T, Thiele HG, Raedler A. Immunoregulatory role of interleukin 10 in patients with inflammatory bowel disease. *Gastroenterology* 1995;108:1434-44.
- Fell JME, Paintin M, Arnaud-Battandier F, Beatties RM, Hollis A, Kitching P, et al. Mucosal healing and a fall in mucosal pro-inflammatory cytokine mRNA induced by a specific oral polymeric diet in paediatric Crohn's disease. *Aliment Pharmacol Therap* 2000;14:281-9.
- Pritchett CJ, Senior PV, Sunter JP, Watson AJ, Wilson, RG, Appleton DR. Cell proliferation in human colorectal mucosa in organ culture: the early adaptive changes. *J Anat* 1985;141:171-9.
- Astrup H, Barrett LA, Jackson FE, Jesudason ML, Stoner G, Phelps P, et al. Explant culture of human colon. *Gastroenterology* 1978;74:1248-57.
- Senior PV, Pritchett CJ, Sunter JP, Appleton DR, Watson, AJ. Crypt regeneration in adult human colonic mucosa during prolonged organ culture. *J Anat* 1982;134:459-69.
- Wilson RG, Smith AN, Bird CC. Immunohistochemical detection of abnormal cell proliferation in colonic mucosa of subjects with polyps. *J Clin Pathol* 1990;43:744-7.
- Green JA, Edwards RE, Manson MM. Immunohistochemical detection of bromodeoxyuridine-labeled nuclei for in vivo cell kinetic studies. *Methods Mol Biol* 1998;80:251-4.
- Gregory L, Eastwood MD, Jerry S, Trier MD. Organ culture of human rectal mucosa. *Gastroenterology* 1973;64:375-82.
- Hyams JS, Fitzgerald JE, Wyzga N, Muller R, Treem WR, Justinich CJ, et al. Relationship of interleukin-1 receptor antagonist to mucosal inflammation in inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 1995;21:419-25.
- Akagi S, Hiyama E, Imamura Y, Takesue Y, Matsuura Y, Yokoyama T. Interleukin-10 expression in intestine of Crohn's disease. *Int J Mol Med* 2000;5:389-95.
- Ishizuka K, Sugimura K, Homma T, Matsuzawa J, Mochizuki T, Kobayashi M, et al. Influence of interleukin-10 on the interleukin-1 receptor antagonist/interleukin-1 β ratio in the colonic mucosa of ulcerative colitis. *Digestion* 2001;63:22-7.
- Gasche C, Bakos S, Dejaco C, Tillinger W, Zakeri S, Reinisch W. IL-10 secretion and sensitivity in normal human intestine and inflammatory bowel disease. *J Clin Immunol* 2000;20:362-70.
- Wong CW, Seow HF, Liu AH, Husband AJ, Smithers GW, Watson DL. Modulation of immune responses by bovine beta-casein. *Immunol Cell Biol* 1996;74:323-9.
- Cross ML, Gill HS. Modulation of immune function by a modified bovine whey protein concentrate. *Immunol Cell Biol* 1999;77:345-50.
- Kohyama M, Kakehi M, Totsuka M, Hachimura S, Hisatsune T, Kaminogawa S. Selective induction of CD8+ T cell functions by single substituted analogs of an antigenic peptide: distinct signals for IL-10 production. *FEBS Letters* 1998;423:138-42.
- Woywodt A, Ludwig D, Neustock P, Kruse A, Schwarting K, Jantschek G, et al. Mucosal cytokine expression, cellular markers and adhesion molecules in inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 1999;11:267-76.

Modification of enteral diets in inflammatory bowel disease

Marian C. Aldhous*, Doris Meister and Subrata Ghosh

Gastro-intestinal Laboratory, Western General Hospital, Edinburgh EH4 2XU, UK

The provision of food is thought to promote the maintenance of gut integrity. Nutrients are able to elicit and affect both systemic and mucosal immune responses. Enteral diet therapy has long been known to be efficacious in inflammatory bowel disease (IBD), particularly in childhood Crohn's disease. However, the mechanisms of action of these diets are not clear. Nutritional repletion, direct effects on the gut mucosa or decreased intestinal permeability have all been postulated as being important in nutritional therapy. There is some evidence that the enteral diet has a direct effect on the gut mucosa by reducing cytokine production and the accompanying inflammation, thus leading to decreased intestinal permeability. Modifications of enteral diet composition have been evaluated in many studies. Such modifications include fat and/or protein content and the addition of bioactive peptides. The fatty acid composition of the enteral diet seems to have a much greater impact on its efficacy than modification of the N source. As specific fatty acids are precursors of inflammatory mediators derived from arachidonic acid, the reduction in these components may be beneficial in nutritional therapy for IBD. Addition of bioactive peptides to enteral diet formulas may also have a role; such peptides may have specific growth factor or anti-inflammatory actions. There is still much work to be done to define disease-specific enteral diet formulas that are effective as therapies for both Crohn's disease and ulcerative colitis.

Enteral diet formulas: Crohn's disease: Nutritional therapy: Inflammation

There is plenty of evidence to suggest that food and nutritional factors affect immune responses in the gut. Nutritional status, both at the macro- and micronutrient level, affects both systemic and mucosal immunity. Early work in our laboratory, under the auspices of the late Professor Anne Ferguson, showed that in protein-deprived mice there was a profound reduction in the induction of oral tolerance (Lamont *et al.* 1987*a,b*, 1988). It follows, therefore, that immunological functions of the gut and nutrient intake would be particularly important in gut disease (Ferguson, 1994). A breakdown of oral tolerance to gut bacteria has been hypothesised as a mechanism by which patients develop inflammatory bowel disease (IBD). Thus, if such immune responses are profoundly affected by nutrition, then nutritional therapy may have a role in the treatment of IBD.

IBD can be divided into two disease patterns, Crohn's disease (CD) and ulcerative colitis (UC), with distinct clinical, histological and immunological features. Both disease patterns show dysregulated cytokine balance; CD tends to be shifted towards a T-helper 1 (pro-inflammatory) phenotype and UC towards a T-helper 2 (anti-inflammatory)

phenotype. This distinction in cytokine phenotype between the two disease states is not totally consistent with a T-helper 1–T-helper 2 split. Both diseases show an increase in inflammation of the gut mucosa, together with increased production of the pro-inflammatory cytokine interleukin (IL)-1 β and a decrease in the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1ra). Indeed, in both CD and UC a decrease in IL-1ra:IL-1 β production has been described in isolated colonic biopsies compared with those from normal controls or patients with infectious colitis (Dionne *et al.* 1998). This imbalance has been proposed as being of pathogenic importance in IBD (Casini-Raggi *et al.* 1995).

The intestine is a metabolically active organ which maintains gut function through various mechanisms, e.g. peristalsis, secretory immunoglobulin A, mucin secretion. Starvation, and possibly parenteral feeding, predisposes the gut to metabolic and immunological deterioration of barrier function, leading to the release of pro-inflammatory cytokines and decreased gut permeability, allowing other macromolecules to cross. Gut contents stimulate immunological mechanisms such as intestinal immunoglobulin A

Abbreviations: CD, Crohn's disease; ED, enteral diet; IBD, inflammatory bowel disease; IL, interleukin; IL-1ra, interleukin 1 receptor antagonist; UC, ulcerative colitis.

***Corresponding author:** Dr Marian C. Aldhous, fax +44 131 537 1007, email maldhous@ed.ac.uk

production, which blocks the binding of bacteria to epithelial cells. Enteral feeding, as opposed to parenteral feeding, appears to maintain these mechanisms by providing food (Donnell *et al.* 1998; Minard & Kudsk, 1998; Rowlands & Gardiner, 1998).

Enteral diets in inflammatory bowel disease

There is little doubt that an enteral diet (ED) is effective in CD, particularly in children, although there is some question as to the clinical efficacy of ED in UC. ED are important in re-establishing nutritional repletion in malnourished patients. There is also some evidence that ED have some effect on the disease activity itself, comparable with steroid therapy, although this factor has been debated widely. A meta-analysis (Griffiths *et al.* 1995) of trials comparing ED with steroids showed that although ED was effective in CD, it was still less effective than steroid treatment. However, other studies have shown that ED are equally as effective (Zoli *et al.* 1997). Either way, it is known that ED is a possible alternative in those patients who may have contraindications for steroid therapy in CD.

Mechanism of action

What really is not clear is the mechanism by which these diets have their action. There is still much discussion as to the best approach for trying to investigate such mechanisms. Although *in vivo* studies are the most relevant, as regards clinical efficacy and side effects, the actual mechanisms by which ED have their effect are hard to deduce, mainly because the gut is a difficult organ to study directly. Such studies may require repeated invasive or unpleasant procedures for patients in order to define a 'before and after' treatment effect, and subtle or transient changes may not be detected. Thus, knowing the best way to modify ED in order to increase clinical efficacy and reduce side effects becomes more difficult. *In vitro* studies are simpler to perform but, by their very nature of being *in vitro*, do not reproduce the many factors that affect gut function, and can only investigate defined and measurable effects such as cytokine production or changes in expression of particular molecules. However, *in vitro* studies of ED, looking at such specific mechanisms of action, may be useful in defining macro- and micronutrient effects on gut tissue, which can then be assessed further by *in vivo* studies.

Various hypotheses as to the action of ED in IBD have been proposed. There is still some discussion as to whether ED has a direct effect on the gut, or whether the efficacy of ED is due to indirect effects such as improvement in nutritional status. In one study of ED in CD, both nourished and malnourished patients responded equally (Gorard *et al.* 1993). Other researchers have found that clinical improvement in disease activity occurred before changes in nutritional status were detected (Teahon *et al.* 1995; Croft *et al.* 2001), suggesting that ED has a direct effect on the gut.

Decreased intestinal permeability has also been proposed as a mechanism through which ED therapy may act. Clinical improvement of CD has been accompanied by decreases in urinary ^{51}Cr EDTA excretion and faecal excretion of

^{111}In -labelled leucocytes (Teahon *et al.* 1991), or a decrease in quantitative leucocyte scintigraphy (Mansfield *et al.* 1995). Zoli *et al.* (1997), in a study comparing ED with steroids, showed a significant decrease in intestinal permeability with ED ($P < 0.05$), but not with steroids, even though both treatment groups went into clinical remission. Studies from our own laboratory have also indicated a decrease in intestinal permeability in CD patients, through decreased protein (immunoglobulin G) loss in whole-gut lavage fluid (Ferguson *et al.* 1998), after treatment with an elemental ED for 2 weeks. This decrease in intestinal permeability was accompanied by a concomitant decrease in IL-1 β levels. These results provide further evidence that ED have a direct effect on the gut, by changing cytokine levels, thus resulting in decreased inflammation, which in turn leads to decreased permeability.

Elemental or polymeric enteral diets?

More recent studies in our laboratory have begun to investigate the possible direct anti-inflammatory effect of ED, in an *in vitro* organ culture model, by monitoring changes in cytokine production (Shand *et al.* 2000). Ileal or colonic biopsies from patients with CD and UC were incubated with ED or medium alone for 24 h. Supernatant fractions were collected and cytokines (IL-1 α , IL-1 β and IL-10) were measured by ELISA. Initial studies used an elemental formula ED (E028; Scientific Hospital Supplies, Liverpool, UK), in which the N source was free amino acids, as opposed to polymeric ED, in which the N source was whole proteins or oligopeptides. We established that the biopsies maintained viability in culture, and we looked for changes in IL-1 α :IL-1 β ; an increase in the ratio would indicate a decrease in IL-1 β or an increase in IL-1 α production, i.e. an anti-inflammatory effect of ED in these experiments. Results showed that an increase in IL-1 α :IL-1 β was obtained in biopsies from CD patients incubated with ED, but no changes in cytokine production were seen in biopsies from UC patients. Further studies used ED with oligopeptides (whey) or whole protein (casein) as the N source and found that the increased IL-1 α :IL-1 β was not abolished. Indeed, if anything, for casein the effect was more pronounced. These results agree with those from clinical studies of elemental, oligopeptide or polymeric ED, which showed that there appears to be little difference between diet efficacy and the N source (Griffiths *et al.* 1995; Mansfield *et al.* 1995; Verma *et al.* 2000).

It has been proposed that elemental ED are effective as they provide gut rest or a low antigen load, through decreased protein content. However, the efficacy of polymeric ED and maintenance of remission through the supplementation of a normal diet with ED (Wilschanski *et al.* 1996) bring these theories into dispute. Indeed, in animal models too much 'gut rest', through use of parenteral nutrition or long-term treatment with an elemental ED, increases the likelihood of bacterial translocation or gut atrophy (Evers *et al.* 1990; Serizawa *et al.* 1994; Xu *et al.* 1998). Many polymeric ED are based on milk proteins and there is some evidence that casein and whey themselves have direct immune modulatory functions (Wong *et al.* 1996; Cross & Gill, 1999).

Modification of fat composition of enteral diets

ED, particularly those of an elemental formula, often have a low fat content, which is another possible mechanism of efficacy. However, studies comparing high- and low-fat ED have found no differences in achieving clinical remission of CD (Royall *et al.* 1994; Leiper *et al.* 2001). Although there may be little effect by changing the amount of fat in ED, there may be some benefit from modification of the fat composition. We have carried out experiments in which colonic tissues from IBD patients were incubated with ED containing specific oils. Preliminary results indicated that incubation of tissue from UC patients with ED containing fish oil led to a significant increase in IL-1 α :IL-1 β ($P < 0.05$). However, for tissue derived from CD patients, incubation with ED containing fish oil had little effect on cytokine production profiles (Meister *et al.* 2001b).

There has been much interest in dietary supplementation with different fats in inflammatory conditions (de Pablo & de Cienfuegos, 2000; James *et al.* 2000). Previous studies of the use of *n*-3 dietary fats in IBD have given conflicting results (for review, see Beluzzi *et al.* 2000). In UC several studies have indicated that supplementation of ED with fish oil may be beneficial, particularly as concomitant decreases were seen in markers of inflammation (e.g. leukotriene B₄ levels) in rectal mucosa. However, not all studies showed significant clinical differences, possibly due to small numbers (Lorenz *et al.* 1989; Aslan & Triadafilopoulos, 1992; Hawthorne *et al.* 1992; Stenson *et al.* 1992). In animal models of UC administration of *n*-3 fatty acids by oral and topical enema routes indicated that reduced pathology and decreases in leukotriene levels could be achieved (Yuceyar *et al.* 1999). Moreover, there may also be a decrease in the colonic antioxidant defence systems, which may promote oxidative injury at the site of inflammation (Nieto *et al.* 1998), and thus explain the clinical results which showed decreases in pathology or inflammation but less effect on clinical remission rates. Although some *in vivo* studies have indicated little effect of fish oil in CD (Lorenz *et al.* 1989; Lorenz-Meyer *et al.* 1996), other studies have indicated that fish oil may be beneficial in CD (Beluzzi *et al.* 1996). The discrepancy between our *in vitro* results for fish oil-supplemented ED on CD and UC may be due to the fact that, as already mentioned, *in vitro* studies use a reductionist model, which may not parallel effects that occur *in vivo*. Our studies have only monitored one mechanism, i.e. alterations in cytokine production, albeit an important pathogenic mechanism in IBD (Casini-Raggi *et al.* 1995). It has been shown that fish oil reduced production of inflammatory cytokines from mononuclear cells (Endres *et al.* 1989). However, other inflammatory mechanisms which are altered by dietary fat intake and composition may be differentially important in CD and UC. For example, macrophage cytotoxic function has been shown to be affected by dietary fat composition (Wallace *et al.* 2000), as has the antigen-presenting function of monocytes (Hughes & Pinder, 2000), the production of inflammatory mediators (for review, see James *et al.* 2000) and cellular immune functions (for review, see Calder, 1998).

We have also cultured colonic tissues from IBD patients with ED containing sunflower oil, which gave significant

increases in IL-1 α :IL-1 β in both CD and UC tissues ($P < 0.05$ in both cases) but was not seen when tissues were incubated with ED containing safflower oil (Meister *et al.* 2001a). These results indicate that substitution by other fats in ED may be important for CD and UC therapy. Although in these ED preparations safflower oil-substituted ED contains more polyunsaturated fatty acids in total, the main difference in the fatty acid composition is that the sunflower oil-substituted ED used here contained no linoleic acid. Linoleic acid is a precursor of arachidonic acid, which in turn is a precursor of the inflammatory eicosanoid mediators leukotriene B₂, prostaglandin E₂ and thromboxane A₂, all of which have been implicated in IBD pathogenesis. Mucosal phospholipids derived from polyunsaturated fatty acids have been shown to be increased in the plasma membranes of active CD, possibly contributing to eicosanoid synthesis and inflammation (Pereira *et al.* 1996). Studies from Japan have implicated that dietary intake of *n*-6 polyunsaturated fatty acids, compared with *n*-3 polyunsaturated fatty acids, was a risk factor for development of CD (Shoda *et al.* 1996). However, a case-control study of CD patients, both newly diagnosed and with longstanding disease, indicated that intake of fat (both quality and quantity) was not different between patients and controls, but that fat metabolism in CD patients was changed, so that different fatty acids were present in plasma membrane phospholipids of CD patients compared with controls (Geerling *et al.* 1999). In addition, increased lipid oxidation has also been reported in CD (Mingrone *et al.* 1996), indicating that increased lipid intake may be beneficial in CD.

Bioactive peptides

Most studies of ED in IBD have concentrated on the macronutrients, i.e. protein and fat content or composition. It is possible that bioactive peptides may be important, and should perhaps be included in ED formulas. There are growth factors and peptides that could be very important in the maintenance of gut integrity in inflammatory diseases, many of which are present in colostrum (Playford *et al.* 2000). One such peptide is epidermal growth factor, which stimulates intestinal epithelial cell growth. A recent study in which patients with UC were treated with enemas containing epidermal growth factor indicated the efficacy of such growth factors in IBD (Sinha *et al.* 2001). Another bioactive peptide is transforming growth factor- β , which also occurs in colostrum and is known to have an anti-inflammatory effect on intestinal cell lines (Donnet-Hughes *et al.* 2000). Its potential benefit has been borne out in a study by Fell *et al.* (2000) who used a commercially available casein-based diet, which also contained transforming growth factor- β ₂ in the treatment of paediatric CD and found a decrease in pro-inflammatory cytokine mRNA in the gut mucosa.

Conclusion

In summary, many studies have shown that ED is an effective therapy in patients with CD. Modification of ED with different fatty acids, bioactive peptides and, to a lesser extent, N source may have a direct effect on the

inflammatory mediators produced in both CD and UC, thus increasing the efficacy of ED in both forms of IBD. Further studies are required to ascertain how ED formulas should be modified in ways that are disease-specific for CD and UC.

Acknowledgements

We acknowledge Sandra Giffen and Wendy Johnston of Scientific Hospital Supplies, Liverpool, UK for their donation of the enteral diet formulas used in our experiments. D. M. is funded by Scientific Hospital Supplies research grant to Intestinal Immunology Research Fund.

References

- Aslan A & Triadafilopoulos G (1992) Fish oil fatty acid supplementation in ulcerative colitis: a double-blind, placebo-controlled, crossover study. *American Journal of Gastroenterology* **87**, 432–437.
- Beluzzi A, Boschi S, Brignola C, Munarini A, Cariani G & Miglio F (2000) Polyunsaturated fatty acids and inflammatory bowel disease. *American Journal of Clinical Nutrition* **71**, Suppl. 1, 339S–342S.
- Beluzzi A, Brignola C, Campieri M, Pera A, Boschi S & Miglioli M (1996) Effect of an enteric-coated fish-oil preparation on relapses in Crohn's disease. *New England Journal of Medicine* **334**, 1557–1560.
- Calder PC (1998) Dietary fatty acids and the immune system. *Nutrition Reviews* **56**, S70–S83.
- Casini-Raggi V, Kam L, Chong YJT, Fiocchi C, Pizarro TT & Cominelli F (1995) Mucosal imbalance of IL-1 and IL-1 receptor antagonist in inflammatory bowel disease. A novel mechanism of chronic intestinal inflammation. *Journal of Immunology* **154**, 2434–2440.
- Croft NM, Kelly EJ, Bannerjee K, Dryhurst K & Sanderson I (2001) Diets can modulate the inflammatory response in vivo and in vitro. *Gut* **48**, Suppl. 1, A2.
- Cross ML & Gill HS (1999) Modulation of immune function by a modified bovine whey protein concentrate. *Immunology and Cell Biology* **77**, 345–350.
- de Pablo M & de Cienfuegos GA (2000) Modulatory effects of dietary lipids on immune system functions. *Immunology and Cell Biology* **78**, 31–39.
- Dionne S, D'Agata ID, Hiscott J, Vanounou T & Seidman EG (1998) Colonic explant production of IL-1 and its receptor antagonist is imbalanced in inflammatory bowel disease. *Clinical and Experimental Immunology* **112**, 435–442.
- Donnell SC, Taylor N, van Saene HKF, Pierro A & Lloyd DA (1998) Nutritional implications of gut overgrowth and selective decontamination of the digestive tract. *Proceedings of the Nutrition Society* **57**, 381–387.
- Donnet-Hughes A, Duc N, Serrant P, Vidal K & Schiffrin EJ (2000) Bioactive molecules in milk and their role in health and disease: the role of transforming growth factor- β . *Immunology and Cell Biology* **78**, 74–79.
- Endres S, Ghorbani R, Kelley VE, Georgilis K, Lonnemann G, van der Meer JWM, Cannon JG, Rogers TS, Klempner MS, Weber PC, Schaefer EJ, Wolff SM & Dinarello CA (1989) The effect of dietary supplementation with *n*-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. *New England Journal of Medicine* **320**, 265–271.
- Evers BM, Izukuru M, Townsend CM Jr, Uchida T & Thompson JC (1990) Differential effects of gut hormones on pancreas and intestinal growth during administration of an elemental diet. *Annals of Surgery* **211**, 630–638.
- Fell JME, Paintin M, Arnaud-Battandier F, Beattie RM, Hollis A, Kitching P, Donnet-Hughes A, MacDonald TT & Walker-Smith JA (2000) Mucosal healing and a fall in mucosal pro-inflammatory cytokine mRNA induced by a specific oral polymeric diet in paediatric Crohn's disease. *Alimentary Pharmacology and Therapeutics* **14**, 281–289.
- Ferguson A (1994) Immunological functions of the gut in relation to nutritional state and mode of delivery of nutrients. *Gut* **35**, Suppl. 1, S10–S12.
- Ferguson A, Glen M & Ghosh S (1998) Crohn's disease: nutrition and nutritional therapy. *Baillière's Clinical Gastroenterology* **12**, 93–114.
- Geerling BJ, van Houwelingen AC, Badart-Smook A, Stockbrügger RW & Brummer R-JM (1999) Fat intake and fatty acid profile in plasma phospholipids and adipose tissue in patients with Crohn's disease compared with controls. *American Journal of Gastroenterology* **94**, 410–417.
- Gorard DA, Hunt JB, Payne-James JJ, Palmer KR, Rees RGP, Clark ML, Farthing MJG, Misiewicz JJ & Silk DBA (1993) Initial response and subsequent course of Crohn's disease treated with elemental diet or prednisolone. *Gut* **34**, 1198–1202.
- Griffiths AM, Ohlsson A, Sherman PM & Sutherland LR (1995) Meta-analysis of enteral nutrition as a primary treatment of active Crohn's Disease. *Gastroenterology* **108**, 1056–1067.
- Hawthorne AB, Daneshmend TK, Hawkey CJ, Belluzi A, Everitt SJ, Holmes GKT, Malkinson C, Shahhen MZ & Willars JE (1992) Treatment of ulcerative colitis with fish oil supplementation: a prospective 12 month randomised controlled trial. *Gut* **33**, 922–928.
- Hughes DA & Pinder AC (2000) *n*-3 polyunsaturated fatty acids inhibit the antigen-presenting function of human monocytes. *American Journal of Clinical Nutrition* **71**, Suppl. 1, 357S–360S.
- James MJ, Gibson RA & Cleland LG (2000) Dietary polyunsaturated fatty acids and inflammatory mediator production. *American Journal of Clinical Nutrition* **71**, Suppl. 1, 343S–348S.
- Lamont AG, Gordon M & Ferguson A (1987a) Oral tolerance in protein-deprived mice. I. Profound antibody tolerance but impaired DTH tolerance after antigen feeding. *Immunology* **61**, 333–337.
- Lamont AG, Gordon M & Ferguson A (1987b) Oral tolerance in protein-deprived mice. II. Evidence of normal 'gut processing' of ovalbumin, but suppressor cell deficiency in deprived mice. *Immunology* **61**, 339–343.
- Lamont AG, Gordon M & Ferguson A (1988) T lymphocyte function in protein deprived mice. *Clinical and Experimental Immunology* **72**, 113–117.
- Leiper K, Woolner J, Mullan MMC, Parker T, van der Vleit M, Fear S, Rhodes JM & Hunter JO (2001) A randomised controlled trial of high versus low long chain triglyceride whole protein feed in active Crohn's disease. *Gut* **48**, Suppl. 1, A2.
- Lorenz R, Weber PC, Szimnau P, Heldwien W, Strasser T & Loeschke K (1989) Supplementation with *n*-3 fatty acids from fish oil in chronic inflammatory bowel disease – a randomised, placebo-controlled, double-blind, cross-over trial. *Journal of Internal Medicine* **225**, Suppl. 1, 225–232.
- Lorenz-Meyer H, Bauer P, Nicolay C, Schulz B, Purmann J, Fleig WE, Scheuren C, Koop I, Pudel V, Carr L & German Crohn's Disease Study Group (1996) Omega-3 fatty acids and low carbohydrate diet for maintenance of remission in Crohn's disease. *Scandinavian Journal of Gastroenterology* **31**, 778–785.
- Mansfield JC, Gjaffer MH & Holdsworth CD (1995) Controlled trial of oligopeptide versus amino acid diet in treatment of active Crohn's disease. *Gut* **36**, 60–66.

- Meister D, Aldhous MC, Bode J, Shand A, Johnson W, Giffen S & Ghosh S (2001a) Anti-inflammatory effect of elemental diet *in vitro* depends on fatty acid composition. *Gut* **48**, Suppl. 1, A2.
- Meister D, Aldhous MC, Bode J, Shand A, Johnson W, Giffen S & Ghosh S (2001b) Fish oil substituted elemental diet in ulcerative colitis. *Gut* **48**, Suppl. 1, A73.
- Minard G & Kudsk KA (1998) Nutritional support and infection: does the route matter? *World Journal of Surgery* **22**, 213–219.
- Mingrone G, Greco AV, Benedetti G, Capristo E, Semeraro R, Zoli G & Gasbarrini G (1996) Increased lipid oxidation in Crohn's disease. *Digestive Disease and Sciences* **41**, 72–76.
- Nieto N, Fernandez MI, Torres MI, Ríos A, Suarez MD & Gil A (1998) Dietary monounsaturated *n*-3 and *n*-6 long-chain polyunsaturated fatty acids affect cellular antioxidant defence system in rats with experimental ulcerative colitis induced by trinitrobenzene sulphonic acid. *Digestive Disease and Sciences* **43**, 2676–2687.
- Pereira SP, Cassel TB, Engelman JL, Sladen GE, Murphy GM & Dowling RH (1996) Plasma arachidonic acid-rich phospholipids in Crohn's disease: response to treatment. *Clinical Science* **91**, 509–512.
- Playford RJ, Macdonald CE & Johnson WS (2000) Colostrum and milk-derived peptide growth factors for the treatment of gastrointestinal disorders. *American Journal of Clinical Nutrition* **72**, 5–14.
- Rowlands BJ & Gardiner KR (1998) Nutritional modulation of gut inflammation. *Proceedings of the Nutrition Society* **57**, 395–401.
- Royall D, Jeejeebhoy KN, Baker JP, Allard JP, Habal FM, Cunnane SC & Greenberg GR (1994) Comparison of amino acid v peptide based enteral diets in active Crohn's disease: clinical and nutritional outcome. *Gut* **35**, 783–787.
- Serizawa H, Miura S, Tashiro H, Imaeda H, Shiozaki H, Ohkubo N, Kimura H, Tanaka S & Tsuchiya M (1994) Alteration of mucosal immunity after long-term ingestion of an elemental diet in rats. *Journal of Parenteral and Enteral Nutrition* **18**, 141–147.
- Shand AG, Meister D, Aldhous M, Anderson N & Ghosh S (2000) Direct anti-inflammatory effect of elemental diet on Crohn's tissue *in vitro*. *Gut* **46**, Suppl. 11, W42.
- Shoda R, Matsueda K, Yamato S & Umeda N (1996) Epidemiologic analysis of Crohn disease in Japan: increased dietary intake of *n*-6 polyunsaturated fatty acids and animal protein relates to the increased incidence of Crohn disease in Japan. *American Journal of Clinical Nutrition* **63**, 741–745.
- Sinha A, Nightingale JMD, West KP, Berlanga-Acosta J & Playford RJ (2001) Can left-sided ulcerative colitis be treated with epidermal growth factor enemas? *Gut* **48**, Suppl. 1, A1.
- Stenson WF, Cort D, Rodgers J, Burakoff R, DeSchryver-Keeskemeter K, Gramlich TL & Beeken W (1992) Dietary supplementation with fish oil in ulcerative colitis. *Annals of Internal Medicine* **116**, 609–614.
- Teahon K, Pearson M, Smith T & Bjarnason I (1995) Alterations in nutritional status and disease activity during treatment of Crohn's disease with elemental diet. *Scandinavian Journal of Gastroenterology* **30**, 54–60.
- Teahon K, Smethurst P, Pearson M, Levi AJ & Bjarnason I (1991) The effect of elemental diet on intestinal permeability and inflammation in Crohn's disease. *Gastroenterology* **101**, 84–89.
- Verma S, Brown S, Kirkwood B & Gjaffer MH (2000) Polymeric versus elemental diet as primary treatment in active Crohn's disease: a randomised double-blind trial. *American Journal of Gastroenterology* **95**, 735–739.
- Wallace FA, Neely SJ, Miles EA & Calder PC (2000) Dietary fats affect macrophage-mediated cytotoxicity towards tumour cells. *Immunology and Cell Biology* **78**, 40–48.
- Wilschanski M, Sherman P, Pencharz P, Davis L, Corey M & Griffiths A (1996) Supplementary enteral nutrition maintains remission in paediatric Crohn's disease. *Gut* **38**, 543–548.
- Wong CW, Seow HF, Liu AH, Husband AJ, Smithers GW & Watson DL (1996) Modulation of immune responses by bovine beta-casein. *Immunology and Cell Biology* **74**, 323–329.
- Xu D, Lu Q & Deitch EA (1998) Elemental diet-induced bacterial translocation associated with systemic and intestinal immune suppression. *Journal of Parenteral and Enteral Nutrition* **22**, 37–41.
- Yuceyar H, Ozutemiz O, Huseyinov A, Saruc M, Alkanat M, Bor S, Coker I & Batur Y (1999) Is administration of *n*-3 fatty acids by mucosal enema protective against trinitrobenzene-induced colitis in rats? *Prostaglandins, Leukotrienes and Essential Fatty Acids* **61**, 339–345.
- Zoli G, Carè M, Parazza M, Spanò C, Biagi PL, Bernardi M & Gasbarrini G (1997) A randomised controlled study comparing elemental diet and steroid treatment in Crohn's disease. *Alimentary Pharmacology and Therapeutics* **11**, 735–740.