Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

Molecular Characterisation of a New Variant of Inflammatory Bowel Disease in Children with Autism.

By

Cara Martin, B.Sc. (Hons), M.Sc.

Department of Histopathology and Morbid Anatomy,
Trinity College, Dublin.

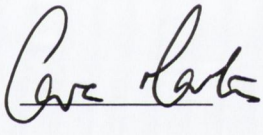
A thesis submitted to Trinity College,
University of Dublin,
for the degree of
Doctor of Philosophy.

January 2004.

Under the supervision of Professor John J. O'Leary.

Declaration

I declare that this thesis is my own work, and has not been submitted previously for a PhD degree at this or any other university. I agree that the library may lend or copy this thesis on request.



Cara Martin

Table of Contents.

3.0 **Detection of measles virus in children with new variant inflammatory bowel disease "autistic enterocolitis"**

List of Figures

List of Tables

Acknowledgements

Firstly I would like to extend my sincerest thanks to my supervisor Professor John O'Leary. His endless support, encouragement and enthusiasm are greatly appreciated.

I would also like to thank Dr Andrew Wakefield, Dr Andrew Anthony and Dr Simon Murch from the Royal Free Hospital, London for supplying the samples for this study. I must also thank the staff in the Gastroenterology Department at AMNCH, in particular Professor O'Morain for giving me an insight into the clinical side of endoscopy and inflammatory bowel disease.

To everyone in the laboratory at the Coombe Women's Hospital, in particular Aoife, Niamh, Richie and Loretto, for all their help and for making this an enjoyable place to work. Thanks also to Catriona, Juanita, Edgar, Martina and Helena for sharing many coffee and lunch breaks.

Thanks also to all the researchers in the James's lab, Paul, Steve and Esther and Dr Orla Sheils for her help with reading this manuscript.

To my housemates past and present, Eimear, Sandrene, Tina, John and Drew thanks for being good friends and for putting up with me throughout this. And to everyone else thanks.

Finally, a special thanks to my parents for their constant encouragement and support. through all my college years.

Summary

A new variant of inflammatory bowel disease, provisionally termed "autistic enterocolitis" has been described in a cohort of children with autistic spectrum disorders. The intestinal pathology includes ileo-colonic lymphonodular hyperplasia and non-specific colitis, which lacks the specific diagnostic features of Crohn's disease or ulcerative colitis. The level of gastrointestinal dysfunction in these children is poorly understood and studies to date have focused primarily on macroscopic and microscopic features of the condition. In addition, the aetiology remains unknown, although an association between measles virus infection or vaccination, onset of gastrointestinal symptoms and developmental regression in these children, has been speculated.

The purpose of this study was to further examine the characteristics of this condition. Initially, the association between measles virus and this condition was investigated. Following this, the cytokine expression profiles in the intestine and peripheral blood were examined, to assess the functional immunology of intestinal mucosa in these children. To further characterise the intestinal condition, gene expression profiles were examined using Affymetrix GeneChip technology.

Measles virus genomes (F, H and N genes) were detected in the intestinal tissue of 74% of affected children with the disorder compared with 14% of control children ($p<0.000$) using a combination of TaqMan RT PCR and in situ RT PCR. The virus was localized to dendritic cells and some lymphocytes within reactive lymphoid follicles in ileal biopsies from affected children. Validation of the TaqMan RT PCR assays was performed in a CD46 transgenic mouse model of measles infection.

Analysis of the mRNA expression profiles of 24 cytokines in intestinal tissues from autistic enterocolitis cases, compared with lymphonodular hyperplasia and IBD controls was performed using TaqMan Microfluidics Card technology. The results demonstrated elevated expression of pro-inflammatory cytokines TNF-α, IL-6 and IL-1 and the anti-inflammatory cytokine IL-10 in the autistic enterocolitis cohort. These findings are indicative of intestinal inflammation and an abnormal innate immune response in these children.

In the final part of the study, the gene expression profiles of autistic enterocolitis, Crohn's disease and ulcerative colitis were examined using Affymetrix GeneChip technology. In total 2161 differentially regulated genes were identified across all disease groups. These included cytokines, chemokines and related genes. In addition there was evidence of a disrupted intestinal mucosa in the autistic enterocolitis cohort as indicated by decreased expression of E-cadherin and increased expression of MMP14. Other interesting differentially regulated genes, not previously described in IBD included oligophrenin 1 and the serotonin 5'-hydroxytryptamine 2C (5'HT 2C) receptor gene. This data identifies genes that may play a crucial role in the pathogenesis of autistic enterocolitis and IBD.

List of Abbreviations

A	Adenine
ADHD	Attention deficit hyperactivity disorder
AE	Autistic enterocolitis
AEC	Amino-ethyl carbazole
AEC	Amino-ethyl carbazole
AP	Alkaline phosphatase
APC's	Antigen presenting cells
APES	Aminopropyltriethoxysaline
BCIP	Bromo-chloro-indolyl-phosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine Serum Albumin
BSA	Bovine serum albumin
C	Cytidine
CARD	Caspase recruitment domain
CD	Crohn's disease
cDNA	Complementary DNA
CNS	Central nervous system
$CoCl_2$	Cobalt chloride
cRNA	copy RNA
Ct	Threshold cycle
CYT	Cytoplasmic tail domain
ddNTP's	Dideoxynucleotide triphosphate
DEPC	Diethyl pyrocarbonate
DIG	Digoxgenin
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNP	Dinitrophenol
DNTPs	Deoxynuclotide triphosphate
DSM IV	Diagnostic and Statistical Manual of Mental Disorders, fourth edition
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate

dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dUTP	Deoxyuridine triphosphate
FACS	Fluorescence-activated cell sorting
Kb	Kilobase
L	Litre
NCBI	National Center for Biotechnology Information
NO	Nitric oxide
ELISA	Enzyme linked immunosorbent assay
EST	Expressed sequence tag
F	Fusion
FA	Formaldehyde agarose
FAM	6-carboxy fluoroscein
FDRC's	Follicular dendritic reticulum cells
g	Gram
G	Guanidine
GABA	Gamma-aminobutryric acid
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde –3-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte colony stimulating factor
GI	Gastrointestinal
GM-CSF	Granulocyte macrophage colony stimulating factor
H	Haemaglutinin
M	Matrix
H&E	Haematoxylin and eosin
H_2O	Water
H_2O_2	Hydrogen Peroxide
HCL	Hydrochloric acid
HEV	High endothelial venules (HEV)
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase

IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IKK	Iκβ kinase
Iκβ	NF-κβ inhibitors
IL	Interleukin
IPTG	Isopropyl-β-D-thiogalactopyranoside
JAK	Janus Kinases
KCL	Potassium chloride
KOAc	Potassium acetate
L	Large
LB	Luria-Bertani
LNH	Lymphonodular hyperplasia
LPS	Lipopolysaccharide
LT	Lymphotoxin
M	Molar
MAPK	Mitogen activated protein kinase
MCP	Membrane cofactor protein
MEKK	Mitogen activated protein kinase kinase
MGB	Minor Groove Binder
MgCl2	Magnesium chloride
MgOAc	Magnesium acetate
$MgSO_4$	Magnesium sulphate
MHC	Major histocompatibility complex
MIBE	Measles inclusion body encephalitis
mM	Millimolar
MM	Mismatch
MMP	Matrix mettalloproteinase
MMR	Measles mumps rubella
MnOAc	Manganese acetate
MOPS	Morpholino propanesulfonic acid
mRNA	messenger RNA
MV	Measles virus

N	Nucleocapsid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBT	Nitro-bromo-tertrazolium
NF	Nuclear factor
ng	Nanogram
NH_4AC	Sodium acetate
NIK	NF-κβ inducing kinase (NIK)
NV-IBD	New variant inflammatory bowel disease
OD	Optical density
ORF	Open reading Frame
P	Phosphoprotein
P13K	Phosphatidylinositol 3-kinase
PAP	Pancreatitis associated protein
PBMC's	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDAR	Pre-developed Assay Reagents
PDH	Pyruvate dehydrogenase
pg	Picogram
PM	Perfect match
pmol	Picomole
RCA	Regulators of complement activation
Reg1α	Lithostathine
RNA	Ribonucleic acid
RNP	Ribonucleoprotein particles
rpm	Revolutions per minute
rRNA	Ribosomal RNA (rRNA),
RT	Reverse transcription
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCR	Short conserved domain
SD	Standard deviation

SDS	Sequence detection system
SDS	Sodium dodecyl sulphate
5' HT-2C	Serotonin hyroxytryptamine
SLAM	Signalling lymphocyte activation molecule
SSC	Sodium citrate
SSPE	Subacute sclerosing panencephalitis
STAT1	Signal transducer and activator of transcription
T	Thymidine
TAMRA	6-carboxy-tetramethyl rhodamine
TBE	Tris-boric acid EDTA
TBS	Tris Buffered Saline
TGF	Transforming Growth factor
Th	T helper
TLR	Toll like receptor
Tm	Melting temperature
TNF	Tumour necrosis factor
TOLLIP	Toll interacting protein (Tollip).
TRADD	TNFR-associated death domain
TRAF	TNF receptor associated protein
TRIS	Tris(hydroxymethyl)methylamine
tRNA	Transfer RNA
TSA	Tyramide Signal Amplification
UC	Ulcerative colitis
µg	Microgram
µm	Micrometer
µM	Micromolar
UTP	Uridine 5' triphosphate
UV	Ultra violet
X-gal	5-bromo-4-chloro-3indolyl-β-D-galactopyranoside

Publications

Book Chapters

O'Leary, J.J., **Martin, C**., Sheils, O. 2003. The *In-Situ* Polymerase Chain Reaction. *In* Molecular Biology in Cellular Pathology (Editors; Crocker, J., Murray, P.G.). John Wiley & Sons, Ltd.

O'Leary, J.J., Sheils, O**, Martin, C**., Crowley A. 2003. TaqMan Technology and Real-Time Polymerase Chain Reaction. *In* Molecular Biology in Cellular Pathology (Editors; Crocker, J., Murray, P.G.). John Wiley & Sons, Ltd.

O'Leary, J.J., **Martin, C**., Sheils, O. 2003. Blots dots, amplification and sequencing. *In* Molecular Biology and Pathology of Paediatric Cancer (Editors; Cullinane, C. J., Burchill, S.A., Squire, J.A., O'Leary, J.J.) Oxford University Press.

Journal Articles

Cotter A.M. **Martin, C.M**., O'Leary, J.J. Daly, S.F. Increased fetal DNA trafficking in early pregnancy is associated with an increased risk of pre- eclampsia. *American Journal of Obstetrics and Gynaecology*, 2004:**191(2):**515-20.
PMID: 15343229

Cotter A.M. **Martin, C.M**., O'Leary, J.J. Daly, S.F. Increased maternal fetal cell trafficking and Rh D gene immunization in pre- eclampsia. *Submitted to Clinical Chemistry 2003.*

Bofin, A.M., Ytterhus, B., **Martin. C.M.,** O'Leary, J.J., Hagmar, B.M. Detection and Detection and quantitation of HER-2 gene amplification and protein expression in breast carcinoma. *American Journal of Clinical Pathology*. 2004;**122**(1):110-9.
PMID: 15272539

Turner, M.J., **Martin C.M.**, O'Leary, J.J. Detection of fetal Rhesus D gene in whole blood of women booking for antenatal care. *European Journal of Obstetrics Gynecology and Reproductive Biology*. 2003;**110**:118
PMID: 12932888

Turner, M.J., **Martin C.M.**, O'Leary, J.J. Detection of fetal Rhesus D gene in whole blood of women booking for antenatal care. *European Journal of Obstetrics Gynecology and Reproductive Biology*. 2003;108: 29-32.
PMID: 12694966

Murphy N, Ring M, Killalea AG, Uhlmann V, O'Donovan M, Mulcahy F, Turner M, McGuinness E, Griffin M, **Martin, C**, Sheils O, O'Leary JJ. p16INK4A as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and ThinPrep smears. *Journal of Clinical Pathology*. 2003;**56**:56-63.
PMID: 12499437

Martin CM, Uhlmann V, Killalea A, Sheils O, O'Leary JJ. Detection of measles virus in children with ileo-colonic lymphoid nodular hyperplasia, enterocolitis and developmental disorder. *Molecular Psychiatry*. 2002;**7** :S47-8.
PMID: 12142949

Uhlmann*, V., **Martin*, C.M.**, Sheils, O., Pilkington, L., Silva, I., Killalea, A., Murch, S.B.,Walker-Smith, J., Thomson, M., Wakefield, A.J., O'Leary, J.J. Potential viral pathogenic mechanism for new variant inflammatory bowel disease. *Molecular Pathology*. 2002 ;**55**:84-90.
PMID: 11950955

Smyth, P., Sheils, O., Finn, S., **Martin, C.**, O 'Leary, J., Sweeney, E.C. Real time Quantitative Analysis of E-cadherin expression in ret/PTC-1-Activated Thyroid Neoplasms. *International Journal of Surgical Pathology*, 2001; **9**:265-272.

O'Donovan, M., Silva, I., Uhlmann, V., Bermingham, N., Luttich, K., **Martin, C.**, Sheils, O., Killalea, A., Kenny, C., Pileri, S., O'Leary, J.J. Expression profile of human herpesvirus 8 (HHV-8) in pyothorax associated lymphoma and in effusion lymphoma. *Molecular Pathology*. 2001 ;**54**:80-5.
PMID: 11322168

Uhlmann, V., Prasad, M., Silva, I., Luettich, K., Grande, L., Alonso, L., Thisted, M., Pluzek, K.J., Gorst, J., Ring, M., Sweeney, M., Kenny, C., **Martin, C.**, Russell, J., Bermingham, N., O'Donovan, M., Sheils, O., O'Leary, J.J. Improved in situ detection method for telomeric tandem repeats in metaphase spreads and interphase nuclei. *Molecular Pathology*. 2000; **53**:48-50.
PMID: 10884922

O'Leary, J.J., Kennedy, M., Luttich, K., Uhlmann, V., Silva, I., Russell, J., Sheils, O., Ring, M., Sweeney, M., Kenny, C., Bermingham, N., **Martin, C.**, O'Donovan, M., Howells, D., Picton, S., Lucas, S.B. Localisation of HHV-8 in AIDS related lymphadenopathy. *Molecular Pathology*. 2000 ;**53**:43-7.
PMID: 10884921

Martin, C., Roberts, D., van Der Weide, M., Rossau, R., Jannes, G., Smith, T., Maher, M. Development of a PCR-based line probe assay for identification of fungal pathogens. *Journal of Clinical Microbiology*. 2000 ;**38**:3735-42.
PMID: 11015393

Published Abstracts.

Martin C.M., Sheils, O., Murch, S., O'Leary, J.J. Cytokine mRNA expression profile in children with lymphonodular hyperplasia and autistic spectrum disorders. United States and Canadian Academy of Pathology, Annual Meeting. 2004 (Platform presentation).

Martin C.M., Hickey, R., Sheils, O., Wakefield, A.J., Murch, S., O'Leary, J.J. Cytokine Gene Expression Analysis in Autistic Enterocolitis. *Journal of Pathology*. 2003; **201**: 1A-60A.

Martin, C.M., Sheils, O., Wakefield, A.J., Murch, S., O'Leary, J.J. Gene Expression Profiling of Inflammatory Bowel Disease. *Journal of Pathology*. 2003; **201**: 1A-60A.

Crowley, A., **Martin, C.**, O'Herlihy, C., O'Leary, J.J., Byrne, B. The dynamics of fetal trafficking in pregnancy – more questions than answers. *Journal of the Society of Investigation.* 2003; **10**: A207-A306.

Silva, I., Smyth, P., **Martin, C.**, Sheils, O., O'Leary, J.J. Common endothelial cell genetic events occur during HHV 8 transformation: evidence from primary transformed endothelial and co-hybrid transformed culture model systems. *Journal of Pathology*. 2003; **201**: 1A-60A.

Murphy, N., Long, C., Mulcahy, F., McGuinness, E., Griffin, M., **Martin, C.M.,** Sheils, O, O'Leary, J.J. Absolute Quantitation of CDC6 and MCM5 mRNA in Cervical Intraepithelial Neoplasia and Invasive Squamous Cell Carcinoma of the Cervix. *Journal of Pathology*. 2003; **201**: 1A-60A.

Crowley, A., **Martin, C.,** Sheils, O., O'Herlihy, C., O'Leary, J.J., Byrne, B. The Dynamics of Fetal Trafficking in Pregnancy. *Journal of Pathology*. 2003; **201**: 1A-60A.

Cotter, A.M. **Martin, C.M.**, O'Leary, J.J. Daly, S.F. Increased maternal fetal cell trafficking and Rh D gene immunization in pre- eclampsia.
Journal of Pathology. 2002; **198**: 1A-58A.

Cotter A.M. **Martin, C.M.**, O'Leary, J.J. Daly, S.F. Increased fetal DNA trafficking in early pregnancy is associated with an increased risk of pre- eclampsia.
Journal of Pathology. 2002; **198**: 1A-58A.

Sheils, O, Smyth, P, **Martin, C,** O'Leary, J.J. Development of an allelic discrimination type assay to differentiate between strain origins of measles virus detected in intestinal tissue of children with ileocolonic lymphonodular hyperplasia and concomitant developmental disorder. *Journal of Pathology.* 2002; **198**: 1A-58A.

O'Donovan, M., Silva, I., **Martin, C.,** Lucas, S.B, Sheils, O.M., O'Leary, J.J. Expression Profiling of Kaposi's sarcoma reveals dysregulation of immunomodulatory, pro-inflammatory, connective tissue and vascular/haematological related genes. *Journal of Pathology.* 2002; **198**: 1A-58A.

O'Donovan, M., Silva, I., **Martin, C.,** Sheils, O.M. O'Leary, J.J. Molecular Analysis of Primary Effusion Lymphoma using mFISH and CGH analysis: demonstrates common molecular genetic pathways. *Journal of Pathology.* 2002; **198**: 1A-58A.

Hughes, C., **Martin, C.**, Sheils, O., Loftus, B., O'Leary, J.J. Development of a detailed molecular profile for prostate cancer. *Journal of Pathology.* 2002; **198**: 1A-58A.

Crowley, A., O'Leary, J.J., Sheils, O., **Martin, C.**, Tauol, F., Anthony, J., O'Herily, C., Byrne, B..M. Quantitation of Fetal DNA in the peripheral blood of women with preeclampsia in South Africa and Ireland- A useful marker of disease severity? *Journal of Pathology.* 2002; **198**: 1A-58A.

Silva, I., O'Donovan, M., **Martin, C.**, Murphy, C., Sheils, O., Lucas, S.B., O'Leary, J.J. Genome wide evaluation of HHV8 virally transformed co-hybrid cell culture model system. *Journal of Pathology.* 2002; **198**: 1A-58A.

Murphy, D., Uhlmann, V., **Martin, C.**, Sheils, O., O'Leary, J.J. Cytokine Profile analysis in multiple sclerosis. *Journal of Pathology.* 2002; **198**: 1A-58A.

Martin, C.M., Uhlmann, V., Sheils, O., Pilkington, L., Silva, I., Killalea, A., Murch, S.B.,Walker-Smith, J., Thomson, M., Wakefield, A.J., O'Leary, J.J. Potential viral pathogenic mechanism for new variant inflammatory bowel disease. *Journal of Pathology*. 2002; **198**: 1A-58A.

Murphy, N., Ring, M., Killalea AG, Mulcahy F, McGuinness E, Griffin M, **Martin, C**, Sheils O, O'Leary JJ. p16INK4A, CDC6, MCM5 and HPV status: predicitive biomarkers in cervical dysplasia. *Journal of Pathology*. 2002; **198**: 1A-58A.

Cotter, A., **Martin, C.**, O'Leary, J., Daly, S. Increased maternal fetal cell trafficking and RhD gene immunization in pre-eclampsia. *American Journal of Obstetrics and Gynecology*. 2001; **185**:S191.

Cotter A, **Martin C**, O'Leary J, Daly S. Increased maternal fetal cell trafficking in early pregnancy is associated with an increased risk of pre-eclampsia. *American Journal of Obstetrics and Gynecology*. 2001; **185**:S192.

Chapter 1

General Introduction

1.0 General Introduction

The association between autism, inflammatory bowel disease and MMR has become one of the biggest medical controversies of our time. The controversy first exploded into the public domain following The Lancet publication by Wakefield and colleagues in 1998 describing a new variant of inflammatory bowel disease in children with autistic disorders. Onset of gastrointestinal symptoms and behavioural changes in these children were linked to MMR vaccination. Since then the medical, scientific and lay public have been bombarded with confusing and contentious reports, which have not solved the ongoing debate but merely fuelled it. The drastic effects of this have resulted in falling MMR vaccination rates, which were reported as low as 82 % in the UK this year. There is clearly a need for more scientific research in this area. Our own group have been involved in describing the pathological aspects of this new variant of inflammatory bowel disease in the setting of autism (Martin *et al.*, 2002; Uhlmann *et al.*, 2002). It is this that forms the basis of this thesis.

This first chapter provides an overview of our knowledge in this subject area to date. The chapter covers three main topics; to begin with we describe autism, gastrointestinal symptoms in autism and new variant inflammatory bowel disease. This is followed by a review of conventional inflammatory bowel disease and its pathogenesis. The final part of the chapter describes measles virus and measles virus induced immunosuppression.

1.1 Autism

Autism was first described by Kanner in 1943 and has since been defined as a neuropsychiatric disorder characterised by impairments in social development, deviance in communication and repetitive patterns of behaviour (Volkmar *et al.*, 2003; Rapin, 1997). Autism is currently classified as one of five pervasive developmental disorders under the DSM IV diagnostic criteria (Diagnostic and Statistical Manual of Mental Disorders, fourth edition) (American Psychiatric Association, 1994). Rett's disorder, Asperger's syndrome, childhood disintegrative disorder and pervasive developmental disorders (not otherwise specified) are also included in this classification. Diagnosis of autism usually occurs before 3 years of age, with children failing to advance developmentally or after a period of apparently normal development, suffering loss of acquired skills and deterioration in behaviour. This is commonly referred to as "regressive autism". In recent years, there appears to be an increase in the frequency of autism with recent studies reporting incidence rates of 60 per 10,000 children (Fombonne, 2002). Boys are three to four times more likely to be affected than girls (Volkmar *et al.*, 2003). A number of factors including genetic, environmental, neurodevelopmental abnormalities and immunological factors have been implicated in the disease aetiology.

1.1.1 Genetic markers in autism

The genetic basis for autism has been well established. Epidemiological studies have demonstrated a strong familial basis for the disorder with a higher prevalence among siblings. In addition twin studies have shown high concordance rates of more than 90% among monozygotic twins (Bailey *et al.*, 1995). Several genetic loci have been implicated in autism, including regions on chromosomes 7q, 2p, 4p, 15q11-13 and 19p.

Alterations in the neurotransmitter systems for GABA (gamma-aminobutryric acid) and serotonin have also been described. Platelet hyperserotonemia is a common finding in autistic children with most studies reporting a 25-50 % increase in platelet serotonin levels compared with normal controls (Singh *et al.*, 1997; Piven *et al.*, 1991). Serotonin is known to play a key role in neurodevelopment and behavioural processes (Chugani, 2002). Its relevance in autism is not yet understood. However serotonin transporter re-uptake inhibitors have proven successful in the treatment of autism behavioural symptoms (McDougle *et al.*, 1996). In addition, a polymorphism in the transcriptional control region (5-HTTLPR) of the serotonin transporter gene on 15q11-q13 has been associated with autism (Cook *et al.*, 1997; Klauck *et al.*, 1997).

Polymorphisms in the GABRB3 receptor subunit gene (c receptor 3 subunit) located on 15q11-13 have been implicated as susceptibility markers for autistic disorders (Buxbaum *et al.*, 2002; Martin *et al.*, 2000) and plasma gamma-aminobutryric acid (GABA) levels are increased in autism (Dhossche *et al.*, 2002). GABA is a major inhibitory neurotransmitter in the brain and alterations in the GABRB3 receptor

subunit could have major implications during brain development and function, however this has not yet been determined in autism.

Persico *et al.*, (2001) have recently described a trinucleotide repeat polymorphism within the reelin gene on 7q22 that is associated with autism susceptibility. However two subsequent studies have failed to confirm this association (Bonora *et al.*, 2003; Krebs *et al.*, 2002). The reelin gene codes for a protein involved in neuronal migration during development, and reduced blood levels of reelin protein have been reported in children with autism (Fatemi *et al.*, 2002).

1.1.2 Brain abnormalities

The high rates of epilepsy and the mental retardation (75 %) in autism provide direct evidence for brain involvement (Rapin, 1997). Brain abnormalities have been described in autistic patients, including reduced numbers of unusually small neurons in the amygdala and the hippocampus regions of the brain (Kemper *et al.*, 2002). In addition, the overall brain size has been reported larger than in developmentally normal controls (Kemper *et al.*, 2002). The alterations in neurotransmitters serotonin and GABA described above may also be important in brain development and function in autism.

1.1.3 Immune response

An autoimmune response to the central nervous system (CNS) has been suggested in the pathogenesis of autism. Elevated levels of antibodies to the CNS protein, myelin basic protein, which may contribute to altered neuron functioning, have been detected in children with autism (Singh *et al.*, 1998; Singh *et al.*, 1993). There is also evidence that the immune system plays a role in the pathogenesis of autism.

Decreased T cell subsets and increased serum IgM and IgE have been reported in autistic children (Gupta *et al.*, 1998). Recent investigations of possible pro-inflammatory cytokine dysregulation in children with autistic spectrum disorders have reported dysregulation of IL-2, IL-6, IL-1 β and TNFα in the peripheral blood (Jyonouchi *et al.*, 2001; Gupta *et al.*, 1998), suggesting a role for Th2 like cytokines in the pathogenesis of the disease.

1.1.4 Gastrointestinal symptoms in autism

For many years, gastrointestinal symptoms in autism and autistic spectrum disorders have been overlooked and attributed to the behavioural abnormalities associated with the disorders. Anecdotal reports by parents have described evidence of gastrointestinal disturbances, including constipation, diarrhoea, abdominal discomfort and certain food intolerances in particular to wheat and cow's milk. Aside from these parental observations, a number of groups have reported gut abnormalities in autistic children that are associated with disease aetiology (Wakefield *et al.*, 2000; Horvath *et al.*, 1999; D'Eufemia *et al.*, 1996).

The studies by Wakefield and colleagues (1998, 2000) describing a new variant of inflammatory bowel disease in children with regressive autism have attracted the most attention and these are fully discussed below. Another study by Horvath *et al.*, (1999) reported a 69% prevalence of histological oesophagitis and decreased activity of intestinal disaccharidases (digestive enzymes) in 36 autistic children evaluated by upper gastrointestinal endoscopy. While these studies consisted of a highly selected series of children specifically referred to a gastroenterologist for investigation of gastrointestinal symptoms, a number of non-referral studies have also been

performed. Melmed *et al.*, (2000) have looked at a non-referral population of 379 children with pervasive developmental disorders and found that 46% of these children reported chronic diarrhoea and or chronic constipation, compared with 18% of unaffected siblings and 10% of unrelated developmentally normal controls. A similar study by Molloy *et al.*, (2003) found that 24% of children from a general population of autism had a history of chronic GI symptoms. While Black *et al.*, (2002) reported that only 9% of children with autism had GI symptoms prior to diagnosis of autism. The results of the various studies taken together indicate that gastrointestinal symptoms are present in a substantial number of children with autistic spectrum disorders. It is interesting to speculate that this may play a significant role in the aetiology of the disorder.

1.1.5 Autistic enterocolitis

A new variant of inflammatory bowel disease, provisionally named "autistic enterocolitis" has been described in a cohort of children with developmental disorders (Wakefield *et al.*, 2000; Wakefield *et al.*, 1998). The intestinal pathology includes ileo-colonic lymphonodular hyperplasia and non-specific colitis, which lacks the specific diagnostic features of Crohn's disease (e.g. granulomata) or ulcerative colitis (distal colonic inflammation) (Wakefield *et al.*, 2000). The disorder combines elements of a motility disorder including oesophageal reflux, constipation with overflow and an intestinal mucosal inflammation (normally associated with diarrhoea). It has been hypothesised that this new syndrome is associated with the combined measles mumps and rubella (MMR) vaccine (Wakefield *et al.*, 1998). The developmental disorder has been defined as regressive autism where patients have apparently developed normally for the first few years of life before degenerating to

an autistic condition, accompanied by loss of acquired skills and behavioural changes. The onset of gastrointestinal symptoms in these children has been associated with developmental regression, which has been reported by both parents and doctors between a few days to months from the time of vaccination.

The initial study by Wakefield *et al.,* (1998) described prominent ileal lymphoid nodular hyperplasia (LNH) in 10 of 12 patients and histological examination showed the presence of chronic non specific colitis in 11 of 12 patients, while nine age matched control tissues were found to be histologically normal. Each of the 12 patients examined had gastrointestinal symptoms including abdominal pain, diarrhoea, bloating and, in some cases, food intolerance. The study suggested a link between these gastrointestinal symptoms and MMR vaccination in 8 of the 12 cases investigated.

A subsequent study by the same group described the endoscopic and pathological characteristics of ileocolonic lymphonodular hyperplasia (LNH) and mucosal inflammation in 60 autistic children including the 12 patients in the original report (Wakefield *et al.*, 2000). The authors concluded that a new variant of inflammatory bowel disease was present in this group of children with developmental disorders. Of the 60 patients investigated the developmental diagnosis were as follows: autism (50 patients), Asperger's syndrome (5), disintegrative disorder (2), attention deficit hyperactivity disorder ADHD (1), schizophrenia (1) and dyslexia (1). Controls included ileocolonic biopsies from 22 histologically normal children and 20 children with ulcerative colitis. Endoscopy findings showed that ileal LNH was significantly more common in affected children (93%) than in controls (14.3%) and colonic LNH was present in 30% of affected children compared with 5.4% of controls. LNH was

classified according to the prominence and extent of the nodules; no LNH (grade 0), mild (grade 1), moderate (grade 2) and severe (grade 3) (Figure 1.1), and was predominantly moderate (grade 2) in the affected children.

The histological findings reviewed blindly by three pathologists identified reactive follicular hyperplasia in ileal biopsies from 92% of affected children, 29% of ulcerative colitis patients. Reactive hyperplasia was not present in any of the non-IBD controls. Lymphoid follicles numbers were increased in ileal biopsies of LNH (4-5 follicles per biopsy), compared with normal biopsies (2-3 follicles per biopsy); the germinal centres were grossly enlarged and reactive as indicated by tingible body macrophages. Often the outer margins of the T cell zone were not clearly defined as in normal ileal follicles with the lymphoid compartment extending into the adjacent villi. Neutrophil and lymphocyte infiltration of the epithelium overlying the follicles and often the crypt epithelium was also observed with active ileitis (neutrophilic infiltration) seen in 8% of affected patients.

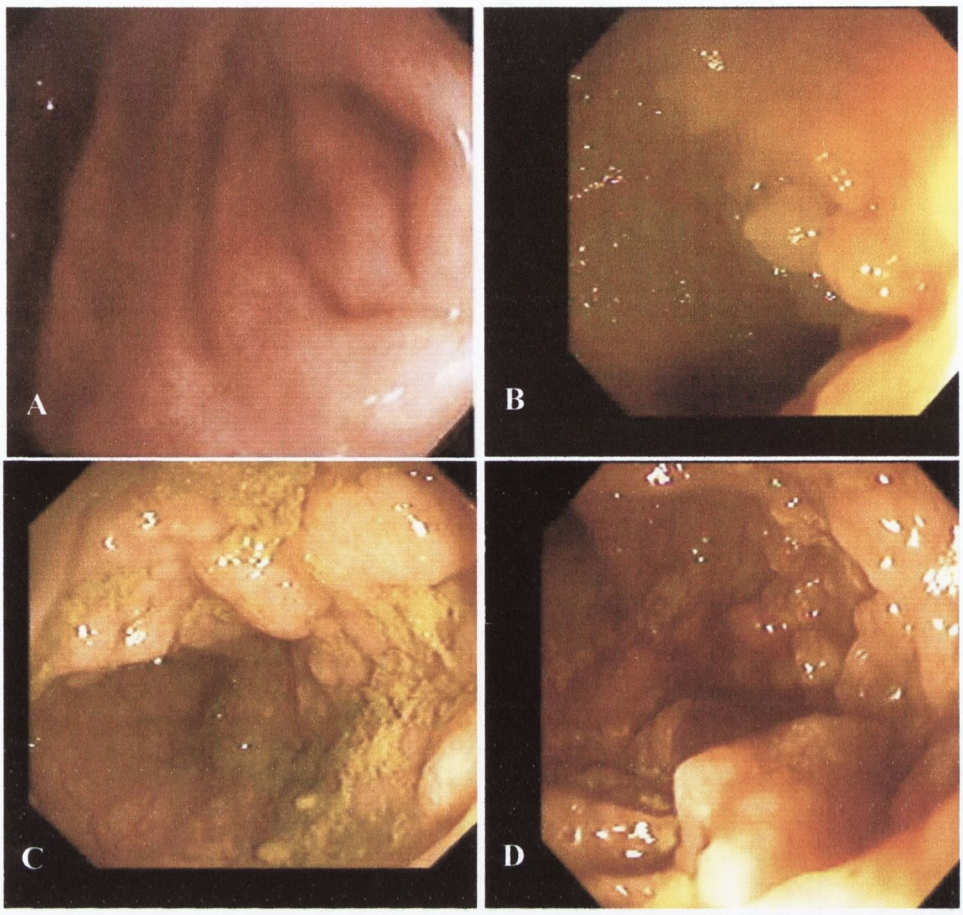


Figure 1.1 Endoscopic evaluation of terminal ileum in affected children showing the representative grades of lymphonodular hyperplasia (LNH).

A. Grade 0- no LNH normal, **B**. Grade 1-mild, **C**. Grade 2- moderate, and **D** Grade 3- severe. (Photographs presented with the permission of Dr Wakefield).

Chronic inflammation was observed in colonic biopsies in 88% of affected children compared with 4.5% of non-IBD controls and 100% of ulcerative colitis patients. This was characterised by increased intraepithelial lymphocytes (13% of affected patients), eosinophilic infiltration of the lamina propria (40% of affected cases) and crypt abscess formation (Wakefield *et al.*, 2000) (Figure 1.2). More recently, a comparison of the lesion with established inflammatory bowel diseases has revealed that the colonic lesion consists of a mucosal infiltrate of intraepithelial lymphocytes; CD3+ cells and in particular CD8+ and γδ T cells, which is significantly greater than that seen in normal or IBD controls (Furlano *et al.*, 2001). Human leukocyte antigen (HLA-DR) was absent in the colonic epithelium suggesting a T helper 2 type immune response (Furlano *et al.*, 2001). Further studies on the small intestinal lesion indicate a cell mediated immune response with increased CD3 and CD8 cells in the lamina propria compared with controls and immunoglobulin G deposition was seen on the epithelial surface which was not seen in any of the normal or disease controls (Torrente *et al.*, 2002).

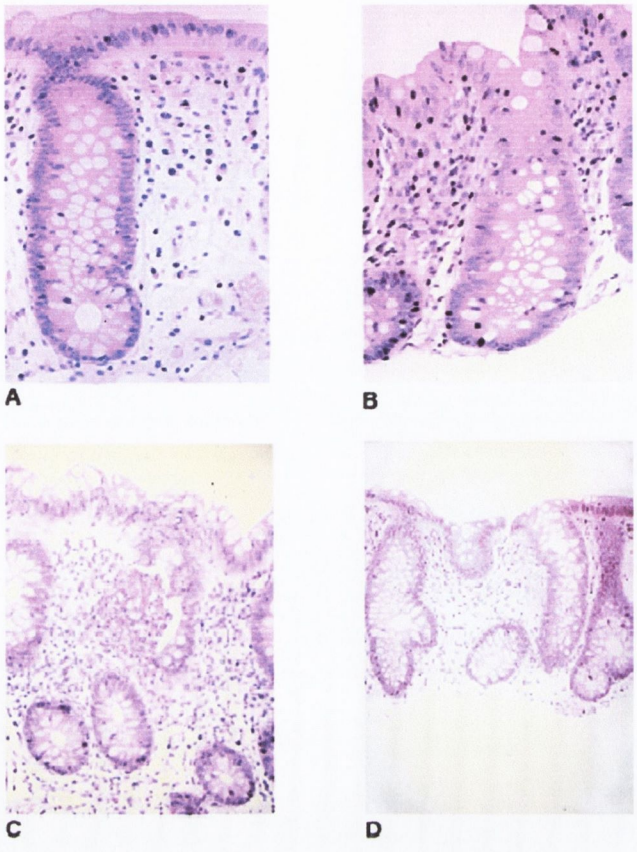


Figure 1.2. Histology of colonic mucosa in autistic enterocolitis.

Images from (Wakefield *et al.*, 2000, permission obtained).

A. Normal colonic mucosa from a non-IBD control child.

B. Colonic mucosa from autistic enterocolitis child, mild disruption and lymphocytic infiltration of epithelium.

C. Crypt abscess formation in autistic enterocolitis.

D. Disruption of crypts in autistic enterocolitis.

In addition, the sulphated glycosaminoglycan (GAG's) content of the basement membrane and epithelium was reduced in the colonic lesion in the autistic cohort (Furlano *et al.*, 2001), providing further evidence of a disrupted mucosal intestinal barrier in autism. These glycosaminoglycans are highly anionic polysaccharide components of the connective tissue matrix and walls of blood vessels. They restrict proteins from exiting the interstitial space and the intestinal lumen. In IBD, in particular in Crohn's disease, disruption of these glycosaminoglycans has been reported with loss of blood proteins into the lumen of the intestine (Murch *et al.*, 1993a).

The presence of reactive follicular hyperplasia and LNH in the small intestine of autistic children suggests an antigen driven response and numerous hypotheses have been described in an attempt explain how intestinal function becomes impaired in autism and how it may influence the disease aetiology. These include the "opioid peptide excess" theory, and the measles/MMR vaccine autism link.

1.1.6 Measles, MMR vaccine, autism, IBD hypothesis

The initial report by Wakefield (1998) suggested a link between the measles mumps and rubella (MMR) vaccine, intestinal symptoms and developmental regression in the autistic children investigated. In eight of the twelve children investigated, parental reports linked the onset of behavioural regression with administration of the trivalent MMR vaccine with an average interval between exposure to vaccine and first behavioural symptoms of 6.4 days. Five of the children had adverse reactions to the vaccine including rashes, fever and convulsions, and one child received monovalent measles vaccine at 15 months and trivalent MMR at 4.5 yrs after which

behavioural problems were noted. Major criticisms of this initial study include firstly the fact that the patient cohort consisted of a highly selected population of children, secondly it was not a case control study and thirdly the study lacked any statistical power calculations to prove either a causal or association effect of the vaccine and the onset of autism. Two previous studies also reported associations between administration of the MMR vaccine and the onset of autism (Fudenburg *et al.*, 1996; Gupta *et al.*, 1996). In addition, the widespread use of the MMR vaccine has reportedly coincided with an increase in the incidence of autism in California (Department of Developmental Services, 1999). The initial Wakefield study has caused great controversy among medical and scientific communities and provoked a series of studies, which examined the relationship between autism, gastrointestinal disease and vaccinations.

A number of epidemiological studies have been performed that failed to find a correlation between MMR and autism (Madsen *et al.*, 2002; Taylor *et al.*, 2002; Fombonne *et al.*, 2001; Kaye *et al.*, 2001; Taylor *et al.*, 1999). The first such study by Taylor (1999) reviewed the clinical records of 498 autistic patients in North East London over a 20 year period starting from 1979. The authors reported a steady increase in the number of cases of autism with no significant difference noted after the introduction of MMR vaccination; similar results were obtained analysing UK general practitioners databases (Kaye *et al.*, 2001). A subsequent study by Taylor's group on 278 autistic patients born between 1979 and 1998, found no difference in the incidences of bowel symptoms and regression before and after the introduction of MMR vaccination (Taylor *et al.*, 2002). Criticism of the above studies suggests the evidence was weak with insufficient statistical power to detect an association and

lacking a population based cohort design (Madsen *et al.*, 2002). The Danish group (Madsen *et al.*, 2002) reported no association between MMR and autism in a study of all children born in Denmark between 1991 and 1998. In this study only 40 of the 316 autistic patients identified were reviewed. These epidemiological studies have been described as fundamentally flawed due to inadequate sample numbers with a bias towards a finding of no association (Spitzer, 2003; Spitzer *et al.*, 2001).

While the epidemiological evidence against a causal effect for all types of autism is strong, with millions of children successfully vaccinated every year without adverse effects, the possibility remains that a small subgroup of susceptible children may be at increased risk of autism if vaccinated. Molecular and immunological studies have suggested evidence of an association and these are discussed below.

Elevated levels of measles but not rubella or mumps antibodies as measured by enzyme linked immunosorbent assays have been detected in the serum of autistic patients. Antibodies to measles virus haemaglutinin (H) protein from vaccine strain were identified in 83% of autistic cases but not in normal or sibling controls (Singh *et al.*, 2003).

Measles virus RNA has been detected in peripheral blood of three of nine autistic enterocolitis patients (Kawashima *et al.*, 2000). In 2002, our group published papers reporting the detection of measles virus genes in small bowel tissue from 82% of patients with new variant IBD compared with 7% of controls (Martin *et al.*, 2002; Uhlmann *et al.*, 2002). In these tissues, components of measles virus were localised within follicular dendritic cells and lymphocytes (Martin *et al.*, 2002; Uhlmann *et al.*,

2002). These findings are presented in Chapter 3 of this thesis. More recently, another group have described detection of measles virus nucleocapsid (N) protein by flow cytometry and immunohistochemistry in follicular dendritic cells within the germinal centres of reactive lymphoid follicles in ileal biopsies from 20 of 22 autistic enterocolitis patients compared with 4 of 21 developmentally normal controls (Ashwood *et al.*, 2003).

Further concerns have been raised that there may be an association between thimerosol, a mercury containing compound that is present in vaccines, and autism (Nelson *et al.*, 2003; Bernard *et al.*, 2001). Thimerosol is an organic compound of ethyl mercury, sodium ethyl mercury thiosalicylate, and was included in vaccines to protect from bacterial and fungal contamination. The clinical symptoms associated with ethyl mercury poisoning mimic those of methyl mercury poisoning, which at high concentrations and prenatal exposure, causes toxic encephalopathy with severe congenital abnormalities (Aschner *et al.*, 2002). Intestinal permeability and the immune system of the gut in rats have been affected by high doses of ethyl mercury (Watzl *et al.*, 1999; Bohme *et al.*, 1992), however data on lower dosages is limited. There are unconfirmed and unpublished reports that autistic children have abnormal levels of mercury. In Denmark, where thimerosol containing vaccines were discontinued in 1992, a population based study investigating whether a parallel decrease in the incidence of autism occurred, indicated no correlation between thimerosol and incidence of autism (Madsen *et al.*, 2003). Thimerosol is currently being eliminated from all vaccine formulations so exposure of children to this toxin will be reduced but currently there is insufficient evidence to associate thimerosol with the increased incidence of autism.

1.1.7 The "Opioid Peptide Excess" theory

In the early 1980's, urinary peptide abnormalities were first reported in autistic spectrum disorders (Reichelt *et al.*, 1981). Specifically, increased levels of the breakdown products of casein, gluten and gliadin were detected in urine of autistic patients. Increased levels of immunoglobulin A antibodies for casein, gluten, and gliadin have also been detected in serum from autistic patients (reviewed in Reichelt *et al.*, 2003; Lucarelli *et al.*, 1995). Gluten and gliadin are found in wheat and cereals and casein is found in milk products. These proteins are digested into amino acids and peptides by pancreatic enzymes and intestinal peptidases in the small intestine. These peptides are structurally similar to endorphins and include β-caseomorphines and gliadmorphines (exorphins due to their dietary origin), which are normally digested by peptidases such as dipeptidyl peptidase IV. Defects in these peptidases, such as that seen in coeliac disease (Smith *et al.*, 1990) can result in increased uptake of peptides in the intestine. Evidence exists that numerous peptidases may be defective as different chain length exorphins have been detected in urine from different autistic children (Reichelt *et al.*, 2003).

In instances where the intestinal mucosa is impaired, with increased epithelial permeability such as that seen in coeliac disease, absorption of these dietary peptides across the intestine can occur. Opioids are known to exert effects on motor and secretory activity in the gastrointestinal tract, increasing the gastric clearing time causing spasms and constipation (Kromer, 1988). On the basis of this, Wakefield (2002) has proposed that opioid mediated intestinal dysfunction may provide an explanation for the paradoxical enterocolitis and chronic constipation observed in autistic enterocolitis.

It has also been hypothesised that some forms of autism may arise as a consequence of the toxic effects of these intestinal products on the developing brain (reviewed Wakefield *et al.*, 2002; Reichelt *et al.*, 2003). Leakage or absorption of these dietary exorphins (β-caseomorphine and gliadmorphin) into the systemic system can potentially affect the central nervous system. There is no evidence to date of altered exorphin levels in cerebrospinal fluid from autistic patients. However evidence does exist that these exorphins can cross the human blood brain barrier; β-caseomorphines have been identified in the cerebrospinal fluid of healthy lactating women (Nyberg *et al.*, 1989) and administration of β-caseomorphine to rats has shown that it crosses the blood brain barrier and binds to areas of the brain that are implicated in autism (Sun *et al.*, 1999). The effect of gluten on the central nervous system has also been suggested in coeliac disease (Reichelt *et al.*, 2003). Untreated coeliac disease is associated with intestinal permeability, increased absorption and urinary excretion of dietary peptides and in some patients ataxia (lack of coordination) and psychotic behaviours.

Further supporting this hypothesis are studies reporting behavioural improvements in autistic children on casein free and or gluten free diets (Knivsberg *et al.*, 2002; Lucarelli *et al.*, 1995). Results of these studies were astounding, with significant behavioural improvements noted in autistic patients within 8 weeks on a diet free of cow's milk. Deterioration in behaviour was subsequently reported when these children were given casein again for a period of 2 weeks (Lucarelli *et al.*, 1995). The incidence of epileptic seizures was also reduced in autistic children on elimination diets that relapsed when the diet was broken (Reichelt *et al.*, 2003). Knivsberg (2002) has also reported improved communication, cognitive and developmental

skills in a group of 10 autistic children (with abnormal urinary peptides) on a casein and gluten free diet for a period of one year compared with 10 autistic patients (also with abnormal urinary peptides) not on an elimination diet. The results of these dietary intervention studies are very promising for the treatment of autistic disorders.

1.2 Lymphonodular hyperplasia (not in autism)

Many endoscopists report that lymphonodular hyperplasia (LNH) in the mucosa of the lower GI tract and terminal ileum is a common finding during paediatric colonoscopies, the exact significance of which remains unclear. However, there are very few reports on LNH in the literature. Kokkonen *et al.*, (1999, 2002a) are among the first studies to describe the prevalence and significance of lymphonodular hyperplasia in the mucosa of the colon, duodenal bulb and the terminal ileum in children undergoing colonoscopy for investigation of gastrointestinal symptoms. LNH was diagnosed in the colon of 46 of 140 patients and was associated with IBD in ten of these patients. In the terminal ileum, LNH was found in 50 of 74 cases (Kokkonen *et al.*, 2002a) and 32 of 55 cases, nine of which had colitis (Kokkonen *et al.*, 2002b). In these and subsequent studies (Bellanti *et al.*, 2003; Kokkonen *et al.*, 2002b; Kokkonen *et al.*, 2000; Kokkonen, 1999), the authors report an association between the presence of lymphonodular hyperplasia and food allergies. Ileal LNH was detected in all patients with food allergies and colonic LNH in 66% of patients with food allergies (Bellanti *et al.*, 2003). Higher densities of intraepithelial $\gamma\delta$ + T cells together with LNH were observed in children with untreated food allergies compared to children on an elimination diet for food allergies (Kokkonen *et al.*, 2002b; Kokkonen *et al.*, 2000).

Immunological abnormalities associated with an imbalance of Th1 function may be the basis of the pathogenesis of non-IgE associated food allergies. Bellanti *et al.*, (2003) reported a predominance of CD4+ cells with a decreased Th1 cytokine profile and a normal Th2 cytokine profile in patients with non IgE mediated food allergies and associated LNH.

1.3 Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) describes a chronic relapsing inflammatory intestinal condition. Ulcerative colitis (UC) and Crohn's disease (CD) are the two main forms of IBD, although other forms do exist, and they are classified on the basis of clinical, endoscopic, radiological and pathological criteria. Both diseases often resemble each other so closely that often they cannot be distinguished pathologically, and are termed indeterminate colitis (Karlinger *et al.*, 2000; Price, 1992). Onset of IBD usually occurs in early adulthood between 15 and 25 yrs although it can occur at any age and can affect both sexes equally (Karlinger *et al.*, 2000). Clinical characteristics include abdominal pain, weight loss and diarrhoea with long term anti-inflammatory treatment usually required. Periods of relapse and remission are features of both disease entities, with surgical intervention often necessary to prevent further spread of the disease.

1.3.1 Pathology of inflammatory bowel disease

Crohn's disease can affect any part of the gastrointestinal tract from the mouth to anus, although the terminal ileum and the colon are more commonly affected. It is characterised by patchy, transmural inflammation (also known as skip lesions) (Price, 1992). The clinical features are determined by the site and behaviour of the disease. Almost 50% of patients have ileocaecal involvement, while others have disease limited to the colon. The histology is characterised by the presence of granulomas and fissuring. These fissures extend from the ulcerated mucosal surface into the submucosa, are lined with inflammatory cells and are the histological basis for the formation of fistulae, which are a characteristic feature of Crohn's disease (Price, 1992).

Ulcerative colitis on the other hand is a disease of the large bowel involving the rectum and colon in a continuous manner. In 30% of patients, the disease is confined to the rectum but may develop more extensively. In 20% of patients the disease involves the whole colon from the outset and patients fall into four symptomatic categories; severe acute colitis, intermittent relapsing colitis, chronic persistent colitis and asymptomatic disease. The clinical severity of the disease is determined using the Truelove and Witts classification (Truelove *et al.*, 1959). The disease is characterised histologically by the presence of crypt abscesses, abundant mucosal neutrophils and goblet cell depletion. Ulceration is also a feature of active disease and can involve the full thickness of the mucosa (Price, 1992).

1.3.2 Prevalence of inflammatory bowel disease

In recent years, the incidence of inflammatory bowel disease has increased dramatically, particularly in childhood. Recent population based studies in adults suggest that the combined prevalence of these diseases in the United Kingdom approaches 400 individuals per 100,000 (Rubin, *et al.*, 2000). Epidemiological, clinical and molecular data has provided evidence that genetic and environmental factors are important in both disease susceptibility and also as determinants of disease progression.

1.3.3 Genetic susceptibility

There is strong evidence that genetic factors are important in the pathogenesis of Crohn's disease and ulcerative colitis. These include wide variations in the incidence and prevalence of CD and UC among different populations and co-segregation in families with rare genetic disorders. Between 6% and 32% of patients with inflammatory bowel disease have an affected first or second degree relative, with the greatest risk to siblings and the least risk to offspring or second-degree relatives (Watts *et al.*, 2002; Parkes *et al.*, 2001). The genetic contribution to the pathogenesis of Crohn's disease is greater than for ulcerative colitis with a relative risk ratio in siblings of affected patients of 20-35 for Crohn's disease and 8-15 for ulcerative colitis (Parkes *et al.*, 2001). Studies in the UK and US have shown high concordance rates for the type, location of disease and extra-intestinal manifestations among members of families with multiple affected individuals (Satsangi *et al.*, 1996) suggesting that the disease phenotype may also be influenced by genetics (Bayless *et al.*, 1996). Epidemiological data from the United States has shown the risk of familial disease is particularly increased among Caucasians especially Jewish

populations, being 2-4 times more prevalent than other racial groups (Yang *et al.*, 1993; Farmer *et al.*, 1980).

1.3.4 Twin studies

Twin studies have been the most valuable source of data for determining the contribution of genetic elements to the development of IBD. Since twins have presumably been exposed to very similar environmental factors, differences between monozygotic and dizygotic twin pairs enables the extent of the genetic contribution to be determined. In total 322 twin pairs have now been reported. In 1988 and in a follow up study in 2003, Tysk *et al,* studied 80 affected twin pairs identified from the Swedish registry of twin births and reported a substantially higher rate of disease concordance in monozygotic twins (18.8 % in ulcerative colitis and 50 % for Crohn's disease) than in dizygotic twins. These results have been replicated by scientists in the UK (Thompson *et al.*, 1996) and Denmark (Orholm *et al*, 1996). Combining data from these European studies shows concordance rates in Crohn's disease of 37% in monozygotic twin pairs and 7% in dizygotic twin pairs. In ulcerative colitis, the equivalent results in monozygotic and dizygotic twins were 10% and 3%, respectively (Watts *et al.*, 2002; Ahmad *et al.*, 2001). The data also indicates that environmental contribution is likely to be greater in ulcerative colitis than in Crohn's disease. These twin studies have complemented the familial inflammatory bowel disease studies and a positive family history of inflammatory bowel disease has been established as the best risk factor for the development of disease.

1.3.5 Genetic markers of inflammatory bowel disease

The main focus of IBD research to date has concentrated on identifying disease susceptibility genes. The current popular genetic model of IBD suggests that ulcerative colitis and Crohn's disease are related polygenic diseases sharing some, but not all, susceptibility loci, a hypothesis that has been supported by linkage studies. The disease phenotype is likely to be determined by the interactions of several gene mutations and the environment.

Genome wide scanning using microsatellite markers and candidate gene association studies have been used to identify disease susceptibility loci. Several candidate regions have been identified and replicated on chromosomes 1, 3, 6, 12, 14 and 16 (Hugot *et al.*, 1996; Satsangi *et al.*, 1996). Three of these regions of linkage received the most attention specifically those on chromosome 16, 12 and 6 and became known as IBD1, IBD2 and IBD3 respectively. The IBD3 region encodes genes within the HLA region and a number of genes within this region have been linked with IBD. TNFα in particular, as well as mutations in the TNF-α promoter region on chromosome 6p21 are associated with susceptibility and pathogenesis of IBD (van Heel *et al.*, 2002). Studies looking at candidate genes within these loci are still ongoing.

The most exciting findings to date have been the identification of the first susceptibility gene ($CARD_{15}$/ NOD2) for Crohn's disease, which is mapped to the IBD1 region of chromosome 16 (Hugot *et al.*, 2001; Ogura *et al.*, 2001a; Hampe *et al.*, 2001). Three main mutations of $CARD_{15}$ have been associated with Crohn's disease but not ulcerative colitis (Hugot *et al.*, 2001; Ogura *et al.*, 2001a; Hampe *et*

al., 2001) and confirmed by several other groups (Bairead *et al.*, 2003; Vavassori *et al.*, 2002). While the precise functional consequences of mutations in the $CARD_{15}$/NOD2 gene are still not clear, it is thought to be responsible for defective recognition of luminal bacteria leading to an abnormal T cell response, cytokine production and tissue inflammation.

1.3.6 Environmental factors

Epidemiological studies have provided strong evidence that environmental and genetic factors are important for both disease susceptibility and progression. Data from twin studies indicates that environmental contribution plays a significant role in the development of ulcerative colitis and to a lesser extent Crohn's disease. Potentially relevant environmental factors include diet, smoking, oral contraceptives, prenatal events, childhood infections and microbial agents. Among these the most established associations are with smoking which appears to be a consistent feature of Crohn's disease, while non-smoking is a feature of UC (Cosnes *et al.*, 2001; Fiocchi, 1998).

1.3.7 Microbial agents

There is little doubt that bacteria are involved in the pathogenesis of IBD. There are several lines of evidence to support this. Firstly, bowel lesions in IBD tend to occur predominantly in areas with the highest bacterial counts (i.e. in the ileocaecum or the large bowel). Secondly, diversion of the fecal stream in Crohn's disease patients following ileostomy is associated with disease improvement (Fiocchi, 1998; Rutgeerts *et al.*, 1991). Similarly, in ulcerative colitis, patients that undergo ileo pouch–anal anastomosis surgery develop mucosal inflammation after colonisation of

the ileal pouch (Fiocchi, 1998). In support of this, studies in genetically engineered rodents have shown that tissue damage or colitis does not occur in the absence of intestinal flora and that commensal bacteria in the intestine are required to maintain the ongoing inflammation in IBD (Rath *et al.*, 2001). More recently, it has been discovered that increased susceptibility to Crohn's disease is associated with a defect in the NOD2/$CARD_{15}$ gene (Hugot *et al.*, 2001; Ogura *et al.*, 2001a), which as discussed below is involved in activation of nuclear factor κβ immune response to bacterial LPS (Ogura *et al.*, 2001b).

Mycobacterium paratuberculosis causes granulomatous enterocolitis in Johne's disease in cattle, similar to that seen in Crohn's disease. There is increasing evidence that *Mycobacterium avium* subspecies *paratuberculosis*, which is found in pasteurised milk, is implicated in Crohn's disease (Greenstein, 2003). Detection of *Mycobacterium* is difficult. It has been suggested that the bacterium may exist in a cell wall deficient form which is not detectable by standard immunohistochemistry (Greenstein, 2003; Wall *et al.*, 1993), however *M. avium* DNA and RNA have been detected in the intestines of Crohn's disease patients (Greenstein, 2003; Mishina *et al.*, 1996; Moss *et al.*, 1992). In a recent study, isolation of granuloma by laser capture microdissection and subsequent PCR amplification has resulted in detection of mycobacterium in significantly more Crohn's disease patients than controls (Ryan *et al.*, 2002).

Other microbes, including *Listeria* and *Esherichia coli,* and viral agents, including herpes virus, rotavirus and in particular, measles virus, have also been implicated in

the aetiology of IBD (Huijsdens *et al.*, 2003; Glasser *et al.*, 2001; Karlinger *et al.*, 2000; Wakefield *et al.*, 1995).

1.3.8 Paramyxovirus and inflammatory bowel disease

Inflammatory bowel disease, in particular Crohn's disease has been linked to persistent measles virus infection (Pardi *et al.*, 2000; Thompson *et al.,* 1995; Wakefield *et al*, 1995; Ekbom *et al.,* 1994; Wakefield *et al.*, 1993). Initial studies by Wakefield *et al.*, (1993) identified paramxyovirus like particles in the vascular endothelium at sites of vascular injury in intestinal tissue from Crohn's disease patients. Subsequent analysis to characterise these particles using immunohistochemistry (using a measles virus monoclonal antibody that was subsequently found to recognise host protein also (Iizuka *et al.*, 2000; Iizuka *et al.*, 1997), electron microscopy and in situ hybridisation, confirmed the presence of measles virus nucleocapsid protein and RNA in intestinal tissue from Crohn's disease patients and ulcerative colitis patients (Lewin *et al.*, 1995; Wakefield *et al.*, 1993).

These observations prompted a population-based study in Sweden that provided strong evidence that perinatal exposure to measles virus was as a strong risk factor for development of Crohn's disease but not ulcerative colitis (Ekbom *et al.*, 1994). Thompson *et al.*, (1995) further extended this to find an association between measles vaccination and both Crohn's disease and ulcerative colitis. A follow up study by Ekbom *et al.*, (1996) found an increased risk of *in utero* exposure to measles virus and the development of Crohn's disease in later life. Others have been unable to detect this association (Pardi *et al.*, 1999; Jones *et al.*, 1997). Pardi (2000) have

since found an association between early exposure to measles infection in children under 5 years old and development of inflammatory bowel disease, while Morris *et al.*, (2000) found no association with Crohn's disease. The discrepancies in the epidemiological studies are most likely related to the different population based methods used to analyse the data.

Another possibility put forward was that mumps virus is associated with IBD, (Montgomery *et al.*, 1999). No difference has been observed in the levels of mumps antibodies between IBD patients or controls (Iizuka *et al.*, 2001). Similarly, no evidence of mumps virus genome has been detected in intestinal tissues from IBD patients (Iizuka *et al.*, 2001; Folwaczny *et al.*, 1999).

Experimental evidence for the persistence of measles infection in inflammatory bowel disease is also conflicting. Miyamoto *et al.*, (1995) reported on the presence of immunoreactive cells in the tissues of patients with Crohn's disease using monoclonal antibodies to measles virus Matrix (M) protein, the specificity of which has come into question by others (Liu *et al.*, 1995). Lewin *et al.*, (1995) identified Nucleocapsid (N) protein in five of six Crohn's disease patients using immunogold electron microscopy, confirmed by Daszak *et al.*, (1997). Serology studies have not detected an increase in measles virus antibody concentration in IBD patients (Van Kruiningen *et al.*, 2000; Fisher *et al.*, 1997).

PCR based methods for detection of various measles virus genes have also been examined. Measles virus N gene was not detected in tissue biopsies or peripheral blood samples from IBD patients using one step PCR, two-step PCR or nested PCR

despite the fact that all patients had detectable measles virus antibodies in their serum (Afzal *et al.*, 1998). Similarly, surgically resected specimens from healthy and diseased tissue were negative for measles virus N, M and H genes (Afzal *et al.*, 2000; Haga *et al.*, 1996) and N and H genes by RT PCR or hybrid capture (Chadwick *et al.*, 1998). In contrast to these studies measles virus F and H genes have been detected by nested PCR and sequencing in the peripheral blood of one of eight patients with Crohn's disease and one of three patients with ulcerative colitis (Kawashima *et al.*, 2000). The discrepancies in all of the studies are most likely related to differences in the techniques applied, assay sensitivities, specific measles targets and tissue specimens examined. The experimental data available to date suggests that measles virus persistence in the intestine of IBD patients is probably at low level and more sensitive assay techniques such as real time PCR may be the solution required to answer this ongoing debate.

1.3.9 Pathogenesis of inflammatory bowel disease

Chronic intestinal inflammation such as that seen in Crohn's disease and ulcerative colitis is a result of mucosal immune activation by commensal bacteria in the lumen of the intestine, dietary antigens or perhaps viral agents. There are two mechanisms of immune activation. Firstly, antigens from luminal bacteria can penetrate the mucosal barrier and directly interact with immune system cells such as dendritic cells and lymphocytes to promote an adaptive immune response. Alternatively, the antigens can stimulate cells of surface epithelium through receptors, which are components of the innate immune response and stimulate the epithelium to produce cytokines and chemokines, which recruit and activate mucosal immune cells (Podolsky, 2002; van Montfrans *et al.*, 2002).

The intestinal mucosa contains a large number of antigen presenting cells (APC's) such as dendritic cells for uptake, processing and transport of antigens to the regional lymph nodes. Activation of these APC's or direct stimulation through pattern recognition receptors (toll like receptors) stimulates these cells through the nuclear factor κβ signalling pathway and promotes differentiation of immature T cells into effector or memory CD4+ cells or cytotoxic CD8+ cells. After antigen uptake, activated dendritic cells migrate to the lymph node where specialised high endothelial venules (HEV) express the chemokine CCR7 ligand CCL2. Naïve T cells express the chemokine CCR7 and migrate into the nearby lymph node via a selectin mediated cascade. Within the lymph node they adhere by integrin mediated adhesion to the endothelium and subsequently interact with dendritic cells, where they differentiate into CD4+ cells or CD8+ cytotoxic T cells (van Montfrans *et al.*, 2002).

The cytokine environment to which CD4+ T cells are exposed determines the differentiation towards a Th1 or Th2 type response. Cytokines such as interleukin 18 and interleukin 12 both produced by antigen presenting cells induce Th1 differentiation, while interleukin 4 produced by natural killer cells and mature CD4+ cells induces Th2 differentiation (van Montfrans e*t al.*, 2002; Roitt *et al.*, 2001). CD4+ lymphocytes have been divided into two sub-populations based upon their profile of cytokine secretion. The Th1 cell population produces IL-2 and IFN γ, and Th2 cells produce IL-4, IL-5, IL-6 and IL-10 (Roitt *et al.*, 2001; Romagnani, 1995). Activated T cells producing Th1 cytokines are responsible for cell-mediated immunity, interacting with mononuclear phagocytes helping them to destroy intracellular pathogens, while those producing Th2 type cytokines are responsible for providing help to B cells to produce immunoglobulins, including IgE (Roitt *et al.*, 2001). CD 8+ T cells in the intestine are thought to be more important in the first line of defence in the epithelium but have also been shown to produce both Th1 and Th2 cytokines (Roitt *et al.*, 2001). A number of cytokines, including interleukin-10 (IL-10) and transforming growth factor β (TGF-β), are secreted by both Th1 and Th2 T cell subsets (Roitt *et al.*, 2001).

The general consensus is that the mucosa of patients with established Crohn's disease is dominated by CD4+ lymphocytes, with a type 1 helper T cell phenotype characterised by the production of IFNγ and IL-2 (Monteleone *et al.*, 1999, Monteleone *et al.*, 1997, Parronchi *et al.*, 1997; Fuss *et al.*, 1996). Conversely, the mucosa of patients with ulcerative colitis may be dominated by CD4+ lymphocytes with a type 2 helper T cell phenotype characterised by production of TGF-β and IL-5 but not IL-4 (Fuss *et al.*, 1996). Increased numbers of dendritic cells producing

IL-12 and IL-18 cytokines have been detected in the intestinal mucosa of Crohn's disease patients (van Montfrans *et al.*, 2002). Additionally, in both forms of IBD, activated macrophages produce a series of non-specific pro-inflammatory cytokines including IL-1, IL-6, and TNF, which act as general inflammatory mediators (Podolsky, 2002). In healthy intestine the anti-inflammatory cytokine IL-10, which is produced predominantly by activated macrophages and T cells, is known to inhibit the antigen presenting function of macrophages and their production of pro-inflammatory cytokines IL-1, IL-6 and TNF-α (Asadullah *et al.*, 2003). Reduced production of IL-10 has been reported in Crohn's disease and levels may be insufficient to downregulate pro-inflammatory cytokines resulting in sustained intestinal inflammation. (Gasche *et al.*, 2000; Autschbach *et al.*, 1998). Disruption of the IL-10 gene in mice leads to development of colitis (Kuhn *et al.*, 1993) with marked increases in concentrations of the IL-1β, IL-6, TNFα and IFN-γ in the lesions (Berg *et al.*, 1996), while treatment by IL-10 gene transfer improved the intestinal inflammation (Barbara *et al.*, 2000). The down regulatory cytokine TGF β may also be dysregulated in IBD in particular in Crohn's disease patients. Increased expression of TGF β has been reported in the mucosa of Crohn's disease patients (Babyatsky *et al.*, 1996), which may be related to the over expression of SMAD7 (an inhibitor of TGFβ) in Crohn's disease (Monteleone *et al.*, 2001). The aberrant immune responses in IBD are characterised by inappropriate T cell responses and dysregulated production of inflammatory and regulatory cytokines.

1.3.10 Nuclear factor κβ signalling in IBD

The mucosal immune responses in gut inflammation are characterised by secretion of proinflammatory cytokines and bacterial LPS that activate the NF-κβ pathway in the gut wall. Activation of NF-κβ proteins play a key role in inflammation in IBD through the regulation of genes including cytokines, chemokines, and growth factors. NF-κB is a heterodimeric complex consisting of two subunits p50 and p65, belonging to the Rel family of transcription factors, which are involved in regulation of gene transcription. In its inactive form NF-κβ exists as an inactive cytoplasmic complex bound to inhibitory components of the Iκβ family.

The NF-κβ complex is activated in response to various stimuli including viral and bacterial infection, exposure to pro-inflammatory cytokines, mitogens and growth factors. Binding of bacterial products and LPS with toll-like receptors on the cell surface trigger the recruitment of the death domain-containing adaptor protein MyD88 (Myeloid differentiation primary response gene 88) and Toll interacting protein (Tollip). (Santoro *et al.*, 2003). Similarly, the proinflammatory cytokine TNF-α interacts with its receptor TNFR1 on the cell surface, via an adaptor protein TRADD (TNFR-associated death domain) activating the NF- κβ signalling cascade. The resulting stimulation of kinases such as NF-κβ inducing kinase (NIK) or mitogen activated protein (MAP) kinase kinase 1 or 3 (MEKK1, MEKK3) (Figure 1.3) initiates Iκβ phosphorylation at specific amino terminal serine residues and dissociation of the NF-κβ-Ikβ complex. The phosporylated Iκβ is then ubiquinated and is subsequently degraded by the 26S proteosome releasing NFκβ dimers (NF-κβ p65 and NF-κβ p50) from the cytoplasmic NFκβ-Iκβ complex allowing them to

translocate to the nucleus where it regulates gene transcription of various cytokines, chemokines and adhesion molecules (Figure 1.3). Cytokines that are stimulated by NF-κβ such as TNF-α and Il-1β, are also inducers of NF-κβ and start a positive auto-regulatory loop that can amplify the inflammatory response and lead to chronic inflammation.

In IBD there appears to be sustained activation and upregulation of NF-κβ (Schreiber *et al.*, 1998), possibly due to cytokines IL-1 and TNF-α, which are produced in excess in inflamed bowel (Fiocchi, 1998). There is also evidence that negatively regulated NF-κβ is involved in inflammation in IBD. A recent study has shown that TGF-β is a negative regulator of transcription factor NF-κβ in the intestine and defective TGF-β signalling due to exaggerated production of SMAD 7 (a substrate for TGF-β receptors) helps to maintain NF-κβ activation (Monteleone *et al.*, 2003; Monteleone *et al.*, 2001).

Mutations in the NOD2 gene in Crohn's disease give rise to a truncated NOD2 protein, which is thought to result in impaired binding of endotoxins and decreased activation of NF-κβ transcription (Ogura *et al.*, 2001a), paradoxical in a disease that is characterised by excess production of TNF-α and NF-kβ. The exact mechanisms of this are not yet understood.

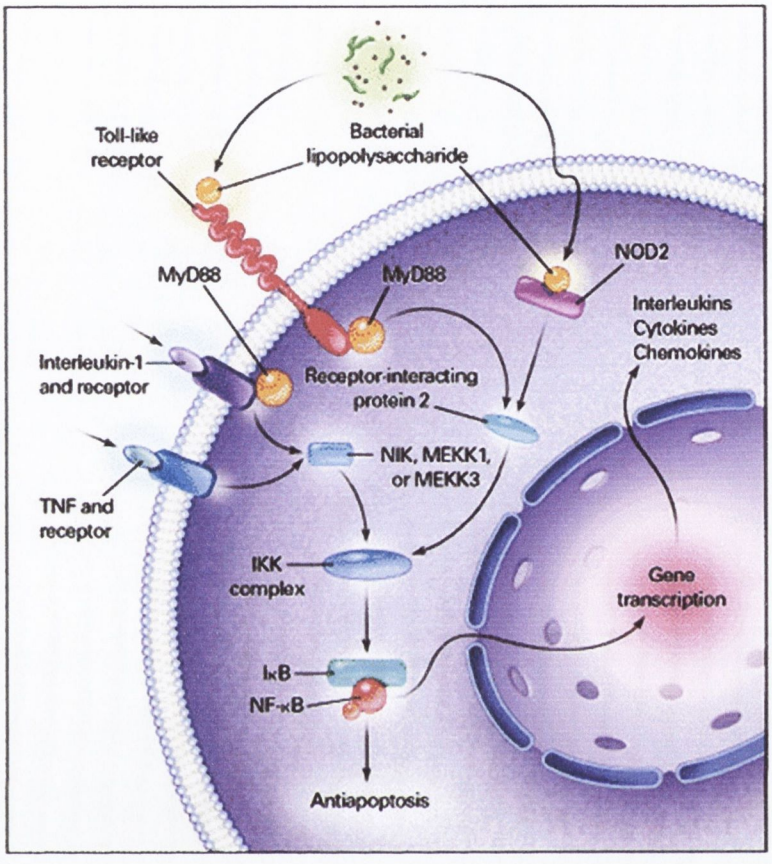


Figure 1.3. Activation in NF-κβ signalling in Inflammatory Bowel Disease

(Image obtained from the following review; Podolsky, D. N.Engl J Med, 2002)

1.4 Measles virus

Measles virus remains one of the leading causes of childhood mortality and morbidity infecting more than 40 million children and leading to more than one million deaths each year (Schneider-Schaulies *et al.*, 2002a). The high morbidity and mortality rate has been associated with measles virus induced suppression of immune functions, which favour the establishment of secondary infections. Vaccination with attenuated virus has greatly reduced the number of cases but measles remains a leading cause of morbidity and mortality in developing countries.

1.4.1 Acute measles infection

Measles virus is transmitted through the respiratory route and exposure leads to local respiratory tract replication. Infection of the local lymphatic tissues then occurs followed by viremia and systemic dissemination as revealed by the characteristic skin rash. The measles rash marks the onset of the immune response indicated by the appearance of virus specific T cells and measles antibodies IgM followed by measles IgG. The major antibody response is directed to measles virus nucleoprotein while antibodies against the haemaglutinin protein are neutralising antibodies, which limit spread of the virus (Oxman, 1997). Elevated levels of CD8+ cytotoxic T cells and CD4+ cells have been detected in the blood during acute infection, and increased production of cytokines IL-2 and IFNγ during the rash is followed by elevated levels of IL-4 that can last for several weeks (Griffin *et al.*, 1993; Ward *et al.*, 1993). This is indicative of a switch from an initial T helper type 1 (Th1) response to a long lasting T helper type 2 (Th2) immune response. This contributes to the prolonged immunosuppression that is associated with measles virus infection.

1.4.2 Persistent measles infections

Measles virus specific immunity is induced during an acute measles infection. Persistence of some component of the virus is thought to be essential to stimulate and maintain adequate antibody titres. However, there is little evidence of widespread persistence of infectious measles in the immune population. Persistence of measles virus in the neuronal and macroglial cells of the central nervous system (CNS) can cause fatal neurological disorders known as subacute sclerosing panencephalitis (SSPE) and measles inclusion body encephalitis (MIBE).

SSPE typically presents in children 6-8 years after the initial measles infection and results in severe mental deterioration. Measles virus is thought to gain entry into the CNS during an acute infection and infects neuronal and glial cells, which are not fully permissive for measles virus. This results in reduced synthesis of envelope glycoproteins (Oxman, 1997), and the establishment of a persistent infection that can slowly progress by cell-to-cell interactions. No single strain of measles virus has been associated with SSPE and persistence of the virus has been linked to mutations following viral replication within the host. There is a high mutation rate during measles virus replication, which may be due to the lack of a proof reading viral RNA polymerase (Rima *et al.*, 1995). Mutations in the measles virus matrix (M) gene leading to a defective M protein (Cattaneo *et al.*, 1989), and in the cytoplasmic tail region of the fusion (F gene) (Schmid *et al.*, 1992) are believed to be the hallmarks of persistent measles infection in SSPE patients which affect the virus's replication. Supporting this, measles virus antigens and RNA have been detected in SSPE tissue (Allen *et al.*, 1996). MIBE, which usually develops 1-7 months after acute measles

infection is also associated with replicative deficient strains of measles virus (Bitnun *et al.*, 1997).

1.4.3 Measles virus structure

Measles is an enveloped RNA virus belonging to the genus *Morbillovirus* within the family *Paramyxoviridia*. Measles virions are generally spherical but pleomorphic enveloped ribonucleoprotein particles (RNP) consisting of the RNA genome tightly encapsulated by nucleocapsid proteins and the viral polymerase complex.

The viral envelope consists of a lipid bilayer derived from the plasma membrane of the infected host cells that carries surface projections (peplomers) with a length 9-15 nm. These peplomers are composed of two different transmembrane glycoproteins, the fusion (F) and haemaglutinin (H) proteins. The H protein is a type II glycoprotein, with two major functions. It binds to the MV receptor (membrane cofactor protein, CD46) on the surface of susceptible host cells, and associates with the F protein to mediate fusion of the viral membrane with the host cell membrane (Nussbaum *et al.*, 1995). The F protein is synthesised as a precursor protein Fo and is proteolytically cleaved and activated into the disulfide bonded F1-F2 heterodimer. Interaction of the H protein with its cellular receptor CD46 or CD150 triggers a conformational change within the F1-F2 heterodimer that leads to the insertion of the hydrophobic fusion domain into the target cell membrane (Figure 1.4) (Schneider-Schaulies *et al.*, 2002a; Oxman, 1997).

On the inside surface of the envelope is the M protein, which appears to interact with the cytoplasmic tails of the F and H transmembrane glycoproteins, and with the

nucleocapsid to play a key role in the virus maturation. The helical nucleocapsid is packed within the envelope in the form of a symmetrical coil. It consists of about 2500 copies of the nucleoprotein bound to the genomic RNA together with a small number of P (phospoprotein) and L (large) proteins (Figure 1.4) (Oxman, 1997).

Three proteins (N, P and L) are complexed with viral RNA to form the nucleocapsid structure. The nucleocapsid protein is the most abundant of all proteins and is synthesized on free ribosomes, folded in the cytoplasm, and phosphorylated on serine and threonine residues. In measles infected Vero cells, the N protein surrounds genomic and antigenomic RNAs that possess a leader sequence to form nucleocapsid structures, which are the required template for both mRNA transcription and RNA replication. The phosphoprotein (P) protein binds to nucleocapsids and is required for RNA encapsidation and cellular location of the N protein. The large (L) protein interacts with the P protein and is required for mRNA transcription and replication (Oxman, 1997). Virus replication is via an RNA dependant polymerase encoded by measles virus phosphoprotein (P) and large protein (L) genes (Oxman, 1997).

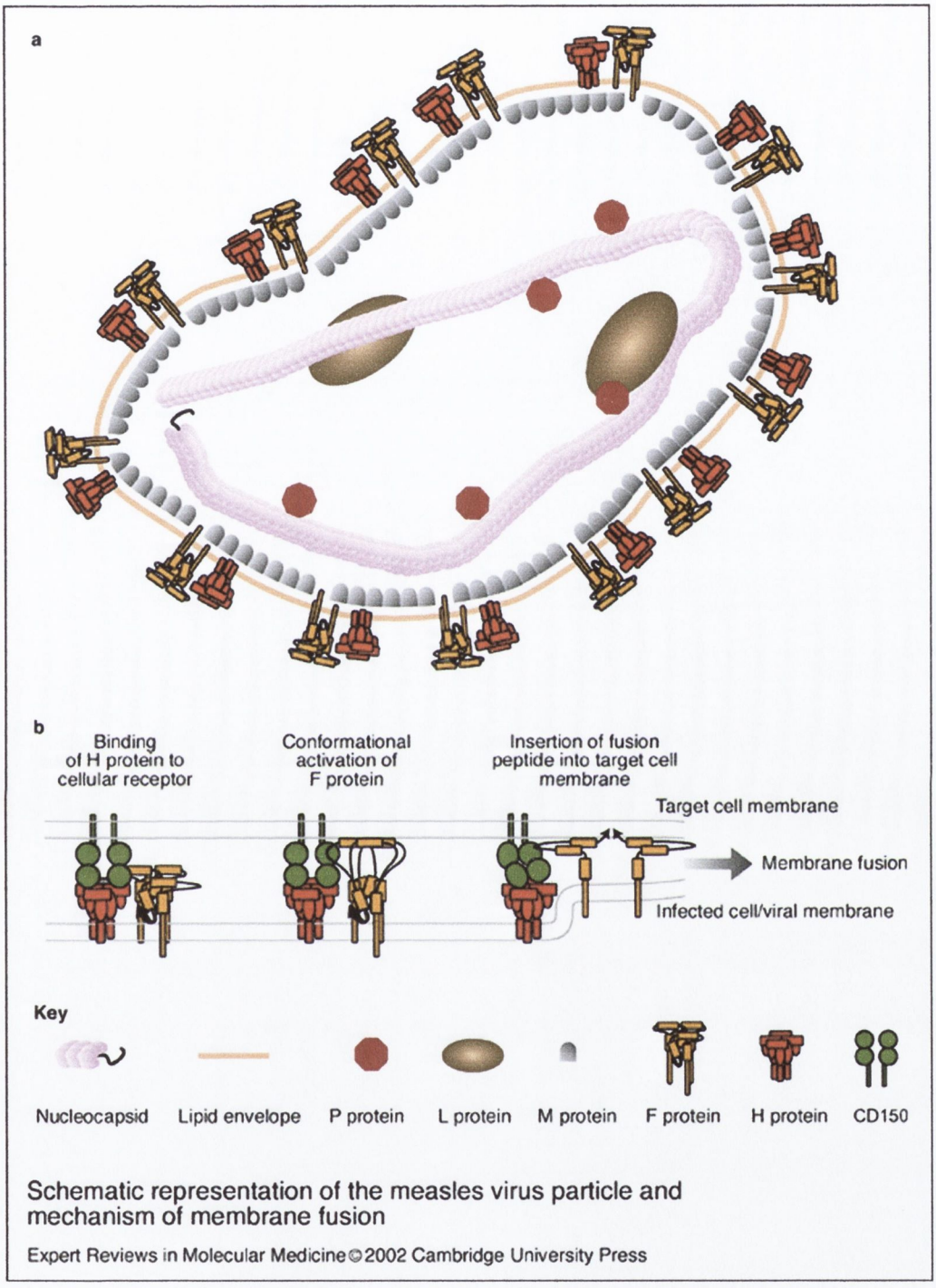


Figure 1.4. Structure of measles virions and mechanism of membrane fusion. (Schneider-Schaulies and ter Meulen, Expert Reviews in Molecular Medicine; 2002a, 4:1-18).

After fusion of the viral envelope with the cell membrane, the viral nucleocapsid is released into the cell cytoplasm and the viral RNA dependant RNA polymerase is activated. The activated polymerase binds to a leader sequence at the 3' end of the negative strand of the measles genome (containing the N gene) and sequentially transcribes the individual genes towards the 5' end (containing the Large protein gene). During transcription the polymerase detaches from the genomic RNA at the intergenic regions following polyadenylation of mRNA and then reattaches to transcribe the next gene (Oxman, 1997). The polymerase lacks proof reading activity, which may account for the large sequence variation between measles strains (Rima *et al*, 1995).

1.4.4 Genomic structure of measles virus

The measles virus genome is a single stranded, negative sense, non-segmented RNA molecule. The 15,900 bp genome encodes six structural proteins and begins at the 3' end with a 53 bp leader nucleotide sequence that shows a high degree of complementarity to the extragenic 40 nucleotide trailer sequence at the 5' end of the genome (Oxman, 1997). After the leader sequence there are 6 non-overlapping consecutive cistrons that encode N, P, M, F, H and L proteins. The intergenic sequences consisting of a single trinucleotide GAA are flanked by consensus sequences. In addition, there is an untranslated region of ~1000 nt at the M-F boundary that spans the 5' end of the M gene and the 3' end of the F gene. The viral polymerase binds to the leader sequence and sequentially transcribes the individual genes. During transcription the polymerase detaches from the genomic RNA at the intergenic regions, following polyadenylation of the mRNA and reattaches to transcribe the next gene. The P cistron codes for three non-structural polypeptides;

P, C and V. The (P) phosphoprotein is a subunit of the viral RNA polymerase and C and V proteins are non structural proteins (Oxman, 1997).

1.4.5 CD46 and CD150 receptors

Initially, CD46 (membrane cofactor protein, MCP) was identified as a cellular receptor for MV (Dorig *et al*, 1993; Naniche *et al.*, 1993). CD46 is a widely expressed cell membrane protein expressed in four isoforms on all human nucleated cells. The protein is a member of the regulators of complement activation (RCA) superfamily, which inhibit complement activation on host cells by binding activated complement components and preventing their deposition on the host cell surface (Casasnovas *et al.*, 1999). The extracellular portion of the CD46 receptor consists of four short conserved domains (SCR's) and a serine threonine rich (ST) domain. The intracellular domain is made up of a transmembrane region and a cytoplasmic tail. The cytoplasmic tail of the receptor possesses motifs that are involved in protein targeting and recruitment (Naniche *et al.*, 1993) and is composed of two cytoplasmic tail domains CYT1 and CYT 2, which arise from alternative splicing, and consist of 16 and 23 amino acids, respectively. The ability of the virus to interact with the CD46 receptor has been assigned to two amino acids (451 and 481) within the measles virus H protein (Hsu *et al.*, 1998; Lecouturier *et al.*, 1996), which have been shown to interact with the short conserved domains (SCR1 and SCR2) of CD46. Amino acid differences at positions 211 and 481 of the H protein are thought to contribute to virus attenuation (Parks *et al.*, 2001). A tyrosine residue at position 481 and glycine at position 211 has been detected in vaccine strains while asparagine has been detected at these positions in wild type isolates (Parks *et al.*, 2001). Interaction of the H protein with the CD46 receptor has been identified as specific for measles

virus vaccine strains and laboratory adapted strains of measles virus (Erlenhoefer *et al.*, 2002; Bartz *et al.*, 1998). In contrast wild type strains isolated from the Epstein-Barr virus transformed marmoset B lymphocyte cell line (B95a) are unable to use CD46 as a receptor. This is indicated by their inability to infect cell lines that are susceptible to the Edmonston laboratory adapted strain (Bartz *et al.*, 1998; Hsu *et al.*, 1998).

More recently CD150 (also known as SLAM: signalling lymphocyte activation molecule) was identified as a cellular receptor for all measles virus strains including wild type, and thus, represents a common measles virus receptor (Tatsuo *et al.*, 2000). The CD150 receptor is a cell surface glycoprotein that, in contrast to the CD46 receptor, is only found on activated T cells, B cells and dendritic cells. It is a member of the immunoglobulin superfamily and as with the CD46 receptor, the most membrane-distal part of the receptor (variable domain) is essential for measles virus binding. The receptor has diverse immunological functions including T and B cell regulation.

1.4.6 Measles virus signalling pathways

Both CD46 and CD150 have different signalling mechanisms. The cytoplasmic tails of CD46 encode signals for protein kinase, casein kinase 2 and Src kinases. CD46 induced signal transduction in response to interaction with measles virus is via an Src family kinase dependant mechanism (Wang *et al.*, 2000). The CYT2 domain (cytoplasmic tail 2) of the CD46 receptor is phosphorylated by Src tyrosine kinases (Wang *et al.*, 2000) and leads to suppression of IL-12 from monocytes (Karp *et al.*, 1996), while the CYT1 domain has been shown to contribute to increased nitric

oxide production from monocytes in the presence of IFNγ (Katayama *et al.*, 2000) (Figure 1.5).

CD150 induced signal transduction is controlled by a SAP/SH2D1A (SLAM Associated Protein; SLAM-signalling lymphocyte activation molecule) gene, mutations in which give rise to X-linked lymphoproliferative disease (Sayos *et al.*, 1998). Interaction of the CD150 receptor with measles virus or CD150 specific antibodies triggers its activation. Signalling pathways in this receptor in response to measles virus have not been elucidated to date and the mechanism of action is speculative based on the natural functioning of the receptor as discussed by Schneider-Schaulies (2002a). Ligation of the receptor with CD150 antibodies induces IFN-γ production from T cells and antibody production from B cells, which may play a role in the impaired Th1 immune response in measles infection. In T cells the receptor co localizes with the T cell receptor and binds to the SAP (SLAM associated protein) protein in the cell, this interaction leads to tyrosine phosphorlyation by Src-like kinases Lck and Fyn in the cytoplasmic tail of the receptor molecule (Veillette *et al.*, 2003). This phosphorylation results in recruitment of the downstream effector molecule SHIP-1, a phosphatase thought to be involved in regulating intracellular signalling molecules inducing IFN-γ production or cell apoptosis (Figure 1.5) (Veillette *et al.*, 2003; Schneider-Schaulies *et al.*, 2002a).

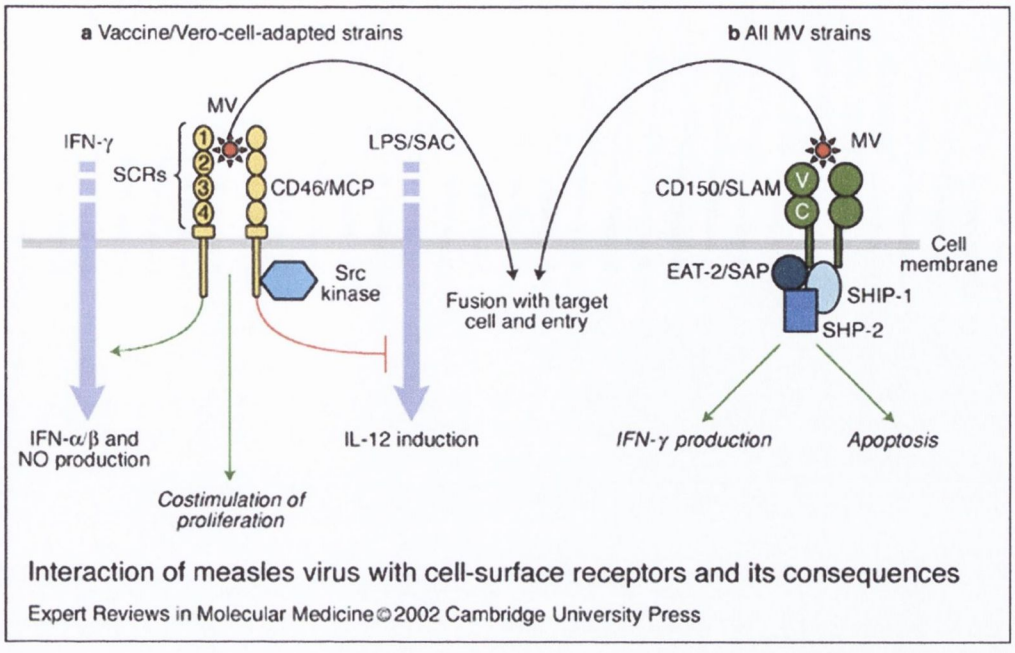


Figure 1.5 Interaction of measles virus with cell surface receptors.

Measles virus H protein interacts with short conserved domains (SCR's) on the CD46 and the variable region on the CD150 receptor. Tyrosine phosphorylation of the CYT2 cytoplasmic domain occurs in an Src kinase dependant manner resulting in suppression of IL-12 production. Binding of measles has been associated with increased production of IFN-γ and increased nitric oxide production. Activation of the CD150 by measles virus has not been determined however, it presumably occurs upon ligation and subsequent interactions with adaptor molecules SAP in T cells, or a similar molecule EAT-2 in APC cells stimulates tyrosine phosphorlyation of the cytoplasmic tail and recruitment of the downstream signalling molecule SHIP2 inducing IFN-γ production or cell apoptosis (Schneider-Schaulies *et al.,* Expert Reviews in Molecular Medicine; 2002a, 4:1-18).

IFN-γ is a pro-inflammatory cytokine well recognised because of its antiviral properties. Signalling of IFN-γ is via STAT1 (signal transducer and activator of transcription) and JAK (Janus Kinases) signalling cascades. After IFN-γ binds to its receptor (IFN-γR) STAT1 is phosphorylated by JAK1 and JAK2, dimerizes and translocates to the nucleus where it induces transcription of IFN-γ inducible genes (Chesler *et al.*, 2002) involved in the control of viral replication. The mechanism through which measles virus replication is inhibited is not yet understood.

Interaction of measles is known to cause down regulation of both CD46 and CD150 from the cell surface (Erlenhoefer *et al.*, 2002; Galbraith *et al.*, 1998). In the case of CD46, down regulation in measles infected cells has been shown to occur by internalisation of the molecule due to its interaction with the H protein (Naniche *et al.*, 1993). Down regulation of CD46 by measles vaccine strains is thought to allow rapid clearance of the virus from the body by facilitating complement lysis of infected cells contributing to attenuation (Schneider-Schaulies *et al.*, 2002a).

1.4.7 Measles virus, dendritic cells and immunosuppression.

The precise role of dendritic cells in natural measles infection is not known. However they are thought to be involved in measles virus induced immunosuppression. Dendritic cells in the respiratory tract mucosa are thought to be the first target cells to encounter the virus and subsequently transport it to local lymph nodes and initiate a virus specific immune response (Klagge *et al.*, 1999).

Wild type strains of virus seem to have a particular tropism for dendritic cells and are more efficient at inducing dendritic cell maturation and immunosuppression than vaccine derived strains (Klagge *et al.*, 2000; Klagge *et al.*, 1999). Uptake of measles virus by dendritic cells induces production of IFN from monocytes, which stimulates maturation of the dendritic cell (Klagge *et al.*, 2000). Cytokines TNF-α, IL-1, IL-6 and early after infection IL-12 have also been detected in measles infected dendritic cells (Servet-Delprat *et al.*, 2000), induction of which are dependant upon activation of the NFκβ transcription factor. Major histocompatibility complex II and costimulatory molecules CD40, CD80, CD86 and CD83 on the dendritic cell surface are also upregulated (Schneider-Schaulies *et al.*, 2002b). Activated dendritic cells migrate to the local lymph nodes where high expression levels of co-stimulatory molecules initiates dendritic cell maturation and signalling to T cells (Schneider-Schaulies *et al.*, 2002b).

As the infection progresses measles infected dendritic cells appear to mediate negative signalling to T cells and a suppression of the immune response as indicated by their failure to stimulate T cell proliferation or to suppress mitogen stimulated T cell proliferation in vitro (Schneider-Schaulies *et al.*, 2002b; Servet-Delprat *et al.*, 2000). A number of mechanisms have been proposed to contribute to this

lymphocyte depletion such as fusion of the infected dendritic cells in the lymph nodes or apoptosis. The measles virus glycoprotein (Fusion/Haemaglutinin) complex expressed on the cell surface of dendritic cells has also been implicated as an effector structure to inhibit T cell proliferation (Schneider-Schaulies *et al.*, 2002a; Schneider-Schaulies *et al.*, 2002b). Surface contact between this glycoprotein complex and another receptor on the lymphocyte (not yet identified) may induce proliferative arrest in the G1 phase of the cell cycle (Schneider-Schaulies *et al.*, 2002a; Schneider-Schaulies *et al.*, 2002b).

The other mechanism for negative signalling of T cells proposed by Schneider-Schaulies involves the IL-2 receptor signalling on T cells. IL-2 is required for the functional differentiation and S phase progression of T cells. IL-2 interaction with the IL2 receptor activates Src kinases and Janus kinases JAK1 /3 which bind to signalling molecules STAT 3 and STAT5 (signal transducers and activators of transcription). P13K (phosphatidylinositol 3-kinase) is also activated which in turn activates the kinase Akt, responsible for regulating downstream targets. This pathway plays a key role in T cell signaling and controls entry of the cells into the S phase. In measles infection *in vitro* the P13K and Akt pathway is disrupted (Schneider-Schaulies *et al.*, 2002a; Avota *et al.*, 2001).

Suppression of IL-12 production from measles infected dendritic cells is associated with the inadequate Th1 immune response and measles induced immunosuppression (Atabani *et al.*, 2001; Karp *et al.*, 1996). Viral antigens may persist in the lymphoid tissue after acute infection and may be responsible for the prolonged IL-12

suppression, however the mechanisms underlying this suppression are currently not known.

1.5. Summary.

A new variant of inflammatory bowel disease has been described in children with autistic spectrum disorders. The intestinal pathology includes ileocolonic lymphonodular hyperplasia and a non-specific colitis that lacks the specific diagnostic features of Crohn's disease or ulcerative colitis. The aetiology remains unknown although it has been suggested that measles virus infection or vaccination may be associated with onset of gastrointestinal symptoms and developmental regression in these children. Studies to date have focussed primarily on macroscopic and microscopic features of the disease. This thesis aims to molecularly characterise the intestinal abnormalities in these children compared with established forms of inflammatory bowel disease.

1.6 Aims of this study.

The overall objective of this study was to examine the pathogenesis of gastrointestinal dysfunction in the new variant inflammatory bowel disease "autistic enterocolitis". To achieve this goal three approaches were examined.

- To investigate the persistence of measles virus infection in children with new variant inflammatory bowel disease "autistic enterocolitis" and in a CD46 transgenic mouse model.

- To investigate the repertoire of pro-inflammatory and anti-inflammatory cytokine genes expressed in the intestine and peripheral blood of patients with new variant inflammatory bowel disease "autistic enterocolitis".

- To examine the gene expression profiles in new variant inflammatory bowel disease "autistic enterocolitis" and inflammatory bowel disease controls, and identify novel genes that may be associated with disease pathogenesis using high-density microarrays.

Chapter 2

Materials and Methods

2.0 Materials and Methods

This chapter provides a full description of all the methodologies employed in this thesis. For some of the newer techniques some background information is also given. Some of the techniques will be referred to in the individual chapters but the full description is limited to this chapter only.

2.1 Specimens

2.1.1 Tissue samples

Tissue biopsies from patients and controls were obtained at endoscopy. Two pinch biopsies were obtained from each site, one biopsy was placed immediately in formalin for routine histology and the other immediately snap frozen in liquid nitrogen and stored at –80 °C until required. Biopsy sites examined in this study included terminal ileum, caecum, descending colon, transverse colon, ascending colon, sigmoidal colon and rectum. Lymph node from one patient and a small bowel surgical resection from one patient were also examined. Formalin fixed paraffin embedded tissue samples were also used in this study. Tissues were fixed in 10% formol saline overnight before embedding in paraffin wax using standard histology.

Towards the latter part of the study samples were taken into RNA*later* (Ambion Inc, USA) preservative to preserve the integrity of the RNA. RNA*later* is an aqueous, non-toxic storage reagent that permeates tissue samples and stabilises and protects cellular RNA in unfrozen tissue specimens. Tissue biopsies were taken directly into approximately 5 volumes of RNA*later* and stored at 4 °C from 1-4 days before being transferred to –80 °C for long term storage.

Tissue samples were obtained from the Department of Gastroenterology, The Royal Free Hospital, London and The Adelaide, Meath and National Children's Hospital, Tallaght, Dublin 24. Archival material was obtained from the Department of Pathology, The Royal Free Hospital, London. Appendix control samples were obtained from Department of Pathology, St James's Hospital, Dublin. Blood and peripheral blood mononuclear cells were obtained from Dr. Colin Fink, Micropathology, UK.

2.1.2 Ethical Approval

Ethical approval for these studies was obtained from the ethical practices committee of the Royal Free, Hampstead, NHS Trust. Fully informed written parental consent was obtained from all trust patients including controls. Ethical approval for tissues samples obtained from the Department of Gastroenterology, The Adelaide, Meath and National Children's Hospital, Tallaght, Dublin 24 and the Department of Pathology, St James's Hospital, Dublin 8, Ireland, was obtained from the Joint Research Ethics Committee at St James's Hospital and Federated Dublin Voluntary Hospitals.

2.1.3 Cell lines

Measles virus (Edmonston B strain) infected and uninfected Vero cells were supplied as a gift from Dr Andrew Anthony at the Royal Free Hospital. For in situ PCR studies infected and uninfected Vero cells were fixed in 10% formol saline overnight and embedded in paraffin wax before being cut onto Perkin Elmer in situ slides. Rubella infected Vero cells (Therien strain) were obtained as a gift from Professor Gregory Atkins at the Department of Microbiology, Trinity College, Dublin.

Measles virus (Hu2 strain) RNA was obtained as a gift from Professor Burt Rima, Department of Virology, Queen's University Belfast.

Vero cell stocks containing 10^6 cells were thawed at 37 °C, added to 10 ml of Dulbecco's Modified Eagles media and centrifuged at 400 g for 10 min. The cell pellets were resuspended in 20 ml of Dulbecco's modified Eagle's media, seeded in sterile plastic flat bottomed 75 cm^2 cell culture flasks and the media changed every three days. At confluence, cells were detached from the flask by addition of 5 ml of trypsin/EDTA solution and incubated at 37 °C for 10 min. 10 ml of Dulbecco's modified Eagle's media was added and the solution transferred to a sterile 50 ml falcon tube. The suspension was centrifuged and the cell pellets resuspended in 10 ml of Dulbecco's modified Eagle's media and counted on a haemocytometer. Cells aliquots were stored in liquid nitrogen until required.

2.1.4 Isolation of Peripheral Blood Mononuclear Cells (PBMCs).

Approximately 10 mls of whole blood taken into EDTA was centrifuged at 2,000 rpm for 10 minutes. The plasma was removed and sterile PBS added to the EDTA tube and cells resuspended by mixing. 9 mls of Histopaque –1077 (Sigma Diagnostics) was added into a sterile tube. The cell suspension from each subject was carefully layered onto the Histopaque solution and centrifuged at 1400 rpm for 30 minutes. The mononuclear layer at the interface of the Histopaque and plasma was carefully aspirated into a fresh tube, washed with 10 ml of PBS and centrifuged at 1200 rpm for 10 minutes. The cell pellet was resuspended in 0.2 ml of PBS and 1.8 ml of RNA*later* was added. The PBMCs were then aliquoted into 0.5 ml aliquots and stored at –80 °C.

2.2 RNA Extraction.

A typical mammalian cytosol contains about 80-85% ribosomal RNA (rRNA), 15-20 % transfer RNA (tRNA) and 1-5% messenger RNA (mRNA). The mRNA population is responsible for encoding virtually all of the polypeptides synthesized by the cell.

RNA is a highly labile molecule and is susceptible to degradation by ribonucleases. Even the smallest amount of RNase in an RNA preparation will cause severe problems. For this reason most reagents (excluding those containing TRIS) used for RNA experiments were treated with 0.1% diethyl pyrocarbonate (DEPC) (a RNase inhibitor) and autoclaved to destroy any ribonucleases present. Sterile RNase and DNase free plastics and aerosol resistant pipette tips were also used. All RNA extractions were performed in a dedicated class II laminar flow hood using dedicated pipettes, aerosol barrier tips and protective clothing.

Total RNA was extracted from fresh frozen material, PBMC'c, infected and uninfected cell lines using the following reagents: UltraSpec-II RNA Isolation system (Biotecx Laboratories, Houston, Texas, USA), Trizol (Life Technologies, Maryland, 21740, USA), and RNeasy Mini kit (Qiagen Ltd, Boundary Court, West Sussex, RH 10 9AX, UK). For the majority of fresh frozen specimens the UltraSpec II RNA Isolation system or Trizol extraction procedure was used. These reagents are a mixture phenol and a high concentration of guanidium isothiocyanate (7M), which helps maintain the integrity of the RNA while disrupting cell membranes. RNA was extracted from PBMCs and cell lines using the Qiagen RNeasy extraction protocol. Archival formalin fixed paraffin embedded tissue samples were extracted using the

Purescript® RNA Isolation Kit (Gentra Systems, Minneapolis, Minnesota, USA).

2.2.1 UltraSpec-II and Trizol Total RNA Isolation Systems

Tissue samples were homogenized in either 400 µl of UltraSpec II RNA reagent or Trizol Reagent using either motorized tube pellet pestles (Anachem, Luton, Bedfordshire, UK) or by bead beating using the Savant BIO 101 FastPrep FP120, (Anachem Ltd, Luton, Bedfordshire, UK) for 40 seconds at a speed of 5. Following homogenization the volume was brought up to 1ml with either UltraSpec-II reagent or Trizol reagent, the samples were vortexed for 1 minute and the homogenate was incubated for 5 minutes at 4 °C. 200 µl of chloroform was added, the homogenate was vortexed for 30 seconds, incubated at 4°C for 5 minutes and centrifuged at 12,000 rpm at 4°C for 15 minutes. The upper aqueous phase was transferred to a sterile 1.5 ml microtube, an equal volume of 100 % isopropanol was added, the sample was vortexed for 30 seconds, incubated on ice for 15 minutes and centrifuged at 12,000 rpm at 4°C for 20 minutes. The supernatant was removed and the pellet was washed twice in 1 ml of 75 % ethanol by vortexing and centrifuging at 12,000 rpm for 1 minute. The RNA pellet was allowed to air dry completely and resuspended in 50 µl of DEPC treated water. RNA was stored at –80 °C until required.

2.2.2 RNeasy Minikit Extraction Protocol.

Fresh frozen biopsy material was homogenized in 600 µl of RLT lysis buffer (Qiagen RNeasy Minikit, Qiagen Ltd, Crawley, West Sussex, RH 10 9AX, UK) containing β-mercaptoethanol, using a bead beater (Savant BIO 101 FastPrep FP120, Anachem

Ltd, Luton, Bedfordshire, UK) for 40 seconds at a speed of 5. Homogenates were placed on ice for 5 minutes and passed through a QiaShredder Column (Qiagen Ltd, Crawley, West Sussex, RH 10 9AX, UK) according to the manufacturer's instructions.

Peripheral blood mononuclear cells or cell pellets were resuspended in 600 µl of RLT buffer containing β- mercaptoethanol and homogenized by passing though a QiaShredder column (Qiagen Ltd, Crawley, West Sussex, RH 10 9AX, UK) according to the manufacturer's instructions.

Total RNA was extracted using the Qiagen RNeasy Minikit, animal tissue/ cells protocol (Qiagen Ltd, Crawley, West Sussex, RH 10 9AX, UK) according to the manufacturer's instructions. An on-column DNase digestion was performed using the RNase free DNase kit (Qiagen Ltd, Crawley, West Sussex, RH 10 9AX, UK) as described below in section 2.4. RNA was eluted in 50 µl of RNase free, DNase free water.

2.2.3 Extraction of RNA from Archival Tissue Specimens.

Total RNA was extracted from formalin fixed paraffin embedded material using the Purescript RNA Isolation Kit (Gentra Systems, Minneapolis, Minnesota, USA). Approximately 20 µm of the embedded tissue specimen was cut using a microtome into a sterile 1.5 ml microfuge tube. Tissue was dewaxed twice in 500 µl of xylene by vortexing for 30 seconds, incubating at room temperature for 5 minutes with constant mixing and centrifuging at 13,000 rpm for 3 minutes to pellet the tissue. The tissue pellet was washed twice in 100% ethanol, as above and allowed to air dry.

The dewaxed tissue was homogenized in 300 µl of cell lysis solution using a motorized microtube pellet pestle until the tissue was sufficiently homogenized. Homogenate was digested with 1.5 µl Proteinase K 20 mg/ml (Finnzyme, Riihitontuntie, FIN-02201, Finland) in a 55°C heating block overnight. Homogenate was cooled to room temperature, 100 µl of protein-DNA precipitation solution was added, mixed and incubated on ice for 5 minutes. The cell lysate was centrifuged at 13,000 rpm for 3 minutes to form a tight pellet of the precipitated proteins and DNA. The supernatant containing the RNA was decanted into a clean 1.5 ml microfuge tube containing 300 µl of ice-cold isopropanol, 1 µl of glycogen (20 mg/ml) was added, the sample mixed and centrifuged at 13,000 rpm for 10 minutes. The RNA pellet was washed twice with 300 µl of 70% ethanol and centrifuged at 13,000 rpm for 1 minute. The pellet was air dried and resuspended in 25 µl of DEPC treated water. RNA was stored at –80 °C until required.

2.3 DNase Digestion

Depending on the RNA extraction protocol used and the subsequent applications, it was often necessary to perform a DNase digestion on the RNA. Two different protocols were used. During Qiagen RNA extraction procedures it was possible to perform an on column DNase digestion step after binding the nucleic acid to the column. The RNase free DNase set from Qiagen (Qiagen Ltd, Crawley, West Sussex, RH 10 9AX, UK) was used for this according to the manufacturer's protocol. Alternatively, DNase digestion was performed on RNA samples by incubating up to 1 µg of RNA with 1X DNase buffer (10X: 500 mM Tris-Cl, pH 8.5, 50 mM $MgCl_2$, 10mM DTT), 10U RNase inhibitor and 0.5 U of DNase I enzyme at 37°C for 15-30 minutes. The reaction was stopped with 2µl of 50mM EDTA. RNA was checked for

DNA contamination by running an aliquot on a 1 % agarose gel. RNA was then cleaned up using the Qiagen RNeasy Minikit RNA clean up protocol (Qiagen Ltd, Crawley, West Sussex, RH 10 9AX, UK).

2.4 Quantitation of Nucleic Acids.

The concentration and quality of extracted RNA and DNA was determined by UV spectrophotometry on a Beckman DU530 UV/VIS spectrophotometer (Beckman Instruments, Inc. Fullteron, CA 92834, USA). The concentration of RNA was determined in water and calculated using the standard: an OD reading of 1 corresponds to 40 μg/ml of single stranded RNA. To assess the RNA quality the OD readings at 260nm /280 nm was calculated. This ratio provides an estimate of the purity of the RNA. Measuring the absorbance in 10 mM Tris-Cl, pH 7.5 provides and accurate measure of purity. Pure RNA has an OD 260nm /280 nm ratio of 1.9-2.1 in 10 mM Tris-Cl, pH 7.5.

In addition to assess the quality, integrity and size distribution of the RNA, the RNA was checked by denaturing/agarose gel electrophoresis on 1 % agarose gels stained with ethidium bromide.

The concentration of DNA was determined in water and calculated using the standard: an OD reading of 1 corresponds to 50 μg/ml of double stranded DNA. To assess the DNA quality the ratio of OD readings at 260nm /280 nm was calculated. This ratio provides an estimate of the purity of the DNA. Pure DNA has an OD 260/280 ratio of 1.7-1.9.

2.5 Agarose Gel Electrophoresis.

Electrophoresis through agarose gels was the method used to separate, identify and purify nucleic acids. 1% agarose gels were prepared by boiling 1 g agarose (Amaresco, Solom, Ohio, 44139, USA) in 100 ml of 1X TBE buffer (10X TBE buffer: 0.45 M Tris–base, 0.89 M boric acid, 0.02M EDTA, pH 8.0) made up in distilled H_2O. The agarose was cooled to 50°C, and ethidium bromide (Sigma Aldrich, St Louis, MO 63178, USA)) was added to a final concentration of 0.5 µg/ml. The agarose was then poured into a BioRad gel casting tray and allowed to set. The solidified agarose was placed in a BioRad electrophoresis tank filled with 1X TBE buffer.

Nucleic acid samples were loaded onto gels in 6X gel loading buffer (Sigma Aldrich, St Louis, MO 63178, USA) and run at 80-110 volts for 1-2hours. Nucleic acids stained with ethidium bromide were visualised on a UV transluminator (Hybaid) and photographed using a Polaroid camera fitted with black and white type 667 film.

Denaturing formaldehyde agarose (FA) gel electrophoresis was used for RNA samples as it gives enhanced sensitivity and helps maintain the integrity of the RNA. A 1.2 % gel was prepared by boiling agarose (Amaresco, Solom, Ohio, 44139, USA) in 1X FA gel buffer (10X FA buffer: 200mM 3-N-morpholino propanesulfonic acid (MOPS), 50 mM Sodium acetate, 10 mM EDTA, pH 7.0) made up in RNase free water. The agarose was cooled to 65 °C, 1.8 ml of formaldehyde and 1 µl of ethidium bromide 10 mg/ml was added, and the gel was poured into the gel casting tray. Once solidified the gel was allowed to equilibrate in 1 X FA running buffer (100 ml of 10X FA gel buffer, 20 ml of 37% formaldehyde, 880 ml of RNase free

water) for 30 minutes prior to running RNA samples. The RNA samples were prepared by adding 1 volume of 5X RNA loading buffer (Sigma-Aldrich, St Louis, MO 63178, USA) per 4 volumes of RNA sample, mixed and incubated at 65 ° C for 5 minutes, cooled on ice and loaded onto the equilibrated FA gel. RNA was visualized and photographed as described above.

2.6 Polymerase Chain Reaction

The polymerase chain reaction first described in 1985 (Saiki *et al.,* 1985) is a simple method for amplification of specific sequences of DNA or cDNA reverse transcribed from RNA. Single stranded oligonucleotide primers, which bind to flanking regions of the target sequence are used to initiate the amplification of the specific DNA strand by a thermostable DNA polymerase enzyme. The reaction is subjected to cycles of heating and cooling which causes denaturation of the double stranded DNA template, primer annealing and strand extension. Each cycle theoretically doubles the target strand copy number leading to an exponential increase in the target sequence. The process is essentially the same for all targets with minor adjustments in magnesium chloride and annealing or cycling conditions.

2.6.1 PCR Primer and Probe Design.

All PCR primers and probes were obtained from Applied Biosystems (Applera UK, Warrington, Cheshire, WA3 7QH, UK). Primer Express Software Versions 1.5 - 1.7 (Applied Biosystems, Lincoln Centre drive, Foster City, CA 94404, USA) were used to design all the PCR primers and probes for this study using the criteria described in the Primer Express user manual. The specificity of the selected sequences was checked using the NCBI Blast program (www.ncbi.nlm.nih.gov/blast). Appendix 1 lists all the primers and probes used in this study.

For controls, the housekeeping gene glyceraldehyde –3-phosphate dehydrogenase (GAPDH) control assay (Applied Biosystems, Lincoln Centre drive, Foster City, CA 94404, USA) was used. The 226 bp mRNA amplicon target starts at base 1457 (of the HUMGAPDH GenBank sequence), spans two introns (intron 2: base 1482-3116, and intron 3 from base 3216-3306) and ends at base 3412.

Pyruvate Dehydrogenase (PDH) was used as an endogenous control for all in situ reverse transcriptase PCRs. These PCR primers and probe were designed across exon 2/exon 3 of the homo sapiens pyruvate dehydogenase sequence (accession number J03576). The primers amplify a 96 bp mRNA amplicon.

The VIC labelled 18s ribosomal RNA (rRNA) endogenous control assay from Applied Biosystems (Applera UK, Warrington, Cheshire, WA3 7QH, UK) was used in multiplex TaqMan Cytokine card assays as described below.

For in situ PCR, oligonuceotide probes were modified at their 5' end by the addition of a biotin moiety. For Southern blot analysis the probes were labelled at the 3' end with digoxigenin. For TaqMan RT PCR, probes were dual labelled with a fluorescent molecule FAM at the 5' end and the quencher TAMRA at the 3' end. In the case of minor groove binding (MGB) TaqMan probes there was a non-fluorescent quencher molecule at the 3' end.

2.6.2 Solution Phase One Step RT PCR

All PCR reagents were supplied by Applied Biosystems, (Lincoln Centre drive, Foster City, CA 94404, USA). Sterile PCR grade water was supplied by Sigma Chemicals or Ambion Inc, UK. PCR cocktails were prepared in a designated room within a Bioflow Class II biological safety cabinet (Germfree Laboratory Inc, Miami, Florida) using dedicated pipettors and aerosol barrier tips. PCR template was added in a separate area. All PCRs were performed on either a GeneAmp 9600 or GeneAmp 9700 thermocycler from Applied Biosystems.

In a one-step RT PCR assay the reverse transcription step and PCR amplification step are performed in a single reaction tube through the use of the dual-functioning enzyme recombinant r*Tth* DNA polymerase (*Thermus thermophilis*). This enzyme possesses both reverse transcriptase and polymerase activity (Orlando *et al.*, 1998). The reverse transcription can be performed at higher temperatures than standard reverse transcriptases and therefore ensures higher specificity. Another added benefit to using the one step system is contamination is minimised.

The *Tth* polymerase enzyme is compatible with both RT and subsequent PCR reactions. Magnesium is a critical parameter in both Reverse transcription and PCR reactions. In the presence of Mg^{2+}, *Tth* functions as a DNA polymerase and as a reverse transcriptase in the presence of Mn^{2+}. The use of Mn^{2+} rather than Mg^{2+} also minimises any problems caused by amplification of re-annealed DNA fragments (Bauer *et al.*, 1997).

For measles virus (MV) specific PCRs, primers were optimized using RNA extracted from measles infected vero cells. In general RT-PCRs were performed using the one step RT and amplification enzyme rT*th* DNA polymerase. The following were typical reaction conditions used to amplify MV F, H and N genes from control RNA: Per 25 µl reaction; 0.4mM dNTPs, 0.4 µM forward and reverse primers; 2.5mM manganese acetate; 2.5-5U rTth DNA polymerase; 0.01U AmpErase, 1X EZ buffer. Buffer consisted of 50mM bicine, 125mM potassium acetate, 40% (w/v) glycerol, pH 8.2 (Applied Biosystems). 5-10 µl of extracted Total RNA was used per reaction. The following RT PCR thermal cycling conditions were used on a 9700 PCR thermocycler (Applied Biosystems, Foster City, CA, USA): Activation of AmpErase at 50 °C for 2 min, reverse transcription at 58 °C for 30 minutes, inactivation of reverse transcriptase at 95 °C for 5 minutes, and PCR amplification for 40 cycles of 94 °C for 20 seconds, 59 °C for 20 seconds, 72 °C for 20 seconds, followed by an extension step at 72 °C for 10 minutes.

To amplify MV F and H genes and GAPDH gene from RNA extracted from terminal ileum tissue samples conditions were slightly altered to reduce smearing. The

manganese acetate was increased to 3-3.5 mM, and the cycling conditions were as follows: 50 °C for 2 min, 58 °C for 30 minutes, 95 °C for 5 minutes, and 40 cycles of 94 °C for 30 seconds, 58-60 °C for 30 seconds, 72 °C for 30 seconds, followed by an extension step at 72 °C for 10 minutes.

The entire N gene was amplified from RNA extracted from measles infected Vero cells and SSPE brain tissue. The following conditions were used; 0.4mM dNTPs, 0.4 μM forward and reverse primers; 3 mM manganese acetate; 2.5 U rTth DNA polymerase; 0.01U AmpErase, 1X EZ buffer. Buffer consisted of 50mM bicine, 125mM potassium acetate, 40% (w/v) glycerol pH 8.2 (Applied Biosystems); 4 μl of extracted RNA was used per reaction. Cycling conditions were as follows on a 9600 thermal cycler: 50 °C for 2 min, 58 °C for 30 minutes, 95 °C for 5 minutes, and 40 cycles of 94 °C for 30 seconds, 57-60 °C for 30 seconds, 72 °C for 30 seconds, followed by an extension step at 72 °C for 10 minutes.

2.6.3 PCR Product Purification.

RT PCR products were purified prior to cloning or sequencing. The products were run on a 1.5-2% agarose gel and the specific DNA band excised. The products were then purified using the Qiagen QiaQuick Gel Extraction Kit (Qiagen Ltd, Crawley, West Sussex, RH 10 9AX, UK), which can be used to purify DNA fragments greater then 100bp. The purified products were eluted in 30 μl of sterile water, left stand at room temperature for 1 minute and centrifuged at 13,000 rpm for 1 minute. Purified PCR products were used immediately for cloning reactions or stored at –20 °C until required.

2.7 TaqMan Quantitative PCR – the 5' nuclease assay

2.7.1 Introduction

TaqMan technology is a quantitative real time PCR technique, based on the 5' nuclease assay first described by Holland *et al.,* (1991), which uses fluorescent probes designed to hybridise within the target sequence and on the annealing/extension phase of PCR generate a signal that accumulates during PCR cycling in proportion to the amount of template prior to the initiation of PCR (Orlando *et al.*, 1998; Gibson *et al.*, 1996). The basis for this system is to continuously measure PCR products as they accumulate using a dual-labelled specific fluorogenic oligonucleotide probe, called the TaqMan probe (Livak *et al.*, 1995; Lee *et al.*, 1993). This probe is composed of a short (~20-25bases) oligonucleotide labelled with two different fluorescent dyes, a 3' quencher dye and a 5' reporter dye and a 3' blocking phosphate to prevent probe extension during PCR (Livak *et al.*, 1995). The oligonucleotide probe sequence is homologous to an internal region present in the PCR product. When the probe is intact, energy transfer of the Förster type occurs between the two fluorophores and results in the suppression of the reporter fluorescence (Gibson *et al.*, 1995; Livak *et al.*, 1995).

The TaqMan assay utilises either *Taq* or *Tth* polymerase, isolated from *Thermus Aquaticus* and *Themus thermophilus* respectively, but any enzyme with 5' nuclease activity can be used. During amplification the non-extendible probe is cleaved by the 5'exonuclease activity of *Taq* or *Tth* DNA polymerase, thereby releasing the reporter from the oligonucleotide quencher and producing an increase in the reporter emission fluorescent intensity, which is monitored in real time during the exponential phase of PCR amplification using the 7700 Sequence Detector System (Applied

Biosystems Lincoln Centre drive, Foster City, CA 94404, USA). Cleavage removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. An overview of the process is illustrated in figure 2.1. Additional reporter dye molecules are cleaved from their respective probes with each cycle, leading to an increase in fluorescence intensity proportional to the amount of amplicon produced. The exonuclease activity of the *Taq* polymerase acts only if the fluorogenic probe is annealed to the target since the activity is double-strand specific therefore the enzyme cannot hydrolyse the probe when it is free in solution and no reporter fluorescence is detected.

Since the polymerase only cleaves probe while it remains hybridised to its complimentary strand, the temperature conditions of the extension of the PCR must be adjusted to ensure probe binding. The TaqMan system uses a combined annealing and polymerisation step at 60-62°C to ensure the probe remains hybridised during amplification. Most probes have a melting temperature (T_M) of around 10°C or at least 5 degrees higher than the PCR primers (Livak *et al.*, 1995), since binding of the TaqMan probe prior to the primers is crucial to the success of the process. Without it PCR products will be formed without generation of fluorescence and thus without detection. This ensures that the probe remains bound to its target during the primer extension step and also ensures maximum 5'-3' exonuclease activity of the *Taq* and *Tth* DNA polymerases.

More recently minor groove binder (MGB) TaqMan fluorogenic probes have been developed that improve the sensitivity of a TaqMan assay. Minor Groove Binders (MGB) (naturally occurring antibiotics and synthetic molecules) are able to fit into

the minor groove of the helix formed by double stranded DNA and stabilise DNA duplexes, increasing mismatch discrimination when bound to an oligonucleotide probe. TaqMan MGB fluorogenic probes have a number of characteristics that make them superior to traditional fluorogenic probes. The MGB can be attached to the 3'-end, the 5'-end, or to an internal nucleotide of the oligonucleotide. MGB probes bind more tightly to their complement, this raises the melting temperature (Tm) of the probes and allows for more flexible assay design. Studies have shown that the introduction of an MGB onto a 12-mer probe with a Tm of 20°C increases its Tm to 65°C. This Tm is equivalent to the Tm of a 27-mer probe without an MGB (Kutyavin *et al.*, 2000).

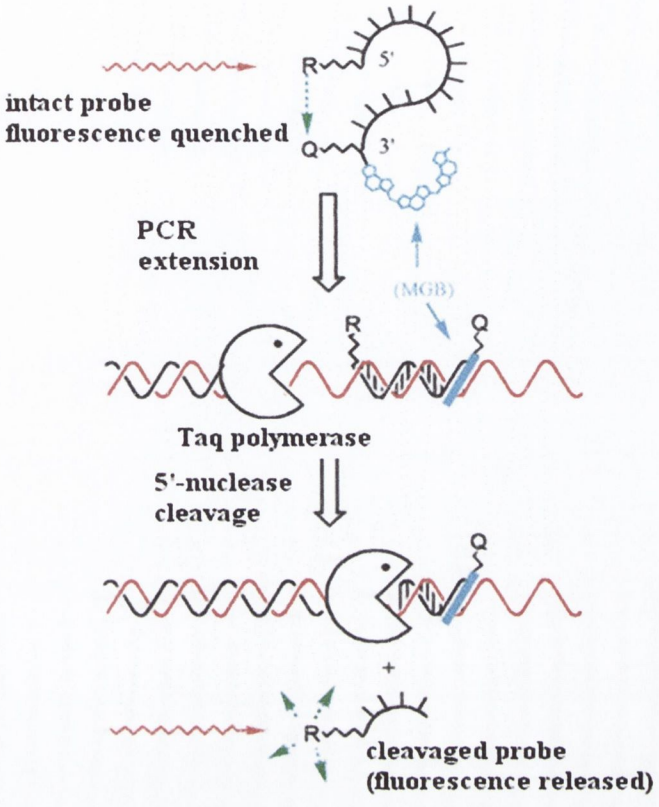


Figure 2.1. Schematic of TaqMan PCR Principle. (Kutyavin *et al.*, 2000).

2.7.2 TaqMan one step RT PCR

In the one-step TaqMan assay the reverse transcription step and PCR amplification step are performed in a single reaction through the use of the dual-functioning enzyme recombinant r*Tth* DNA polymerase (*Thermus thermophilis*) as described above.

Real time quantitative reverse transcriptase (RT) PCR was performed using sequence specific PCR primers and TaqMan probes designed using Primer Express Software as described above. All quantitative PCRs were prepared in a dedicated facility in a class II laminar flow using dedicated pipettors and aerosol resistant pipette tips. Template RNA was prepared and added to the PCR mastermix in a separate facility.

TaqMan RT PCR was performed using the EZ TaqMan RT PCR reagents following manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). RT PCR reactions were performed in duplicate under the following conditions per 25 µl reaction: 1X EZ buffer, 3mM $MnOAc_2$, 200-300 nmol of each primer, 100 –300 nmol of TaqMan probe, 0.01 U of AmpErase, and 0.1 U of r*Tth* polymerase. The thermal cycling conditions on the 7700 were as follows 50 °C for 2 min, 58 -60°C for 30 min, 95 °C for 5 min, followed by 40 cycles of 94 °C for 20 s, 60 °C for 1 min.

Controls for TaqMan RT PCR included the following: No template control (water added as template), no amplification control (omission of r*Tth* polymerase), irrelevant target primers and specific TaqMan probe, probe only controls (omit PCR primers), human RNA control, spiked RNA control, asymmetric TaqMan PCR (TaqMan PCR with one or other primer and specific TaqMan probe).

2.7.3 Reference genes

Reference genes used in this study were VIC labelled GAPDH and 18s rRNA TaqMan primer probe sets (Applied Biosystems, Foster City, CA, USA). The GAPDH endogenous control was assayed in a separate TaqMan PCR reaction to the target gene. GAPDH TaqMan RT PCR reactions were performed in duplicate under the following conditions per 25 µl reaction: 1X EZ buffer, 3.5 mM $MnOAc_2$, 200 nmol of each primer, 100 nmol of TaqMan probe, 0.01 U of AmpErase, and 0.1 U of r*Tth* polymerase. The thermal cycling conditions on the 7700 were as follows 50 °C for 2 min, 60°C for 30 min, 95 °C for 5 min, followed by 40 cycles of 94 °C for 20 s, 60 °C for 1 min.

The 18S rRNA endogenous control was used in a multiplex TaqMan PCR for TaqMan Human cytokine card analysis. This concentration of the 18S rRNA primers was limited to avoid competition between the target and the endogenous control. The protocol is described below under the TaqMan Human cytokine card section.

2.7.4 Relative quantitation.

The objective of a RT PCR relative quantitative experiment is to determine the ratio of a target mRNA molecule to a different target molecule or to itself under different conditions. The result can then be reported as a fold difference relative to a calibrator sample. There are two methods for calculating relative quantitation of target gene expression: the standard curve method, and the comparative Ct method.

2.7.4.1 Standard curve method.

For the standard curve method, standard curves are generated for both the target and the endogenous control. For each test sample the amount of target and control quantity is determined from the appropriate standard curve. Then the target amount is divided by the endogenous control quantity to obtain a normalized target value. To calculate the relative expression levels the normalized target values are divided by the calibrator normalized target values.

Standard curves in this study were generated from cloned copy RNA (cRNA) standards prepared as desribed in section 2.11 below. Briefly, 10 fold serial dilutions from 10^7 –10^1 copies per µl were prepared and amplified in TaqMan one step RT PCR assays. Standard curves were generated and samples were plotted along the curve to determine the quantity of target in the sample.

GAPDH standard curves were generated from 10 fold serial dilutions of human control RNA (50 ng/µl –Applied Biosystems, Foster City, CA, USA), where 1ng of control RNA contains $1X10^5$ copies of GAPDH (Applied Biosystems, Foster City, CA, USA). The quantity of GAPDH in the sample is then calculated from this standard curve.

2.7.4.2 Comparative Ct method

For the comparative Ct method the relative quantification values are calculated from the threshold cycle (Ct) values generated during the PCR. The Ct value is the cycle at which a statistically significant increase in PCR product is first detected. The comparative Ct method for relative quantitation calculates relative gene expression

using the following equation:

$$\text{Relative Quantity} = 2^{-\Delta\Delta Ct}$$

The ΔCt is calculated by normalising the Ct of the target sample with the Ct of the endogenous control (Ct target- Ct endogenous control). The ΔΔCt is then calculated by subtracting the average ΔCt for the calibrator sample from the corresponding average ΔCt for the target sample. The relative levels of the target gene expression are then expressed as a fold change relative to the calibrator sample.

2.8 TaqMan Human Cytokine Cards.

2.8.1 Introduction

The TaqMan human cytokine card is a novel research tool for profiling human cytokine gene expression using the comparative Ct method of relative quantitation described in section 2.7.4.2. The TaqMan Human cytokine card (Applied Biosystems, Foster City, CA, USA) consists of a 96-well consumable divided into 24 sets of replicates, one set for each cytokine assay.

The card evaluates a single cDNA sample generated from human total RNA in a two-step RT PCR experiment. This assay measures the following 24 cytokines TNF–α, TNF-β, IFN-γ, TGF-β, LT-β, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 IL-10, IL-12p35, IL-12p40, IL-13, IL-15, IL-17, IL-18, G-CSF, GM-CSF, and M-CSF. The card layout is outlined in figure 2.2. Each well contains lyophilised FAM labelled TaqMan MGB probes and primers for one human cytokine mRNA. A VIC labelled TaqMan MGB primer and probe set for 18S ribosomal RNA endogenous control was used for multiplex assays. The TaqMan cytokine primers and probes for the 24 cytokine target assays span exon junctions to minimise the contribution of contaminating genomic DNA. The 18S rRNA endogenous control assay is not RNA specific and consequently is affected by genomic DNA contamination. However, because of the extremely high expression level of rRNA, even gross contamination has a negligible effect on the relative quantification values obtained from the card. In multiplexed reactions there is a concern that the PCR will be dominated by high 18S rRNA expression levels. To minimise the competition between the reactions the 18S endogenous control assay is primer limited to prevent it from competing with the amplification of the cytokine target sequences.

Relative RNA quantification assays on the cytokine card are performed in a two-step reverse transcription polymerase chain reaction (RT-PCR). In the first step, cDNA is reverse transcribed from total RNA using random hexamers and MultiScribe™ Reverse Transcriptase. The second step includes amplification of the cDNA product using TaqMan Universal Mastermix and *AmpliTaq Gold* DNA polymerase. All 96 wells of the card are filled simultaneously with a mixture of cDNA and TaqMan Universal mastermix through a single port via a channel system. The reaction volume of each well is 1 µl and the total volume required to fill the card is 250 µl.

2.8.2 Reverse transcription–cDNA generation.

Reverse transcription reactions were set up in 100 µl volume reactions containing the following: 1X TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 µM of each dNTP, 2.5 µM random hexamers, 0.4 U/µl RNase inhibitor, 1.25 U/µl MultiScribe Reverse Transcriptase, 500ng –1µg of RNA template. Reverse transcription reactions were performed on a 9600 thermal cycler under the following conditions: Hexamer incubation at 25°C for 10 min, reverse transcription at 37 °C for 60 min, and inactivation of reverse transcriptase at 95 °C for 5 min.

2.8.3 Amplification of cDNA.

The entire cDNA reaction (100 µl) was used in the PCR amplification. 150 µl of TaqMan Universal master mix (2X), 20 µl of RNase free water, 1 X 18S MGB primer and probe mix and 100 µl of cDNA were added into a microtube, mixed by vortexing and centrifuged briefly to remove any air bubbles. Reactions were kept on ice while preparing the TaqMan Human Cytokine Card.

2.8.4 Cytokine expression card protocol

The adhesive flap attached to the card was folded back and the fill consumable was securely attached, aligning the aperture and pins on the edge of the fill consumable with the holes on the card. The card and fill consumable were loaded into the filling station, and the vacuum was applied. When the vacuum pressure stabilized at 600 microns the 300 µl of sample specific PCR mix was loaded into the fill reservoir, ensuring that no bubbles were introduced. In one motion the actuator was released and the card filled with sample specific PCR mix. After the vacuum is switched off the fill consumable is discarded and the adhesive flap secured over the fill reservoir to seal the card. The filled card was then loaded into an ABI Prism 7700 Sequence Detection System with a card adaptor. The card was then run on the ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA), under the following cycling conditions, 50°C for 2 min, 99°C for 10 min, 35 cycles of 99°C for 15 seconds, 60 °C for 1 min.

The Human TaqMan Cytokine Card data was analysed using the SDS v1.7.1 and SDS v2.1 software.

2.9 In situ RT PCR

2.9.1 Introduction

A major limitation with conventional PCR techniques is the inability to visualize or localize amplified products within particular cells. In situ hybridisation is a useful technique for the specific localisation of nucleic acid sequences at individual cell levels buts its usefulness can be limited by its low detection sensitivity. In situ or in cell PCR is a technique that enables specific sequences to be localized at a cellular level within tissues and facilitates low copy gene detection with a reported sensitivity of one viral genome/ cell (Uhlmann *et al.*, 1998; Boshoff *et al.*, 1995; Haase *et al.*, 1990). For detection of low copy RNA sequences a reverse transcription step has been added to generate cDNA templates from RNA template prior to in situ PCR. Prior to in situ PCR, the cells or tissue sample must be permeablised and fixed to preserve the cell morphology but also to permit access of PCR reagents into the cell. There are several procedures for permeabilising the cell membrane including the use of proteases or detergents. The permeabilisation step is crucial as extended times can cause diffusion of the amplicon and PCR reagents out of the cell and shorter times can cause patchy amplification.

Intracellular PCR products can be detected by two methods; in situ hybridisation with a specific oligonucleotide probe or direct detection through the use of labelled nucleotides incorporated during the PCR. The former approach provides maximum sensitivity.

2.9.2 In situ RT PCR Preparation

Tissue sections, 4 μm thick, were cut using a microtome onto silane coated in situ PCR glass slides (Applied Biosystems, Lincoln Centre drive, Foster City, CA 94404, USA). All pre-PCR steps were performed in Tissue Tek containers containing 250 ml of solutions. All solutions and reagents used for the in situ RT PCR and detection protocol were prepared in autoclaved DEPC treated water and were filter sterilized prior to use.

2.9.3 Cell and Tissue Permeabilisation

Cells must be adequately digested and permeabilised to facilitate the access of PCR reagents into the cell. This can be achieved by protease treatment and/or mild acid hydrolysis. Maximal digestion times and protease concentrations have to be optimised for each particular tissue/cytological preparation.

To remove the paraffin from the tissue sections slides were incubated twice for 15 minutes at room temperature in xylene, followed by once in 100% ethanol for 10 minutes, 75 % ethanol for 2 minutes, and 50 % ethanol for 2 minutes. The slides were then rinsed in DEPC water for 5 minutes.

To digest proteins on the tissue surface and permeabilise the cell membrane the slides were incubated in 0.02 N HCL for 10 minutes, washed in 1X PBS pH 7.4 for 2 min, 1XPBS /0.01% Triton X100 for 2 minute followed by 1X PBS for 2 minutes. Slides were then incubated in preheated (37°C) proteinase K buffer (0.1M Tris, pH 7.5, 5 mM EDTA) for 5 minutes, followed by incubation at 37 °C for 17 min in proteinase K (300 mg/ml)(Roche Molecular Biochemicals, Mannheim, Germany) in

proteinase K buffer (0.1 M Tris, pH 7.5, 5 mM EDTA). Proteinase K was inactivated by soaking slides in 1X PBS /0.2% glycine for 5 minutes and washing in 1X PBS and DEPC treated water.

When using a non-isotopic labelling method, a blocking step must be employed, depending on the method used for post amplification detection of the product. Endogenous intestinal alkaline phosphatase activity was blocked by soaking the slides in ice cold 20 % acetic acid for 15 seconds, or using Levamisole Endogenous AP Inhibitor (DAKO Ltd, Glostrup, Denmark) during chromogenic detection. Endogenous peroxidase activity is quenched by treating sections in 3% H_2O_2 in methanol for 10 minutes. Endogenous avidin and biotin activity was blocked using the DAKO Avidin/Biotin blocking kit (DAKO Ltd, Glostrup, Denmark). Briefly 50µl of avidin blocking solution was applied onto the tissue section and incubated at room temperature for 5 minutes. The section was rinsed in 1xPBS pH 7.4, and 50 µl of biotin blocking solution was added to each section and incubated for 10 minutes at room temperature. Again the sections were rinsed with 1xPBS, and incubated in 50% ethanol for 2 minutes, 75% ethanol for 2 minutes, and 100% ethanol for 2 minutes. Slides were stored in ethanol at room temperature until required.

2.9.4 In Situ RT PCR

All PCR reagents were supplied by Applied Biosystems, (Lincoln Centre drive, Foster City, CA 94404, USA). Pre-sterilized RNase free reagents and disposables were used. PCR cocktails were prepared in a designated room within a Bioflow Class II biological safety cabinet (Germfree Laboratory Inc, Miami, Florida) using dedicated pipettors and aerosol barrier tips.

In situ RT PCRs were performed using a one step RT and amplification enzyme rT*th* polymerase. (Applied Biosystems, Lincoln Centre drive, Foster City, CA 94404, USA). The following typical reaction conditions were used to amplify the measles virus, N gene in tissue sections: Per 50 µl reaction; 0.4 mM dNTPs, 1 µM forward and reverse primers; 2.5- 3.5 mM manganese acetate; 10 U r*Tth* DNA polymerase, 1X EZ buffer. Buffer consisted of 50 mM bicine, 125mM potassium acetate, 40% (w/v) glycerol pH 8.2.

The GeneAmp in situ PCR system 1000 (Applied Biosystems, Foster City, CA, USA) comes with its own slide assembly tool. Briefly, the slide is placed on the preheated assembly tool. The RT PCR mix is applied onto the tissue section. A stainless steel Amplicover clip, with a silcone Amplicover disc attached, is inserted into the handle of the assembly tool, where they are held in place by a magnet. The handle is drawn down over the slide and the Amplicover disc and clip are assembled onto the surface of the slide, creating a liquid tight seal over the tissue section.

The following RT PCR thermal cycling conditions were used on a GeneAmp in situ PCR system 1000 (Applied Biosystems, Foster City, CA, USA): 58 °C for 45 minutes, 94 °C for 5 minutes, and 25 cycles of 94 °C for 45 seconds, 60 °C for 45 seconds, 72 °C for 45 seconds.

2.9.5 Post PCR Reaction/Hybridisation

Once the PCR was complete the slides were disassembled and fixed in 100% ethanol for 2 min. Tissues sections were denatured for 12 minutes at 94°C and hybridised at 37 °C overnight with a 5' biotinylated oligonucleotide probe (1μg /ml –2μg/ml) in hybridisation buffer (5% Dextran sulphate, 2X SSC, 50% Formamide, 0.2 % dried milk powder).

Following hybridisation, slides were rinsed in 2xSSC at 37°C for 15 minutes and incubated in TBT (50 mM Tris-HCl pH 7.2, 50 mM NaCl, 0.05% Triton X-100) at room temperature for 15 minutes.

2.9.6 Amplicon detection

2.9.6.1 Three Step Immunocytochemical Detection

All immunocytochemical detection steps were performed in an immunostainer tray. 50 μl of a 1/100 dilution of mouse anti biotin antibody clone BK-1/39 (DAKO Ltd, Glostrup, Denmark) in TBT (50 mM Tris-HCl pH 7.2, 50 mM NaCl, 0.05% Triton X-100) was applied to each section and incubated at room temperature for 30 minutes. Sections were rinsed in TBS (50 mM Tris-HCl pH 7.2, 50mM NaCl). 50 μl of a 1/100 dilution of rabbit anti-mouse immunoglobulin biotinylated (DAKO Ltd, Glostrup, Denmark) in TBT (50 mM Tris-HCl pH 7.2, 50mM NaCl, 0.05% Triton X-100) was added to each section and incubated at room temperature for 30 minutes. The sections were rinsed again in TBS (50mM Tris-HCl pH 7.2, 50mM NaCl). 50 μl of a 1/100 dilution of streptavidin –alkaline phosphatase conjugate (DAKO Ltd, Glostrup, Denmark) was added to each section and incubated at room temperature for 30 minutes. The sections were rinsed again in TBS and incubated in buffer 2

(0.5M Tris-HCL pH 9.5, 0.5M NaCl, 10mM $MgCl_2$, 0.1% TritonX-100) for 10 minutes. One NBT/BCIP (nitro-bromo-tertrazolium/ bromo-chloro-indolyl-phosphate) tablet (Roche Molecular Biochemicals, Mannheim, Germany) was dissolved in 10ml of sterile water and protected from the light. 500 µl of the NBT/BCIP solution was applied to each section and incubated in the dark for 13 minutes. The sections were then soaked in Proteinase K buffer (0.1M Tris, pH 7.5, 5 mM EDTA) for 2 minutes to stop the colorimetric reaction and rinsed in sterile water.

Sections were counter-stained in Methyl green (Vector, Burlingame, CA 94010, USA) for 5 sec and rinsed immediately in sterile water. Slides were air dried, mounted and coverslipped with mounting medium (DAKO Ltd, Glostrup, Denmark).

2.9.6.2 Dinitrophenol (DNP) Tyramide Signal Amplification

TSA™ (Tyramide Signal Amplification) technology (NEN® Life Science Products, 549 Albany Street, Boston MA 02118, USA) uses horse radish peroxidase (HRP) to catalyse the deposition of dinitrophenol (DNP) labelled amplification reagent onto tissue sections or cell that have been blocked with proteins (Figure 2.3) (Bobrow *et al.*, 1989). The labels can be detected indirectly with significant amplification or enhancement of the signal.

After blocking endogenous HRP and AP activity and permeabilising the cell membrane as described above, pretreated tissue sections were incubated in 100 µl of a 1/50 dilution of avidin-HRP (DAKO Ltd, Glostrup, Denmark) in TBT buffer (50mM Tris-HCL pH 7.2, 50mM NaCl, 0.05% Triton X-100) for 30 minutes at room

temperature. Sections were washed three times for 5 min each with TNB buffer (0.1M Tris-HCL pH 7.5, 0.15M NaCl, 0.5% blocking reagent) (NEN® Life Science Products, 549 Albany Street, Boston MA 02118, USA). Tissue sections were incubated in a 1/50 dilution of TSA-DNP (amplification solution) in amplification diluent for 8-9 minutes and rinsed three times in TBS buffer (0.1M Tris-HCL pH 7.5, 0.15M NaCl). The sections were then incubated in a 1/50 dilution of anti-DNP-AP conjugate in TBT (50mM Tris-HCL, pH 7.2, 50mM NaCl, 0.05% Triton X-100) for 30 minutes and washed in once in TBS (0.1M Tris-HCL pH 7.5, 0.15M NaCl). Slides were then equilibrated in buffer 2 (0.5M Tris-HCL pH 9.5, 0.5M NaCl, 10mM $MgCl_2$, 0.1% TritonX-100) for 10 minutes. Colour development was performed using NBT/BCIP (nitro-bromo-tertrazolium / bromo-chloro-indolyl-phosphate) (Roche Molecular Biochemicals, Mannheim, Germany) as described above.

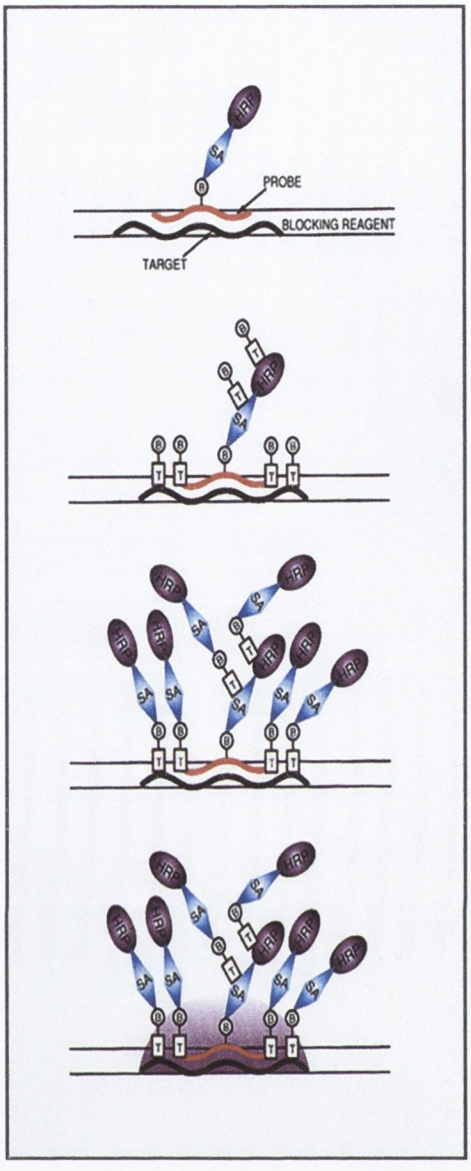


Figure 2.2. Tyramide Signal Amplification.

Tissue section hybridised with biotin labelled probe are incubated with avidin-HRP conjugate. The horse-radish peroxidase catalyses the deposition of dinitrophenol labelled amplification reagent onto the tissue sections, at and adjacent to the immobilised HRP enzyme. These labels can be detected with an anti DNP-Alkaline phoshatase conjugate resulting in enhanced signals.

2.9.7 Reaction, tissue and detection controls

A number of controls were performed with each batch of IS PCR. These included amplification of a reference control gene pyruvate dehydrogenase under the conditions described above, using target primers with an irrelevant probe, irrelevant primers with a specific probe, and RNase and DNase digestion of the tissue sections prior to in situ PCR.

2.9.8 Combined RT in situ PCR and Immunohistochemistry

Follicular dendritic reticulum cells (FDRC's) are present in lymphoid follicles and in germinal centres of lymphoid follicles of lymph nodes, spleen and tonsils. The cells are characterised by numerous cytoplasmic branching processes extending between adjacent lymphoid cells that form a network within which germinal centre lymphoid cells are found. FDRC's are difficult to recognise by conventional histology, however a monoclonal antibody CNA42 has been generated that recognises a 120 kd formalin resistant glycosylated antigen that is mainly expressed by follicular dendritic reticulum cells (Raymond *et al.*, 1997).

To examine the in situ hybridisation signal localization, combined in situ RT PCR and immunohistochemistry was performed. Immunohistochemistry was performed prior to in situ PCR. Briefly, tissue sections were pre-treated as described above. Endogenous peroxidase activity was blocked by soaking the sections in 3% H_2O_2 in methanol. Tissue sections were incubated in a 1/25 dilution of the mouse anti-human follicular dendritic cell clone CNA42 (DAKO Ltd, Glostrup, Denmark) in TBT and a 1/100 dilution of mouse anti biotin antibody clone BK-1/39 (DAKO Ltd, Glostrup, Denmark) for 30 minutes. The dendritic cell signal was developed using the 3-step

detection method described above with alkaline phosphatase (AP) (Dako Ltd, Glostrup, Denmark) and nitro-blue tetrazolium, (NBT) bromo-chloro-indolyl phosphate (BCIP) (Roche Molecular Biochemicals, Mannheim, Germany) as a substrate.

In situ RT PCR and hybridisation was then performed using the 5' biotin labelled oligonucleotide probe as described above except the hybridisation signal was developed using streptavidin-peroxidase HRP and amino-ethyl carbazole substrate kit (AEC: Vector Labs, Burlingame, CA, USA) according to the manufacturer's instructions.

2.9.9 In situ Hybridisation with Histone mRNA probe

Histone H3 is a replication dependant nucleosomal protein involved in the packaging of chromatin. The mRNA of histone H3 accumulates in the cytoplasm of cells, which are undergoing DNA synthesis during the S phase of the cell cycle and is rapidly degraded outside of the S phase. Therefore H3 mRNA can be used as a marker of proliferating cells (i.e. germinal centres of lymphoid tissues).

Pre treated, permeabilised and fixed tissue sections were hybridised with 20 µl of Histone H3 mRNA fluorescein labelled DNA probe pre diluted in hybridisation solution (Dako Ltd, Glostrup, Denmark) for 2 hours at 55 °C. Tissue sections were washed twice for 10 minutes at 50 °C in 2X SSC. Tissue sections were blocked in TBT (50mM Tris-HCl pH 7.2, 50mM NaCl, 0.05% Triton X-100, 0.1%BSA) for 15 minutes. The Histone mRNA signal was developed using the 3-step detection method described in section 2.9.6.

2.10 Southern Blot Analysis.

Southern blot first described in 1975 by E. M. Southern was one of the first techniques for detecting specific sequences within mixtures of DNA. In a Southern blot the DNA is size fractionated by gel electrophoresis and then transferred by capillary action to a nitrocellulose membrane. The immobilized DNA is then hybridised with either a radiolabelled or non- isoptopically labelled probe. The nucleic acid/probe hybrid is detected with an appropriate detection method. This technique was used in this study to confirm the specificity of the TaqMan PCR primers and probes.

2.10.1 Blotting procedure.

RT PCR products electrophoresed on agarose gels as described in section 2.6 were denatured in 2 gel volumes of fresh denaturant (0.5 M NaOH, 1.5 M NaCl) for one hour with frequent agitation. The gel was then neutralized in 2 gel volumes of neutralizer (0.5 M Tris-HCl, pH 7.4, 3 M NaCl) for one hour with frequent agitation.

Six sheets of Whatmann paper (exactly same measurement as gel) were soaked in 20 X SSC, placed onto a piece of clingfilm and rolled over with a clean pipette to remove air bubbles. The gel was placed with the wells uppermost on top of the Whatmann paper and the sides were sealed with parafilm. A sheet of Hybond N + nylon membrane (Amersham) soaked in sterile water, was placed on top of gel and rolled over with a clean pipette to remove air bubbles. Three sheets of Whatmann paper soaked in 6XSSC were placed on top of the nylon membrane and rolled over with a clean pipette to remove any air bubbles. A stack of paper napkins was placed on top of the Whatmann to cover the entire gel. A 1-2 kg weight was placed on top of

the napkins to facilitate capillary transfer of the DNA to the nylon membrane. Gel was left to transfer overnight. Once blotted the DNA was fixed to the membrane UV crosslinking at 254 nm for 3 minutes.

2.10.2 DIG Labelling of Oligonucleotides.

The DIG Oligonucleotide 3' end labelling kit (Roche Molecular Biochemicals, Sandhofer Strasse 116, D-68305 Mannheim, Germany) was used to enzymatically label oligonucleotides at their 3' end, with terminal transferase by incorporation of a single digoxigenin-labelled dideoxyuridine-triphosphate (DIG-ddUTP).

Briefly, the following were added on ice: 100 pmol of oligonucleotide probe, 1 X reaction buffer, 5 mM $CoCl_2$ solution, 0.05 mM DIG-ddUTP solution and 2.5 U/µl of terminal transferase. The reaction was incubated at 37°C for 15 minutes in a 9600 thermalcycler, and placed on ice. 2ul of 0.2M EDTA (pH 8.0) was added to stop the labelling reaction.

2.10.3 Hybridisation and Post Hybridisation Washes.

Membrane was incubated in 10 ml of pre-hybridisation buffer (5X SSC, 0.2 % SDS, 25 X Denhardts solution) in a hybridisation bag for 30 minutes at 50°C. The DIG labelled oligonucleotide probe (100 pmol) was mixed with 10 ml of pre warmed hybridisation buffer (5X SSC, 0.2 % SDS). The membrane was incubated in this buffer for 1 hour at 50°C. After incubation the blots were washed once at 50°C in 5X SSC, 0.2 %SDS for 10 minutes, followed by two washes at room temperature in 2X SSC and once in 1XSSC.

2.10.4 DIG Luminescent detection.

The DIG Luminescent detection kit (Roche Molecular Biochemicals, Sandhofer Strasse 116, D-68305 Mannheim, Germany) was used to detect DIG labelled DNA. After hybridisation and stringency washes the membrane was rinsed briefly in washing buffer (0.1M Maleic acid, 0.15 M NaCl, 0.3 % Tween 20, pH 7.5). The membrane was then incubated for 30 minutes in Buffer 2 (0.1M Maleic acid, 0.15 M NaCl, 1 X blocking reagent). The polyclonal sheep antibody anti- DIG-Alkaline Phosphatase conjugate, diluted 1/10,000 in buffer 2 (0.1M Maleic acid, 0.15 M NaCl, 1X blocking reagent) was added to the membrane and incubated at room temperature for 30 min. After incubation the blot was washed twice for 15 minutes in washing buffer (0.1M Maleic acid, 0.15 M NaCl, 0.3 % Tween 20, pH 7.5) and equilibrated for 5 minutes in detection buffer (0.1M Tris-HCL, 0.1M NaCl, pH 9.5). The membrane was sealed in a hybridisation bag and incubated for 5 minutes in 2 mls of CSPD® diluted 1/100 in detection buffer. The membrane was then sealed into a damp hybridisation bag and incubated at 37°C for 15 minutes to enhance the luminescent reaction. The membrane was transferred to an autoradiography cassette and exposed to Kodak Scientific imaging film for 10- 30 minutes depending on the exposure required.

2.10.5 Stripping and Re-Probing of DNA blots.

It is possible to probe the same blot with a number of different labelled probes. In order to do this the blot was stripped to remove the DIG labelled probe using the following procedure. Briefly the membrane was rinsed in sterile distilled water followed by two 15 minute washes at 37 °C in 0.2 M NaOH, 0.1% SDS. The membrane was then rinsed in 2X SSC and pre-hybridised and hybridised with a second DIG labelled probe.

2.11. Cloning Reactions using the Invitrogen TOPO TA Cloning Technology.

Plasmids are circular molecules of bacterial DNA that can replicate in the host cell independently of the main bacterial chromosome. Plasmid vectors are engineered to contain multiple antibiotic resistance genes, a multiple cloning site and various restriction sites, which allow insertion of DNA into the vector.

For generation of TaqMan PCR standards the Invitrogen TOPO TA Cloning kit and TOP 10 F' One shot™ kits were used. The specific PCR targets were cloned into the TOPO PCR 2.1 vector (Figure 2.4). The *Taq* polymerase used to generate the PCR product has a non-template dependent terminal transferase activity, which adds a single deoxyadenosine (A) on to the 3' ends of PCR products. The linearised TOPO PCR 2.1 vector has a single, overhanging 3' deoxythymidine (T) residue, which allows the PCR insert to ligate efficiently with the vector.

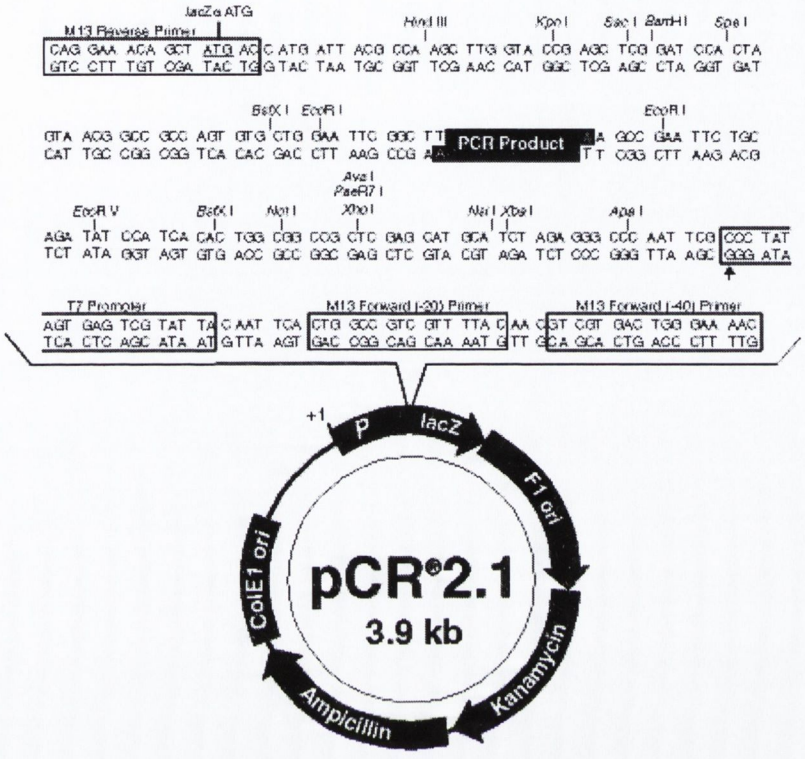


Figure 2.3 pCR 2.1 Vector Map.

The *Lac Z* gene, which codes for the enzyme beta-galactosidase is used as a reporter gene in the TOPO pCR 2.1 vector and confers on the recipient bacterial cell the ability to metabolise the galactose analogue 5-bromo-4-chloro- X – 3 indole -β- D-galactopyranosidase (X-gal). The multiple cloning site within the TOPO II vector is located with the *Lac Z* operon, therefore when a DNA insert has been successfully ligated into the vector, the *Lac Z* gene is interrupted, preventing expression of functional β – galactosidase. Following transformation and growth of transformed bacteria on selective agar plates containing X-gal and the inducer IPTG (isopropyl-β-D-thiogalactopyranoside), bacteria containing a plasmid with an insert will grow as a white colony whereas those with a functional *Lac Z* gene will grow as a blue colony. Antibiotic resistance genes for ampicillin and kanamycin are also present on the vector enabling selection of transformed bacteria containing the TOPO pCR 2.1 vector.

2.11.1 Ligation Reaction.

Topoisomerase 1 is an enzyme isolated from Vaccinia virus that recognizes specific sequences C/TCCTT. Topoisomerase 1 is covalently bound to the TOPO vector and cleaves double stranded DNA at specific sites along the phosphodiester backbone in one strand causing the strand to rotate around the other strand and religating the ends of the cleaved strand and releasing the topoisomerase.

The ratio of product insert to plasmid vector is crucial to obtaining successful recombination. The ligation reactions were performed in a final volume of 6 µl. Agarose gel purified PCR products (section 2.7.3) (2-4µl) were mixed with 1 µl of salt solution and 1µl of TOPO pCR 2.1 vector solution and incubated at room

temperature for 5 minutes. The reactions were held on ice prior to transformation.

2.11.2 Transformation Reaction

2 µl of the ligation reaction was added on ice to a vial of TOPO 10 F' competent *E. coli* cells (Invitrogen BV, 9704 CH Groningen, Netherlands). The reaction was mixed gently and incubated on ice for 20 minutes. The cells were heat shocked 42 °C for 30 seconds and immediately placed on ice for 2 minutes. 250 µl of pre-warmed SOC medium (0.5% Yeast extract, 2% tryptone, 10mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose) was added to the cells and incubated on a shaking incubator at 37 °C for 1 hour. For blue/white plasmid colony screening Luria Bertani (LB) agar plates (1% Tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0, 1.5 % agarose) supplemented with 50 µg/ml of ampicillin, were spread with 40 mg/ml of X- gal and 100 mM IPTG. Transformed cells were plated out onto the selective LB agar and incubated at 37 °C overnight.

2.11.3 Analysis of Positive Clones.

At least 5 white colonies per transformation were analysed. Initially clones were screened by direct PCR from colonies, then plasmids were isolated and inserts were confirmed by PCR and /or restriction digestion. A glycerol stock was made of all positive clones and stored at – 80 ° C.

PCR cocktails (20 µl) for amplification directly from colonies were prepared as follows: 1X PCR buffer, 3 mM $MgCl_2$, 0.4 mM dNTP's, 100 ng PCR primers (M13 Fwd and M13 Rev) Amplitaq Gold DNA polymerase and sterile water. Positive transformants (white colonies) were picked from plate using a sterile toothpick,

patched onto a separate LB agar plate for future use and the colony resuspended in the PCR cocktail. The PCR was performed on a 9600 thermal cycler (Applied Biosystems, Lincoln Centre drive, Foster City, CA 94404, USA) using the following cycling conditions, 95 °C for 10 minutes, followed by 94 °C for 1minutes, 55 °C for 1 minutes, 72 ° C for 1minute for 30 cycles, and a final extension at 72 °C for 10 minutes. Amplified PCR products were analysed by gel electrophoresis as described in section 2.5.

2.11.4 Plasmid DNA Isolation

The QiaAmp Miniprep DNA Kit (Qiagen Ltd, Crawley, West Sussex, RH 10 9AX, UK) was used to purify plasmid DNA from positive clones. Positive transformant colonies were grown at 37 °C overnight in 3-5 ml of selective LB broth. The bacterial culture was centrifuged at 4,000 rpm for 10 minutes, the supernatant removed and the cell pellet was resuspended in PB lysis buffer. Plasmid DNA was isolated using the QiaAmp Miniprep DNA kit according to the manufacturer's instructions and eluted in 50 µl of Tris-HCl pH 8.0. DNA was quantified by UV spectrophotometry as described in section 2.4. Specific inserts were confirmed by PCR as described in section 2.6 or by restriction digestion. All clones used as standard controls for TaqMan RT PCR assays were sequenced as described in section 2.14 to confirm the specific inserts.

2.12 Restriction Enzymes and Digestion.

Restriction enzymes recognize specific sites of different lengths and composition in a DNA sequence. There are three main categories of restriction enzymes Type I, Type II and Type III. Type II are the most common and contain separate restriction and methylation systems and usually have a short recognition sequence, 4-8 bp in length.

Restriction reactions were usually set up in a final volume of 20 µl containing 1µg of DNA, 1 X restriction buffer (specific for restriction enzyme), 1U of restriction enzyme, where 1 U is defined as the amount of enzyme required to completely digest 1ug of DNA in 60 minutes at the optimal assay temperature (37 °C). The restriction enzymes and buffers used in this study were obtained from Promega (Woods Hollow Road, Madison, USA).

2.13 *In vitro* transcription reactions for synthesis of copy RNA standards.

For RNA assays including TaqMan RT PCR copy RNA (cRNA) standards/controls were generated from cloned DNA plasmids. Transcription reactions were performed using the Promega Riboprobe® *In Vitro* Transcription System (Promega, Woods Hollow Road, Madison, USA). During the course of this study it was found that labeling cRNA with biotin helped to stabilise the transcripts.

Prior to transcription DNA plasmids were linearised with the single cutter *BAM*H1 restriction endonuclease as described in section 2.12. The reaction volume was brought up to 100 µl with RNase free water. An equal volume of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1 pH 8.0) was added, the sample was vortexed for 1 minute and centrifuged at 13,000 rpm for 5 minutes. The aqueous

phase was transferred to a fresh tube, one volume of chloroform:isoamyl alcohol (24:1) was added. The sample was vortexed for 1 minute and centrifuged at 13,000 rpm for 5 minutes. The aqueous phase was transferred to a fresh tube and 2.5 volumes of ice cold 100 % ethanol were added. The sample was incubated -70°C for 60 minutes and centrifuged at 13,000 rpm for 30 minutes at 4 °C. The supernatant was discarded and the pellet washed twice with 70 % ethanol. Linearised plasmid DNA was eluted in 5-10 µl of RNase free water.

The transcription reactions were performed in a final volume of 20 µl, containing the following: 1X Transcription buffer, 10 mM DTT, 20 U RNasin inhibitor, 2.5 mM each of ATP. CTP, GTP and UTP stocks (A 10 mM stock of Biotin-UTP was made up with 6.5 mM UTP and 3.5 mM biotin UTP (Roche Molecular Biochemicals, Sandhofer Strasse 116, D-68305 Mannheim, Germany)), 20U T7 RNA polymerase and 1-10µg of linearised plasmid DNA template. Reactions were incubated at 37°C in a waterbath for 5 hours with frequent mixing. RNase–free *DNase* was added to a concentration of 1U/µg of template DNA and incubated for at 37 °C for 15-30 minutes. DNA contamination was checked by gel electrophoresis before proceeding with clean up of the *in vitro* transcription reactions.

2.13.2 Clean up of In Vitro Transcription Reaction.

The *in vitro* transcription reaction was extracted with 1 volume of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1 pH 4.5), vortexed for 1 minute and centrifuged at 13,000 rpm for 5 minutes. The aqueous phase was transferred to a fresh tube, one volume of chloroform:isoamyl alcohol (24:1) was added. The sample was vortexed for 1 minute and centrifuges at 13,000 rpm for 5 minutes. The aqueous phase was transferred to a fresh tube, 0.5 volumes of 7.5 M ammonium acetate and 2.5 volumes of ice cold 100 % ethanol were added. The sample was incubated – 70°C for 60 minutes and centrifuged at 13,000 rpm for 30 minutes at 4 °C. The supernatant was discarded and the pellet washed twice with 70 % ethanol. The cRNA pellet was allowed to dry, resuspended in 20 µl of DEPC treated water and stored at – 70 °C. cRNA was quantified by UV spectrophotometry as described in section 2.4.

2.13.3 Calculation of cRNA copy numbers

For absolute quantitation RNA assays the following formula was used to calculate the number of copies of plasmid in a particular extract. This is a sample calculation to determine the copy number for a TOPO pCR 2.1 vector containing a 150 bp insert.

- Size of plasmid (3931bp) + insert (150 bp) = 4081 bp
- Concentration of plasmid extraction= 100 ng/µl
- Molecular weight of 1bp single stranded RNA= 330 g/l
- Avogadro's constant = 6.02×10^{23} copies/litre.

4081bp x 330g/l = 1.35×10^{6} Concentration of a 1 molar solution.

$6.02 \times 10^{23} / 1.35 \times 10^{6} = 4.14 \times 10^{17}$ copies/g

4.14×10^{8} copies/ng

100ng/µl x 4.14×10^{8} copies/g = 4.14×10^{10} copies/µl

Copy Number = 4.14×10^{10} copies/µl

2.14 Cycle Sequencing.

All sequencing reactions were performed on an ABI Prism 310 Genetic analyser. Fluorescent dye labels were incorporated into the sequencing reactions using 3' dye labelled dideoxynucleotide triphopshates (dye terminators). With dye terminator labelling each of the four dideoxy terminators (ddNTP's) are tagged with a different fluorescent dye and therefore the four sequencing reactions can be performed in a single tube. The extension DNA chain is simultaneously terminated and labelled with the dye that corresponds to that base. ABI prism big dye terminator structures contain a fluorescein donor dye e.g 6-carboxyfluorescein (6-FAM) linked to a dichlororhodamine acceptor dye. The donor dye is optimized to absorb the excitation energy of the argon ion laser in the ABI 310 Genetic Analyser. These dyes are 2-3 times brighter than standard dye terminators when incorporated into cycle sequencing products. The terminators and their corresponding labels in brackets are as follows A (dR6G), C(dROX), G(dR110), and T(dTAMRA).

The ABI Prism BigDye Terminator Cycle sequencing kit employs AmpliTaq DNA Polymerase. This enzyme provide rapid nucleotide incorporation and has no 3'-5'exonuclease activity. The enzyme contains a point mutation in the active site, which results in less discrimination against ddNTP's and leads to more even peak intensity pattern.

2.14.1 Cycle Sequencing Reactions

The ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) was used for all sequencing reactions performed for this study. Sequencing reactions were performed on both strands of DNA using sense and antisense strand primers. pGEM control sequencing reactions were set up with every batch using M13 PCR primers. Reactions were set up in a final volume of 20 µl consisting of 2-8 µl of purified PCR product or 100-500 ng of plasmid DNA, 8 µl of Big Dye terminator mastermix and 3-10 pmol of primer. Cycle sequencing reactions were performed on a 9600 GeneAmp PCR cycler under the following conditions: 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes.

Excess and unincorporated Big Dye terminators were remove by precipitation of the sequencing reaction in 80 µl of 75% isopropanol at room temperature for 15-30 minutes. The reactions were centrifuged at 13,000 rpm for 20 minutes, and the pellet washed twice with 250 µl of 75% ethanol. The pellet was air dried completely and resuspended in 15 µl of template suppression reagent. Reactions were denatured at 95 °C for 2 minutes and cooled on ice before loading onto the ABI 310 genetic analyzer. Sequencing reactions were run using POP 6 polymer and rapid 1ml E run on the ABI 310 Genetic Analyser. Data was analysed using the ABI Sequence Analysis software version 3.1.

2.15 Microarray Technology.

2.15.1 Introduction.

As this is a new technology a full description of the technique is given in this chapter of the thesis and the applications are described in chapter 6. The recent release of the first draft of the human genome sequence and the development of the expressed sequence tag (EST) clone collection, have made available to scientists an enormous number of DNA and mRNA sequences. DNA microarray technology is a powerful tool for exploring this data in many biological systems. Genome wide expression analysis using microarrays offers unique opportunities to study the interactions of genes and pathways, to characterise gene regulatory networks and to identify novel genes implicated in diseases.

Currently there are two technologies for performing genome wide gene expression analysis. One technology uses cDNA microarrays, constructed by robotic spotting and immobilisation of purified cDNA clones, oligonucleotide sequences or PCR products onto glass slides. The second technology is Affymetrix GeneChip technology, which uses prefabricated oligonucleotide chips.

2.15.2 Affymetrix GeneChip Technology

The Affymetrix oligonuceotide arrays are synthesised using a process combining photolithography and combinatorial chemistry (Pease *et al.*, 1994). The arrays are composed of a quartz wafer, which is naturally hydroxylated. A set of photolithographic masks are manufactured that allow the sequential addition of specific nucleotides to particular locations on the chip. When ultraviolet light is shone over the mask in the first step of synthesis the exposed linkers become deprotected and are available for nucleotide coupling. The single type nucleotide solution is then washed over the wafers surface and attaches to the activated linkers. In the next step another mask is placed over the wafer for the next round of deprotection and coupling. The process is sequentially repeated until the probes reach their full length. Figure 2.4 illustrates the process.

Individual genes are represented on the GeneChip using a series (typically 11-20) of different 25-mer "perfect match" oligonucleotides. The HG-U133 set contains 11 probe pairs per probe set. The probe sets are generally located within 600 bp upstream from the poly-A site as this portion of mRNA is most efficiently converted into labelled target. Probes are selected based on their complementarity to the selected gene or EST, uniqueness relative to other related genes and their predicted hybridisation characteristics. For every set of perfect match oligonucleotide probes a paired set of mismatch probes are also present on the array. The oligonucleotide sequence used for the MM probes are identical to the PM probe but with a single nucleotide substitution in the central position.

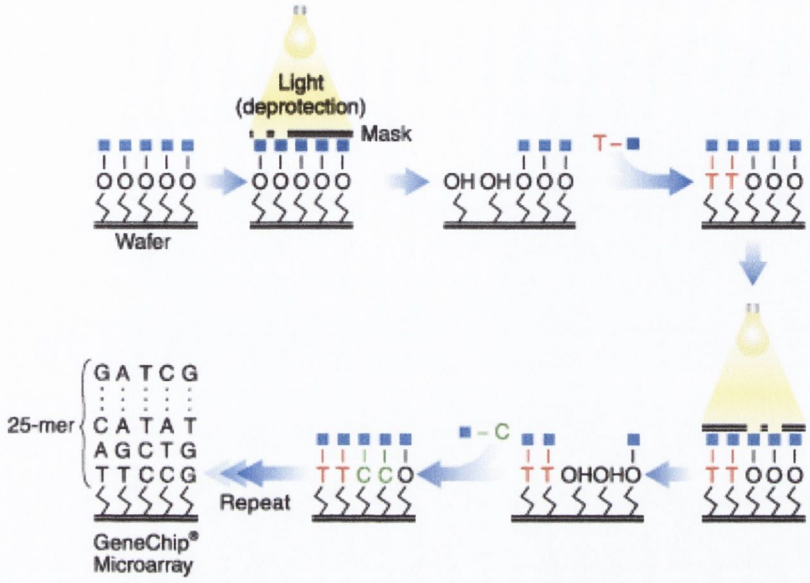


Figure 2.4 Affymetrix uses a combination of photolithography and combinatorial chemistry to manufacture GeneChip Arrays. (Image supplied by Affymetrix).

The specific hybridisation signal is determined by subtracting the fluorescence signal from the MM probes from the PM fluorescence signal and averaged over all the probe pairs representing a particular gene. This average fluorescence signal is a measure of the transcript abundance.

The Affymetrix software detection algorithm uses probe pair intensities to generate a detection P value and assign a present/ absent call for each individual gene. Each probe pair in a probe set has a discrimination score, which is calculated for each probe pair and compared to a predefined threshold *Tau*. The discrimination score is a basic property of a probe pair that describes its ability to detect its intended target. It measures the target specific intensity difference of the probe pair (PM-MM) relative to its overall hybridisation intensity. Probe pairs with scores higher than *Tau* vote for the presence of transcript. Probe pairs with scores lower than *Tau* vote for the absence of transcript. A P value is also generated that reflects the confidence of the detection call. This strategy permits high sensitivity at low target concentrations and preserves the ability to discriminate between closely related sequences.

In contrast to cDNA microarrays, which use a two colour hybridisation approach where labelled target and control are hybridised to the same array, each Affymetrix GeneChip is designed to analyse a single RNA sample. mRNA is converted to double stranded cDNA by reverse transcription using an oligo d(T) primer engineered to contain a T7 RNA promotor site. Biotin labelled nucleotides are directly incorporated into the cRNA target by in vitro transcription with a T7 polymerase. Labelled cRNA is fragmented into < 200 bp fragments and hybridised to the GeneChip. The fluorescent signal is obtained after staining the GeneChip with

streptavidin phycoerythrin. Figure 2.5 outlines the procedure involved.

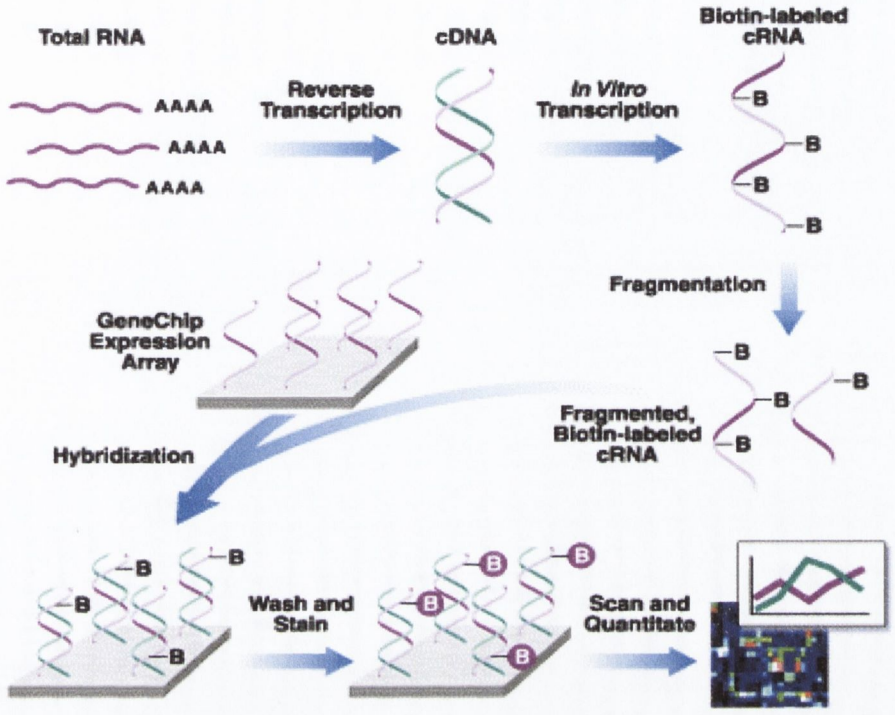


Figure 2.5 Schematic of the procedure for Gene Expression Analysis using Affymetrix GeneChips

2.15.2 Affymetrix GeneChip Analysis Procotol.

2.15.2.1 Double stranded cDNA synthesis.

Probe preparation was performed following the protocols in the GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Double stranded cDNA was prepared from 5-8 μg of total RNA using the Invitrogen Superscript Choice System (Invitrogen BV, 9704 CH Groningen, Netherlands). All reactions were performed on a 9600 thermal cycler (Applied Biosystems). A HPLC purified T7 –(dT) 24 primer (Affymetrix, Santa Clara, CA) was used to prime the first strand cDNA synthesis. The sequence of the T7 promoter primer is as follows;

T7 –(dT) 24 primer sequence:

5' GGCCAG TGAAATTGTAATACGACTCACTATAGGGAGGCGG- (dT)24 3'

The T7 primer (100 pmol) and RNA were mixed, incubated at 70 °C for 10 minutes and cooled on ice. For first strand synthesis, 1X first strand buffer, 0.1M DTT, and 10 mM dNTP mix were added, incubated at 42°C for 2minutes, 200U of *Superscript II* reverse transcriptase was added and the reaction incubated at 42 °C for 1 hour in a 9600 thermal cycler with heated lid.

For second strand cDNA synthesis reactions the following reagents were added to the first stand synthesis reactions, 1X second strand buffer, 200μM of each dNTP, 10 U of DNA Ligase, 40 U of DNA polymerase I and 2 U of RNase H. The reactions were mixed and incubated at 16°C for 2 hours in a 9600 thermal cycler without heated lid. Blunt ends were ligated by adding 20U of T4 DNA polymerase

incubating for 5 minutes at 16°C. The reaction was stopped by adding 10 µl of 0.5 M EDTA.

2.15.2.2 Cleanup of double stranded cDNA.

An equal volume (162µl) of (25:24:1) phenol:chloroform:isoamyl alcohol pH 8.0 was added to cDNA reactions and samples were vortexed and centrifuged at 13,000 rpm for 5 minutes. The aqueous phase was transferred to a fresh 1.5ml microtube, 0.5 volumes of 7.5M NH_4AC and 2.5 volumes of ice cold ethanol were added to the sample. The sample was immediately centrifuged at 13,000 rpm for 20 minutes at room temperature, the supernatant removed and the pellet washed twice with 80 % ethanol. The pellet was air dried and resuspended in 12 µl of RNase free H_2O.

2.15.2.3 Synthesis of Biotin labelled cRNA (IVT)

cRNA was synthesized from cDNA and labelled with biotin using the BioArray high yield RNA Transcript labelling kit (Enzo Diagnostics, Farmingdale, NY). The following reagents were added to 10 µl of the cleaned up cDNA reactions; 1X high yield reaction buffer, 1X biotin labelled ribonucleotides, 1X DTT, 1X RNase inhibitor mix, and 1X T7 RNA polymerase. Reactions were incubated in a 37 °C waterbath for 5 hours, mixing every 45 minutes. Labelled cRNA was cleaned up using the Qiagen RNeasy clean up protocol and eluted in 40 µl of RNase free water. *In vitro* transcription reactions were quantified by spectrophotometry and the adjusted cRNA yield was calculated using the following formula:

Adjusted cRNA= RNAm -(Total RNAi)(y)

Where:
RNAm= Quantity of cRNA after IVT (μg)

Total RNAi= starting amount of total RNA (ug)

y= fraction of cDNA in IVT

2.15.2.4 Hybridisation to GeneChip arrays

Twenty micrograms of cRNA (adjusted concentration) was fragmented by heating at 94 °C for 35 minutes in fragmentation buffer (40 mM Tris acetate (pH 8.1), 125 mM KOAc, 30 mM MgOAc). Fragmented cRNA was analysed by agarose gel electrophoresis.

15 μg of fragmentated cRNA was added to 300 μl of hybridisation cocktail (1X hybridisation buffer: 10mM MES, 1M Na^+, 20 mM EDTA, 0.01 % Tween 20, 50pmol of control oligonucleotide B2, 1 X Eukaryotic hybridisation controls, 0.1 mg/ml Herring sperm DNA, 0.5 mg/ml BSA). The cocktail was heated to 99°C for 5 minutes, 45 °C for 5 minutes and hybridised to U133A GeneChip arrays. Hybridisation was performed at 45 °C for 16 hours in a GeneChip rotating hybridisation oven. After hybridisation each chip was washed and stained with streptavidin phycoerythrin, followed by signal amplification with biotin labelled anti streptavidin phycoerythrin antibodies, restained with streptavidin phycoerythrin using the Affymetrix Fluidic station 400. Fluorescent signals were measured on the arrays using the Affymetrix GeneArray laser scanner (Hewlett Packard). Images were analysed using Microarray Suite software version 4.0.

Chapter 3

Detection of measles virus in children with new-variant inflammatory bowel disease "autistic enterocolitis"

3.1 Introduction

The prevalence of gastrointestinal symptoms in children with developmental disorders has not been fully determined. However a relationship between gastrointestinal dysfunction and developmental disorders has been described based on the identification of a novel form of immune-mediated inflammatory bowel disease in a cohort of children with a developmental disorder (provisionally termed autistic enterocolitis) (Wakefield *et al.*, 1998a). A number of studies have since investigated the problem of gastrointestinal symptoms in autistic and autistic spectrum disorders (Molloy *et al.*, 2003; Black *et al.*, 2002; Mehmed *et al.*, 2000; Horvath *et al.*, 1999; D'Eufemia *et al.*, 1996) and indicate that gastrointestinal symptoms are a common finding in autistic spectrum disorders. These are fully discussed in Chapter 1.

The developmental disorder described by Wakefield *et al.*, (1998a) has been defined as regressive autism where children appear to have developed normally for the first few years of life before regressing to an autistic phenotype with associated behavioural and social difficulties. Gastrointestinal symptoms in these children, which often started around the same time as the characteristic developmental regression include chronic constipation with overflow, abdominal pain, bloating and oesophageal reflux. The intestinal pathology includes ileo-colonic lymphonodular hyperplasia and non-specific colitis, which manifests as neither Crohn's disease nor ulcerative colitis. The histological and clinical aspects of this new disorder have been reported previously (Wakefield *et al.*, 2000). Lymphoid follicles numbers were increased in ileal biopsies of LNH, and compared with normal follicles the germinal centres were grossly enlarged and reactive. Increased intraepithelial lymphocytes in

particular CD8+ and γδ T cells, were observed in the colonic mucosa compared with that seen in normal or IBD controls (Furlano *et al.*, 2001). Studies in the small intestinal lesion indicated a cell mediated immune response with increased CD3 and CD8 cells in the lamina propria compared with controls and immunoglobulin G co localized with complement C1q on the epithelial surface, which was not seen in any of the normal or disease controls (Torrente *et al.*, 2002).

The exact significance of the finding of lymphonodular hyperplasia in the mucosa of the lower GI tract and the terminal ileum remains unclear but it has been reported as a common finding in paediatric colonoscopies (Kokkonen, 1999). A number of studies have reported an association between the presence of lymphonodular hyperplasia on the duodenal bulb and food allergies (Bellanti *et al.*, 2003; Kokkonen *et al.*, 2000; Kokkonen, 1999). Similarily, Kokkonen (2002) have recently reported an association between LNH of the terminal ileum, increased density of $\gamma\delta^+$ T cells and food allergy in developmentally normal children (Kokkonen *et al.*, 2002).

The presence of reactive lymphonodular hyperplasia in the autistic enterocolitis cohort also suggests an antigen driven response, and numerous theories on the nature of the antigen have been explored. These include, dietary and vaccine derived antigens and are discussed fully in chapter 1. The initial report by Wakefield (1998) suggested a link between the measles, mumps and rubella (MMR) vaccine, intestinal symptoms and developmental regression in the autistic children investigated. Parental reports linked the onset of behavioural regression with administration of the trivalent MMR vaccine in eight of the twelve children investigated. This has

sparked a huge controversial public debate on whether the MMR vaccine causes autism.

Measles is a highly contagious childhood disease that was ranked as one of the leading causes of childhood mortality in the late 20th century. In developing countries 1 million deaths per annum are related to measles virus infections (Schneider Schaulies *et al.*, 2002a). The virus is transmitted by the respiratory route and is transported by dendritic cells or macrophages to the local lymphatic tissues. The virus is amplified and spreads by a cell-associated viremia as revealed by the characteristic skin rash. The virus induces profound suppression of immune functions that favours the establishment of secondary infections. Other diseases including subacute sclerosing panencephalitis (SSPE) and measles inclusion body encephalitis (MIBE) develop as a result of persistent measles infection of neuronal and macroglial cells (Bitnun *et al.*, 1999). Several effective vaccines including the MMR vaccine have been derived from measles virus Edmonston strain that have been successful in reducing the incidence and magnitude of measles infections. However the recent scare about a possible link between MMR and autism has resulted in reduced uptake of the MMR vaccine, which was as low as 78% in the UK last year.

Measles virus is an enveloped RNA virus that belongs to the family of *Paramyxoviruses.* The 16kb genome encodes six structural proteins; the nucleoprotein, the phosphoprotein, the matrix protein, the haemaglutinin protein, the fusion protein and the large protein which is a catalytic subunit of the RNA dependant RNA polymerase. There is a transcriptional gradient from the 3' end to

the 5' end of the genome. The N, F and H genes were chosen for analysis in this study. The Nucleocapsid (N) protein is essential for packaging the genome into a ribonucleoprotein complex that served as a template for transcription, replication and packaging of the virions. Haemaglutinin and fusion genes encode envelope glycoproteins that mediate cell surface recognition, membrane fusion and virus entry (Oxman, 1997), as discussed in chapter 1. Changes in these proteins such as those seen in vaccine strains may influence the cell to cell spread of the virus and contribute to the attenuated phenotype (Parks *et al.*, 2001).

A number of population-based studies (Madsen *et al.*, 2002; Taylor *et al.*, 2002; Fombonne *et al.*, 2001; Taylor *et al.*, 1999) in contrast with molecular and immunological studies have failed to find an association between the MMR vaccine and autism. Preliminary immunohistochemistry data suggested the presence of measles virus antigen in the extracellular matrix of the mid-gut mucosal lymphoid tissue in affected children (Wakefield *et al.*, 1998b). Since then a number of studies have detected measles virus antibodies and RNA in autistic children. Elevated levels of measles antibodies have been detected in the serum of autistic patients and are associated with the presence of myelin basic protein (MBP) antibodies, which are related to autoimmunity (Singh *et al.*, 2003; Singh *et al.*, 2002). Measles virus haemaglutinin RNA consistent with vaccine strain virus has been detected by RT PCR in the peripheral blood of three of nine autistic enterocolitis patients and one of three patients with ulcerative colitis, while wild type strain was identified in one of eight patients with Crohn's disease (Kawashima *et al.*, 2000).

Inflammatory bowel disease, in particular Crohn's disease has been previously linked to persistent measles virus infection (Ekbom *et al.,* 1994, Wakefield *et al*, 1995). Both the epidemiological and experimental evidence of an association are conflicting and this is fully discussed in chapter 1. Measles virus nucleocapsid and matrix proteins have been detected by immunohistochemistry and immunogold electron microscopy (Daszak *et al.*, 1997; Lewin *et al.*, 1995; Miyamoto *et al.*, 1995). The majority of studies with the exception of the Kawashima study described above failed to detect measles virus RNA in intestinal tissue from IBD patients. The discrepancies in the findings are most likely related to differences in the RT-PCR techniques applied, assay sensitivities and measles specific targets.

Based on the epidemiological and experimental evidence to date, and in an attempt to resolve the current issues regarding detection of measles virus, the hypothesis that persistent measles virus infection is involved in the pathogenesis of autistic enterocolitis is examined in this study.

3.2 Study Aims

This focus of this chapter was to detect the presence of persistent measles virus infection in children with new variant inflammatory bowel disease (autistic enterocolitis). The chapter has been divided into two sections. Section 1 describes the detection and quantitation of measles virus fusion and haemaglutinin genes using TaqMan RT PCR. Section 2 describes the detection and localisation of measles virus nucleocapsid gene in these patients using reverse transcriptase in situ PCR and immunocytochemistry.

3.3 Section 1

Aim: To determine the presence and quantity of measles virus fusion (F) and haemaglutinin (H) genes in the intestinal tissue and peripheral blood of children with new variant inflammatory bowel disease (autistic enterocolitis) by RT PCR.

3.3.1 Materials and Methods

3.3.1.1 Patient Samples

All patient samples were provided by the Department of Gastroenterology, Royal Free Hospital, London, UK and Department of Pathology, St James's Hospital, Dublin 8, Ireland. Intestinal tissue biopsies from 126 affected children were examined (median age 6 yrs; range 3-20; 106 male). Terminal ileum was examined in the all affected cases with the following exceptions; lymph node only from one patient and duodenal biopsies from one patient. In addition to terminal ileum, transverse colon tissue biopsies were also examined in four cases.

These "affected cases" consisted of autistic children who underwent colonoscopy for investigation of gastrointestinal symptoms. Autistic enterocolitis was specifically diagnosed based on endoscopic and histological features in the intestine. These included the presence of LNH and reactive lymphoid follicles in the terminal ileum with non-specific inflammatory changes throughout the colon, as outlined in Wakefield *et al.*, (2000). All patients in the affected cohort had been vaccinated against measles virus infection with either the MMR or MR vaccines.

The control population were selected from a series of children who underwent colonoscopy for investigation of gastrointestinal symptoms. These consisted of

developmentally normal paediatric controls (n = 89; median 12 yrs; range 0-19yrs; 59 male). Ages were not available for five of the controls. The control group included: 14 children with normal ileal biopsies (endoscopically and histologically normal), 10 children with mild non-specific chronic inflammatory changes, 3 children with ileal lymphonodular hyperplasia (LNH) investigated for abdominal pain, 8 children with Crohn's disease, 2 children with ulcerative colitis and 5 children with colitis. In addition, 47 children who had undergone appendicectomy for abdominal pain including appendicitis were included in the control cohort. Aside from the appendicectomy samples, terminal ileum was examined from the control population. Small bowel resection was examined from one ulcerative colitis patient.

Information on the measles vaccination status or clinical measles infection within the control group was not available. For this reason only individuals born post 1982 were used in this study. The measles vaccination program was introduced in the UK and Ireland in 1982, it was therefore assumed that all patients born after this date had received vaccinations.

Peripheral blood mononuclear cells (PBMCs) from 74 patients including 53 affected patients (median age 8.5 yrs range, 5-20yrs; 44 male), 11 normal controls (median age 12.5 yrs; range 6-15, 9 male), 9 autistic patients without any gut pathology (median age 10.5 yrs; range 6-14yrs; 8 male) and one ulcerative colitis case (age 10yrs, M) were also examined. Peripheral blood mononuclear cells were prepared from 10 ml of peripheral blood using Histopaque 1077 solution (Sigma Diagnostics) as described in section 2.1.3. The cell pellet was resuspended in 0.2 ml of PBS and 1.8 mls of RNA*later* was added to preserve the RNA. The PBMCs were then

aliquoted into 0.5 ml aliquots and stored at –80 °C. One of the four 0.5 ml aliquots was used in this study.

Measles virus (MV) positive control material included tissue specimens from three cases of SSPE and MV infected Vero cells. Negative control material included uninfected Vero cells, human tissues, and control RNA extracted from Raji cells (Applied Biosystems, Foster City, CA, USA). Cells were cultured as described in section 2.1.2.

3.3.1.2 RNA extraction.

Total RNA was extracted from fresh frozen biopsies, tissue specimens, MV infected and uninfected Vero cell lines using the Ultraspec-11 RNA isolation system (Biotecx Laboratories Inc, Texas, USA) and Qiagen RNeasy minikit. Total RNA was extracted from PBMCs using the Qiagen RNA blood kit. Total RNA was extracted from formalin fixed paraffin embedded tissues using the Purescript® RNA Isolation Kit (Gentra Systems, Minneapolis, MN 55441, USA).

3.3.1.3 Primer and Probe Design.

PCR primers and probes to conserved regions of the MV Nucleocapsid (N), Haemaglutinin (H) and Fusion (F) genes were designed using Primer Express Software Version 1.5 (ABI Prism; Applied Biosystems, Foster City, CA, USA) as described in section 2.6.1. Primers and probes were designed based on measles virus (Edmonston strain) GenBank accession number K01711. The specificity of primer/probe sets were confirmed using BlastN (www.ncbi.nlm.nih.gov/blast). Table 3.1 shows the MV primer and probe sequences and amplicon sizes. In some

instances primer sets overlap with each other (e.g. the sequence of amplicon N1 overlaps partially with N2 PCR amplicon).

PCR primers for sequencing of the MV N gene were designed based on measles virus (Edmonston strain) GenBank accession number K01711. The N gene was sequenced using a total of nine different overlapping primer pairs spanning the 1630bp of the entire N gene. These primer pairs and amplicon sizes are listed in Table 3.2. As controls two PCR primers (5F rc and 5R rc- Table 3.2) were designed to "complementary" strand of the N gene.

Glyceraldehyde dehydrogenase (GAPDH) and Pyruvate dehydrogenase (PDH) housekeeping genes were used as controls. The TaqMan GAPDH control set was supplied by Applied Biosystems (Foster City, CA, USA). The PDH primers and probes were designed to amplify from *Homo sapiens* pyruvate dehdrogenase gene accession number J03576 spanning exons 2 and 3. PDH1 and PDH2 will amplify RNA and DNA sequence whereas PDHjunc is designed across the exon 2, intron 3 and exon3 boundaries to specifically amplify mRNA, when used with PDH1 will amplify a 96 bp amplicon specific for mRNA sequence. The sequences are listed below.

PDH1: 5' GGT ATG GAT GAG GAG CTG GA 3'

PDH2: 5' AGA AGG TAT TTC TGC TTG GAG AAG AAG TTG 3'

PDH Junc: 5' CAG CCC TCG ACT AAC CTT GT 3'

For TaqMan quantitative RT PCR, probes were dual labelled with fluorescein molecule FAM at the 5' end and quencher TAMRA at the 3' end. For in situ RT PCR probes were labelled with at the 5' end with biotin, and for southern blot analysis probes were 3' labelled with digoxygenin.

Table 3.1 Measles virus primer and probes

Primer/ Probe	Sequence 5'- 3'	Amplicon Size
N1 Fwd	5' TCA GTA GAG CGG TTG GAC CC 3'	
N1 Rev	5' GGC CCG GTT TCT CTG TAG CT 3'	150BP
N2 Fwd	5' GAG TCG AGG AGA AGC CAG GG 3'	
N2 Rev	5' GCT GGA CTC CGA TGC AGT GT 3'	120BP
H1 Fwd	5' TTC ATC GGG CAG CCA TCT AC 3'	
H1 Rev	5' CTC TGA GGT GTC CTC AGG CC 3'	150BP
H2 Fwd	5' TGG GCA CCA TTG AAG GAT AA 3'	
H2 Rev	5' AAC CGT GTG TGA TCA ATG GC 3'	120BP
F1 Fwd	5' TGA CTC GTT CCA GCC ATC AA 3'	
F1 Rev	5' TGG GTC ATT GCA TTA AGT GCA 3'	150BP
F2 Fwd	5' CCC ACC GGT CAA ATC CAT T 3'	
F2 Rev	5' CCC TCG TGC AGT TAT TGA GGA 3'	150BP
N1 Probe	5' CAA ACA GAG TCG AGG AGA AGC CAG GGA 3'	
H1 Probe	5' CCG CAG AGA TCC ATA AAA GCC TCA GCA C 3'	
F1 Probe	5' CTG CAC GAG GGT AGA GAT CGC AGA ATA CAG 3'	

Table 3.2 Measles virus nucleocapsid (N) gene sequencing primers

Name	Sequence 5'-3'	Amplicon Size
1F	5' GGA TAT CCG AGA TGG CCA CA 3'	
1R	5' ACG TCA GGG TCA TCG GTG AT 3'	300 bp
1F b	5' TGT GGC CAT CTC GGA TAT CC 3'	
1R b	5' ATC ACC GAT GAC CCT GAC GT 3'	Control
2F	5' AGC GGG CCC AAA CTA ACA G 3'	
2R	5' TCT CGA ACC ATC CGA ACC TG 3'	253 bp
3F	5' AGG ATG AGG CGG ACC AAT ACT 3'	
3R	5' AAT CAG CTG CCG TGT CTG G 3'	200 bp
4F	5' CTG GGT ACC ATC CTA GCC CAA 3'	
4R	5' ATC CTG GGT TTG TTT CCG G 3'	250 bp
5F	5' TCC TTA CGC CGA TTC ATG GT 3'	
5R	5' TCC CCA TTT GCT GGT AAA GGT 3'	240 bp
6F	5' CCT GCT CTT GGA CTG CAT GA 3'	
6R	5' GAT CGG CCA AAG TTC AAA CCT 3'	220 bp
7F	5' GTG CAG GAT CAT ACC CTC TGC 3'	
7R	5' TGA TAC TTG GGC TTG TCT GGG 3'	280 bp
8F	5' GTA GAG CGG TTG GAC CCA GA 3'	
8R	5' GGC TTG CAG CCT AAG CAG G 3'	280 bp
9F	5' ATT GAC ACT GCA TCG GAG TCC 3'	
9R	5' GAT GGA GGG TAG GCG GAT GT 3'	200 bp

3.3.1.4 Solution Phase RT PCR

Purified MV RNA extracted from measles infected Vero cells was used as a positive control to optimise PCR assays and for generation of quantitative TaqMan PCR standard controls.

The following optimal reaction conditions were used per 25 µl reaction: 0.4mM dNTPs, 0.4µM forward and reverse primers, 3 mM magnesium acetate, 2.5-5U r*Tth* DNA polymerase, 0.01U AmpErase, 1X EZ buffer. The following RT PCR thermal cycling conditions were used on a 9700 PCR thermocycler (Applied Biosystems, Foster City, CA, USA): 50 °C for 2 min, 58 °C for 30 min, 95 °C for 5 min, and 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, followed by an extension step at 72 °C for 10 min.

3.3.1.5 Southern Blot Analysis.

In order to confirm the reaction specificity, solution phase RT PCR was performed on patients and controls as described above and MV F and H gene amplicons were examined by Southern blotting using sequence specific probes (Table 3.1).

Measles virus F and H gene specific oligonucleotide probes (F1 and H1) were labelled at the 3' end with digoxigenin using a DIG Oligonucleotide 3' end Labelling Kit (Roche Molecular Biochemicals, Mannheim, Germany), hybridised to the Southern blot at 50 °C in 5X SSC/0.2 % SDS for one hour, washed once in 5X SSC at 50 °C for 10 min, once in 2X SCC at room temperature for 10 min, and once in 0.2X SCC at room temperature for 10 min. Hybrids were detected using the DIG Luminescent Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany).

3.3.1.6 Sequencing

RT PCR products for N gene sequencing were generated as follows: per 25 µl reaction: 0.4mM dNTPs, 0.4µM forward and reverse primers, 3mM magnesium acetate, 2.5U r*Tth* DNA polymerase, 0.01U AmpErase, 1X EZ buffer (Applied Biosystems), approximately 1µg of extracted RNA was used per reaction. The following RT PCR thermal cycling conditions were used on a 2400 PCR thermocycler (Applied Biosystems, Foster City, CA, USA): 50 °C for 2 min, 58 °C for 30 min, 95 °C for 5 min, and 40 cycles of 94 °C for 30 s, 58-60 °C for 30 s, 72 °C for 30 s, followed by an extension step at 72 °C for 10 min. The annealing temperature for different primer pairs varied from 58-60°C. RT PCR amplicons were gel purified as described in section 2.6.3. Forward and reverse strand sequencing was performed using the same PCR primers (Table 3.2) and cycle sequencing reactions were performed using the ABI Big Dye Terminator Sequencing chemistry (section 2.14). A consensus sequence was generated from forward and reverse strand sequences, which were assembled and overlapped for each primer set. F gene RT PCR products were amplified and sequenced as described above.

3.3.1.7 TaqMan RT PCR

Real time quantitative Reverse Transcriptase (RT) PCR based on the 5' nuclease assay was performed on an ABI 7700 Sequence detector, using the EZ TaqMan RT PCR reagents (Applied Biosystems, Foster City, CA, USA) as described in section 2.7.

The GAPDH TaqMan control primers and probe from Applied Biosystems (Foster City, CA94404) were used according to the manufacturer's instructions, to amplify the glyceraldehyde dehydrogenase housekeeping gene in all samples.

The concentration of measles virus TaqMan primers and probes used in each assay were optimised on controls by performing PCR titrations of each primer set 50-300 nmol and probe 50-250 nmol. The optimal combination of primer and probes for each TaqMan assay were chosen based on those that generated the lowest threshold cycle and the maximum ΔRn.

TaqMan RT PCR reactions for F and H gene assays were performed in duplicate under the following conditions per 25 µl reaction: 1X EZ buffer, 3mM $MnOAc_2$, 200 nmol of each primer, 100 nmol of TaqMan probe, 0.01 U of AmpErase, 0.1 U of r*Tth* polymerase, and 3 µl of total RNA. The thermal cycling conditions on the 7700 were as follows 50 °C for 2 min, 58-60 °C for 30 min, 95 °C for 5 min, followed by 40-45 cycles of 94 °C for 20 s, 60 °C for 1 min. GAPDH TaqMan assays were performed as described above with 3.5mM $MnOAc_2$ and reverse transcription at 60°C for 30 min.

Controls for TaqMan RT PCR included the following: No template control (water added as template), no amplification control (omission of r*Tth* polymerase), irrelevant target primers and specific TaqMan probe (HPV 16, HHV 8 primers), probe only control (omit PCR primers), human RNA control, spiked RNA control, asymmetric TaqMan PCR (TaqMan PCR with one or other primer and specific TaqMan probe).

Gene dosage correction/ normalization was carried out using Glyceraldehyde Phosphate Dehydrogenase (GAPDH) as a housekeeping gene. Measles virus quantitative TaqMan RT PCR was performed by generating standard curves for F- and H-genes. This was achieved using serial dilutions of cloned copy cRNA transcripts over a linear dynamic range of $1 \times 10^{7} - 1 \times 10^{1}$ copies.

3.3.2 Results

3.3.2.1 Solution Phase RT PCR

A total of six different PCR primer sets were optimised by solution phase RT PCR to amplify measles virus F-, H- and N-genes from RNA extracted from MV infected Vero cells (Figure 3.1). The F1, N1 and H1 primer probe sets listed in table 3.1 were then chosen for all subsequent assays.

The specificity of the primer/probe sets to detect MV F- and H genes was established by RT-PCR and Southern blot analysis of RNA extracted from measles infected Vero cells, SSPE brain (figure 3.2) and RNA extracted from a series of controls including uninfected Vero cells, rubella infected Vero cells, breast carcinoma cell line, buccal swab, and a case of Hashimoto's thyroiditis (data not shown). Positive measles virus signals were observed for RNA extracted from measles infected Vero cells and SSPE brain tissue, while the controls remained negative.

Solution phase RT PCR and Southern blot analysis was also performed on RNA extracted from terminal ileum biopsy material from four affected children (Figure 3.3 I). All four patient samples were positive for MV F- and H-genes by TaqMan RT PCR. Amplicon specificity for measles F gene was confirmed by Southern blot analysis using F gene specific probes (Figure 3.3 II). No template controls run in parallel were negative.

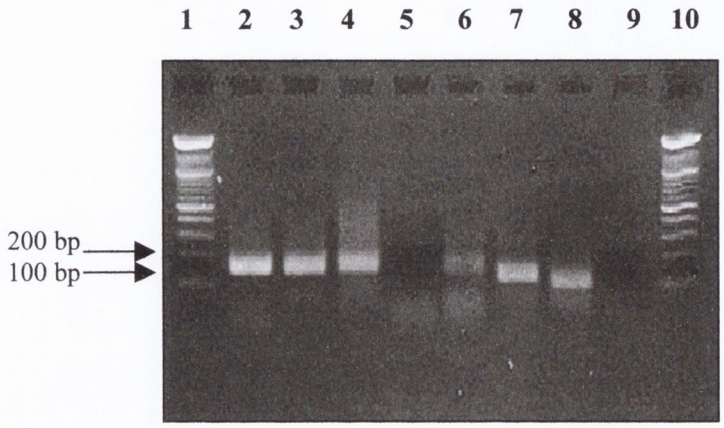


Figure 3.1. Agarose gel electrophoresis of measles virus cDNA amplicons generated from measles infected Vero cell RNA under optimised PCR conditions.

Lanes 1 + 10 represent a 100 base pair DNA ladder (Roche Molecular Biochemicals, Mannheim, Germany). **Lanes 2 + 3**; F gene cDNA amplicons (150 bp) for two different F-gene primer sets (F1 and F2). **Lanes 4 + 5**; N gene cDNA amplicons for two different N-gene primer sets (N1 –150 bp and N2- 120 bp). N2 RT PCR failed in this reaction but has been successfully amplified. **Lanes 6 + 7**; H-gene cDNA amplicons for 2 different H-gene primer sets (H1=150 bp and H2= 120 bp). **Lane 8**; PDH (pyruvate dehydrogenase) RT PCR product (96 bp). **Lane 9**; no template control.

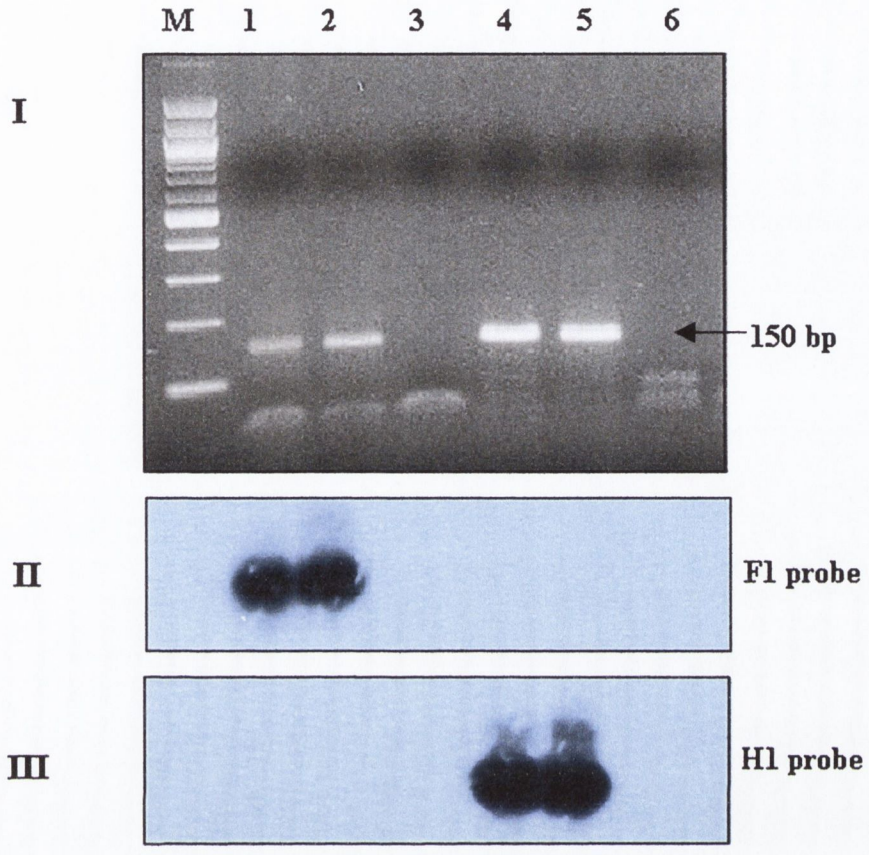


Figure 3.2. Agarose gel electrophoresis and southern blot hybridisation of measles virus F and H gene RT PCR products amplified from total RNA extracted from measles infected Vero cells and SSPE brain tissue.

I. 2% agarose gel showing RT PCR products amplified from: **lane 1**; F1 amplicon from SSPE brain tissue, **lane 2;** F1 amplicon from measles infected Vero cells, **lane 3;** negative water control (F1 primers), **lane 4;** H1 amplicon from SSPE brain tissue, **lane 5;** H1 amplicon from measles infected Vero cells, **lane 6;** negative water control (H1 primers).

II. Southern blot and hybridisation of RT PCR products above hybridised with F1 DIG labelled probe.

III. Southern blot and hybridisation of RT PCR products in gel I above stripped and re-hybridised with H1 DIG labelled probe.

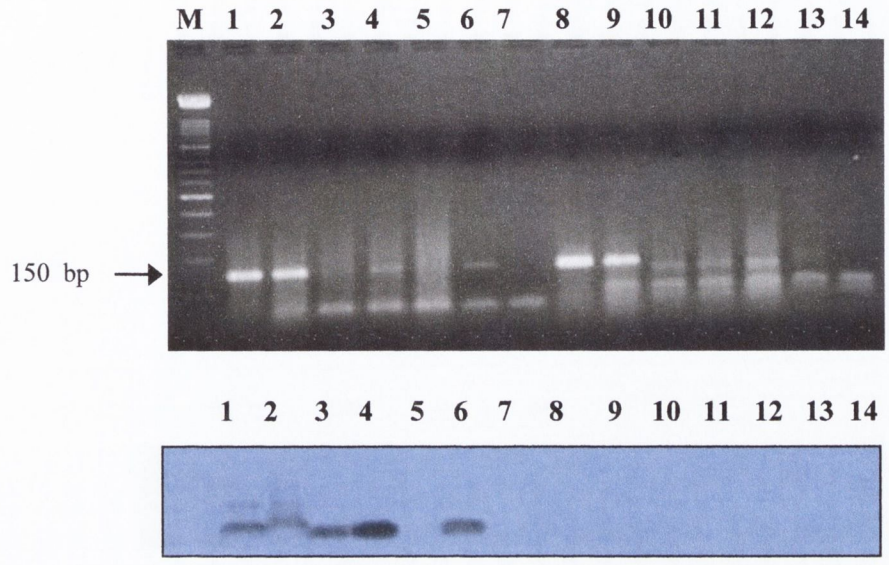


Figure 3.3. Agarose gel electrophoresis and Southern blot analysis of F and H gene RT PCR products amplified from intestinal tissue specimens.

I. 2 % agarose gel of measles virus F-gene and H-gene RT PCR amplicons. **Lanes 1- 6** correspond to F-gene PCR amplicons from the following total RNA samples; measles infected Vero cells, SSPE brain, terminal ileum from 4 affected children. **Lane 7** -no template control. **Lanes 8-14** correspond to H-gene PCR amplicons generated from RNA samples as above. For one patient sample, RT PCR for F- gene failed (Lane 5). **M** = 100 bp molecular weight marker.

II. Southern blot of the agarose gel described in figure 2(I) probed with a dig labelled F-gene specific probe as described in materials and methods.

3.2.3.2 Sequencing.

A large portion of the N gene amplified (using 9 different primer sets) from SSPE brain tissue and measles infected Vero cells was sequenced. RT PCR controls for N gene included primer set 1b, which were designed to amplify the complementary sense strand of the MV N gene genomic RNA. No amplicon was obtained using these primers confirming that N gene sequence present originated from measles virus RNA. Primer set 9 failed to amplify from the SSPE brain RNA samples, however a 200 bp amplicon was amplified from measles infected Vero cell RNA. A 250 bp amplicon was generated for primer set 4, but sequencing reactions consistently failed with these primers. Forward and reverse strand sequences were assembled and overlapped to generate 1424 bases of genetic sequence, a 127 bp portion is missing in the assembled sequence corresponding to that area amplified by primer set 4.

The 1424 bp sequence was blasted against the entire GenBank and EMBL databases using the NCBI blast program. Measles virus N gene sequence was confirmed and multiple sequence alignments with the top six significant homologous sequences were performed using ClustalW (1.82). Figure 3.4 shows this alignment. The N gene sequence appears to be wild type measles virus sequence based upon Blast sequence homology results, which identified significant similarity with a strain of measles virus isolated from a case of SSPE in Nottingham, UK in the 1990's. In addition analysis of the three previously detected nucleotide substitutions at positions 492 (C-A), 550 (A-G) and 1542 (T-A) that result in amino acid substitutions, which discriminate between vaccine and wild type strains were examined (Parks *et al.*, 2001). These substitutions are not conserved across all vaccine strains and no single strain contains all three substitutions.

F gene RT PCR products (150 bp) amplified from RNA isolated from terminal ileal tissue and SSPE brain tissue were sequenced to confirm the specificity of the cDNA amplicon (data not shown). In both cases measles virus F gene sequence was confirmed.

CLUSTAL W (1.82) multiple sequence alignment- Measles Virus Nucleocapsid Gene Sequences

```
AF504048        Wild type virus isolated from a case SSPE (Nottingham)
AB12949         Edmonston B vero cell adapted strain.
AF266289        Rubeovax vaccine strain
AF266288        MV Edmonston wild type.
MVU01996        MV Halonen strain.
U03656          Edmonston B vaccine strain
```

Start codon

```
U03656          ----------------------------------------------ATGGCCACACTTT 120
MVU01996        ----------------------------------------------ATGGCCACACTTT 120
AF266289        ----------------------------------------------ATGGCCACACTTT 120
AF266288        ----------------------------------------------ATGGCCACACTTT 120
AB12949         ----------------------------------------------ATGGCCACACTTT 120
AF504048        ----------------------------------------------ATGGCCACACTTT 120
SSPE            ------------------------------------------------------------

U03656          TAAGGAGCTTAGCATTGTTCAAAAGAAACAAGGACAAACCACCCATTACATCAGGATCCG 180
MVU01996        TAAGGAGCTTAGCATTGTTCAAAAGAAACAAGGACAAACCACCCATTACATCAGGATCCG 180
AF266289        TAAGGAGCTTAGCATTGTTCAAAAGAAACAAGGACAAACCACCCATTACATCAGGATCCG 180
AF266288        TAAGGAGCTTAGCATTGTTCAAAAGAAACAAGGACAAACCACCCATTACATCAGGATCCG 180
AB12949         TAAGGAGCTTAGCATTGTTCAAAAGAAACAAGGACAAACCACCCATTACATCAGGATCCG 180
AF504048        TAAGGAGCTTAGCATTGTTCAAAAGAAACAAGGACAAACCACCCATTACATCAGGATCCG 180
SSPE            -AAGGAGCTTAG-ATTGTTCAAAAGAA-CAAGNACAAACCACCCATTACATCAGGATCCG 57
                  *********** ************** **** ***************************

U03656          GTGGAGCCATCAGAGGAATCAAACACATTATTATAGTACCAATCCCTGGAGATTCCTCAA 240
MVU01996        GTGGAGCCATCAGAGGAATCAAACACATTATTATAGTACCAATCCCTGGAGATTCCTCAA 240
AF266289        GTGGAGCCATCAGAGGAATCAAACACATTATTATAGTACCAATCCCTGGAGATTCCTCAA 240
AF266288        GTGGAGCCATCAGAGGAATCAAACACATTATTATAGTACCAATCCCTGGAGATTCCTCAA 240
AB12949         GTGGAGCCATCAGAGGAATCAAACACATTATTATAGTACCAATCCCTGGAGATTCCTCAA 240
AF504048        GTGGAGCCATCAGAGGAATCAAACACATTATTATAGTACCAATCCCTGGGGATTCCTCAA 240
SSPE            GTGGAGCCATCAGAGGAATCAAACACATTATTATAGTACCAATCCCTGGGGATTCCTCAA 117
                ************************************************** **********

U03656          TTACCACTCGATCCAGACTTCTGGACCGGTTGGTCAGGTTAATTGGAAACCCGGATGTGA 300
MVU01996        TTACCACTCGATCCAGACTTCTGGACCGGTTGGTCAGGTTAATTGGAAACCCGGATGTGA 300
AF266289        TTACCACTCGATCCAGACTTCTGGACCGGTTGGTCAGGTTAATTGGAAACCCGGATGTGA 300
AF266288        TTACCACTCGATCCAGACTTCTGGACCGGTTGGTCAGGTTAATTGGAAACCCGGATGTGA 300
AB12949         TTACCACCCGATCCAGATTACTGGACCGGTTGGTCAGGTTAATTGGAAACCCGGATGTGA 300
AF504048        TTACCACTCGATCCAGACTACTGGACCGGTTGGTCAGGTTAATTGGAAACCCGGATGTGA 300
SSPE            TTACCACTCGATCCAGACTACTGGACCGGTTGGTCAGGTTAATTGGAAACCCGGATGTGA 177
                ******* ********* * ****************************************

U03656          GCGGGCCCAAACTAACAGGGGCACTAATAGGTATATTATCCTTATTTGTGGAGTCTCCAG 360
MVU01996        GCGGGCCCAAACTAACAGGGGCACTAATAGGTATATTATCCTTATTTGTGGAGTCTCCAG 360
AF266289        GCGGGCCCAAACTAACAGGGGCACTAATAGGTATATTATCCTTATTTGTGGAGTCTCCAG 360
AF266288        GCGGGCCCAAACTAACAGGGGCACTAATAGGTATATTATCCTTATTTGTGGAGTCTCCAG 360
AB12949         GCGGGCCCAAACTAACAGGGGCACTAATAGGTATATTATCCTTATTTGTGGAGTCTCCAG 360
AF504048        GCGGGCCCAAACTAACAGGGGCACTAATAGGTATATTATCCTTGTTTGTGGAGTCTCCAG 360
SSPE            GCGGGCCCAAACTAACAGGGGCACTAATAGGTATATTATCCTTGTTTGTGGAGTCTCCAG 237
                ****************************************** ****************

U03656          GTCAATTGATTCAGAGGATCACCGATGACCCTGACGTTAGCATAAGGCTGTTAGAGGTTG 420
MVU01996        GTCAATTGATTCAGAGGATCACCGATGACCCTGACGTTAGCATAAGGCTGTTAGAGGTTG 420
AF266289        GTCAATTGATTCAGAGGATCACCGATGACCCTGACGTTAGCATAAGGCTGTTAGAGGTTG 420
AF266288        GTCAATTGATTCAGAGGATCACCGATGACCCTGACGTTAGCATAAGGCTGTTAGAGGTTG 420
AB12949         GTCAATTGATTCAGAGGATCACCGATGACCCTGACGTTAGCATCAGGCTGTTAGAGGTTG 420
AF504048        GTCAATTGATTCAGAGGATCACCGATGACCCTGACGTTAGCATCAGGCTGTTAGAGGTTG 420
SSPE            GTCAATTGATTCAGAGGATCACCGATGACCCTGACGTTAGCATCAGGCTGTTAGAGGTTG 297
                ****************************************** ****************
```

```
U03656          TCCAGAGTGACCAGTCACAATCTGGCCTTACCTTCGCATCAAGAGGTACCAACATGGAGG 480
MVU01996        TCCAGAGTGACCAGTCACAATCTGGCCTTACCTTCGCATCAAGAGGTACCAACATGGAGG 480
AF266289        TCCAGAGTGACCAGTCACAATCTGGCCTTACCTTCGCATCAAGAGGTACCAACATGGAGG 480
AF266288        TCCAGAGTGACCAGTCACAATCTGGCCTTACCTTCGCATCAAGAGGTACCAACATGGAGG 480
AB12949         TCCAGAGTGACCAGTCACAATCTGGCCTTACCTTCGCATCAAGAGGTACCAACATGGAGG 480
AF504048        TCCAAAGTGACCAGTCACAATCTGGCCTTACCTTCGCATCAAGAGGTACCAACATGGAGG 480
SSPE            TCCAGAGTGACCAGTCACAATCTGGCCTTACCTTCGCATCAAGAGGTACCAACATGGAGG 357
                **** ************************************************** 
                          492 (C-A)
U03656          ATGAGGCGGACCAATACTTTTCACATGATGATCCAATTAGTAGTGATCAATCCAGGTTCG 540
MVU01996        ATGAGGCGGACCAATACTTTTCACATGATGATCCAATTAGTAGTGATCAATCCAGGTTCG 540
AF266289        ATGAGGCGGACCAATACTTTTCACATGATGATCCAATTAGTAGTGATCAATCCAGGTTCG 540
AF266288        ATGAGGCGGACCAATACTTTTCACATGATGATCCAATTAGTAGTGATCAATCCAGGTTCG 540
AB12949         ATGAGGCGGACCAATACTTTTCACATGATGATCCAAGCAGTAGTGATCAATCCAGGTTCG 540
AF504048        ATGAGGCGGACCAATACTTTTCACATGATAATCCAAGTAGTAGTGATCAATCCAGGTCTG 540
SSPE            ATGAGGCGGACCAATACTTTTCACATGATGATCCAAGTAGTAGTGATCAATCCAGGTTCG 417
                **************************** ****** ****************** * 
                          550 (A-G)
U03656          GATGGTTCGAGAACAAGGAAATCTCAGATATTGAAGTGCAAGACCCTGAGGGATTCAACA 600
MVU01996        GATGGTTCGAGAACAAGGAAATCTCAGATATTGAAGTGCAAGACCCTGAGGGATTCAACA 600
AF266289        GATGGTTCGAGAACAAGGAAATCTCAGATATTGAAGTGCAAGACCCTGAGGGATTCAACA 600
AF266288        GATGGTTCGAGAACAAGGAAATCTCAGATATTGAAGTGCAAGACCCTGAGGGATTCAACA 600
AB12949         GATGGTTCGAGAACAAGGAAATCTCAGATATTGAAGTGCAAGACCCTGAGGGATTCAACA 600
AF504048        GATGGTTCGAGAACAAGGAAATCTCAAATATTGAAGTGCAAGACCCTGAGGGATTCAACA 600
SSPE            GATGGTTCGAGAACAAGGAAATCTCAGATATTGAAGTGCAAGACCCTGAGGGATTCAACA 477
                ************************** ******************************** 

U03656          TGATTCTGGGTACCATCCTAGCCCAAATTTGGGTCTTGCTCGCAAAGGCGGTTACGGCCC 660
MVU01996        TGATTCTGGGTACCATCCTAGCCCAAATTTGGGTCTTGCTCGCAAAGGCGGTTACGGCCC 660
AF266289        TGATTCTGGGTACCATCCTAGCCCAAATTTGGGTCTTGCTCGCAAAGGCGGTTACGGCCC 660
AF266288        TGATTCTGGGTACCATCCTAGCCCAAATTTGGGTCTTGCTCGCAAAGGCGGTTACGGCCC 660
AB12949         TGATTCTGGGTACCATTCTAGCCCAAATTTGGGTCTTGCTCGCAAAGGCGGTTACGGCCC 660
AF504048        TGATTCTGGGTACCATTCTAGCCCAAATTTGGGTCTTGCTCGCAAAGGCAGTTACGGCCC 660
SSPE            TGATTCTGGGTACCATTCTAGCCCAAATTTGGGTCTTGCTCGCAAAGGCAGTTACGGCCC 537
                **************** ******************************** ********** 

U03656          CAGACACGGCAGCTGATTCGGAGCTAAGAAGGTGGATAAAGTACACCCAACAAAGAAGGG 720
MVU01996        CAGACACGGCAGCTGATTCGGAGCTAAGAAGGTGGATAAAGTACACCCAACAAAGAAGGG 720
AF266289        CAGACACGGCAGCTGATTCGGAGCTAAGAAGGTGGATAAAGTACACCCAACAAAGAAGGG 720
AF266288        CAGACACGGCAGCTGATTCGGAGCTAAGAAGGTGGATAAAGTACACCCAACAAAGAAGGG 720
AB12949         CAGACACGGCAGCTGATTCGGAGCTAAGAAGGTGGATAAAGTACACCCAACAAAGAAGGG 720
AF504048        CAGACACGGCAGCTGATTCGGAGCTAAGACGGTGGATAAAATACACCCAACAAAGAAGGG 720
SSPE            CAGACACGGCAGCTGATTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 597
                ****************** 

U03656          TAGTTGGTGAATTTAGATTGGAGAGAAAATGGTTGGATGTGGTGAGGAACAGGATTGCCG 780
MVU01996        TAGTTGGTGAATTTAGATTGGAGAGAAAATGGTTGGATGTGGTGAGGAACAGGATTGCCG 780
AF266289        TGGTTGGTGAATTTAGATTGGAGAGAAAATGGTTGGATGTGGTGAGGAACAGGATTGCCG 780
AF266288        TAGTTGGTGAATTTAGATTGGAGAGAAAATGGTTGGATGTGGTGAGGAACAGGATTGCCG 780
AB12949         TAGTTGGTGAATTTAGATTGGAGAGAAAATGGTTGGATGTGGTGAGGAACAGGATTGCCG 780
AF504048        TAGTTGGTGAGTTTAGATTGGAGAGAAAATGGTTGGATGTGGTGAGGAACAGGATTGCCG 780
SSPE            NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 657

U03656          AGGACCTCTCCTTACGCCGATTCATGGTCGCTCTAATCCTGGATATCAAGAGAACACCCG 840
MVU01996        AGGACCTCTCCTTACGCCGATTCATGGTCGCTCTAATCCTGGATATCAAGAGAACACCCG 840
AF266289        AGGACCTCTCCTTACGCCGATTCATGGTCGCTCTAATCCTGGATATCAAGAGAACACCCG 840
AF266288        AGGACCTCTCCTTACGCCGATTCATGGTCGCTCTAATCCTGGATATCAAGAGAACACCCG 840
AB12949         AAGACCTCTCTTTACGCCGATTCATGGTGGCTCTAATCCTGGATATCAAGAGGACACCAG 840
AF504048        AGGACCTCTCCTTACGCCGATTCATGGTCGCTCTAATCCTAAATATCAAGAGGACGCCCG 840
SSPE            NNNNNNNNNNNNNNNNNNNNNNNNNNNTCGCTCTAATCCTAGATATCAAGAGGACACCCG 717
                                          * *********** ********** ** ** * 

U03656          GAAACAAACCCAGGATTGCTGAAATGATATGTGACATTGATACATATATCGTAGAGGCAG 900
MVU01996        GAAACAAACCCAGGATTGCTGAAATGATATGTGACATTGATACATATATCGTAGAGGCAG 900
AF266289        GAAACAAACCCAGGATTGCTGAAATGATATGTGACATTGATACATATATCGTAGAGGCAG 900
AF266288        GAAACAAACCCAGGATTGCTGAAATGATATGTGACATTGATACATATATCGTAGAGGCAG 900
AB12949         GGAACAAACCTAGGATTGCTGAAATGATATGTGACATTGATACATATATCGTAGAGGCAG 900
AF504048        GGAACAAACCTAGGATTGCTGAAATGATATGTGACATTGATACATATATCGTAGAGGCAG 900
SSPE            GGAACAAACCTAGGATTGCTGAAATGATATGTGACATTGATACATATATCGTAGAGGCAG 777
                * ******** ************************************************** 
```

```
U03656          GATTAGCCAGTTTTATCCTGACTATTAAGTTTGGGATAGAAACTATGTATCCTGCTCTTG 960
MVU01996        GATTAGCCAGTTTTATCCTGACTATTAAGTTTGGGATAGAAACTATGTATCCTGCTCTTG 960
AF266289        GATTAGCCAGTTTTATCCTGACTATTAAGTTTGGGATAGAAACTATGTATCCTGCTCTTG 960
AF266288        GATTAGCCAGTTTTATCCTGACTATTAAGTTTGGGATAGAAACTATGTATCCTGCTCTTG 960
AB12949         GATTAGCCAGTTTTATCTTGACTATTAAGTTTGGGATAGAAACTATGTACCCTGCTCTTG 960
AF504048        GATTAGCCAGTTTTATCCTGACTATTAAGTTTGGGATAGAAACTATGTATCCTGCTCTTG 960
SSPE            GATTAGCCAGTTTTATCCTGACTATTAAGTTTGGGATAGAAACTATGTATCCTGCTCTTG 837
                ***************** ****************************** **********

U03656          GACTGCATGAATTTGCTGGTGAGTTATCCACACTTGAGTCCTTGATGAACCTTTACCAGC 1020
MVU01996        GACTGCATGAATTTGCCGGTGAGTTATCCACACTTGAGTCCTTGATGAACCTTTACCAGC 1020
AF266289        GACTGCATGAATTTGCTGGTGAGTTATCCACACTTGAGTCCTTGATGAACCTTTACCAGC 1020
AF266288        GACTGCATGAATTTGCTGGTGAGTTATCCACACTTGAGTCCTTGATGAACCTTTACCAGC 1020
AB12949         GACTGCATGAATTTGCTGGTGAGTTATCCACACTTGAGTCCTTGATGAATCTTTACCAGC 1020
AF504048        GACTGCATGAATTCGCTGGTGAGTTATCCACACTCGAGTCCTTGATGAATCTTTACCAGC 1020
SSPE            GACTGCATGAATTTGCTGGTGAGTTATCCACACTCGAGTCCTTGATGAATCTTTACCAGC 897
                ************* ** ***************** ************** **********

U03656          AAATGGGGGAAACTGCACCCTACATGGTAATCCTGGAGAACTCAATTCAGAACAAGTTCA 1080
MVU01996        AAATGGGGGAAACTGCACCCTACATGGTAATCCTGGAGAACTCAATTCAGAACAAGTTCA 1080
AF266289        AAATGGGGGAAACTGCACCCTACATGGTAATCCTGGAGAACTCAATTCAGAACAAGTTCA 1080
AF266288        AAATGGGGGAAACTGCACCCTACATGGTAATCCTGGAGAACTCAATTCAGAACAAGTTCA 1080
AB12949         AAATGGGAGAAACTGCACCCTACATGGTAATCCTAGAGAACTCAATTCAGAACAAGTTCA 1080
AF504048        AAATGGGAGAAACTGCACCCTACATGGTAATCCTGGAGAACTCAATTCAGAATAAGTTCA 1080
SSPE            AAATGGGAGAAACTGCACCCTACATGGTAATCCTGGAGAACTCAATTCAGAATAAGTTCA 957
                ******* ************************** ***************** *******

U03656          GTGCAGGATCATACCCTCTGCTCTGGAGCTATGCCATGGGAGTAGGAGTGGAACTTGAAA 1140
MVU01996        GTGCAGGATCATACCCTCTGCTCTGGAGCTATGCCATGGGAGTAGGAGTGGAACTTGAAA 1140
AF266289        GTGCAGGATCATACCCTCTGCTCTGGAGCTATGCCATGGGAGTAGGAGTGGAACTTGAAA 1140
AF266288        GTGCAGGATCATACCCTCTGCTCTGGAGCTATGCCATGGGAGTAGGAGTGGAACTTGAAA 1140
AB12949         GTGCAGGATCATACCCTCTGCTCTGGAGCTATGCCATGGGAGTGGGAGTGGAACTTGAAA 1140
AF504048        GTGCAGGGTCATACCCTCTGCTCTGGAGCTATGCCATGGGAGTAGGGGTGGAACTTGAAA 1140
SSPE            GTGCAGGATCATACCCTCTGCTCTGGAGCTATGCCANNNNNNNNNNNNNNNNNACTTGAAA 1017
                ******* ****************************                ********

U03656          ACTCCATGGGAGGTTTGAACTTTGGCCGATCTTACTTTGATCCAGCATATTTTAGATTAG 1200
MVU01996        ACTCCATGGGAGGTTTGAACTTTGGCCGATCTTACTTTGATCCAGCATATTTTAGATTAG 1200
AF266289        ACTCCATGGGAGGTTTGAACTTTGGCCGATCTTACTTTGATCCAGCATATTTTAGATTAG 1200
AF266288        ACTCCATGGGAGGTTTGAACTTTGGCCGATCTTACTTTGATCCAGCATATTTTAGATTAG 1200
AB12949         ACTCCATGGGAGGTTTAAACTTTGGTCGATCTTACTTTGATCCAGCGTATTTTAGATTAG 1200
AF504048        ACTCCATGGGAGGTTTGAACTTTGGTCGATCTTACTTTGATCCAGCATATTTTAGATTAG 1200
SSPE            ACTCCATGGGAGGTTTGAACTTTGGTCGACCTTACTTTGATCCAGCATATTTTAGATTAG 1077
                ***************** ******** *** ***************** *************

U03656          GGCAAGAGATGGTAAGGAGGTCAGCTGGAAAGGTCAGTTCCACATTGGCATCTGAACTCG 1260
MVU01996        GGCAAGAGATGGTAAGGAGGTCAGCTGGAAAGGTCAGTTCCACATTGGCATCTGAACTCG 1260
AF266289        GGCAAGAGATGGTAAGGAGGTCAGCTGGAAAGGTCAGTTCCACATTGGCATCTGAACTCG 1260
AF266288        GGCAAGAGATGGTAAGGAGGTCAGCTGGAAAGGTCAGTTCCACATTGGCATCTGAACTCG 1260
AB12949         GGCAAGAGATGGTGAGGAGGTCAGCTGGGAAGGTCAGTTCCACATTGGCATCTGAACTCG 1260
AF504048        GCCAAGAGATGGTGAGGAGGTCAGCTGGGAAGGTCAGTTCCACATTGGCATCTGAACTCG 1260
SSPE            GCCAAGAGATGGTGAGGAGGTCAGCTGGGAAGGTCAGTTCCACATTGGCATCTGAACTCG 1137
                * *********** ************** ********************************

U03656          GTATCACTGCCGAGGATGCAAGGCTTGTTTCAGAGATTGCAATGCATACTACTGAGGACA 1320
MVU01996        GTATCACTGCCGAGGATGCAAGGCTTGTTTCAGAGATTGCAATGCATACTACTGAGGACA 1320
AF266289        GTATCACTGCCGAGGATGCAAGGCTTGTTTCAGAGATTGCAATGCATACTACTGAGGACA 1320
AF266288        GTATCACTGCCGAGGATGCAAGGCTTGTTTCAGAGATTGCAATGCATACTACTGAGGACA 1320
AB12949         GTATCACTGCCGAGGATGCGAGGCTTGTTTCAGAGATTGCAATGCATACTACTGAGGACA 1320
AF504048        GTATCACTGCCGAGGATGCAAGGCTTGTTTCAGAGATCGCAATGCATACTACTGAGGACA 1320
SSPE            GTATCACTGCCGAGGATGCAAGGCTTGTTTCAGAGATTGCAATGCATACTACTGAGGACA 1197
                ****************** ***************** **********************

U03656          AGATCAGTAGAGCGGTTGGACCCAGACAAGCCCAAGTATCATTTCTACACGGTGATCAAA 1380
MVU01996        AGATCAGTAGAGCGGTTGGACCCAGACAAGCCCAAGTATCATTTCTACACGGTGATCAAA 1380
AF266289        AGATCAGTAGAGCGGTTGGACCCAGACAAGCCCAAGTATCATTTCTACACGGTGATCAAA 1380
AF266288        AGATCAGTAGAGCGGTTGGACCCAGACAAGCCCAAGTATCATTTCTACACGGTGATCAAA 1380
AB12949         GGATCAGTAGAGCGGTCGGACCCAGACAAGCCCAAGTGTCATTTCTACACGGTGATCAAA 1380
AF504048        GGACCAGTAGAGCGGTTGGACCCAGACAAGCCCAAGTGTCATTTCTACACGGTGATCAAA 1380
SSPE            AG-TCAGTAGAGCGGTTGGACCCAGACAAGCCCAAGTATCANNNNNNNNNNNNNNNNNNN 1256
                 *  ************ ******************** ***
```

```
U03656          GTGAGAATGAGCTACCGAGATTGGGGGGCAAGGAAGATAGGAGGGTCAAACAGAGTCGAG 1440
MVU01996        GTGAGAATGAGCTACCGAGATTGGGGGGCAAGGAAGATAGGAGGGTCAAACAGAGTCGAG 1440
AF266289        GTGAGAATGAGCTACCGAGATTGGGGGGCAAGGAAGATAGGAGGGTCAAACAGAGTCGAG 1440
AF266288        GTGAGAATGAGCTACCGAGATTGGGGGGCAAGGAAGATAGGAGGGTCAAACAGAGTCGAG 1440
AB12949         GTGAGAATGAGCTCCCAGGATTGGGGGGCAAGGAAGATAGGAGGGTCAAACAGAGTCGGG 1440
AF504048        GTGAGAATGAGCTACCAGGATTGGGGGGCAAGGAAGACAAGAGGGTCAAACAGGGTCGGG 1440
SSPE            NNNNNNNNNAGCTACCAGGATTGGGGGGCAAGGAAGACAAGAGGGTCAAACAGGGTCGGG 1316
                     **** **  ********************* * ************ **** *

U03656          GAGAAGCCAGGGAGAGCTACAGAGAAACCGGGCCCAGCAGAGCAAGTGATGCGAGAGCTG 1500
MVU01996        GAGAAGCCAGGGAGAGCTACAGAGAAACCGGGCCCAGCAGAGCAAGTGATGCGAGAGCTG 1500
AF266289        GAGAAGCCAGGGAGAGCTACAGAGAAACCGGGCCCAGCAGAGCAAGTGATGCGAGAGCTG 1500
AF266288        GAGAAGCCAGGGAGAGCTACAGAGAAACCGGGCCCAGCAGAGCAAGTGATGCGAGAGCTG 1500
AB12949         GAGAAGCCAGGGAGAGCTACAGAGAGACCGGGTCCAGCAGAGCAAGTGATGAGAGAGCTG 1500
AF504048        GAGAAGCCAGGGAGAGCTACAGAGAAACCGGGTCCAGCAGAGCAGGTGATGCGAGAGCTG 1500
SSPE            GAGAAGCCAGGGAGAGCTACAGAGAAACCGGGTCCAGCAGAGCAAGTGATGCGAGAGCTG 1376
                ************************** ****** ********** ****** ********
                                                  1542 (T-A)
U03656          CCCATCTTCCAACCGGCACACCCCTAGACATTGACACTGCAACGGAGTCCAGCCAAGATC 1560
MVU01996        CCCATCTTCCAACCGGCACACCCCTAGACATTGACACTGCATCGGAGTCCAGCCAAGATC 1560
AF266289        CCCATCTTCCAACCGGCACACCCCTAGACATTGACACTGCAACGGAGTCCAGCCAAGATC 1560
AF266288        CCCATCTTCCAACCGGCACACCCCTAGACATTGACACTGCATCGGAGTCCAGCCAAGATC 1560
AB12949         CCCATCTTCCAACCAGCACACCCCTAGACATTGACACTGCATCGGAGTCAGGCCAAGATC 1560
AF504048        CCCATCTTCCAACCAGCACACCCCCAGACATTGACACTGCATCGGAGTCAGGCCAAGATC 1560
SSPE            CCCATCTTCCAACCAGCACACCCCCAGACATTGACACTGCATCGGAGT------------ 1424
                ************** ********* ***************** ******

U03656          CGCAGGACAGTCGAAGGTCAGCTGACGCCCTGCTTAGGCTGCAAGCCATGGCAGGAATCT 1620
MVU01996        CGCAGGACAGTCGAAGGTCAGCTGACGCCCTGCTTAGGCTGCAAGCCATGGCAGGAATCT 1620
AF266289        CGCAGGACAGTCGAAGGTCAGCTGACGCCCTGCTTAGGCTGCAAGCCATGGCAGGAATCT 1620
AF266288        CGCAGGACAGTCGAAGGTCAGCTGACGCCCTGCTTAGGCTGCAAGCCATGGCAGGAATCT 1620
AB12949         CGCAGGACAGTCGAAGGTCAGCTGACGCCCTGCTCAGGCTGCAAACCATGGCAGGAATCT 1620
AF504048        CGCAGGACAGCCGACGGTCAGCTGACGCCCTGCTCAGGCTGCAAGCCATGGCAGGAATCT 1513
SSPE            ------------------------------------------------------------

U03656          CGGAAGAACAAGGCTCAGACACGGACACCCCTATAGTGTACAATGACAGAAATCTTCTAG 1680
MVU01996        CGGAAGAACAAGGCTCAGACACGGACACCCCTATAGTGTACAATGACAGAAATCTTCTAG 1680
AF266289        CGGAAGAACAAGGCTCAGACACGGACACCCCTATAGTGTACAATGACAGAAATCTTCTAG 1680
AF266288        CGGAAGAACAAGGCTCAGACACGGACACCCCTATAGTGTACAATGACAGAAATCTTCTAG 1680
AB12949         TGGAGGAACAAGGCTCAGACACGGACACCCCTAGGGTGTACAATGACAGTGATCTTCTAG 1680
AF504048        TGGAAGAACAGGGCTCAGACACGGACACCCCTAGGGTGTACAATGACAGAAATCTTCTAG 1680
SSPE            ------------------------------------------------------------

                    Stop codon
U03656          ACTAG 1685
MVU01996        ACTAG 1685
AF266289        ACTAG 1685
AF266288        ACTAG 1685
AB12949         ACTAG 1685
AF504048        ACTAG 1685
SSPE            -----
```

Figure 3.4. Multiple sequence alignment of measles virus nucleocapsid (N) gene sequences in wild type and vaccine derived strains.

The N gene sequence generated in this study from SSPE brain tissue using 9 different primer sets was blasted against NCBI database using BlastN (2.2.6). The top six matches were aligned with this sequence using ClustalW (1.82). The coding sequence of the N gene is shown in the alignment with start and stop codons highlighted. Three nucleotide substitutions that may contribute to virus attenuation (Parks *et al.*, 2001) are highlighted.

3.3.2.3 Standards for quantitative TaqMan RT PCRs.

RT PCR products (F1 and H1 primer sets) amplified from total RNA extracted from measles infected Vero cells (Figure 3.1) were gel purified as described in section 2.6.3 and cloned into TOPO pCR 2.1 vector as described in section 2.11. Plasmid were isolated and inserts were confirmed by PCR (section 2.11) and sequencing (section 2.14) (data not shown). cRNA transcripts were generated from the plasmids containing F and H gene inserts using a T7 RNA polymerase (Figure 3.5). Standard curves for F and H TaqMan RT PCR assays were generated by amplifying serial dilutions of the specific cloned cRNA standards ($1x\ 10^7$-$1x10^1$copies/μl) (Figure 3.6). Standard curves for GAPDH TaqMan RT PCR assays were generated by amplifying serial dilutions of Human Control RNA (Applied Biosystems) ($5\ x\ 10^6$-$5x10^1$ copies/μl).

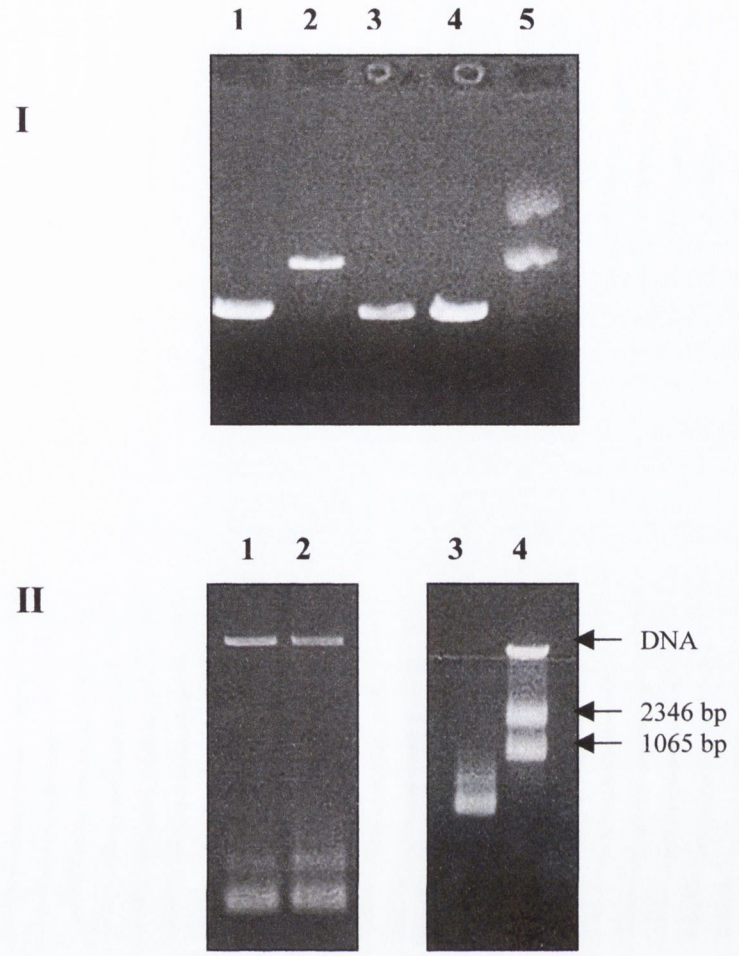


Figure 3.5. Plasmid and cRNA standards for TaqMan quantitative RT PCR.

I. A 1% agarose gel showing restriction digestion of plasmid constructs (pCR 2.1 vector + F1 insert or pCR 2.1 +H1 insert). **Lanes 1-4** *Bam*H1 digested F gene and H gene plasmid constructs. **Lane 5** undigested plasmid. Restriction digest in lane 2 was unsuccessful.

II. cRNA transcripts generated from *Bam*H1 digested constructs pre and post DNase digestion.

Lane 1 represents F gene cRNA before DNase digestion; **Lane 2**, H gene cRNA before DNase digestion; **Lane 3**, F gene cRNA post DNase digestion; **Lane 4** PGEM cRNA control reaction.

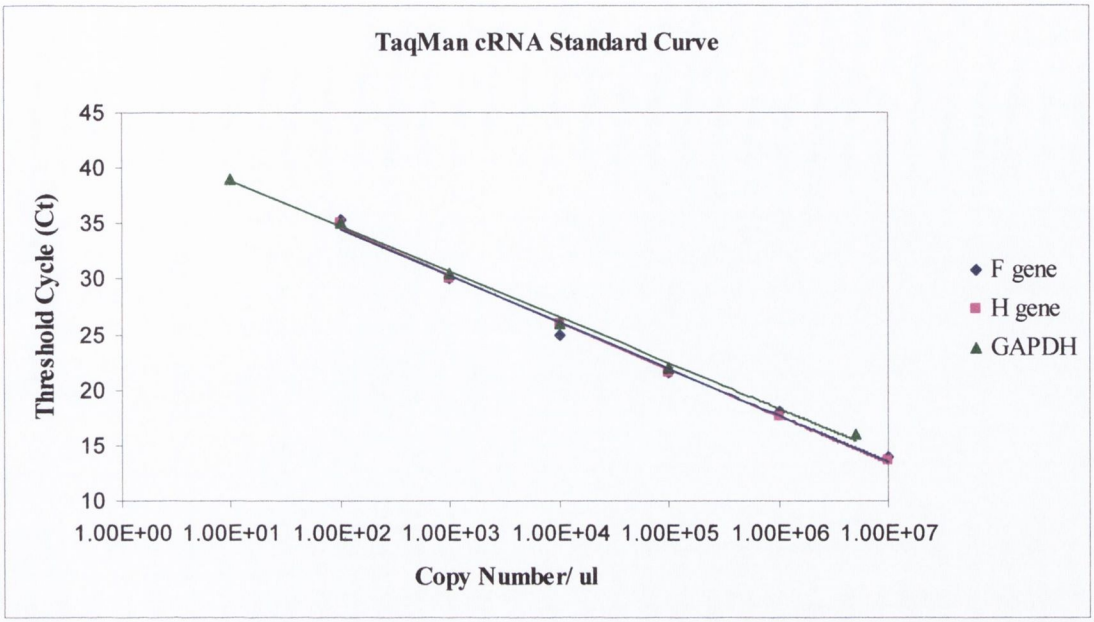


Figure 3.6: Quantitative Standard Curves Generated for each TaqMan RT PCR Assay.

The slope, which is a measure of the reaction efficiency, is similar for all three standard curves. The intercept where the graph crosses the Y axis is the cycle of PCR at which one copy of target is detected. R is the correlation coefficient for each curve. The specific viral gene load in each sample was calculated from these standard curves.

3.3.2.4 TaqMan RT PCR assay for detection of measles virus in tissue specimens.

F and H gene TaqMan RT PCRs were optimised on RNA extracted from measles infected Vero cells and cRNA standards. Quantitative TaqMan RT PCR for measles virus F and H genes and human GAPDH endogenous controls were performed using standard curves (figure 3.5 above).

Tissue samples from three SSPE cases were examined by TaqMan RT PCR. Brain tissue specimens from all three patients were positive for both F and H gene by TaqMan RT PCR. Additional tissue samples from two of the cases (thymus, lymph node and terminal ileum) were also positive for F and H gene by TaqMan RT PCR. Measles virus copy numbers in these cases were high ranging from $1x\ 10^2$ to $8x10^5$ copies/ng of total RNA. A selection of these results were confirmed by Southern blot analysis. Figure 3.2 above shows RT PCR and Southern blots for F and H genes in brain tissue from one of the cases.

In total, 126 patients with ileocolonic lymphonodular hyperplasia and developmental disorders and 89 controls patients were examined by TaqMan RT PCR for the presence of measles virus in the intestinal tissue. All samples included in the analysis were positive for GAPDH housekeeping gene. Measles virus copy number in positive biopsies was generally low, but ranged from 1 copy to 1.3×10^6 copies of MV per ng of total RNA. Statistical representation of measles vius copy number distribution is shown in table 3.3. In the affected cohort, 74.6 % contained MV genomes in ileal lymphoid tissue compared with 8.9 % in the control patient cohort. (Chi Squared χ^2 Test: $p<0.000$: SPSS, Version 11.0). Of the paediatric control group,

MV was not detected in normal children. However, 6 of 47 appendicectomy samples, 1 of 2 ulcerative colitis patients and one of five patients with non-specific colitis harboured MV genome. Table 3.4 provides a summary of TaqMan RT PCR results.

All samples were tested for measles virus F gene and a smaller subset were tested for measles virus H gene. The H gene was detected in 20 of 51 (39%) of affected cases and 2 of 19 (10.5%) controls (Chi Squared χ^2 Test: $p<0.000$: SPSS, Version 11.0). The 20 affected cases that were positive for H gene were all positive for MV F gene, however H gene was not detected in 21 cases that were positive for F gene. The F gene copy number in these samples was low $< 10^3$ copies/ng. In the control group H gene was not detected in normal or children with mild non-specific changes. However one case of ulcerative colitis (positive for MV F gene) was positive for MV H gene. One patient with isolated ileal LNH that was negative for MV F gene was positive for MV H gene (Table 3.4).

In addition to terminal ileum tissue, transverse colon tissue biopsies were examined from four cases of autistic enterocolitis. Two cases were positive for MV F gene in both ileum and transverse colon biopsies, one case was negative for MV F gene in both biopsies, and in one case the transverse colon was positive for F gene while the terminal ileum was negative for F gene. Lymph node examined from one patient also tested positive for MV F gene.

Table 3.3 Statistical representation of measles virus copy number distribution.

Statistics	Affected	Control
N	126	89
Number positive for MV	94	8
Q1 (copies/ng)	0	0
Min (copies/ng)	0	0
Median (copies/ng)	79.5	0
Max (copies/ng)	1300000	350000
Q3 (copies/ng)	1300	0

Table Key

N= number of patients in each group.

Q1= lower quartile

Min= minimum value in each group.

Max= maximum value in each group.

Q3 = upper quartile.

Table 3.4. Summary of TaqMan RT PCR results on intestinal tissues.

	Patient Groups	F gene Positive	H gene Positive
Affected	Autistic enterocolitis	94 /126 * (74.6 %)	20/51 ** (39 %)
Controls	Appendicectomies	6/47 (13 %)	NT
	Crohn's disease	0/8 (0 %)	0/1 (0 %)
	Ulcerative colitis	1/2 (50 %)	1/2 (50%)
	Lymphonodular hyperplasia	0/3 (0 %)	1/3 (33.3 %)
	Mild Changes	0/10 (0 %)	0/6 (0%)
	Normals	0/14 (0%)	0/2 (0%)
	Colitis	1/5 (20 %)	0/5 (0%)

*Affected compared to controls; Chi Squared $\chi 2$ Test: $p<0.000$: SPSS, Version 11.

** Affected compared to controls; Chi squared $\chi 2$ Test: $p<0.000$: SPSS, Version 11.

NT- not tested

3.3.2.5 TaqMan RT PCR assay for detection of measles virus in PBMCs.

Peripheral blood mononuclear cells (PBMCs) from 53 affected patients, eleven normal controls, nine autistic children with no gut pathology and one child with ulcerative colitis were examined by TaqMan RT PCR for the presence of MV F gene. All samples tested positive for GAPDH housekeeping gene. In the affected cohort 34% were positive for measles virus F gene in their peripheral blood compared with 23% of the controls (Table 3.5). The presence of measles virus in the affected group was not statistically different from that in the control group (Chi squared $\chi 2$ Test: p=0.395: SPSS, Version 11). Of the control group, MV F gene was not detected in the normal samples, however four of the autism control patients and one ulcerative colitis patient were positive for measles virus. As with the intestinal tissues the F gene copy number in the peripheral blood mononuclear cells was low ranging from 1- 3 $x10^2$ copies/ng.

Thirteen of the eighteen F gene positive affected patients and the one ulcerative colitis patient were also positive for F gene in their terminal ileal tissues. Two patients that were positive for F gene in PBMCs were negative for F gene in the terminal ileum.

Table 3.5. Summary of TaqMan RT PCR results in PBMCs .

Patient Groups	F gene Positive	F gene Negative	Total No
Affected [a] (Autistic enterocolitis)	18 (34%)	35 (66 %)	53
Controls[b]	5 (23.8%)	16 (76 %)	21

[a] Detection of MV in the affected patients compared with controls was not statistically significant (Chi squared $\chi 2$ Test: p=0.395).

[b] Four autism control patients and one ulcerative colitis patient were positive for MV F gene.

3.4 Section 2.

Aim: To localise measles virus in intestinal tissues biopsies from children with new variant inflammatory bowel disease (autistic enterocolitis) using in situ RT PCR for MV N gene and immunocytochemistry.

3.4.1 Materials and Methods.

3.4.1.1 Specimens.

Formalin fixed paraffin embedded ileal lymphoid tissue biopsies from 74 affected children (median age 6yrs; range 3-13 yrs, 63 male). Developmentally normal paediatric controls (n= 12) (median age 15 yrs; range 3-19 yrs, 8 males) including 5 normal controls, 4 Crohn's disease and 3 ulcerative colitis patients were examined in this study. Sections (4μm) were cut onto silane coated in situ PCR slides from Applied Biosystems as described in section 2.9. Control material consisted of formalin fixed paraffin embedded SSPE brain material from three SSPE cases and formalin fixed paraffin embedded measles infected and uninfected Vero cells. Thymus, lymph node and terminal ileum tissues from one SSPE case were also examined.

3.4.1.2 RT in situ PCR

Formalin fixed paraffin embedded tissue sections were pre-treated in xylene and taken through a series of graded alcohol. Endogenous avidin and biotin activity was blocked using DAKO Biotin Blocking System (DAKO, Glostrup, Denmark). Sections were digested with proteinase K (300μg/ml) for 17 minutes at 37 °C.

Following pre-treatment, measles virus N gene RNA was amplified using the following protocol: 58 °C for 45 min, 94 °C for 5 min, followed by 25 cycles of 94 °C for 45 secs, 60 °C for 45 secs, 72 °C for 45 secs as described in section 2.10. Measles virus N1 primers (Table 3.1) were used for in situ RT PCR. After amplification, sections were fixed in 100% ethanol and air-dried.

Hybridisation was carried out with the 5'biotinylated N1 oligonucleotide probe (sequence is given in table 3.1) using previously published protocols (Uhlmann *et al.*, 1998). Hybrid detection was achieved using a 3-step immunocytochemical method (Herrington *et al.*, 1998) or dinitrophenol (DNP) tyramide signal amplification (TSA) (Bobrow *et al.*, 1989). Alkaline phosphatase was detected with nitro-bromo-tertrazolium and bromo-chloro-indolyl-phosphate BCIP chromogen. Endogenous alkaline phosphatase was blocked using Levamisole Endogenous AP Inhibitor DAKO (DAKO Ltd, Glostrup, Denmark) during chromogenic detection. Full details of this methodology are given in section 2.9.

Reaction optimisation experiments were initially carried out using formalin fixed paraffin embedded, measles infected Vero cells. A variety of manganese concentrations (2.5mM - 4mM) and probe concentrations (1 μg/ml, 1.5 μg/ml, 2 μg/ml) were tested. 1 μg/ml of probe yielded optimal signals and was used in subsequent experiments.

Controls for RT in situ PCR included the following: Measles virus infected and uninfected Vero cells, MV N- gene primers and an irrelevant probe (nonsense pyruvate dehyrogenase probe) and irrelevant primers and N gene specific probe.

Other control experiments included RNase digestion of MV infected Vero cells prior to RT in situ PCR. Histone mRNA in situ hybridisation controls were performed with the Histone H2 mRNA fluorescein labelled probe (Dako Ltd, Glostrup, Denmark) and detected using the 3 step immunocytochemical detection method as described in section 2.9.6.

3.4.1.3 Combined RT in situ PCR and Immunohistochemistry.

To examine MV signal localization, RT in situ PCR for MV N gene was performed as described above on a terminal ileum tissue section from one affected child following immunohistochemistry using the follicular dendritic cell CNA 42 monoclonal antibody (Dako Ltd, Glostrup, Denmark) (Section 2.9.8). For in situ hybridisation a 5' biotin labelled N1 oligonucleotide probe was used described in section 2.9.

The MV hybridisation signal was developed with horseradish peroxidase (HRP) chromogen using amino-ethyl carbazole (AEC: Vector Labs, Burlingame, CA, USA) and the dendritic cell signal was developed using a 3-step detection method with alkaline phosphatase (AP) (Dako Ltd, Glostrup, Denmark) and nitro-blue tetrazolium, (NBT) bromo-chloro-indolyl phosphate (BCIP) (Roche Molecular Biochemicals, Mannheim, Germany) as a substrate.

3.4.2 Results

RT in situ PCR optimisation experiments were performed as described above. The optimal manganese concentration (Mn^{2+}) for the RT PCR was determined by performing Mn^{2+} titrations on formalin fixed measles infected Vero cells, a concentration of 3.5 mM was chosen for subsequent experiments. The optimal probe concentration was also determined on formalin fixed measles infected Vero cells. 1 µg/ml probe concentration yielded optimal signals and was used in subsequent experiments (Figure 3.7). Higher concentrations of probe showed increasing non-specific nuclear staining.

Optimal cell permeabilisation conditions were determined in formalin fixed SSPE material. A mild acid hydrolysis using 0.01N HCL followed by digestion with Proteinase K for 17 min was required to permeablise the cell membrane. The concentration and duration of the proteinase K treatment is critical as excessive digestion can cause amplicon diffusion within the tissue section, patchy amplification, destroy the tissue architecture and cause the tissue to detach from the slide.

In MV infected Vero cells, MV amplicons were identified as a cytoplasmic signal (Figure 3.7). In SSPE brain material, discrete, intense foci of MV amplicons were detected in grey matter using RT in situ PCR (Figure 3.8A, 3.8B). Signal was not detected in the control sections where irrelevant PCR primers and the MV N1 specific probe were used (Figure 3.8C). Similarly, no signal was detected where MV N gene specific primers and an irrelevant probe (e.g. Pyruvate Dehydrogenase oligonucleotide probe), or where no primers were used (data not shown).

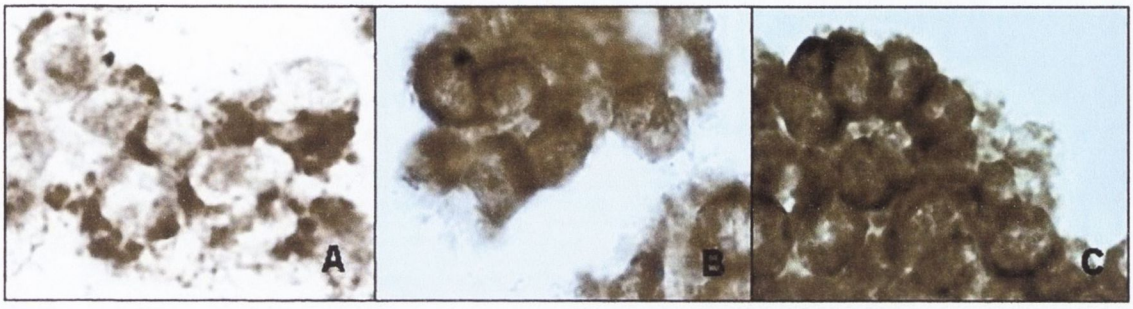


Figure 3.7. In situ RT PCR and hybridisation in measles infected Vero cells using different concentrations of N1 biotinylated oligonucleotide probe.

Optimal cytoplasmic staining is achieved in **panel A** (1ug/ml), higher probe concentrations in **panels B** (1.5ug/ml) and **C** (2ug/ml) showed increasing, non-specific nuclear staining.

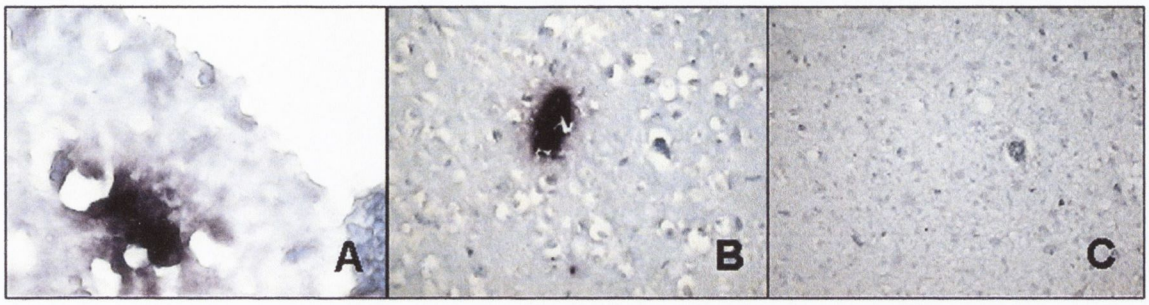


Figure 3.8. In situ RT PCR combined with in situ hybridisation for measles virus N gene in sub acute sclerosing panencephalitis (SSPE) brain tissue.

Panel A: Measles virus N gene amplicon detected with NBT/BCIP chromogen.

Panel B: shows intense, discrete black signals within the brain parenchyma.

Panel C: Negative control section of panel A, treated identically but employing irrelevant PCR primers. No MV signal was observed (original magnification x 400).

Tissue samples from three SSPE cases were examined by in situ RT PCR. Positive MV signals were observed in brain material (Figure 3.8A and B above) from all three cases and in thymus and lymph node in one of the cases. These samples were all positive for MV F and H genes by TaqMan RT PCR (Section 1).

In total 74 biopsies from affected children, 5 controls, 4 Crohn's disease patients and 3 ulcerative colitis cases were examined by in situ hybridisation. A number of tissue sections (22%) from the affected cohort showed severe fragmentation and architectural distortion despite reducing the digestion times, and therefore were omitted from the results. The quality of the tissue sections post in situ PCR and counterstaining was poor compared to the parallel H&E sections. This is most likely due to the repeated exposure to high and low temperatures during the PCR thermocycling.

Of the 57 tissue biopsies successfully examined from affected children, 46 (80%) were positive for MV N gene by RT in situ PCR and amplicons could be detected in serial sections of ileal biopsies. Eleven biopsies were negative for MV. TaqMan RT PCR for MV F gene was performed on all cases. MV was confirmed in positive biopsies with the exception of two cases, which were positive by in situ RT PCR but negative by TaqMan RT PCR. Similarly, four cases that were negative by in situ PCR were positive by TaqMan PCR.

Of the control group, one of the five (20%) children with histologically normal small and large bowel mucosa, three of four (75%) of the Crohn's disease group and two of

three ulcerative colitis patients (66.6%) had detectable MV N genome, present in a distribution that was identical to that seen in biopsies from affected children.

MV amplicons in the terminal ileum from one affected child were localised to the reactive follicle centres (Figure 3.9, 3.10, 3.11), and were associated with cells possessing dendritic processes (Figure 3.12) and lymphocytes. No MV signal was seen in serial control sections where irrelevant PCR primers were employed.

Additional RT in situ PCR control experiments were performed on measles infected Vero cells. No signal was obtained in MV infected Vero cells following RNase digestion (Figure 3.13). Similarly, no signal was obtained following omission of r*Tth* polymerase enzyme or in uninfected Vero cells.

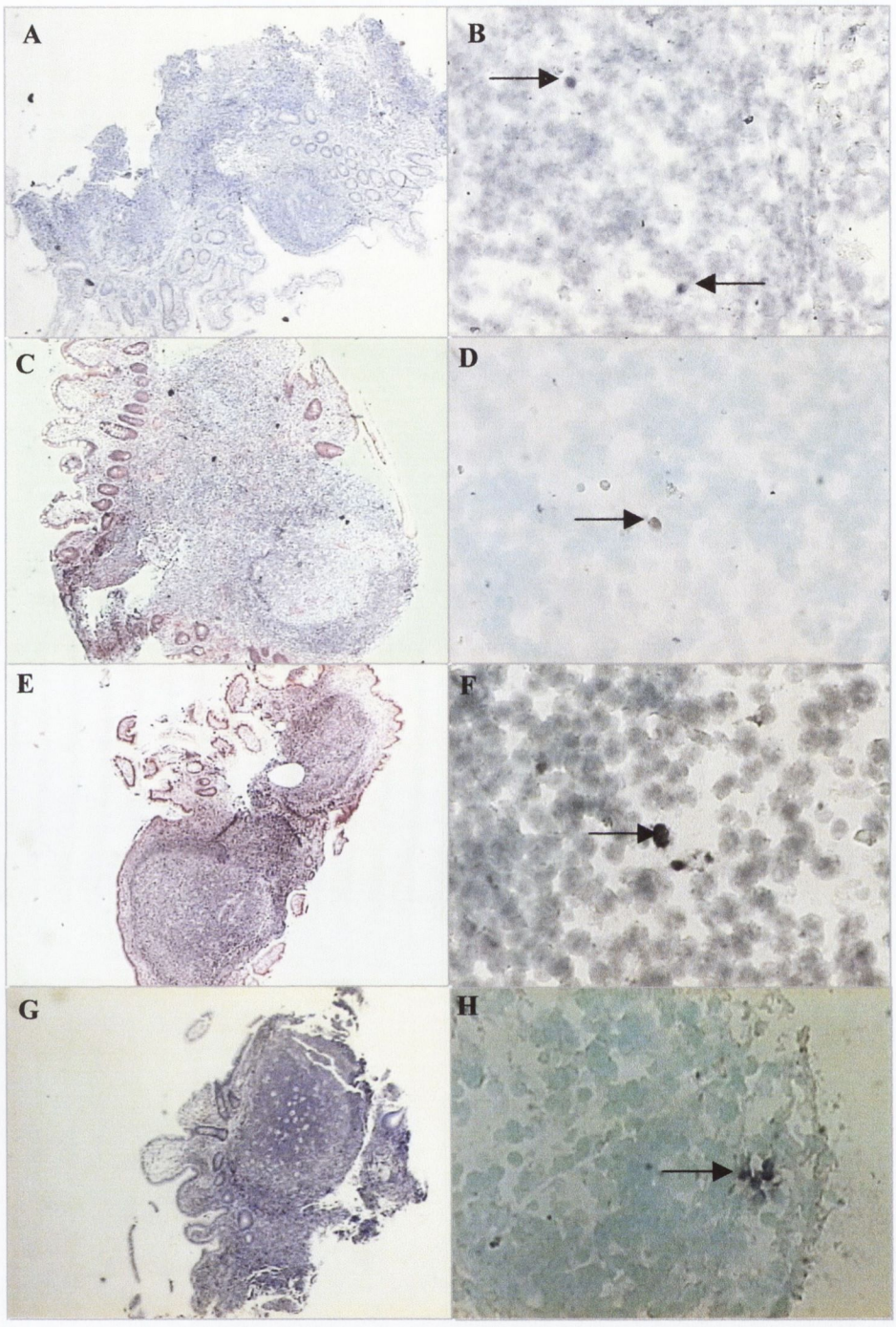


Figure 3.9. H&E stain and corresponding in situ RT PCR for MV N gene in terminal ileum samples from autistic enterocolitis children. Slides from four affected patients are shown AB, CD, EF and GH. Measles N gene amplicons are indicated by the arrows.

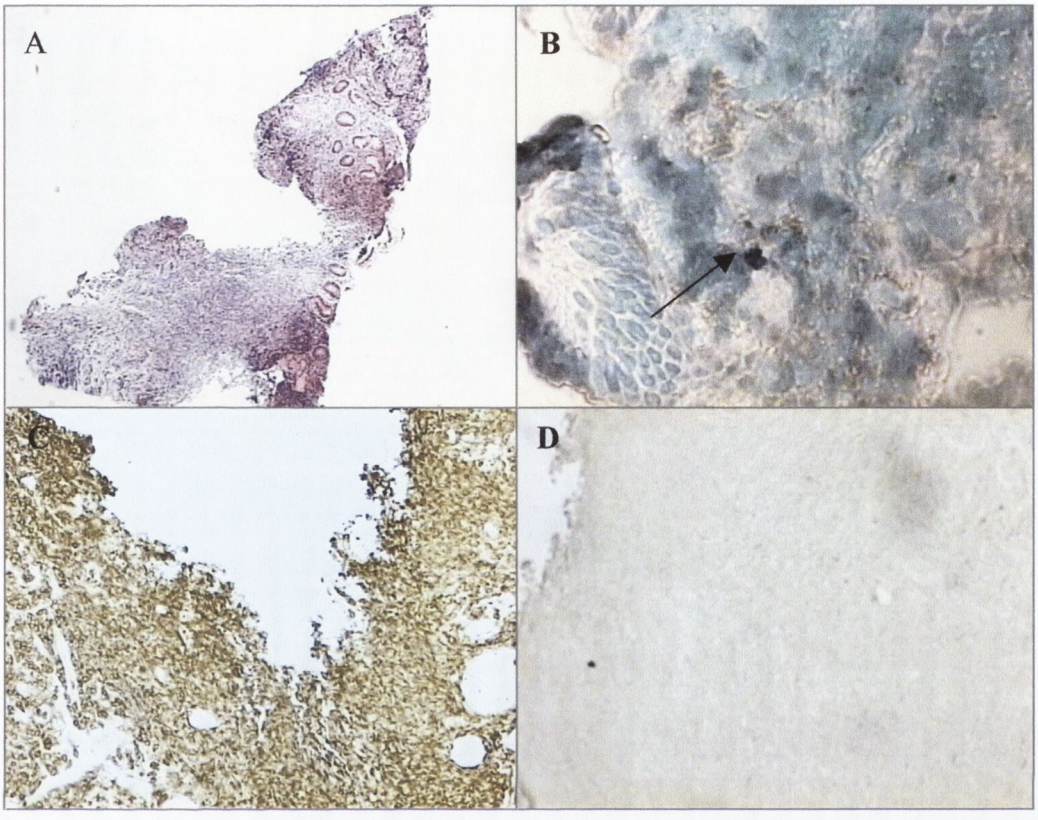


Figure 3.10. Immunohistochemistry and in situ RT PCR on terminal ileum from an autistic enterocolitis child.

A. Haematoxylin and Eosin stain of terminal ileum tissue section from affected child.

B. In situ RT PCR and hybridisation for measles virus N gene using NBT/BCIP chromogen. There is slight background staining on this section. Positive measles virus signals in lymphoid cells (indicated by arrow).

C. Histone mRNA in situ hybridisation.

D. Negative control section for histone mRNA in situ hybridisation.

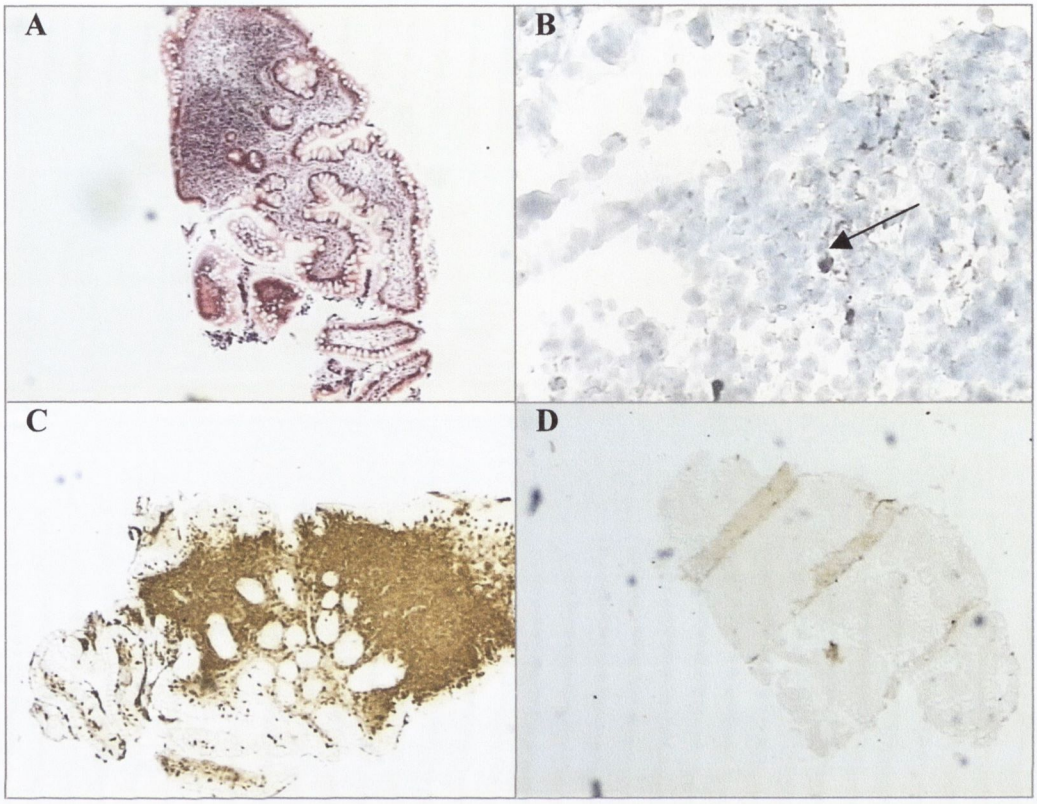


Figure 3.11. Immunohistochemistry and in situ RT PCR on terminal ileum from an autistic enterocolitis child.

A. Haematoxylin and Eosin stain of terminal ileum tissue section from affected child.

B. In situ RT PCR and hybridisation for measles virus N gene using NBT/BCIP chromogen. Positive measles virus amplicon is indicated by arrow.

C. Histone mRNA in situ hybridisation.

D. Negative control section for histone mRNA in situ hybridisation.

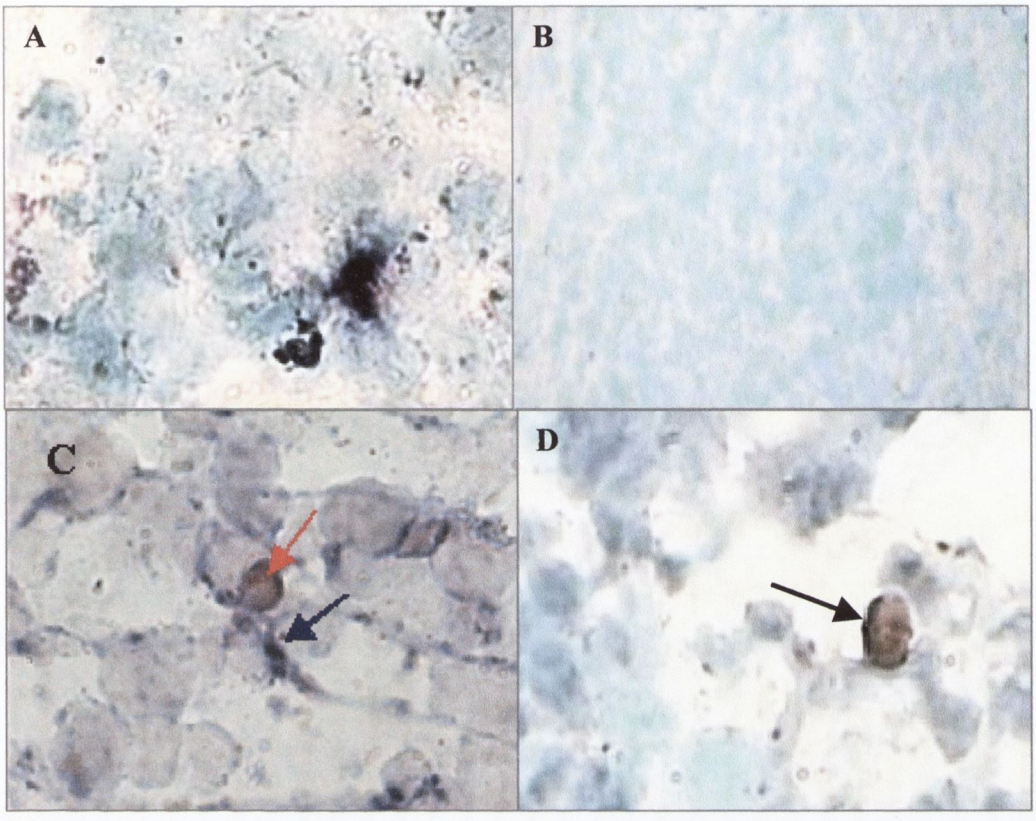


Figure 3.12. In situ RT PCR and combined immunocytochemistry.

A. MV N-gene RT in situ PCR combined with ISH showing in a reactive ileal lymphoid follicle centre. Positive signal radiates from an intense central core in an apparently beaded, fibrillary pattern (original magnification x 1000).

B. RT in situ PCR on a serial section of panel A constituting a negative control (irrelevant PCR primers) (original magnification x 1000).

C. Combined RT in situ PCR for MV N-gene and immunohistochemistry with Mab CNA 42, a follicular dendritic cell marker. A single cell is shown, present in a reactive follicle centre within the terminal ileum of an affected child. The MV genomic signal (red) appears to exhibit a central pattern that would be consistent with the cell body (Red Arrow), while CNA 42 (blue-black) delineates dendritic processes radiating from the cell body (blue arrow) (enlarged from original magnification of x1000).

D. RT in situ PCR shows a single mature lymphocyte (indicated with arrow) infected with measles virus RNA in a hyperplastic area.

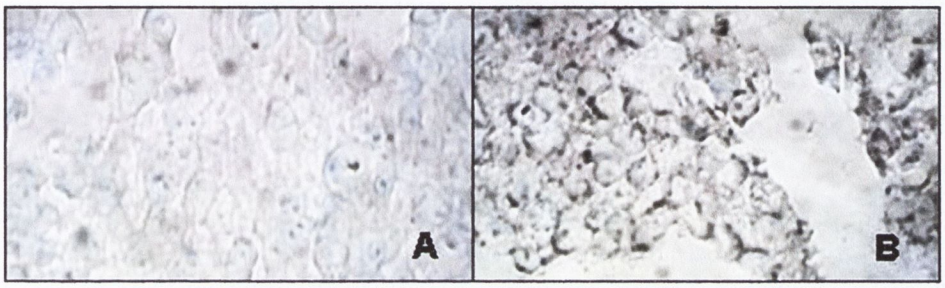


Figure 3.13. In situ RT PCR with and without RNAse digestion.

RT in situ PCR control experiment for MV N- gene on MV infected Vero cells with **(panel A)** and without **(panel B)** an RNase digestion step. No signal is observed for measles virus in panel A where RNase digestion was performed prior to in situ RT PCR. Panel B shows MV cytoplasmic signal in the absence of an RNase digestion step.

3.5 Summary of TaqMan RT PCR and in situ RT PCR results

Overall using a combination of both techniques, 99 of 133 (74.4%) affected children contained MV genomes in ileal lymphoid tissue compared with 14 of 100 (14 %) in the control patient cohort. ($\chi 2$ Test: $p<0.000$: SPSS Software, version 11). Table 3.6 below provides a summary of results.

Table 3.6 Summary of TaqMan RT PCR and in situ RT PCR results in intestinal tissues specimens.

	TaqMan RT PCR	In Situ PCR	Total Number
	Positive	**Positive**	**Positive cases ***
Affected Patients	94/126	46 /57	99/133 **
	(74.6%)	80 %	(74.4 %)
Controls			
Lymphonodular Hyperplasia	1/3 (33.3 %)	NT	1/3 (33.3 %)
Appendicectomies	6 /47 (12.7 %)	NT	6/47 (12.7 %)
Normal Controls	0 /14 (0 %)	1 /5 (20 %)	1/19 (5 %)
Mild non-specific changes	0/10 (0 %)	NT	0/10 (0 %)
Ulcerative colitis	1/2 (50 %)	2/3 (66.6 %)	2/4 (50 %)
Crohn's disease	0/8 (0%)	3/4 (75 %)	3/12 (25 %)
Colitis	1/5 (20 %)	NT	1/5 (20 %)

*In some instances cases were not tested for MV using both techniques. The overall number tested using either a combination or one of both techniques is listed in this column.

** The presence of MV in affected population as compared with the control population was highly significant ($\chi 2$ Test: $p<0.000$).

NT; not tested.

3.6 Discussion.

In this chapter an association between persistent measles virus infection and ileocolonic lymphonodular hyperplasia and ileocolitis in children with developmental disorders is described (Martin *et al.*, 2002; Uhlmann *et al.*, 2002; Wakefield *et al*, 1998a). The molecular data demonstrates the presence of measles virus genomes in the intestinal tissue of 74% of affected children with the disorder compared with 14% of control children. In addition, in the autistic cohort there appears to be a strong segregation of the disease phenotype with male children in keeping with the reported male predominance of developmental disorder (Volkmar *et al.*, 2003).

In this study measles virus F and H genes were detected by TaqMan RT PCR and N gene by in situ RT PCR. In general, for both groups (affected and controls), there was good correlation for detection of the three different genes. However, 21 of the affected cases, which were positive for MV F gene by TaqMan RT PCR, were negative for H gene. F gene copy number was low in these cases and 14 of these were also positive for N gene by in situ RT PCR. The low or undetectable expression of MV H gene in F/N gene positive samples is likely to be related to the low abundance of mRNA of genes encoded at the 5' end of the genome. There is a transcriptional gradient from the 3' end of the measles genome, in the following order N, P, M, F, H, and L, where N is the most abundant mRNA. Sidhu (1994) have demonstrated a steep mRNA transcription gradient in SSPE tissue with significantly lower levels of H gene mRNA than F or N gene mRNA.

Overall the reproducibility of our findings has been confirmed by using more than one molecular technique to detect different measles virus gene targets. In total 57 of the affected cases were examined using more than one technique, of these 44 cases were positive for measles virus by both TaqMan RT PCR and in situ RT PCR, 11 were negative for measles virus using both techniques. In addition, three different measles virus genes were detected using these techniques. This in conjunction with the strict anti-contamination measures and controls (described in chapter 2 and section 3.3.1) that were taken for each technique ensure confidence in the data obtained.

Measles virus localisation was demonstrated in tissue biopsies by RT in situ PCR and combined immunohistochemistry. In measles infected Vero cells the signal was predominantly cytoplasmic in keeping with previous studies (Ray *et al.*, 1996). The signal observed in tissue biopsies had a fibrillary character, and appeared to associate with the dendritic cell matrix confirmed by immunohistochemical staining using a dendritic cell specific antibody (CNA42), which recognizes a 120 kd formalin resistant glycosylated antigen (Figure 3.12).

Measles virus was detected in dendritic cells within reactive follicular hyperplastic centres in ileal biopsies from affected children (Figure 3.9, 3.10, 3.11) and also in mature lymphocytes in these hyperplastic areas (Figure 3.12). The number of positive cells per biopsy was low, typically in the range of 1-5 cells per tissue section, indicating that the virus is persisting but not replicating in the terminal ileum in these children. This result indicates a possible interaction between measles virus and the immune response in the pathogenesis of ileocolitis in these children.

Measles virus may be a potential 'immunological trigger' in the pathogenesis of lymphoid hyperplasia and ileocolitis. Potential initiators for this type of immunological response are dendritic cells. Dendritic cells capture and process viral antigens in the periphery, express co-stimulatory molecules and serve as vehicles for viral antigens to the mediators of immunity (B and T-cells) in lymphoid tissue. As a result cytokines are released initiating an immune response (Schneider-Schaulies *et al.*, 2002; Banchereau *et al.,* 1998).

Measles virus localisation in follicular dendritic cells mirrors HIV-1 infection patterns observed in HIV-1 enteropathy (Klagge *et al.*, 1999). The presence of measles virus antigen in follicular dendritic cells may reflect a transient stage in the progression from latent to persistent measles virus infection (Klagge *et al.*, 1999; Tachetti *et al.*, 1997; Pantaleo *et al.*, 1994). The similarity with HIV infection is interesting: HIV, like measles virus, potentially disrupts cellular immunity, and induces follicular hyperplasia and lymphadenopathy in the early stages of infection. This is associated with expansion of the follicular dendritic cell network and trapping of HIV within germinal centers (Klagge *et al,* 1999, Taccheti *et al.*, 1997). Tachetti *et al.*, (1997) demonstrate HIV antigens on the surface of follicular dendritic cells in the early latent phase of infection, in a pattern similar to that reported here for measles virus. Such a location may favour the induction of immunological tolerance and failure of viral clearance.

The mechanism by which measles virus, immunological abnormalities and chronic intestinal pathology may be linked, are currently not known. Precedents of delayed intestinal and immunological responses to measles virus exposure include chronic

immunodeficiency, diarrhoeal disease, and death, following early natural measles exposure (Aaby *et al.*, 1990). Natural measles infection induces an initial Th1 response and protective cytotoxic immunity (characterised by the classical measles rash and gastro-intestinal upset) followed by a prolonged Th2 response with antibody production (Griffin *et al.*, 1996). In the majority of the population, this has led to lifelong immunity without delayed pathological events. Nonetheless, immune activation during the measles infection causes a reduction in the non-specific cellular immune response, reflecting the potent immunosuppressive properties of measles virus (Schneider-Schaulies *et al.*, 2002b). Recent studies suggest that this may be achieved through impaired interleukin 12 production by infected dendritic cells (Atanabi *et al.*, 2001; Karp *et al.*, 1996) and blocking of interleukin 2 receptor α (IL-2Rα) expression by activated T cells (Schneider Schaulies *et al.*, 2002b; Bell *et al.*, 1997). In such circumstances, impaired type-1 immunity with a shift towards a dominant type-2 response, might contribute to establishing persistent infection and delayed immunopathology.

Interestingly, in contrast to what was observed in the intestine, the presence of measles virus F gene in the peripheral blood of 34% of affected and 23% of control children were not significantly different between both groups. A recent study describes elevated levels of measles antibodies in the serum of autistic patients (Singh *et al.*, 2003). Detection of measles virus genome in the PBMCs in the control population was not surprising. Vaccination and measles infection provide lifelong immunity to measles infection however it is well recognized that measles virus continues to circulate in highly immunized populations. Asymptomatic measles virus infection has been described in both immune adults and children (Sonoda *et al.*,

2002; Sonoda *et al.*, 2001). Measles virus H gene was detected by RT-PCR in 15.6% of bone marrow aspirates taken from adults for diagnosis of malignant disease (Sonoda *et al.*, 2002) and in PBMCs from 71% of children within two months of vaccination and 46% of children vaccinated or immunized in the past (Sonoda *et al.*, 2001).

Measles virus was detected in the terminal ileum of 14% of the control population. In appendices from 47 children only six harboured measles virus genomes. The presence of persistent measles virus in the intestine of a small minority of apparently healthy children is not surprising in light of the finding of Warthin-Finkeldy giant cells in inflamed appendices in children with measles infection (Gaulier *et al,* 1991). However, the prevalence of persistent measles virus infection in the general population remains unclear and warrants further investigation.

In the inflammatory bowel disease cohort, measles virus was detected in 25 % of Crohn's disease patients and two of four ulcerative colitis patients. These results provide some support for the controversial hypothesis that measles virus may be involved in the pathogenesis of IBD (Pardi *et al.*, 2000; Wakefield *et al.*, 1995; Pounder, 1995). While there is substantial epidemiological evidence that measles virus, in particular early infection, may be implicated in IBD (Pardi *et al.*, 2000; Ekbon *et al.*, 1994) the lack of experimental evidence has argued against such an hypothesis. Measles virus particles specific staining has been shown in the foci of granulomata inflammation in Crohn's disease by immunogold electron microscopy, immunohistochemistry and in situ hybridisation (Wakefield *et al.*, 1997; Lewin *et al.*, 1995). Similarly, measles virus F and H genes have been detected in the peripheral

blood of three of nine patients with autism and associated IBD, one of eight patients with Crohn's disease and one of three patients with ulcerative colitis (Kawashima *et al.*, 2000). In contrast to these studies a number of groups have failed to demonstrate the presence of measles virus RNA in clinical samples of inflammatory bowel disease (Afzal *et al.*, 2000; Afzal *et al.*, 1998; Chadwick *et al.*, 1998; Iizuka *et al.*, 1995). The discrepancies in the findings are most likely related to differences in the techniques applied, assay sensitivities, specific targets and patient samples.

A comparative evaluation of measles virus specific RT PCR methods has been performed between 7 different groups including those mentioned above (Afzal *et al.*, 2003). This study highlighted the major differences in different RT PCR assay sensitivities (as much as 1000 fold) between the different assays and different measles gene targets. Unfortunately, no real time PCR assay was evaluated in the study. The TaqMan real time PCR assays used in the present study employs shorter PCR amplicons and a sequence specific probe, which improves the assay sensitivity and specificity.

The author acknowledges the fact that the affected and control groups were poorly matched in relation to disease state, sample numbers and subjects ages. The reasons for this relate to the difficulties encountered in obtaining an appropriate "normal" control group. A truly "normal" diagnosis is rare in children who have undergone colonoscopy. In addition paediatric colonoscopies, resulting in a diagnosis of IBD tend to be performed at an older age than the mean age observed for the autistic group.

We have described the presence of measles virus RNA genes in new variant inflammatory bowel disease. One of the major questions raised by the study was whether the measles virus detected was wild type or vaccine in origin. Vaccine strains and wild type Edmonston strain are known to differ by at most 0.3% (Parks *et al.*, 2001) and several conserved amino acid coding substitutions have been identified which may help discriminate between vaccine and wild type strains (Parks *et al.*, 2001). Analysis of the N gene sequence amplified from a case of SSPE in the present study identified wild type measles infection (Appendix 2). The three predicted amino substitutions in the N gene at nucleotide positions 492, 550 and 1542 were consistent with wild type virus. None of these substitutions are conserved across all vaccine strains and no single vaccine strain contains all three substitutions (Parks *et al.*, 2001). The exact function of these substitutions is not fully understood, it is likely they play a role in RNA binding, formation of the nucleocapsid structure and interaction with P protein and subsequently influence the virus attenuation. Our group has designed a TaqMan assay to detect one of the conserved amino acid substitutions at nucleotide position 7901, amino acid position 211 on the H gene region of the measles genome (Sheils *et al.*, 2002), which is associated with interaction with the measles CD46 receptor molecule. Of the cases presented in this study, measles virus detected in 14 of 23 (60%) intestinal tissue samples from autistic enterocolitis patients was identified as vaccine strain. Wild type measles virus was detected in brain from one SSPE case.

This study has focused principally on measles virus. We have not excluded the presence of alternative infections. Viruses may persist elsewhere, or exert a transient effect not requiring subsequent persistence. One such transient risk may be that

concurrent exposure to measles and another infection may increase the risk of measles virus establishing persistent infection.

This study raises many questions. Firstly, does measles virus play an aetiological role in intestinal inflammation in developmental disorder? There is not sufficient evidence in this study to suggest that measles virus is a causative agent for this new variant of inflammatory bowel disease. However the study does report for the first time an association between persistent measles virus infection and ileocolonic lymphonodular hyperplasia and ileocolitis in children with developmental disorder.

Chapter 4

Detection of measles virus in a CD46 transgenic mouse model system

4.1 Introduction.

Measles virus is one of the most contagious diseases in humans. Despite the development and use of the combined MMR vaccine comprising attenuated live measles, mumps and rubella viruses, it is estimated that over 40 million people become infected and over one million die each year (Schneider-Schaulies *et al.*, 2002a). Measles virus infection is restricted to primates and typically infects the immune system and the central nervous system, causing profound suppression of the immune response and favouring the establishment of secondary infections. In rare cases the virus can persistently infect the central nervous system causing a number of fatal neurological disorders including measles inclusion body encephalitis (Bitnun *et al.*, 1999) and sub acute sclerosing panencephalitis (Schneider Schaulies *et al.*, 2002a).

This focus of this thesis is to characterise and describe a new variant of inflammatory bowel disease "autistic enterocolitis", which has been identified in a group of children with autistic disorders. The intestinal pathology includes ileocolonic lymphonodular hyperplasia and a non-specific colitis (Wakefield *et al.*, 2000). Chapter 3 describes an association between persistent measles virus infection and this new variant of inflammatory bowel disease (Martin *et al.*, 2002, Uhlmann *et al.*, 2002). Similarly, Kawashima *et al.*, 2000 have reported measles virus H gene in PBMCs from autistic patients. Aside from these two studies, attempts to detect measles virus genome sequences in intestinal tissues from inflammatory bowel disease patients, using various RT PCR methods, have been unsuccessful (Afzal *et al.*, 2000; Afzal *et al.*, 1998; Chadwick *et al.*, 1998).

In recent years, major advances have been made in our understanding of measles virus pathogenesis and mechanisms of virus persistence, largely due to the development of a non-primate animal model that is permissive to measles infection. The identification of the CD46 or membrane co factor protein (MCP) as one of the human receptors for measles virus (Naniche *et al.*, 1993, Dorig *et al.*, 1993) has made this possible. The ability of the measles virus to interact with the extracellular domain (SCR1 and SCR2) of the CD46 receptor has been assigned to two amino acids (451 and 481) within the measles virus H protein (Hsu *et al.*, 1998; Lecouturier *et al.*, 1996). Amino acid substitutions at these positions affects the interaction of the measles virus H protein with the CD46 receptor. It appears that this receptor is specific for laboratory adapted measles virus (Edmonston) strains and vaccine strains derived from it (Erlenhoefer *et al.*, 2002; Bartz *et al.*, 1998). An additional measles virus receptor signalling lymphocyte activation molecule (SLAM also known as CDw150) has recently been identified as a cellular receptor for many wild type and laboratory strains of measles virus (Tatsuo *et al.*, 2000).

The human CD46 receptor has 45% homology with the mouse CD46 receptor and therefore rodents are generally are non permissive to measles infection (Tsujumura *et al.*, 1998; Manchester *et al.,* 1994). A CD46 transgenic mouse line has been developed in which expression of the CD46 receptor closely mimics the location and amount of CD46 found in humans (Rall *et al.*, 1997). Full length genomic CD46, from a yeast artificial chromosome library was microinjected into fertilised mouse eggs to allow expression of the four major isoforms of CD46 (BC1, BC2, C1 and C2). In CD46 transgenic mice inoculated with measles virus, replicating virus was recovered from cells in the immune system and central nervous system (Patterson *et*

al., 2001, Oldstone *et al.*, 1999). The virus was found in T and B lymphocytes, the T cell enriched areas of the spleen, peyers patches and peripheral blood lymphocytes. The same group have recently created a SLAM transgenic mouse that is permissive to wild type measles infection (Hahm *et al.*, 2003). These mouse models provide the opportunity to investigate mechanisms of measles virus immunosuppression, pathogenesis and persistence in viral infection.

4.2 Study Aims

In collaboration with the Department of Neuropharmacology at The Scripps Research Insititute, our laboratory was recruited to validate the sensitivity and specificity of the TaqMan technique described in chapter 3 for detection of measles virus Fusion (F) and Haemaglutinin genes in a CD46 transgenic mouse model. In addition the efficiency of measles virus uptake and inoculation in the CD46 transgenic mouse model was examined. The information relating to the animals and RNA samples is limited as the study was terminated before completion. However the findings of our laboratory are reported in this chapter.

4.3 Materials and Methods.

4.3.1 Mouse RNA Samples

Two RNA aliquots from each of 24 transgenic mouse tissue samples and two control samples were obtained from Professor Michael Oldstone, Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California. Transgenic mice were inoculated with measles virus, intracerebrally, intraperitoneally or orally. Tissue samples were taken from mice, snap frozen in liquid nitrogen and stored at –70°C. Various tissues from 4 transgenic mice infected with measles virus and one uninfected transgenic control animal (Table 4.2) were tested. One transgenic animal had been crossed with a homozygous RAG (recombinant activating gene) deficient mouse to generate a lymphocyte deficient mouse. RNA was extracted using Trizol (Life Technologies), from the tissue samples at The Scripps Institute, the samples were coded and sent to the Department of Pathology, Coombe Women's Hospital. RNA extracted from normal mouse liver using Qiagen RNeasy extraction procedure was used a control. The quality and concentration of total RNA was quantified by uv spectrophotometry and agarose gel electrophoresis.

4.3.2 TaqMan RT PCR

Real time quantitative Reverse Transcriptase (RT) PCR based on the 5' nuclease assay was performed on an ABI 7700 Sequence detector, using the one step EZ TaqMan RT PCR reagents (Applied Biosystems, Foster City, CA, USA) as described in section 2.7. TaqMan Primers and probes for measles virus F and H gene were designed as described in chapter 3. The sequence of these primers and probes are listed in table 4.1 below.

Table 4.1. Primer and Probe Sequences.

Name	5'-3' Sequence
H1 Fwd	5' TTC ATC GGG CAG CCA TCT AC 3'
H1 Rev	5' CTC TGA GGT GTC CTC AGG CC 3'
H1 Probe	5' CCG CAG AGA TCC ATA AAA GCC TCA GCA C 3'
F1 Fwd	5' TGA CTC GTT CCA GCC ATC AA 3'
F1 Rev	5' TGG GTC ATT GCA TTA AGT GCA 3'
F1 Probe	5' CTG CAC GAG GGT AGA GAT CGC AGA ATA CAG 3'
Rodent GAPDH Forward	5' TGC ACC ACC AAC TGC TTA G 3'
Rodent GAPDH Reverse	5' GGA TGC AGG GAT GAT GTT C 3'
Rodent GAPDH Probe	5' CAG AAG ACT GTG GAT GGC CCC TC 3'

TaqMan RT PCR reactions for measles virus F and H gene assays were performed in duplicate under the following conditions per 25 µl reaction: 1X EZ buffer, 3mM $MnOAc_2$, 200 nmol of each primer, 100 nmol of TaqMan probe, 0.01 U of AmpErase, 0.1 U of r*Tth* polymerase, and 0.1–1µg of total RNA. The thermal cycling conditions on the 7700 were as follows 50 °C for 2 min, 58 °C for 30 min, 95 °C for 5 min, followed by 40 cycles of 94 °C for 20 s, 60 °C for 1 min.

Measles virus quantitative TaqMan RT PCR was performed by generating standard curves for F- and H-genes. The F and H gene 150 bp PCR fragments generated from RNA extracted from measles infected Vero cells as described in chapter 3 were cloned into pCR 2.1 vector (Invitrogen, The Netherlands), using the TOPO TA cloning technology described in section 2.11. The resulting plasmid and insert was sequenced by dideoxynucleotide chain termination method using the ABI Big Dye Terminator Ready Mix (Applied Biosystems, Foster City, CA, USA). Copy (c)RNA was synthesised from the plasmids using the Promega *In vitro* Transcription System (Promega, Woods Hollow Road, Madison, USA. Standard curves were generated using serial dilutions of the copy (c)RNA transcripts over a linear dynamic range of 5 x 10^{7} –5 x 10^{1} copies for F gene and 1 x 10^{7} –1 x 10^{1} copies for H gene as described in Chapter 3.

GAPDH TaqMan RT PCR control assays were performed using the VIC labelled TaqMan Rodent GAPDH Control (Applied Biosystems, Foster City, CA, USA). The rodent GAPDH TaqMan assay amplifies both RNA and DNA, therefore TaqMan assays were performed both with (RT+) and without (RT-) the reverse transcriptase step. GAPDH TaqMan RT PCR assays were performed as described

above using 3.5 mM $Mn(OAc)_2$. GAPDH TaqMan PCR assays without the reverse transcription step were performed using 1X TaqMan Universal PCR mastermix containing Amplitaq Gold, 100 nmol of each primer and 100 nmol of the GAPDH rodent TaqMan probe. The thermal cycling conditions for PCR on the 7700 were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by followed by 40 cycles of 94 °C for 20 s, 60 °C for 1 min. GAPDH RNA standard curves were generated using serial dilutions of total RNA extracted from normal mouse liver from 300 ng/µl- 3 pg/µl. GAPDH DNA standard curves were generated using serial dilutions of rat genomic DNA from 100 ng/µl- 10 fg/µl.

4.4 Results.

This study was blinded in that all samples tested were coded and their identity only revealed to the investigator after all TaqMan PCR analysis was complete. Two separate aliquots of RNA extracted from 24 tissues from five transgenic animals were examined (Table 4.2). The total RNA sent from the Scripps Institute was deemed to be of good quality, intact and free from impurities (Figure 4.1). All RNA samples tested positive for GAPDH RNA by TaqMan RT PCR. There was also some DNA contamination of the RNA preparations as determined by the TaqMan PCR without the reverse transcription step. The samples were analysed to assess the presence and quantity of measles virus F and H genes. Standard curves were established with cRNA synthesised from plasmids containing the 150bp F and H gene PCR inserts. cRNA was diluted in water from 10^7 to 10 copies per microliter. Each dilution was amplified in duplicate and a standard curve of threshold cycle plotted against copy number was constructed (Figure 4.2). The quantification was linear over the range amplified.

Duplicate RNA samples were tested for both F and H genes and the overall results are presented in Table 4.2. A scoring system 0-5 was applied to measles virus positivity, where a negative sample with 0 copies of F or H gene /µl of template RNA = 0, <10=1, <100 =2, <1000=3 and <10000=4, > 10,000=5. The uninfected transgenic mouse and the measles infected Rag-/- transgenic mouse were both negative for measles virus F and H genes.

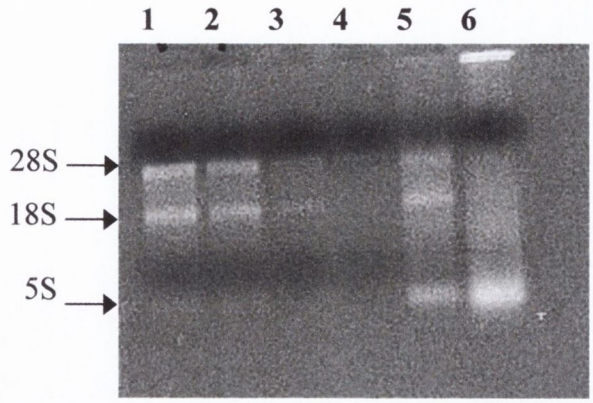


Figure 4.1: Gel electrophoresis of RNA extracted from mouse tissue samples.

Lanes 1-4: Total RNA extracted from normal mouse liver tissue used as control for GAPDH TaqMan RT PCRs, 1µg, 500 ng, 250 ng and 100 ng respectively.

Lane 5: 1µg of total RNA extracted from transgenic mouse 1462-11 liver tissue. Intact rRNA bands are visible.

Lane 6: 1µg of total RNA extracted from transgenic mouse 1465-18 liver tissue. There is some RNA degradation in this sample as indicated by smearing. Genomic DNA contamination is also present.

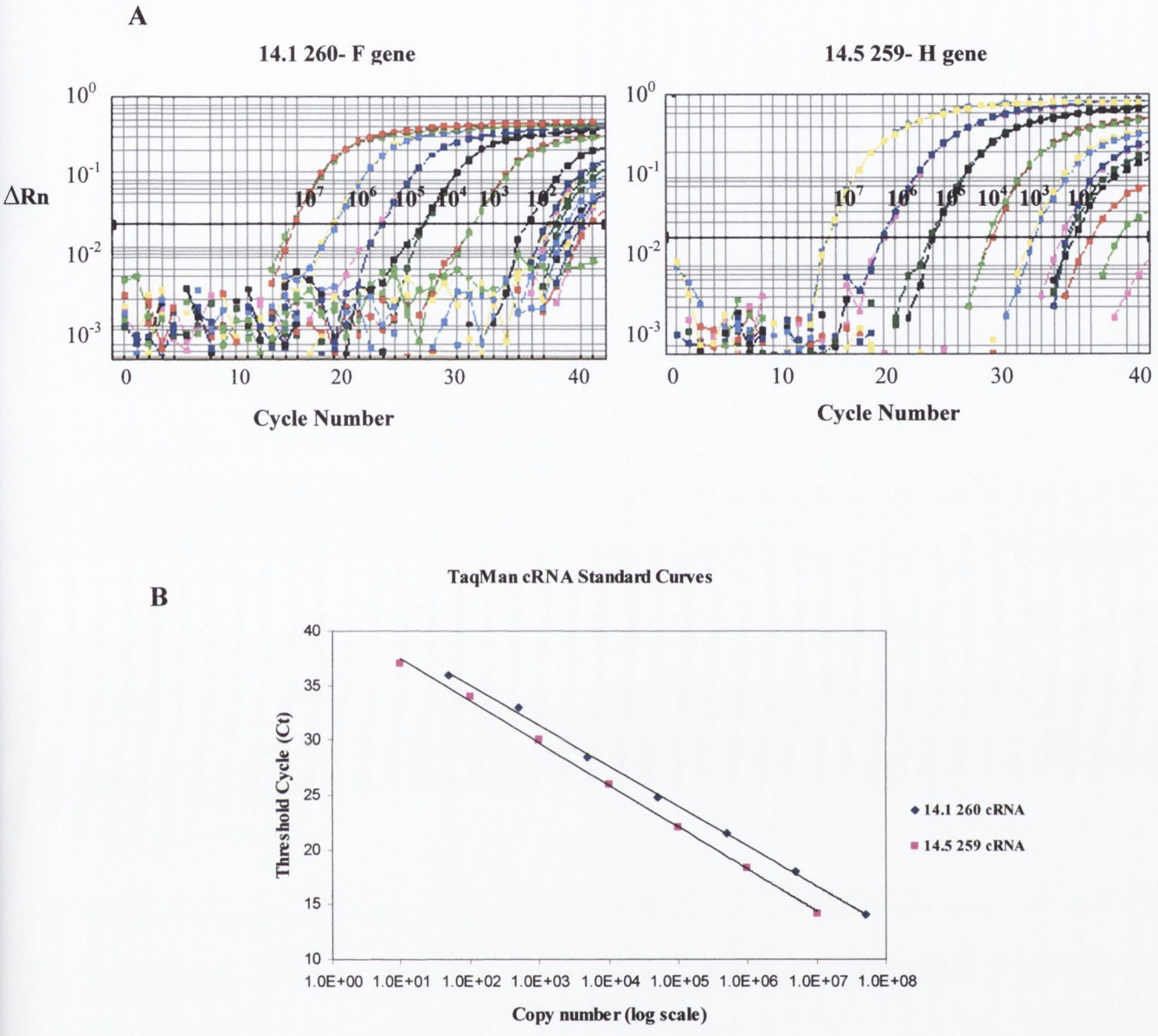


Figure 4.2. Amplification plots (A) and Standard curves (B) obtained with measles virus cRNA controls and tissue samples. Serial dilutions from 10^7 to 10 copies per µl were used as template for TaqMan RT PCRs. Amplifications were performed in duplicate for each sample. The ΔRn is amount of normalised reporter minus the amount of reporter signal at the start of the PCR.

Two separate RNA aliquots from each RNA sample were tested for measles virus F and H genes. In samples with low copy measles virus there were some discrepancies in the results and these are indicated by an asterisk in Table 4.2. At least two replicate TaqMan RT PCRs (each replicate performed in duplicate) were performed for each gene on each RNA aliquot. Occasionally in tissue samples with low copy measles virus between 100-10 copies/ul, a positive PCR result was not obtained in all replicate TaqMan RT PCRs. The viral load in brain tissue from the animals inoculated with measles virus intracerebrally and intraperitoneally was high (in the range of 10^5 and 10^3 copies/μl) while the majority of other tissue samples were lower (Table 4.2). Tissues from the uninfected transgenic animal were negative for measles virus F and H genes. The lymphocyte deficient CD46 transgenic mouse crossed with a homozygous RAG (recombinant activating gene) deficient mouse was also negative for measles virus (Table 4.2).

Measles virus infected and uninfected cell lines were also included as controls in the study. Measles virus copy number in the RNA from infected Vero cells was high (10^6 copies/μl). One of four aliquots of uninfected Vero cells was positive for both measles virus F and H gene by TaqMan RT PCR, and by Southern blot analysis on repeated occasions for measles virus F and H gene. The second aliquot was repeatedly negative for measles virus F and H genes. Uninfected Vero cells are consistently used as controls for measles virus F and H gene TaqMan PCR assays and this is the only occasion where a positive result was obtained. It was concluded that the positive aliquot had been contaminated with measles virus. As two separate measles gene targets tested positive in this aliquot it is unlikely that the

contaminating event occurred during the PCR set up. A more likely explanation is during extraction of the RNA.

Table 4.2 Summary of TaqMan RT PCR Results on Transgenic Animals.

Transgenic Mouse infected with MV orally			
RNA	**Tissues**	**MV F gene**	**MV H gene**
266-1	Mesenteric lymph node	0	0
266-2	Peyers patches	0	0
266-3	Spleen *	2*	0
266-4	Lung *	2*	1*
266-5	Large intestine *	2*	1*
266-6	Brain*	1	1
Transgenic Mouse crossed with Rag-/- Mouse given MV orally			
RNA	**Tissues**		
6436-7	Spleen	0	0
6436-8	Lung	0	0
6436-9	Small intestine	0	0
6436-10	Brain	0	0
Transgenic Mouse infected with MV intracerebral			
RNA	**Tissues**		
1462-11	Liver	0	0
1462 -12	Small intestine	0	0
1462-13	Kidney	2	2
1462-14	Heart *	2	1
1462-15	Large intestine	2	2
1462-16	Brain	5	5
1462-17	Lung *	2	2
Transgenic Mouse infected with MV intraperitoneally			
RNA	**Tissues**		
1465-18	Liver	2	2
1465-19	Small intestine	0	0
1465-20	Kidney *	2	2
1465-21	Heart*	2	2
1465-22	Large intestine*	1	1
1465-23	Brain	3	2
1465-24	Lung	2	2
CD46 Transgenic Mouse Uninfected			
RNA	**Tissues**		
9439-1	Brain	0	0
9439-2	Liver	0	0
1000-3	Large intestine	0	0
9439-4	Small intestine	0	0
9439-5	Spleen	0	0
9439-3	Kidney	0	0

* Indicates samples in which not all TaqMan RT PCR replicates tested positive.

A scoring system from 0-5 for MV positivity where 0= 0, 1= <10, 2= <100, 3= <1000, 4 = <10000 and 5= >10,000 copies/µl of MV.

4.5 Discussion.

Major discrepancies exist in the published literature describing the presence of measles virus in inflammatory bowel disease. Clinical samples have been examined for the presence of measles virus genome sequence with most studies failing to detect virus in inflammatory bowel disease patients (Afzal *et al.*, 2000; Afzal *et al.*, 1998; Chadwick *et al.*, 1998). Chapter 3 describes the detection of measles virus F and H genes in intestinal tissues from children with autistic enterocolitis and inflammatory bowel disease patients using real time quantitative PCR (Martin *et al.*, 2002; Uhlmann *et al.*, 2002). The reasons for these discrepancies may be related to differences in sensitivities and measles gene targets in the different RT-PCR assays. The present study evaluates and validates the TaqMan assays described in chapter 3 for detection of measles virus in a CD46 transgenic mouse model, with the added strength of a blinded study protocol.

Tissues from the CD46 transgenic animals infected with measles virus, intracerebrally, intraperitoneally and orally expressed measles virus F and H genes at varying amounts ranging from 10^5 to <10 copies/µl. High levels of measles virus F and H gene mRNA were detected in brain tissue from the CD46 transgenic animal inoculated with measles virus intracerebrally and intraperitoneally, corroborating the findings of Oldstone *et al.*, (1999), who report the presence of MV N gene mRNA and protein in multiple sites of the brain in these animals. Similarly, the detection of low levels of virus in other tissues including spleen, lung, large intestine, kidney, heart and liver was not surprising given the findings of Oldstone *et al.*, (1999) that CD46 receptor was expressed in these tissues in the CD46 transgenic mice. The route of inoculation will undoubtedly play a role in the dissemination of the virus.

Mice that were inoculated intracerebrally and intraperitoneally had higher levels of measles in their tissues than those given measles orally.

In four of the tissue samples (266-3, 266-4, 266-5, 1462-14 and 1465-23) there were differences in the amount of measles virus F and H genes in individual tissues. This is in keeping with the findings in chapter 3 and may be related to the low abundance of mRNA of genes encoded at the 5' end of the genome (Sidhu *et al.*, 1994). In samples with low levels of measles virus, in particular in animal 266, TaqMan RT PCR failed to amplify MV mRNA in duplicate RNA samples and in all replicate PCRs. This intermittent detection of virus is a common finding with low copy viral gene detection and is related to variations in template aliquots. The confidence and reproducibility of detection is improved by increasing the number of replicates analysed. In this study we performed a minimum of two replicates (all in duplicate), and in many cases three replicates, for both measles virus genes. A positive result in any replicate is significant, given that all of the controls tested negative for measles virus. In addition animals that tested positive for the virus were all infected with measles virus.

Measles virus was not detected in the immunosuppressed transgenic animal that was administered measles virus orally. However virus was co-cultured from spleen tissue harvested from this animal (Oldstone-personal communication). Measles induced CNS disease has previously been observed in such mice infected with measles virus intracerebrally (Lawrence *et al.*, 1999). Intracerebral infection of CD46 transgenic mice with measles virus Edmonston strain produced severe CNS disease associated with extensive neuronal infection in neonates, while adult transgenic animals were

resistant to infection and disease (Rall *et al.*, 1997). Failure to detect measles virus RNA in tissues harvested from the immunosuppressed transgenic animal in this study may be related to low levels of virus present in the animal and associated with the route of inoculation with virus. In the normal transgenic animal that was infected with measles virus orally (animal #266), viral RNA was not amplified in all aliquots or replicate PCRs.

In summary measles virus was detected in tissue samples from measles infected CD46 transgenic mice. Tissues examined from the uninfected CD46 transgenic animal and uninfected Vero cells were negative for measles virus confirming the specificity of the TaqMan RT PCR assay. The results also highlight the problems associated with low level viral detection. The CD46 mouse model of measles infection provides an ideal system to investigate the mechanisms of viral persistence and immune suppression by measles virus. The data presented in this study validates our TaqMan system for detection of measles virus and examines the effect of measles virus inoculation and dissemination in the CD46 transgenic mouse model. We can conclude that the route of inoculation with measles virus clearly has implications for tissue distribution and the ultimate amount of measles virus present in individual tissues.

Chapter 5

Cytokine mRNA expression analysis in children with new-variant inflammatory bowel disease "autistic enterocolitis"

5.1 Introduction

Autism is a neuropsychiatric disorder characterised by deficient language, social and communication skills and is accompanied by mental retardation in three out of four patients (Rapin, 1997). It is the most prevalent in a subset of disorders known as pervasive developmental disorders. A number of factors including genetic, environmental, neurodevelopmental abnormalities and immunological factors have been implicated. There is evidence that the immune system plays an important role in the pathogenesis of autism, decreased T cell subsets and increased serum IgM and IgE have been reported in children with autism (Gupta *et al.*, 1998). Recent investigations of possible proinflammatory cytokine dysregulation in children with autistic spectrum disorders have reported dysregulation of IL-2, IFN-γ, IL-6, IL-1β and TNF-α in the systemic system (Jyonouchi *et al.*, 2001; Gupta *et al.*, 1998; Singh *et al.*, 1996) suggesting a role for Th2 like cytokines in the pathogenesis of the disease.

A relationship between gastrointestinal immune dysregulation and autistic spectrum disorders has been postulated based on the identification of a new variant of inflammatory bowel disease (autistic enterocolitis) in a cohort of autistic children (Wakefield *et al.,* 2000, Wakefield *et al.*, 1998a). Ileal lymphonodular hyperplasia was detected in 93% of affected children compared with 14.3 % of the control group. The histological findings revealed reactive follicular hyperplasia in 92 % of the affected children with increased numbers of lamina propria lymphocytes, macrophages and neutrophils reported in the autistic cohort compared with controls.

The ileocolonic lymphonodular hyperplasia (LNH), which is a feature of this new variant IBD, has also been observed in children with attention deficit hyperactivity disorder (ADHD) (Wakefield *et al.*, 2000) and non-IgE mediated food allergies (Bellanti *et al.*, 2003; Kokkonen *et al.*, 2002a; Kokkonen *et al.*, 2002b; Sabra *et al.*, 1998). Bellanti *et al.*, (2003) have recently suggested an immunological dysfunction associated with an imbalance of Th1 function in the pathogenesis of non-IgE associated food allergies in which a predominance of CD4+ cells with a decreased Th1 cytokine profile and a normal Th2 cytokine profile was reported.

Intestinal inflammation such as that seen in patients with established inflammatory bowel disease (IBD) is also caused by dysregulation of the intestinal immune system. Several immunological abnormalities have been described in IBD patients that are reflected by an abnormal or imbalance of pro-inflammatory and inhibitory cytokines. Cytokines are regulatory proteins secreted by cells of the immune system (lymphocytes and macrophages) and other cells including endothelial cells, epithelial cells and smooth muscle cells, which act in an autocrine, paracrine or endocrine manner to modify immune responses (Roitt *et al.*, 2001). Complex cytokine profiles exist in both Crohn's disease and ulcerative colitis. The general consensus is that the mucosa of patients with established Crohn's disease is dominated by CD4+ lymphocytes and is predominantly a T helper cell type 1 response with exaggerated production of interleukin 12, interleukin 18 and interferon γ (Podolsky, 2002, Monteleone *et al.*, 1999, Monteleone *et al.*, 1997, Parronchi *et al.*, 1997). In contrast the mucosa in patients with ulcerative colitis is thought to be dominated by CD4+ lymphocytes with a type 2 helper T cell response, characterised by production of

TGF-β, and interleukin 5, but not interleukin 4 (Fuss *et al.*, 1996), however there are conflicting reports on this (Melgar *et al.*, 2003;).

While major advances have been made in the understanding of the immune dysregulation in established inflammatory bowel disease, the majority of evidence of gastrointestinal inflammation in children with autistic spectrum disorders has been based on macroscopic and microscopic findings. Increased numbers of CD8(+) cell, γδ T cells and intraepithelial lymphocytes have been reported in transverse colonic biopsies from patients with new variant inflammatory bowel disease (autistic enterocolitis) compared with Crohn's disease, LNH (not in autism) and normal controls (Furlano *et al.*, 2001). A recent publication by DeFelice (2003) reports no significant difference in production of cytokines IL-6, IL8 and IL-1β in intestinal tissue biopsies from children with pervasive developmental disorders using an organ culture technique. Aside from this study there are no other reports in the literature evaluating cytokines or regulatory proteins in the intestine of these patients.

5.2 Study Aims.

The focus of this chapter was to investigate the repertoire of inflammatory and pro-inflammatory cytokine genes expressed in the intestine and peripheral blood of patients with new variant inflammatory bowel disease "autistic enterocolitis" compared with controls including, isolated lymphonodular hyperplasia (not associated with autism), ulcerative colitis and Crohn's disease using TaqMan Human Cytokine Microfluidics Card technology. The TaqMan Human cytokine card is a novel technology for evaluating and quantitating the expression profile of 24 cytokines including IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 IL-10, IL-12p35, IL-12p40, IL-13, IL-15, IL-17, IL-18, G-CSF, GM-CSF, M-CSF, TNF–α, TNF-β, IFN-γ, TGF-β, and LT-β from a single cDNA sample.

5.3 Materials and Methods

5.3.1 Patient samples and medications

Intestinal tissue biopsies (snap frozen) taken from 8 patients with histologically confirmed autistic enterocolitis, 4 patients with histologically confirmed lymphonodular hyperplasia (not associated with autism), 5 patients with histologically confirmed Crohn's disease and 5 patients with histologically confirmed ulcerative colitis were examined in this study.

Terminal ileal tissue was examined in most cases with the exception of the following; transverse colon from a case of autistic enterocolitis (#354), which was endoscopically normal while histology indicated a mild increase in chronic inflammatory cells with occasional lymphoid aggregates. A small bowel tissue resection from one histologically confirmed ulcerative colitis patient was examined.

Peripheral blood mononuclear cells (PBMCs) from 14 patients including four normal controls, six autistic patients with gut pathology and four autistic patients with no gut pathology were also examined. Peripheral blood mononuclear cells were prepared from 10 ml of peripheral blood using Histopaque 1077 solution (Sigma Diagnostics) as described in section 2.1.3. The cell pellet was resuspended in 0.2 ml of PBS and 1.8 mls of RNA*later* was added to preserve the RNA. The PBMCs were then aliquoted into 0.5 ml aliquots and stored at –80 °C. One of the four 0.5 ml aliquots was used in this study.

None of the autistic enterocolitis or LNH (not associated with autism) patients were taking any medications at the time of endoscopy. Three of the Crohn's disease

patients were taking corticosteroids, azathioprine or methotrexate and three of the ulcerative colitis patients were taking azathioprine.

5.3.2 RNA extraction

RNA was extracted from terminal ileum biopsies using the Qiagen RNeasy RNA isolation kit or the UltraSpec II RNA isolation system (Biotecx, Texas, USA). RNA was extracted from PBMCs using the QIAmp RNA blood extraction kit. Biopsies were homogenized in 600 µl of lysis buffer using the BIO 101 bead beater (Savant) or mechanical pellet pestle. An on-column DNase digestion step was performed during RNA purification. RNA was eluted in 50 µl of RNase free water. The OD260/280 nm ratio of the extracted RNA was determined using a UV spectrophotometer.

5.3.3 TaqMan RT PCR.

Quantitative TaqMan RT PCRs for measles virus F gene and human GAPDH gene were performed as described in chapter 3. Briefly, one step TaqMan RT PCR assays were performed using the EZ TaqMan RT PCR reagents following manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). RT PCR reactions were performed in duplicate under the following conditions per 25 µl reaction: 1X EZ buffer, 3mM $MnOAc_2$, 200 nmol of each primer, 100 nmol of TaqMan probe, 0.01 U of AmpErase, 0.1 U of r*Tth* polymerase, and 3 µl (5-50 ng) of total RNA. The thermal cycling conditions on the 7700 were as follows 50 °C for 2 min, 58 °C for 30 min, 95 °C for 5 min, followed by 40 cycles of 94 °C for 20 s, 60 °C for 1 min. Standard curves for measles virus F gene were generated using cloned cRNA

standards (section 2.11) and for GAPDH using human control RNA (Applied Biosytems, Foster City, CA, USA).

5.3.4 TaqMan Human Cytokine Card.

The TaqMan human cytokine card uses the comparative Ct method of relative quantitation described in section 2.7.4.2. The card evaluates a single cDNA sample generated from human total RNA in a two-step RT PCR experiment. The TaqMan card assay measures the following 24 cytokines IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 IL-10, IL-12p35, IL-12p40, IL-13, IL-15, IL-17, IL-18, G-CSF, GM-CSF, M-CSF, TNF–α, TNF-β, IFN-γ, TGF-β, and LT-β. The card consists of a 96 well plastic consumable where each well contains lyophilised FAM labelled TaqMan MGB probes (section 2.7) and primers for one human cytokine mRNA. All primers and probes sets were designed across exon junctions to avoid amplification of genomic DNA. There are four replicates of each cytokine mRNA target on a TaqMan Human Cytokine card. A multiplex PCR assay is performed on the card with a VIC labelled TaqMan MGB primer and probe set for 18S ribosomal RNA endogenous control. For multiplex PCR the primer concentration of the 18S ribosomal RNA control assay was limited to minimise competition for PCR reagents.

5.3.5 cDNA synthesis and multiplex TaqMan PCR.

cDNA was synthesized from 0.5- 1ug of total RNA in a final reaction volume of 100 µl. For PBMC samples 0.5 µg of RNA was used to synthesize cDNA and for intestinal tissues 1 µg of total RNA was used. cDNA reactions were set up as follows; 1X TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 µM of each dNTP, 2.5 µM random hexamers, 0.4 U/µl RNase inhibitor, and 1.25 U/µl MultiScribe Reverse Transcriptase. All reverse transcription reagents were supplied by Applied Biosystems (Foster City, CA, USA). Reverse transcription reactions were performed on a 9600 thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: 25°C for 10 min, 37 °C for 60 min, 95 °C for 5 min.

The entire cDNA reaction (100 µl) was used in the PCR amplification. A PCR mastermix was prepared in a final volume of 300 µl for each card containing the following, 1X TaqMan Universal master mix (Applied Biosystems, Foster City, CA, USA), 1 X 18S MGB primer and probe mix (Applied Biosystems) and 100 µl of cDNA. The PCR mastermix was loaded into the cytokine card as described in section 2.8. The final volume in each well of the TaqMan Human cytokine card was 1 µl, the excess mastermix is required to ensure all wells are filled. TaqMan Human Cytokine cards were run on a 7700 thermal cycler under the following cycling conditions; 50°C for 2 min, 99°C for 10 min, 35 cycles of 99°C for 15 secs, 60 °C for 1 min. Each card evaluates a single cDNA sample with four replicates of each cytokine target amplified. The TaqMan card assay performance was determined using TaqMan Human Control Total RNA from Applied Biosystems.

5.3.6 Relative Quantitation Analysis.

Human TaqMan Cytokine Card data was analysed using the SDS v1.7.1 and SDS v2.1 software (Applied Biosystems, Foster City, CA, USA). The comparative Ct method for relative quantitation was performed as described in section 2.7.4.2. For all relative quantitation the threshold settings for the individual dye layers were set to the same value for all cards used in the comparison. For PBMC comparative analysis the threshold was set to 0.06 for the both the VIC and FAM dye layers. For the intestinal tissue analysis the thresholds were set to 0.08 for VIC and 0.03 for the FAM dye layer. The threshold cycle (Ct) is the cycle number at which the amount of amplified gene reached a fixed threshold. Prior to relative quantitation analysis, any wells within a replicate group with a Ct value outside the average for that group or samples that amplified too weakly to be reproducible were omitted. To calculate the fold change the ΔCt was calculated by subtracting the average Ct of the 18S endogenous control from the average Ct of the target cytokine. Relative quantities of the target were calculated using the following formula $2^{-\Delta\Delta Ct}$ where the ΔΔCt is calculated by subtracting the ΔCt of the calibrator sample from the ΔCt of the test sample. The range of expression was calculated using the ΔCt standard deviations (+/-) for each disease group.

5.4 Results

5.4.1 Study population.

A total of 36 patients were examined in this study, including intestinal tissue from 22 patients and controls and 14 peripheral blood samples from patients and controls as detailed below. The median ages of the autistic enterocolitis cohort was 7 yrs (range 3-15 yrs), the Crohn's disease cohort was 14 yrs (range 10-15 yrs), the ulcerative colitis cohort was 12 yrs (range 8-14 yrs), the LNH (not associated with autism) cohort was 9 yrs (range 4-13 yrs), the normal control cohort was 13 yrs (range 6-15 yrs), and the autistic cohort with no gut pathology was 11.5 yrs (range 10-14yrs) (Table 5.1). The median age of the entire study population was 10.5 yrs (range 3-15 yrs) and a total of 27 males (72.9 %) and 10 females (27 %).

Table 5.1 Patient details

Disease group	Sample type	Male: Female	Median Age (Range)
1. Autistic enterocolitis (n=8)	Terminal ileum (n=7) Transverse colon (n=1)	8:0	7 yrs (3-15)
2. Autistic enterocolitis (n= 6)	PBMC	6:0	8 yrs (6-13)
2. LNH (n=4)	Terminal ileum	2:2	9 yrs (4-13)
3. Crohn's disease (n=5)	Terminal ileum	1:4	14 yrs (10-15)
4. Ulcerative colitis (n=5)	Terminal ileum (n=4) Small bowel resection (n=1)	3:2	12 yrs (8-14)
5. Autism with no gut pathology (n=4)	PBMC	4:0	11.5 yrs (10-14)
6. Normal Controls (n=4)	PBMC	4:0	13 yrs (6-15)

5.4.2 GAPDH and measles virus TaqMan RT PCR.

Total RNA was extracted from specimens, DNase digested and the quality and quantity determined by gel electrophoresis and UV spectrophotometry. All samples tested positive for the GAPDH housekeeping gene by quantitative TaqMan RT PCR prior to cytokine mRNA analysis. Total RNA samples from autistic cases were also tested for measles virus F gene by quantitative TaqMan RT PCR. These results are shown in table 5.2 below. In the positive intestinal tissues samples, measles virus copy number was low ranging from 3.5×10^5 - 3×10^2 copies/ng of total RNA. The copy number in the positive PBMC samples was even lower ranging from 1 copy – 8×10^1 copies/ng of total RNA.

Table 5.2 Measles virus F gene TaqMan RT PCR results.

Intestinal Tissues	**MV F gene Positive**	**MV F gene Negative**
Autistic enterocolitis	4	4
PBMCs		
Autism with gut pathology	4	2
Autism without gut pathology	1	3
Normal Controls	0	4

5.4.3 Validation of TaqMan Cytokine Card Technology.

Prior to analysis of any samples the performance of the TaqMan Human Cytokine Card Assay was assessed using human control RNA supplied by Applied Biosystems. cDNA synthesized from 1 μg of control RNA was amplified in a multiplex PCR assay on the TaqMan cytokine card. The average threshold cycle (Ct) for the 18S rRNA endogenous control was below 12 cycles and the ΔCt for the following five cytokine mRNA targets were as follows IL-10 <20, lymphotoxin β <20, TGF-β <17, TNF-α <20 and TNF-β <17. These cytokines are significantly over expressed in this RNA control and the ΔCt's obtained were in keeping with those recommended by the manufacturer's. The control card experiment was replicated three times to confirm reproducibility of the assay.

The individual cytokine assays on the TaqMan Cytokine card (Applied Biosystems) have been optimised by the manufacturers for use in a multiplex PCR with 18S rRNA control. The efficiency of both the VIC labelled 18S endogenous control and the FAM labelled cytokine target PCRs were validated in this study. cDNA generated from three dilutions of the human control total RNA (1ug, 500 ng and 100 ng) were amplified in multiplex PCRs on the TaqMan human cytokine card. The ΔCt for TNF α and TNFβ cytokines were calculated for each dilution and plotted as a function of the log of the input RNA concentrations. When the amplification of the target and endogenous control genes are linear and approximately equal in efficiency the slope of the line is zero or close to zero (Livak, *et al.*, 2001). Amplification of TNFα and TNF-β were linear over the range of RNA concentrations examined and the slopes calculated by the equations y=-0.069+17.1(TNF-α) and y=0.55+16.2 (TNF-β) were not different from zero (Figure 5.1)

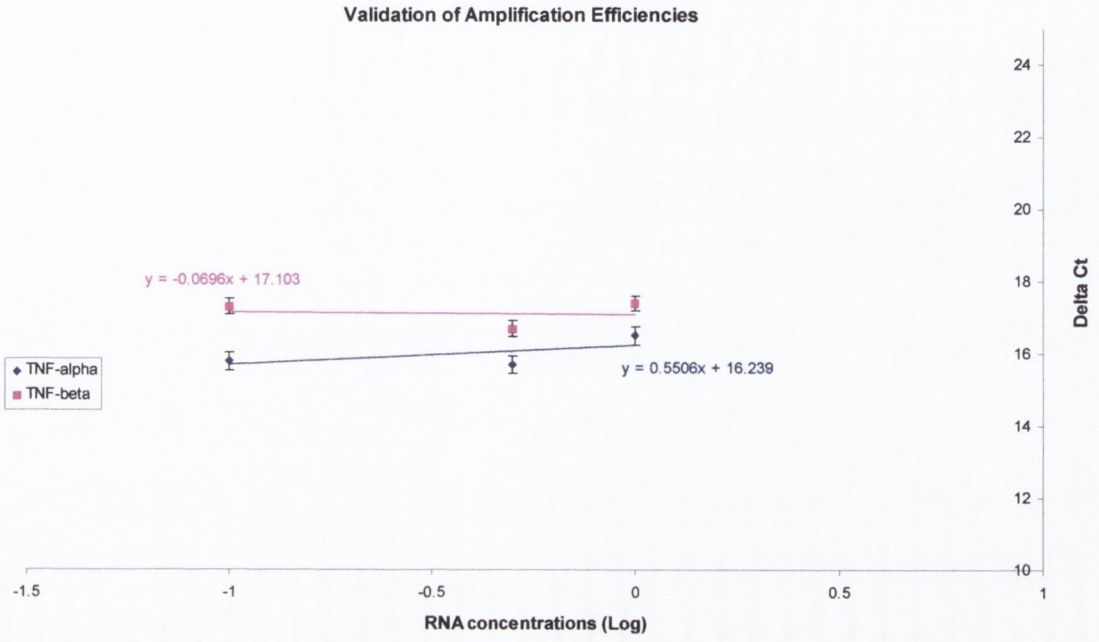


Figure 5.1. Validation of the 2-ΔΔCt method.

cDNA synthesised from different concentrations of RNA (1 μg, 500 ng and 100 ng) was amplified using the TaqMan Human cytokine cards in a multiplex PCR with the 18SrRNA endogenous control and cytokine targets. The ΔCt was calculated for TNF-α and TNF-β cytokines and plotted against the log of input RNA concentration. Slopes close to zero demonstrate that the amplification efficiencies of the 18S rRNA endogenous control and cytokine targets are approximately equal.

5.4.4 Cytokine mRNA analysis in intestinal tissue biopsies from autistic enterocolitis patients and controls.

The expression profiles of 24 inflammatory and proinflammatory cytokines were examined in the intestinal tissue biopsies from 22 patients (table 5.1 above). One μg of total RNA (determined by spectrophotometry) extracted from intestinal tissue samples was converted to cDNA and used as template in a multiplex TaqMan PCR for detection of the VIC labelled 18S rRNA endogenous control and the FAM labelled cytokine mRNA target. The amplification threshold was set at 0.08 for the VIC dye layer and 0.03 for the FAM dye layer in all cards to allow for accurate comparison across all cards.

The 18S rRNA was successfully amplified in all samples with Ct's ranging from 4-12 cycles. The average Ct's for the 18S rRNA endogenous control in each of the disease groups are as follows; Crohn's disease 6.6, ulcerative colitis 7.5, lymphonodular hyperplasia (not in autism) 6.5 and autistic enterocolitis 9. In general amplification of the 18S rRNA target was uniform within all wells of a particular card, however there were some instances where a difference of up to 3 cycles in the 18S Ct's were observed on an individual card. In these cases any outliers within the four replicates were removed from the analysis. These differences are most likely caused by problems with filling the cytokine card with the cDNA mixture using a vacuum, or manufacturing problems with the card itself (as wells within the card with dramatically different Ct's to other wells tended to correspond to particular rows and channels).

Four replicates of each cytokine target were amplified in each cDNA sample. The amount of the individual targets in each well was normalized to the endogenous control amplified in that well by calculating the ΔCt (Ct target – Ct 18S rRNA endogenous control), the average ΔCt for each target was then determined. The cytokine expression profiles were then calculated relative to a calibrator sample. Four different calibrator samples were used; lymphonodular hyperplasia (not associated with autism), ulcerative colitis, Crohn's disease and the human RNA control from Applied Biosystems.

The autistic enterocolitis patient cohort was divided into two groups based on the presence or absence of measles virus in the tissue specimens and these were plotted on separate charts. In the disease groups that were used as calibrators, the average ΔCt from each cytokine in all of the patients within the disease group were calculated and used to determine the ΔΔCt and subsequent relative expression profile. Cytokine targets that produced an average Ct ≥ 35 in both test and calibrator samples were excluded from the relative quantitation analysis. In instances where a particular cytokine target had a Ct ≥ 35 in the test sample and a Ct < 35 in the calibrator sample, or *vice versa* a Ct ≥ 35 the calibrator sample and a Ct < 35 in the test sample, these cases did not have enough target for accurate comparison and are indicated by a asterisks on the relative quantitation graphs.

Relative cytokine mRNA expression profiles in individual autistic enterocolitis patients were compared with pooled average values from lymphonodular hyperplasia (not associated with autism) (Figure 5.2 and 5.3), ulcerative colitis (Figure 5.4 and 5.5) and Crohn's disease (Figure 5.6 and 5.7) as calibrators and are expressed as a

fold difference. Transverse colon was examined from one of the autistic enterocolitis patients (#354) and the cytokine expression profile was dramatically different to that observed in the terminal ileum from other patients (Figure 5.2, 5.4, 5.6).

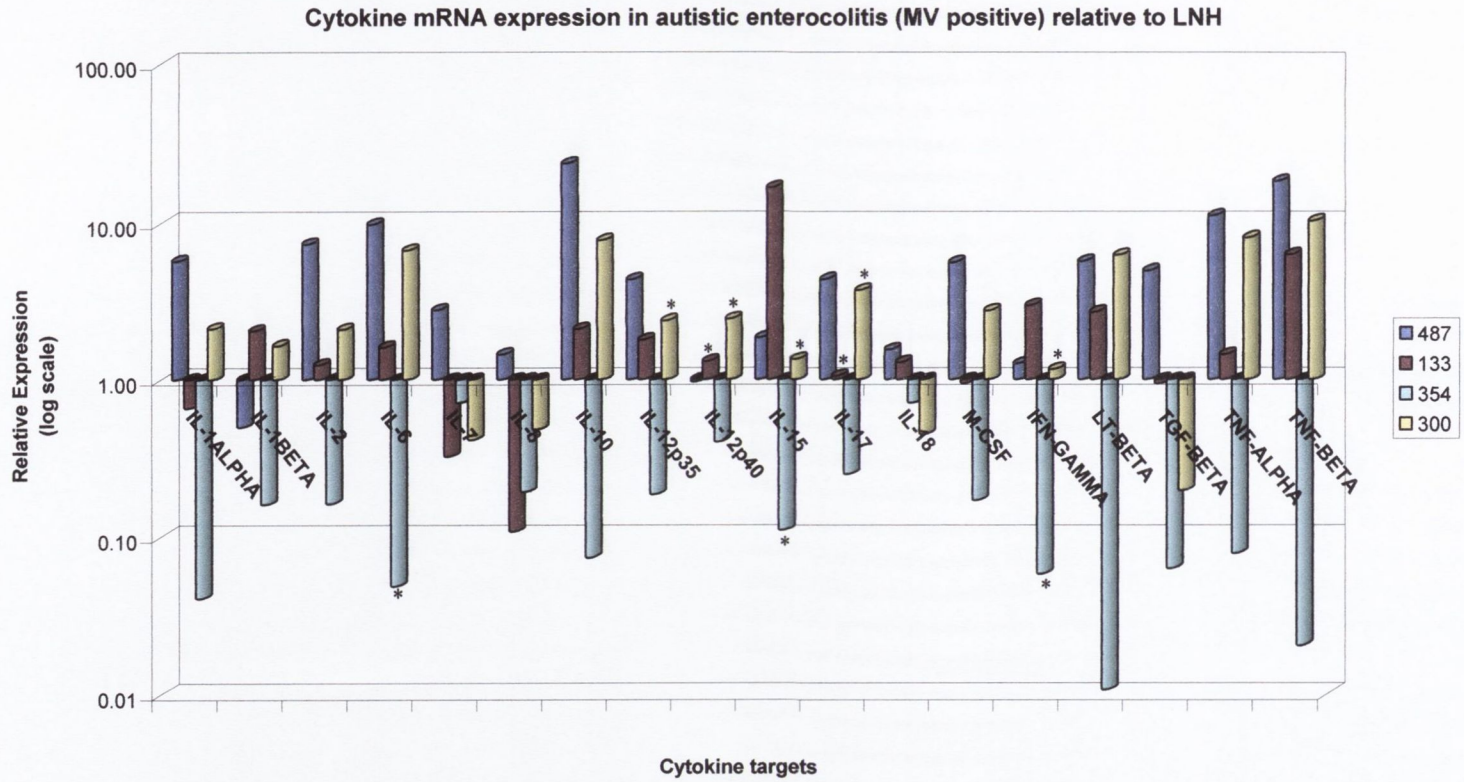


Figure 5.2. Relative cytokine expression profile in intestinal tissue from four cases of autistic enterocolitis (which were positive for measles virus) expressed relative to the average cytokine profile of four cases of lymphonodular hyperplasia (not in autism). Terminal ileum tissue was examined from all LNH cases, autistic cases 487, 133, and 300 and transverse colon from case no. 354.

* Indicates cytokine targets where either the calibrator or test sample had Ct ≥ 35.

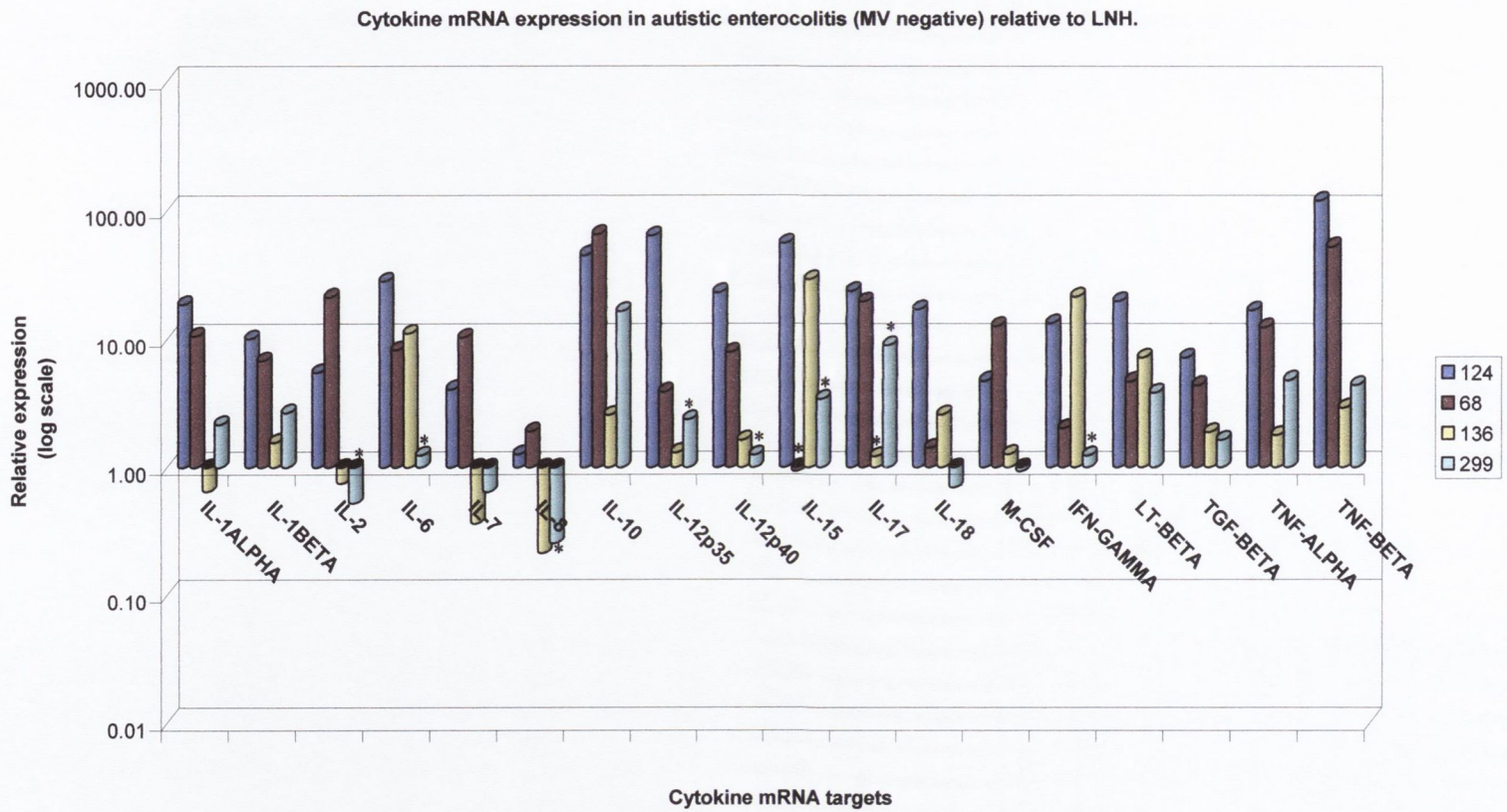


Figure 5.3. Relative cytokine expression profile in the intestinal tissue from four cases of autistic enterocolitis (which were negative for measles virus) expressed relative to the average cytokine profile of four cases of lymphonodular hyperplasia (not in autism). Terminal ileum tissue was examined from all LNH and autistic enterocolitis patients.

* Indicates cytokine targets where either the calibrator or test sample had Ct ≥ 35.

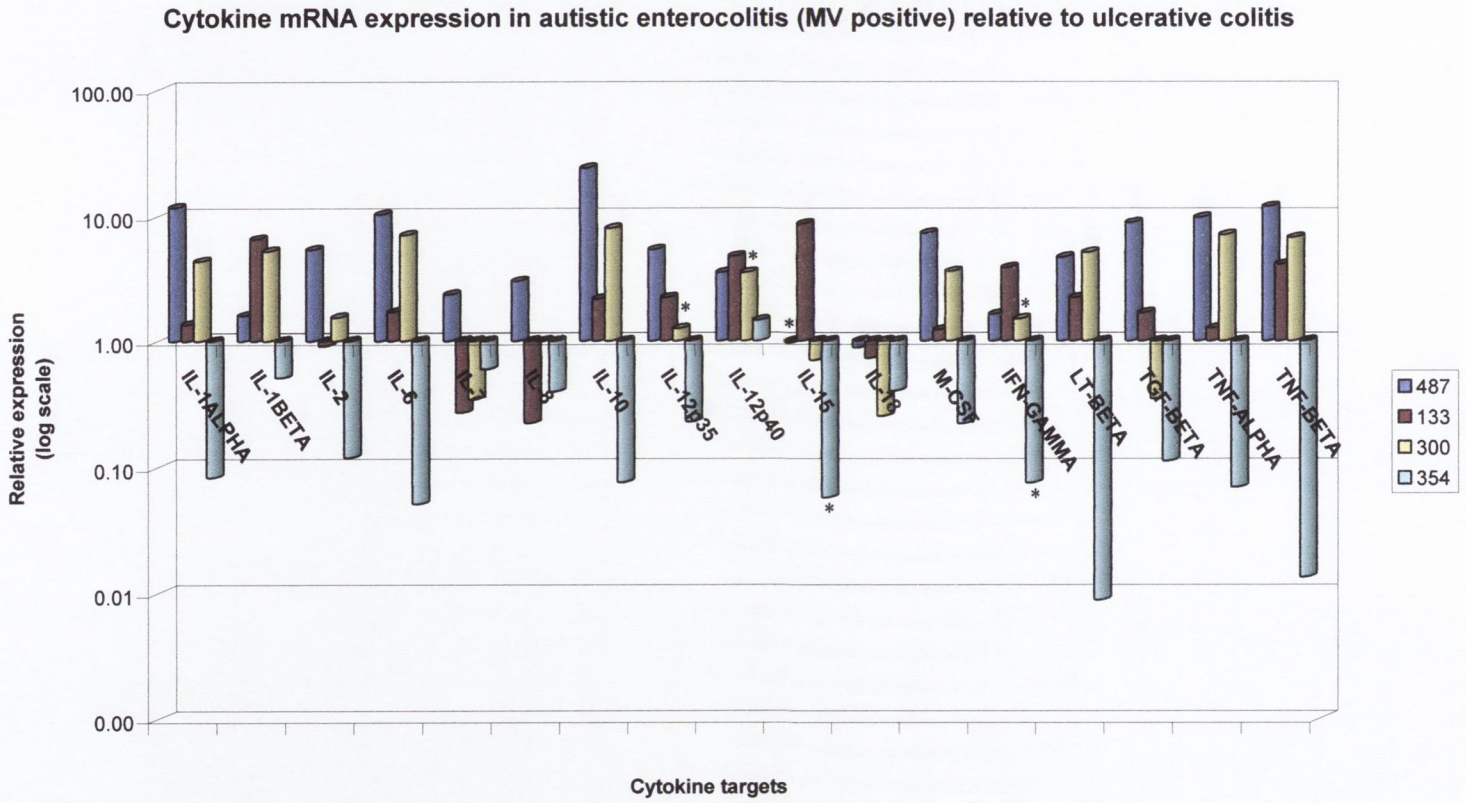


Figure 5.4. Relative cytokine expression profile in four cases of autistic enterocolitis (which were positive for measles virus) expressed relative to the average cytokine profile of five cases of ulcerative colitis. Terminal ileum tissue was examined from cases 487, 133, and 300 and transverse colon from case no. 354. Terminal ileum was investigated in four cases of ulcerative colitis and one small bowel resection was examined.

* Indicates cytokine targets where either the calibrator or test sample had $Ct \geq 35$.

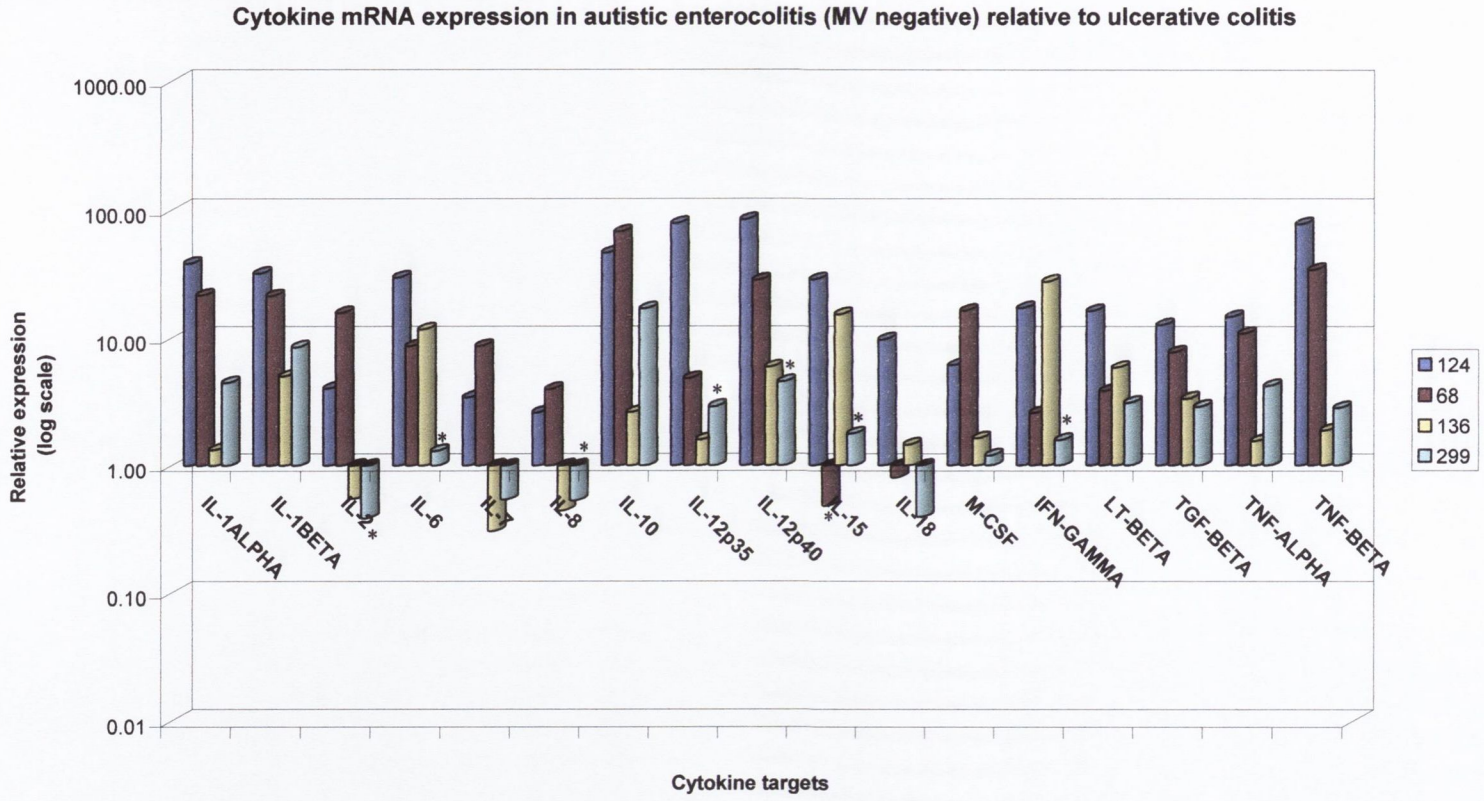


Figure 5.5. Relative cytokine expression profile in the intestinal tissue from four cases of autistic enterocolitis (which were negative for measles virus) expressed relative to the average cytokine profile of five cases of ulcerative colitis. Terminal ileum tissue was examined from all cases of autistic enterocolitis and in four cases of ulcerative colitis. A small bowel resection was examined from one ulcerative colitis case. *Indicates cytokine targets where either the calibrator or test sample had Ct $\geq$ 35.

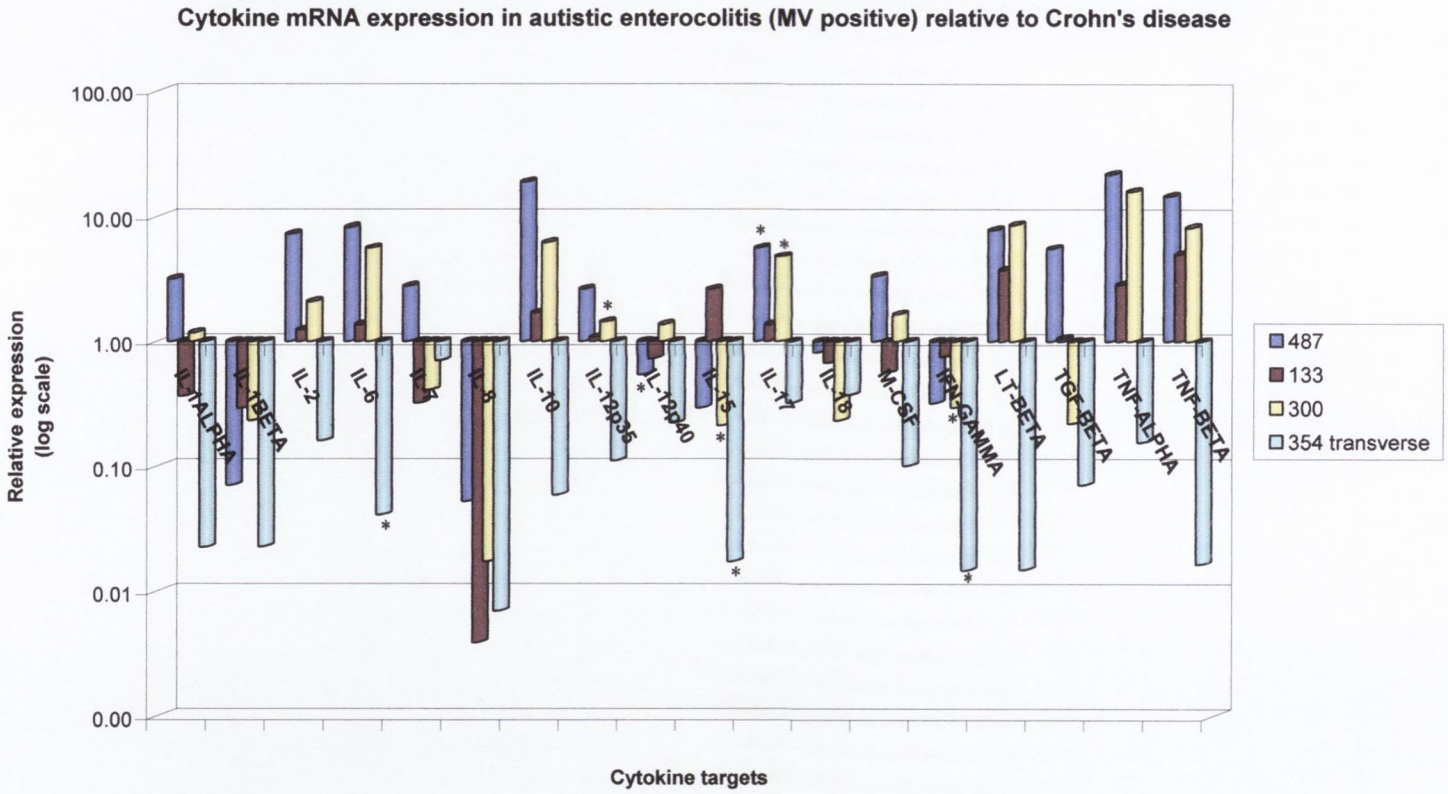


Figure 5.6. Relative cytokine expression profiles in the intestinal tissue from four cases of autistic enterocolitis (which were positive for measles virus) compared with five cases of Crohn's disease. Terminal ileum tissue was examined from all Crohn's disease cases, autistic cases 487, 133, and 300 and transverse colon from case no. 354.

* Indicate cytokine targets where the Ct of the test sample or calibrator sample was ≥ 35 cycles.

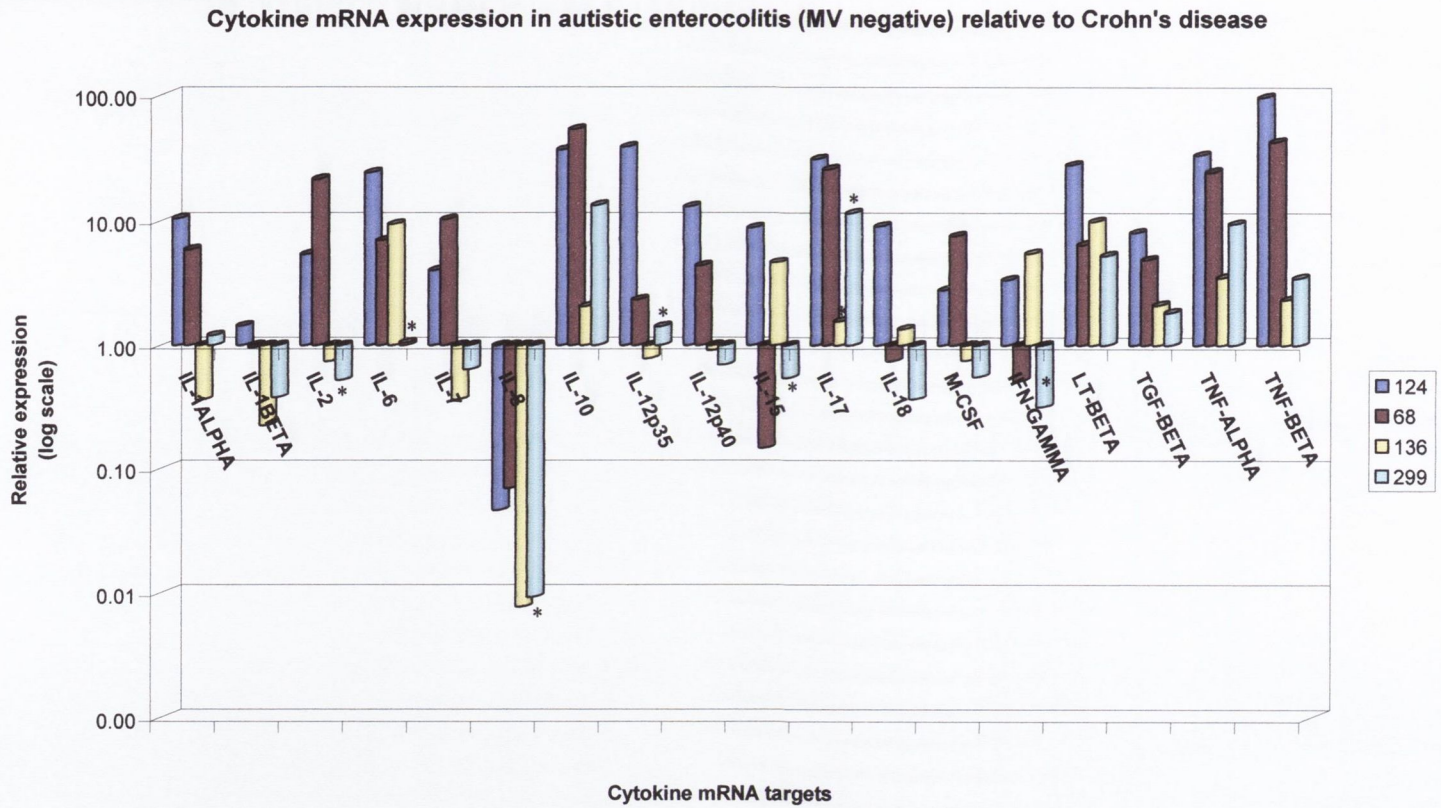


Figure 5.7. Relative cytokine expression profile in the intestinal tissues of four cases of autistic enterocolitis (which were negative for measles virus) expressed relative to the average cytokine profile of five cases of Crohn's disease. Terminal ileum tissue was examined from all cases of autistic enterocolitis and Crohn's disease.

* Indicates cytokine targets where either the calibrator or test sample had Ct ≥ 35.

5.4.5 Average cytokine expression profiles in intestinal tissues from autistic enterocolitis patients.

The overall relative cytokine mRNA expression profile (average across all disease groups) of autistic enterocolitis cohort (n=7) compared with Crohn's disease (n=5), ulcerative colitis (n=5), and LNH (n=4) cohorts are shown in table 5.3. The autistic enterocolitis patients were grouped into two groups based of the presence (n=3) or absence (n=4) of measles virus in the tissue biopsies. The fourth measles virus positive sample from patient # 354 was omitted from the overall relative expression profiles as it showed a very different pattern of expression than all other patients within this cohort (figure 5.2, 5.4 and 5.6) and was considered an outlier. Transverse colon was examined from this patient whereas small bowel was examined in all other patients. The upper and lower levels of target expression are also indicated in table 5.3. These were calculated using the standard deviation calculated from the average ΔCt values in each patient group. In some instances the range of expression was quite variable reflecting the differences in expression profiles within the individual patients.

There was no amplification of cytokines IL-3, IL-4, IL-5, and IL-13 in any of the disease groups. Cytokines G-CSF and GM-CSF amplified weakly or not at all in some patients and therefore were omitted from the analysis. Cytokines IL-12p35, IL-12p40, IL-15, IL-17 and IFN-γ did not amplify in some of the autistic patients and therefore accurate comparison across the disease group was not possible. These cytokines are marked with asterisks in Table 5.3. Cytokines with a fold change greater than 4, which is equivalent to a two-cycle difference between the delta Ct from a test and calibrator sample, were considered significant. These are highlighted

in tables 5.3. Cytokines IL-1α, IL-1β, IL-6, IL-8, IL-10, LT-β, TNF α and TNF-β showed differential expression patterns in autistic enterocolitis patients compared with controls.

Table 5.3 Average Relative Expression Profiles of Autistic Enterocolitis.

	AE MV (Positive) Rel to LNH	AE MV (Negative) Rel to LNH	AE MV (Positive) Rel to Crohn's	AE MV (Negative) Rel to Crohn's	AE MV (Positive) Rel to UC	AE MV (Negative) Rel to UC
	n=3	n=4	n=3	n=4	n=3	n=4
IL1 ALPHA	2.00 (0.68-5.92)	4.11 (0.89-19.03)	1.11 (0.38-3.28)	2.28 (0.49-10.54)	4.10 (1.39-12.12)	8.40 (1.81-38.90)
IL1 BETA	1.19 (0.56-2.55)	4.12 (1.76-9.66)	0.17 (0.08-0.36)	0.59 (0.25-1.37)	3.79 (1.77-8.12)	13.10 (5.59-30.71)
IL2	2.68 (1.07-6.69)	2.61 (0.46-14.96)	2.66 (1.07-6.65)	2.60 (0.45-14.89)	1.96 (0.79-4.90)	1.91 (0.33-10.96)
IL6	4.74 (1.83-12.32)	7.58 (2.03-28.26)	4.00 (1.54-10.38)	6.39 (1.71-23.82)	4.98 (1.92-12.94)	7.96 (2.13-29.69)
IL7	0.72 (0.22-2.35)	1.78 (0.37-8.48)	0.71 (0.22-2.33)	1.76 (0.37-8.4)	0.60 (0.18-1.97)	1.49 (0.31-7.11)
IL8	0.42 (0.11-1.55)	0.60 (0.20-1.83)	0.02 (0.00-0.06)	0.02 (0.01-0.07)	0.87 (0.24-3.22)	1.25 (0.41-3.79)
IL10	7.32 (2.18-24.60)	19.03 (4.44-81.58)	5.87 (1.75-19.76)	15.28 (3.56-65.51)	7.5 (2.23-25.22.)	19.50 (4.55-83.62)
IL12 p35 *	2.66 (1.69-4.19)	5.28 (0.94-29.73)	1.59 (1.01-2.51)	3.17 (0.56-17.83)	-	
IL12 p40 *	1.46 (0.91-2.35)	4.41 (1.11-17.56)	0.81 (0.51-1.31)	2.46 (0.62-9.79)	-	
IL15*	3.47 (0.88-13.64)	8.55 (1.28-57.30)	0.55 (0.14-2.15)	1.35 (0.20-9.02)	-	
IL17*	2.59 (1.18-5.67)	8.53 (2.18-33.39)	3.34 (1.53-7.31)	10.99 (2.81-43.03)	-	
IL18	0.96 (0.49-1.89)	2.58 (0.65-10.24)	0.51 (0.26-0.99)	1.35 (0.34-5.37)	0.55 (0.28-1.07)	1.46 (0.37-5.81)
M-CSF	2.46 (1.0-6.02)	2.91 (0.86-9.77)	1.46 (0.60-3.58)	1.73 (0.51-5.81)	3.18 (1.30-7.80)	3.76 (1.12-12.65)
IFN GAMMA*	1.64 (0.97-2.76)	5.19 (9.77-21.13)	0.42 (0.25-0.70)	1.32 (0.32-5.36)	2.11 (1.25-3.57)	6.70 (1.65-27.27)
LT BETA	4.6 (2.9-7.28)	7.14 (3.41-14.93)	6.37 (4.02-10.08)	9.88 (4.72-20.67	3.75 (2.73-5.94)	5.82 (2.78-12.18)
TGF BETA	0.97 (0.19- 4.9)	3.14 (1.56-6.32)	1.10 (0.22-5.51)	3.53 (1.75-7.11)	1.73 (0.34-8.72)	5.58 (2.77-11.24)
TNF ALPHA	5.07 (1.69-15.23)	6.51 (2.36-17.97)	10.03 (3.34-30.10)	12.88 (4.67-35.52)	4.44 (1.48-13.34)	5.71 (2.07-15.74)
TNF BETA	10.61 (6.21-18.31)	16.93 (2.71- 105.86)	8.51 (4.98-14.53)	13.57 (2.17-84.87)	6.92 (4.05-11.81)	11.03 (1.76-68.98)

Relative expression is calculated using the formula $2^{-\Delta\Delta Ct}$. The range of expression is displayed in brackets and calculated using the formula above +/- ΔCt standard deviation.

* indicates samples where Ct>=35 in one or more of the test or calibrator patients

5.4.6. Cytokine expression profiles in autistic enterocolitis patients and controls using human RNA control as a calibrator.

Choosing an appropriate calibrator sample is of crucial importance in relative quantitation analysis. In this study the cytokine mRNA expression profiles of autistic enterocolitis cohort were compared with those in LNH (not associated with autism), ulcerative colitis and Crohn's disease controls as described above. Unfortunately as "normal" intestinal tissue was not available to analyse the expression profiles in each disease group, they were compared to that in the human RNA control from Applied Biosystems. The following cytokines were significantly expressed in the RNA control; IL1- α, IL-7, IL-8, IL-10, LT-β, TGF-β, TNF-α and TNF-β. The average ΔCt for each of these cytokines in the disease groups was calculated and the relative quantities compared with the RNA control sample was determined as described previously. These relative expression profiles in each disease group; autistic enterocolitis, Crohn's disease, ulcerative colitis and LNH (not in autism) are shown in figure 5.8 and the range of expression given in table 5.4. Cytokines IL-10, TGF-β, TNF-α and TNF-β were all significantly down regulated in each of the disease groups (i.e. there was a fold change greater than 4). IL-8 was up regulated in Crohn's disease but down regulated in all the other groups.

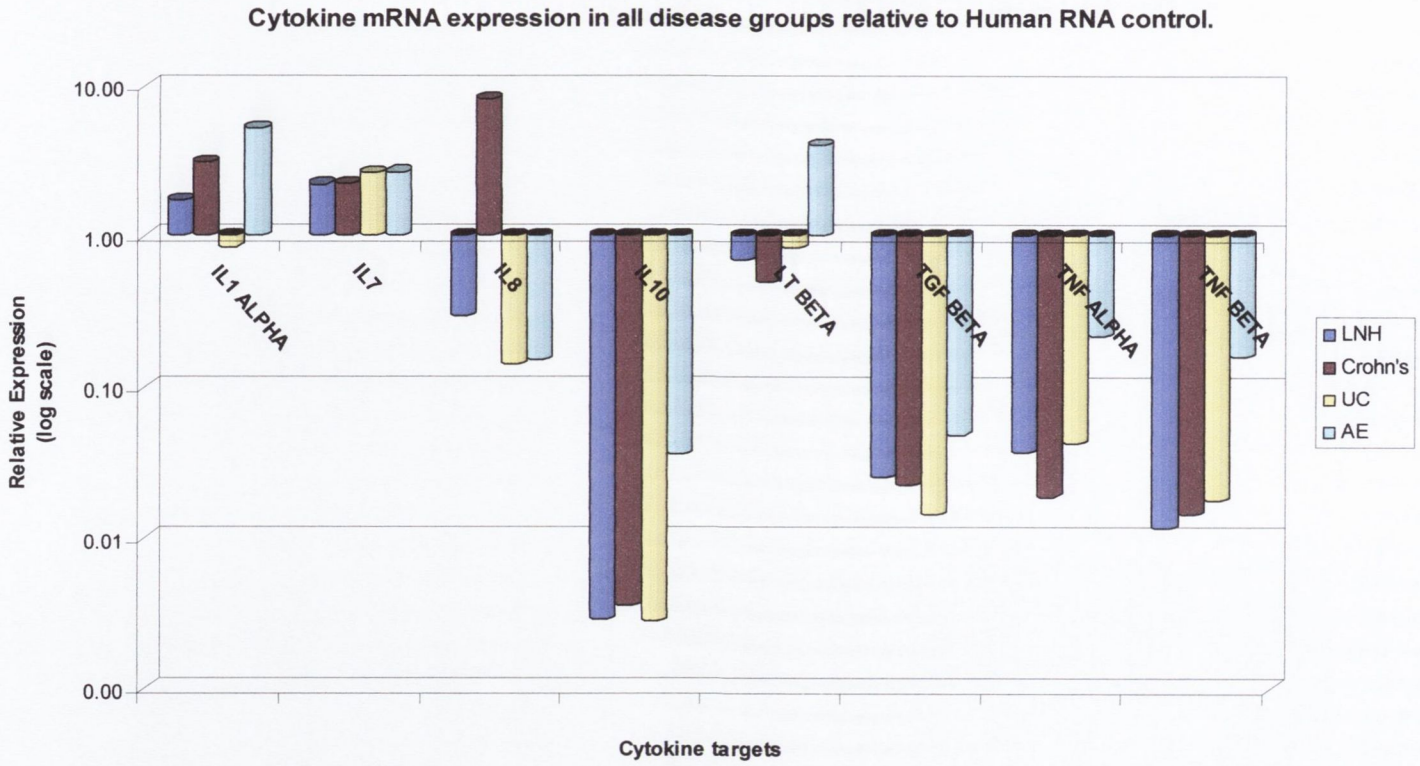


Figure 5.8. Relative cytokine expression profile in the intestinal tissues of patients with autistic enterocolitis (n=7), Crohn's disease (n=5), ulcerative colitis (n=5) and lymphonodular hyperplasia (not in autism) (n=4) expressed relative to the cytokine profile in human control RNA (Applied Biosystems).

Table 5.4. Relative cytokine expression profiles in each disease group expressed relative to human control RNA (Applied Biosystems).

Targets	LNH n=4	UC n=5	CD n=5	AE n=7
IL1 ALPHA	1.73 (1.2-2.48)	0.85 (0.11-6.80)	3.12 (3.04-3.2)	5.22 (1.41-19.34)
IL7	2.23 (0.82-6.06)	2.66 (0.87-8.15)	2.24 (1.46-3.4)	2.68 (0.67-10.73)
IL8	0.30 (0.11-0.83)	0.14 (0.008-2.47)	8.18 (2.71-24.7)	0.15 (0.05-0.46)
IL10	0.003 (0.001-0.01)	0.00 (0.000-0.03)	0.003 (0.003-0.004)	0.04 (0.01-0.14)
LT BETA	0.69 (0.16-2.95)	0.84 (0.108-6.54)	0.50 (0.11-2.16)	4.06 (2.16-7.63)
TGF BETA	0.03 (0.02-0.04)	0.01 (0.004-0.05)	0.02 (0.02-0.03)	0.05 (0.01-0.17)
TNF ALPHA	0.04 (0.01-0.11)	0.04 (0.003-0.53)	0.02 (0.01-0.03)	0.22 (0.08-0.58)
TNF BETA	0.01 (0.003-0.05)	0.02 (0.002-0.2)	0.01 (0.02-0.12)	0.16 (0.04-0.63)

Relative expression is calculated using the formula $2^{-\Delta\Delta Ct.}$ The range of expression is displayed in parenthesis and calculated using the formula above +/- ΔCt standard deviation.

5.4.7. Cytokine expression profiles in control patients.

As mentioned above the choice of calibrator greatly influences the results of a relative quantitation experiment, therefore to explore all aspects of the expression profiles generated for the autistic enterocolitis cohort, the control groups were compared to each other. Cytokine expression profiles in the ulcerative colitis cohort were expressed relative to Crohn's disease and LNH (not in autism). The profile in Crohn's disease was expressed relative to LNH (not in autism) (Figure 5.9 table 5.5). IL1- α, IL1-β, IL-8 and IFN -γ were down regulated in ulcerative colitis compared with Crohn's disease, while IL1-β, IL-8, IL-15 and IFN-γ were up regulated in Crohn's disease compared with LNH. There was no difference in the cytokine expression profiles between the ulcerative colitis and LNH (not associated with autism) patients.

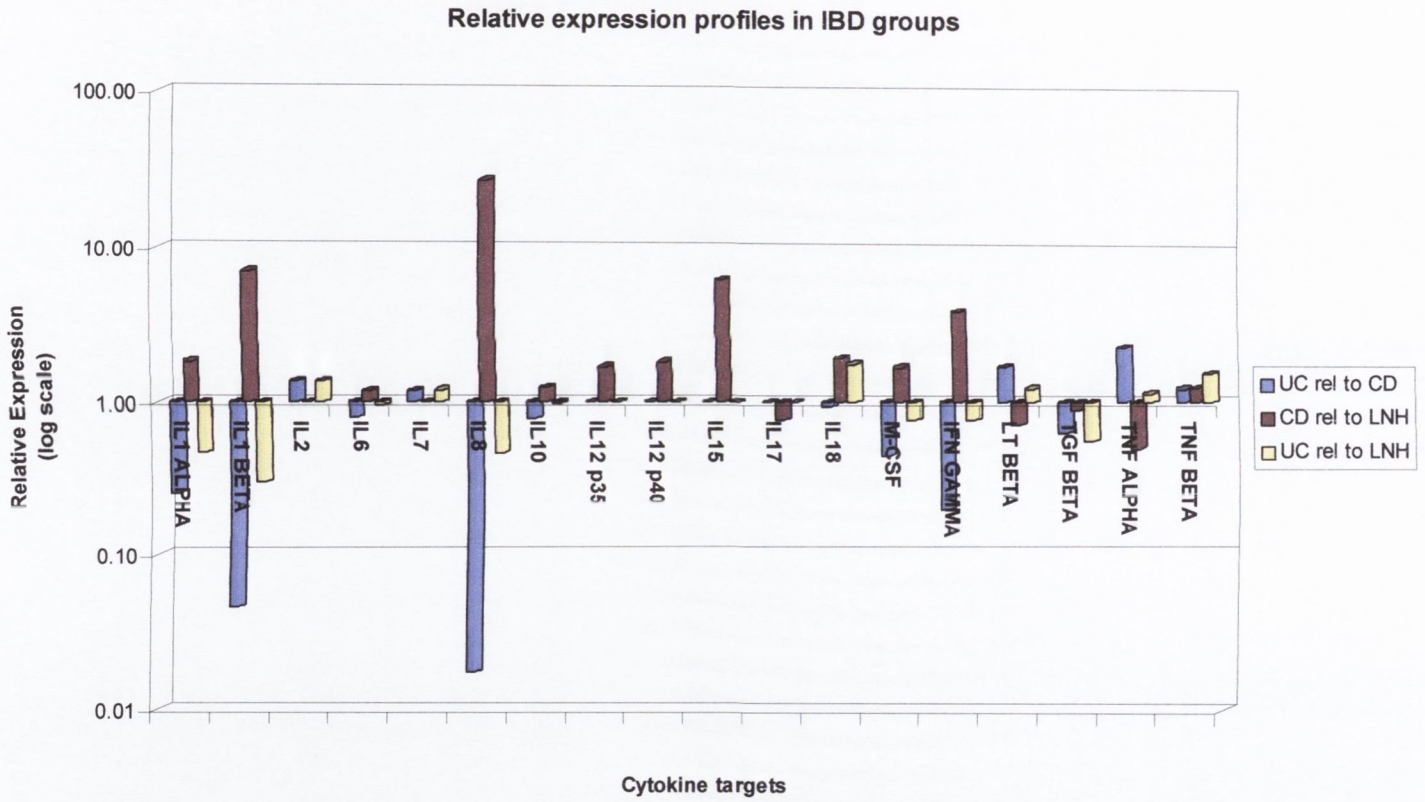


Figure 5.9. Comparison of cytokine expression profiles in the control disease groups; ulcerative colitis (n=5) expressed relative to Crohn's disease (n=5), and LNH (not in autism) (n=4), and Crohn's disease expressed relative to LNH (not in autism).

Table 5.5. Relative expression profiles across different IBD groups.

	UC relative to CD	CD relative to LNH	UC relative to LNH
IL1 ALPHA	0.27 (0.03-2.18)	1.80 (1.76-1.85)	0.49 (0.06-3.94)
IL1 BETA	0.04 (0.002-0.79)	7.04 (4.47-11.09)	0.31 (0.02-5.55)
IL2	1.36 (0.26-6.98)	1.00 (0.58-1.73)	1.36 (0.27-7.02)
IL6	0.80 (0.04-14.96)	1.19 (0.43-3.25)	0.95 (0.65-17.75)
IL7	1.19 (0.39-3.64)	1.01 (0.66-1.54)	1.19 (0.39-3.66)
IL8	0.02 (0.001-0.3)	27.63 (9.14-83-48)	0.48 (0.03-8.33)
IL10	0.78 (0.078-7.8)	1.25 (1.12-1.38)	0.98 (0.1-9.72)
IL12 p35	NA	1.67 (0.87-3.18)	NA
IL12 p40	NA	1.79 (1.02-3.15)	NA
IL15	NA	6.35 (3.38-11.95)	NA
IL17	NA	0.78 (0.10-6.33)	NA
IL18	0.92 (0.25-3.37)	1.91 (1.62-2.24)	1.76 (0.48-6.42)
M-CSF	0.46 (0.07-2.95)	1.68 (1.5-1.89)	0.77 (0.12-4.96)
IFN GAMMA	0.20 (0.03-1.24)	3.94 (2.93-5.30)	0.77 (0.12-4.88)
LT BETA	1.70 (0.21-13.19)	0.72 (0.17-3.15)	1.23 (0.16-9.53)
TGF BETA	0.63 (0.18-2.24)	0.89 (0.59-1.34)	0.56 (0.16-1.99)
TNF ALPHA	2.26 (0.18-28.07)	0.51 (0.32-0.79)	1.14 (0.09-14.2)
TNF BETA	1.23 (0.11-13.68)	1.2 (0.15-10.13)	1.53 (0.14-17.07)

Relative expression is calculated using the formula $2^{-\Delta\Delta Ct}$. The range of expression is displayed in brackets and calculated using the formula above +/- ΔCt standard deviation.

NA-Not applicable, insufficient target for accurate comparison.

5.4.8. Cytokine mRNA analysis in PBMCs from autistic enterocolitis patients

The expression profiles of 24 inflammatory and proinflammatory cytokines were examined in peripheral blood mononuclear cells for 14 age and sex matched patients and controls (table 5.1) consisting of 6 autistic enterocolitis patients, 4 autistic patients with no gut pathology and 4 normal control patients. 500 ng of total RNA extracted from PBMC samples was converted to cDNA and used as template in a multiplex TaqMan PCR for detection of the VIC labelled 18S rRNA endogenous control and the FAM labelled cytokine mRNA target. The amplification threshold was set at 0.06 for both the VIC dye layer and the FAM dye layer in all cards. The 18S rRNA was successfully amplified in all samples with Ct's ranging from 4-6 cycles. Four replicates of each cytokine target were amplified in each cDNA sample. The amount of the individual targets in each well was normalized to the endogenous control amplified in that well by calculating the ΔCt as described above. Relative cytokine expression profiles were expressed relative to the average profile for the normal control group (n=4) as described above. The overall expression profiles for each disease group relative to the normal control group are shown in Table 5.6 and Figure 5.10. The range of expression for each target within the disease groups was determined using the standard deviations calculated from the individual patient ΔCt's.

Table 5.6. Cytokine mRNA expression profiles in PBMC samples compared with normal controls.

Cytokines	Autistic enterocolitis (Neg MV) (n=2)	Autistic enterocolitis (Pos MV) (n=4)	Autism with no gut pathology (n=4)
IL1 ALPHA*	0.33	4.18 (0.66-26.36)	2.17 (0.22-21.7)
IL1 BETA	0.21 (0.09-0.49)	1.18 (0.29-4.82)	0.90 (0.42-1.93)
IL4*	0.28 (0.05-1.60)	0.71 (0.22-2.26)	0.10 (0.01-0.87)
IL6 *	1.51* (0.70-3.24)	6.02 (1.68-21.57)	3.04* (0.23-41.16)
IL7	1.12 (0.89-1.43)	1.23 (0.61-.2.51)	0.48 (0.22-1.06)
IL8	0.04 (0.00-1.74)	2.62 (0.58-11.76)	0.87 (0.09-8.31)
IL10	1.31 (0.53-3.23)	1.78 (0.84-3.78)	1.18 (0.41-3.39)
IL12 p35	0.61 (0.31-1.17)	1.29 (0.55-3.04)	0.90 (0.47-1.74)
IL15	0.81 (0.51-1.29)	1.28 (0.57-2.84)	0.49 (0.24-1.02)
IL18	0.96 (0.56-1.63)	1.26	0.05 (0.00-4.12)
M-CSF	0.38 (0.07-2.07)	0.70 (0.26-1.94)	0.85 (0.27-2.66)
IFN GAMMA	0.95 (0.74-1.23)	2.98 (1.18-7.51)	1.80 (0.53-6.18)
LT BETA	0.78 (0.70-0.87)	1.02 (0.55-1.86)	0.73 (0.44-1.23)
TGF BETA	0.68 (0.54-0.86)	1.25 (0.44-3.51)	0.71 (0.50-1.00)
TNF ALPHA	0.54 (0.54-0.55)	2.03 (0.84-4.88)	1.39 (0.76-2.53)
TNF BETA	1.00 (0.91-1.09)	1.81 (1.00-3.26)	1.66 (1.46-1.89)

Relative expression is calculated using the formula $2^{-\Delta\Delta Ct.}$ The range of expression is displayed in brackets and calculated using the formula above +/- ΔCt standard deviation.

* indicates samples where Ct ≥ 35 one or more of the test patients.

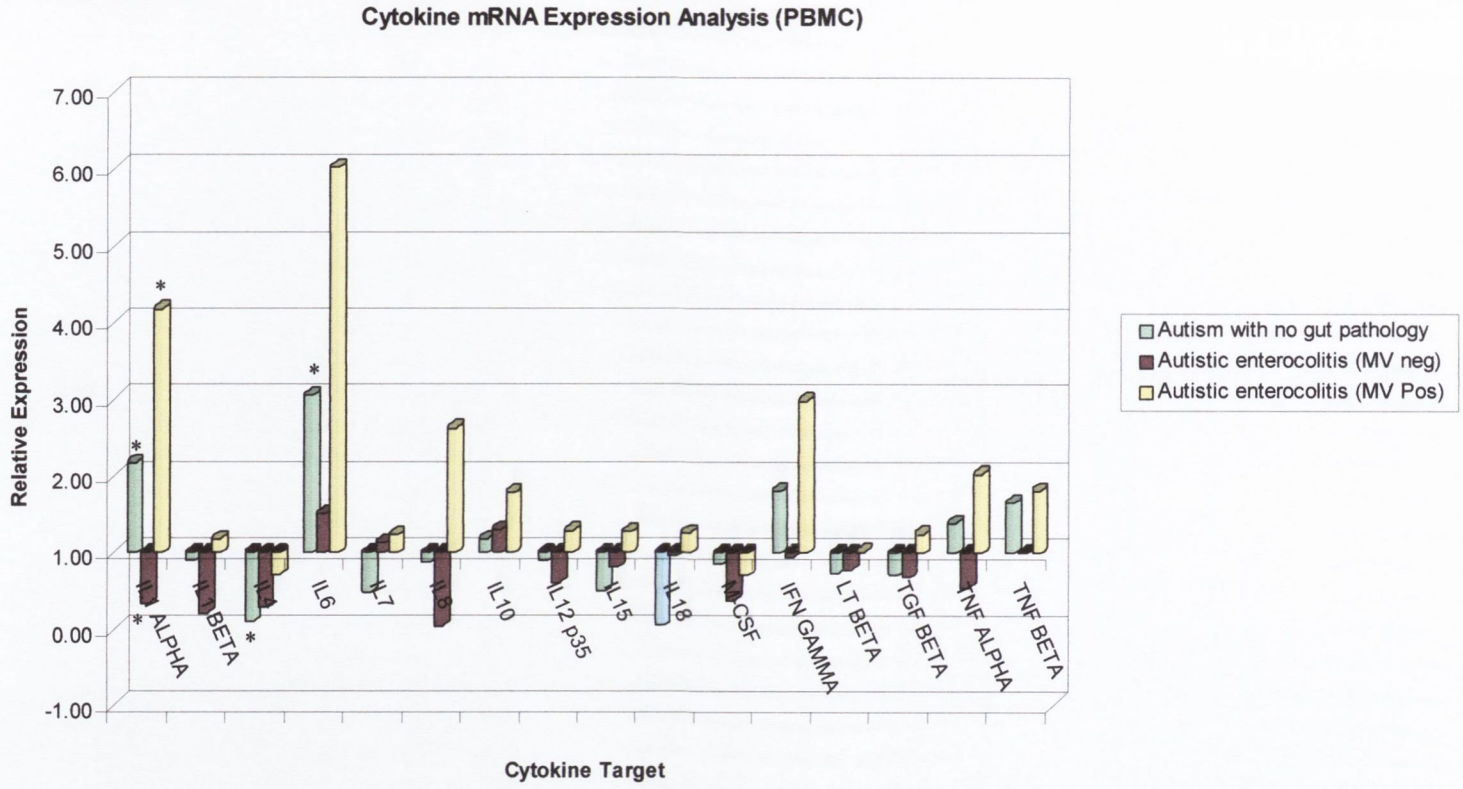


Figure 5.10. Relative cytokine expression profile in PBMCs from six cases of autistic enterocolitis (four were positive for measles virus, and two were negative for measles virus) and autistic patients with no gut pathology (n=4) were expressed relative to the average cytokine profile of normal controls (n=4).

* Indicates cytokine targets where either at least one of the tests sample had Ct ≥ 35 and therefore accurate comparisons was not possible

There was no amplification of cytokines IL-2, IL-3, IL-5, IL12p40, IL-13, IL-17, G-CSF and GM-CSF in any of the PBMC samples tested. Cytokines IL-1α, IL-4 and IL-6 did not amplify in some patients and therefore accurate comparison across the disease groups was not possible, these cytokines are marked with asterisks in Table 5.6. Cytokines with a fold change greater than 4, which is equivalent to a two cycle difference between the delta Ct from a test and calibrator sample were considered significant. These are highlighted in table 5.6. In general there was no significant differential regulation of cytokines observed in PBMC samples from any of the patient cohorts with the following exceptions; IL-6 was up regulated in the autistic enterocolitis cohort that were positive for measles virus, and IL-8, was down regulated in the autistic enterocolitis cohort that were negative for measles virus.

5.5 Discussion.

In this study we describe the mRNA expression profiles of 24 inflammatory and pro-inflammatory cytokines observed in the intestinal tissue from patients with autistic enterocolitis (new variant inflammatory bowel disease) compared with lymphonodular hyperplasia (not associated with autism), Crohn's disease and ulcerative colitis controls and in the peripheral blood of autistic enterocolitis compared with autistic patients without gut pathology and developmentally normal controls. The results indicate a pattern of immunological dysregulation in the terminal ileum of patients with autistic enterocolitis that is not as apparent in the peripheral blood of these patients.

Analysis of cytokine gene expression is becoming increasingly common, as tissue samples are often too small to allow quantification of cytokines at the protein level. In this study real time quantitative RT PCR was performed using TaqMan Human Cytokine microfluidics cards to investigate the expression profiles of 24 cytokines in an individual cDNA sample. This technology relies on relative quantitation of specific cytokines using the comparative Ct method (Livak *et al.*, 2001), whereby the amount of cytokine target was normalized to the 18s rRNA endogenous control housekeeping gene, and expressed relative to a calibrator sample. This housekeeping gene is ubiquitously expressed in all tissues and has been shown to be relatively stable in human tissues under varying experimental conditions (Schmittgen *et al.*, 2000). Amplification of the endogenous control and cytokine targets were performed in a multiplex PCR under primer-limited conditions to avoid competition between the target gene and the 18S rRNA endogenous control. Furthermore, as relative quantitation using the comparative Ct method is dependant upon similar PCR

amplification efficiencies for both the target and the endogenous control genes (Livak *et al.*, 2001; Giulietti, *et al.*, 2001), the linearity and efficiency of the 18S and a selection of target gene assays were confirmed over a range of RNA concentrations (Figure 5.1).

This present study was undertaken to evaluate the repertoire of cytokines involved in autistic enterocolitis compared with IBD and non-IBD controls. As discussed in chapter 1, parental reports have linked the onset of developmental regression with gastrointestinal symptoms and adverse reactions to environmental factors including viral infection, vaccination and dietary antigens. In the study, increased mRNA expression of pro-inflammatory cytokines IL-1, IL- 6, and TNF-α and the anti-inflammatory cytokine IL-10 in the intestinal tissue of autistic patients when compared with IBD and LNH controls (Table 5.3) was demonstrated. In peripheral blood IL-6 mRNA was more subtly increased in autistic enterocolitis patients and autistic patients without gut pathology when compared with normal children (Tables 5.6). The results suggest an abnormal innate immune response in the intestine of these patients.

Supporting these findings Jyonouchi *et al.*, (2001) have demonstrated increased production of these pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) by enzyme linked immunosorbent assay (ELISA), in PBMCs from children with autistic disorders compared with healthy siblings and controls, following stimulation with LPS and dietary antigens (Jyonouchi *et al.*, 2002). Others have also reported increased production of IL-6 and TNF-α in peripheral blood from autistic children compared with normal children (Croonenberghs *et al.*, 2002). In the intestine,

DeFelice *et al.*, (2003), have recently reported no difference in levels of cytokines IL-6, IL-8 and IL-1β in children with developmental disorders. In this study the authors use organ culture and ELISA to measure cytokines, they describe no histological evidence of inflammation in these patients and appear to have grouped data from all biopsy sites (terminal ileum-rectum). The above studies have measured protein rather than mRNA, therefore direct comparison with our study is difficult. Nonetheless, overall the findings of increased production of pro-inflammatory cytokine proteins and mRNAs in autism are suggestive of an abnormal innate immune response.

Expression of cytokines TNF-α, IL1-α, IL-6, IL-10 and the chemokine IL-8 is regulated by the NF-κβ signalling pathway, and increased levels of NF-κβ transcription factor have been demonstrated in lamina propria cells from IBD patients in particular Crohn's disease (Schreiber *et al.*, 1998). In the intestinal mucosa NF-κβ is activated by numerous stimuli including TNF-α, IL-1, LPS and bacterial endotoxins, which induces proinflammatory cytokines, chemokines, cell adhesion and MHC molecules (Jobin *et al.*, 2003). Activated monocytes are the predominant source of TNF-α in IBD and recently mutations found in the NOD2/ CARD15 protein in these cells have been associated with Crohn's disease (Hugot *et al.*, 2001; Ogura *et al.*, 2001a). These mutations are thought to alter the interaction of bacterial components with these cells, resulting in altered NF-κβ activation and TNF-α signalling and ultimately sustained intestinal inflammation. In autistic enterocolitis the elevated expression of TNF-α, pro-inflammatory cytokines IL-6, and IL-1 may reflect an abnormal inflammatory response to bacteria in the intestine. In addition, mutations in the TNF-α promoter region on chromosome 6p21, which is

involved in binding of the NF-κβ transcription factor, have been associated with susceptibility and pathogenesis of IBD (van Heel *et al.*, 2002). To date there is no evidence of an association between autism and the chromosome regions 6p and 16q, however it would be interesting to investigate these regions in the autistic enterocolitis subgroup of autism.

The association between measles virus and gastrointestinal pathology was described in Chapter 3. The cytokine mRNA profiles observed in the autistic enterocolitis cohort were similar between patients with and without detectable persistent measles virus RNA in their intestinal tissues. The immune response in measles infection is characterised by an initial Th1 type immune response with increased production of IL-2 and IFN-γ followed by a delayed Th2 type response with elevated levels of IL-4 and suppression of IL-12. This is likely to contribute to the profound immunosuppression associated with measles infection (Schneider-Schaulies *et al.*, 2002a,b). The lack of a typical measles type immune response in the autistic patients that tested positive for the virus was not surprising, given the low levels of measles virus RNA persisting in dendritic cells and lymphocytes within the lymphoid follicles in the intestinal tissues from the autistic patients (Chapter 3). The persisting virus may however contribute to the dysregulated innate immune response through disruption of the NF-κβ signalling pathway. This hypothesis requires further investigation.

Interactions of viruses with their cellular receptors, or accumulation of viral proteins or RNA within the cell are known to activate NF-κβ signalling and induce an antiviral response via the well established IFN-β induced janus kinase/ signal

transducers and activators of transcription pathway (Santoro *et al.*, 2003). RNA viruses, TNF-α and Il-1β have recently been shown to activate NF-κβ signalling, and an antiviral innate immune response in a manner that is independent of interferon (Bose *et al.*, 2003). It is therefore possible that the elevated TNF-α and IL-1β observed in autistic enterocolitis may represent an antiviral innate immune response. Alternatively failure of measles virus to initiate an appropriate innate immune response may be responsible for the persistence of measles virus in the intestine. Recent studies have shown that measles virus fails to phosphorylate the IKK component of the NF-κβ complex in neuronal cells infected with virus (Fang *et al.*, 2002; Fang *et al.*, 2001). This has been suggested as a mechanism for viral persistence in neuronal cells, such as that seen in SSPE.

Several studies have shown that local production of proinflammatory cytokines such as IL-1, IL-6 and TNF -α are increased in inflammatory bowel disease and may be important for inducing and maintaining intestinal inflammation (Brown *et al.*, 2002; Nikolaus *et al.*, 1998; Reimund *et al.*, 1996). In this study TNF-α expression was increased between four and eight fold (range of relative expression 1.5-35) in intestinal tissue in the autistic enterocolitis cohort compared with IBD and non-IBD controls. Elevated levels of TNF-α mRNA and protein have been detected in serum, mucosa and stools from IBD patients by immuno PCR, immunohistochemistry and ELISA (Komatsu *et al.*, 2001; Murch *et al.*, 1993b; Braegger *et al.*, 1992). In addition, the role of TNF-α in intestinal inflammation is highlighted by the administration of monoclonal anti-TNF antibody which has been highly effective in treatment of IBD (D'Haens *et al.*, 1999).

Overall IL-6 was significantly increased between four and eight fold in the intestinal tissue (range of relative expression 1-29) and peripheral blood samples from the autistic enterocolitis cohort compared with IBD and non-IBD controls. In inflammatory bowel disease IL-6 is produced by activated macrophages, monocytes and epithelial cells (Kusugami *et al.*, 1995) in response to IL-1 and TNF, and stimulates growth and differentiation of activated B cells and T cells. This is known to induce hepatic release of acute phase proteins (C reactive protein, serum amyloid A protein, albumin) and control responses to tissue injury and inflammation (Brown *et al.*, 2002; Nielsen *et al.*, 2000). The source of IL-6 in this study has not been confirmed as whole biopsies were examined but epithelial cells in the intestinal mucosa and macrophages in the lamina propria may be stimulated by luminal flora that permeate the intestinal mucosal barrier and activate the NF-κβ signalling pathway as described above. The increased expression of IL-6 in the peripheral blood may arise from peripheral blood monocytes, which has been described in active IBD (Suzuki *et al.*, 1990) or may be related to migration of macrophages to the lymphatic system via the systemic system. Unfortunately, we did not have matched peripheral blood and biopsy data from the individual patients to compare systemic and intestinal levels of IL-6.

Inconsistent IL-6 production has been reported in patients with inflammatory bowel disease. A number of studies have shown elevated levels of IL-6 protein and mRNA correlating with histological inflammation in patients with Crohn's disease but not ulcerative colitis (Mahida *et al.*, 1991). While others report correlation with histological inflammation in ulcerative colitis patients but not in Crohn's disease patients (Ishiguro, 1999). Further confusion arises from studies that report enhanced

production of IL-6 in both Crohn's disease and ulcerative colitis (Brown *et al.*, 2002; Reimund *et al.*, 1996; Kusugami *et al.*, 1995). It is likely that reasons for this conflicting data relate to disease activity, the cell types investigated and the methods used to evaluate cytokines expression. However the general consensus is that IL-6 is increased in active IBD and in the context of autistic enterocolitis reflects the inflammatory nature of the intestinal condition.

The increased expression of proinflammatory cytokines may also be due to dysregulated secretion of the anti-inflammatory cytokine IL-10, which was increased in intestinal tissues from the autistic enterocolitis cohort compared with IBD and LNH controls. IL-10 cytokine produced predominantly by activated macrophages but also T and B cells, is known to inhibit the antigen presenting function of macrophages and their production of pro-inflammatory cytokines IL-1, IL-6 and TNF-α (Asadullah *et al.*, 2003). Similarly disruption of the IL-10 gene in mice leads to development of colitis (Kuhn *et al.*, 1993) with marked increases in concentrations of the IL-1β, IL-6, TNF-α and IFN-γ in the lesions (Berg *et al.*, 1996). This highlights the importance of IL-10 in controlling intestinal inflammation. In the present study elevated expression levels of both pro-inflammatory cytokines and the anti-inflammatory cytokine IL-10 were observed in the autistic enterocolitis group compared with IBD and non-IBD controls. Quantitation of cytokines is expressed relative to a calibrator sample and without *in vitro* stimulation. Alternative comparisons between ulcerative colitis and Crohn's disease showed no alteration in IL-10 expression (Table 5.5), while comparisons with human control RNA showed reduced expression of IL-10 (Table 5.4), highlighting the importance of the calibrator sample.

The majority of studies to date have examined IL-10 production in the lamina propria and it is well recognised that Crohn's disease is characterised by reduced production of bioactive IL-10 in these cells (Gasche *et al.*, 2000; Autschbach *et al.*, 1998). Similarily, low ileal concentrations of IL-10 mRNA have been associated with endoscopic recurrence of the disease after surgery (Meresse *et al.*, 2002). However, elevated IL-10 expression has been detected by immunohistochemistry in epithelial cells within the submucosa in IBD, while reduced expression has been detected in the lamina propria (Autschbach *et al.*, 1998). A more recent study by Melgar (2003) has reported increased levels of IL-10 mRNA in mucosal T cells, and corresponding reduced levels of IL-2, IFN-γ and TNF-α using real time PCR in lamina propria T cells from inflamed large intestinal mucosa and non inflamed terminal ileum from ulcerative colitis patients. In this study we examined mRNA extracted from whole tissue biopsies and therefore direct comparisons cannot be made.

The chemokine interleukin 8 was decreased in the intestinal tissue from autistic enterocolitis cohort compared with Crohn's disease patients (Table 5.3). Elevated expression of IL-8 mRNA was detected in Crohn's disease patients compared with ulcerative colitis and LNH. (Figure 5.3, 5.5). These findings are consistent with others reporting increased levels of IL-8 mRNA (detected by in situ hybridisation and RT-PCR) and protein have been detected in IBD that correlated with histological inflammation (Katsuta *et al.*, 2000; Nielson *et al.*, 1997; Ina *et al.*, 1997). IL-8 gene expression is also regulated by NF-κβ transcription factor (Jobin *et al.*, 2000). Chemokines recruit immune cells to inflammatory sites and cause inflammation by generating metalloenzymes and reactive oxygen radicals that cause tissue damage. IL-10 has been shown to inhibit IL-8 production in LPS stimulated PMN's (Wang *et*

al., 1994), which may explain the reduced IL-8 mRNA expression observed in the autistic enterocolitis patients.

Immune dysregulation is also a feature of autism. An imbalance of Th1 and Th2 like cytokines has been reported in PBMCs in autistic patients whereby the proportion of CD4+ and CD8+ T cells that produce IFN-γ and IL-2 were significantly reduced whereas those that produce IL-4 were elevated compared with controls (Gupta *et al.*, 1998). On the other hand the presence of autoantibodies to myelin basic protein and a possible association with viral infection triggered autoimmunity in the CNS mediated by Th1 cells has been reported (Singh *et al.*, 1998; Singh *et al.*, 1996; Singh *et al.*, 1993). In T cells IL-10 is known to down regulate the production of T helper 1 cytokines IFN-γ and IL-2, through the suppression of IL-12 synthesis in the antigen presenting cells, promoting the development of a Th2 cytokine pattern. Expression of IL-12 was significantly increased (fold change > 4) in terminal ileum biopsies from two of seven autistic enterocolitis patients (patients # 124 and 68), (both negative for measles virus by TaqMan RT PCR), compared with LNH, Crohn's disease and ulcerative colitis controls (Table 5.3, Figure 5.2-5.7). IFN-γ mRNA was also elevated in two of seven autistic enterocolitis patients (Patients 124 and 136) compared with controls. Overall there was no evidence of a Th2 cytokine pattern in the intestinal tissues or PBMCs from autistic patients examined in this study. This correlates with the Jyonouchi *et al.*, (2001) study in which the ratio of IFNγ/IL-5 produced by PBMCs in response to antigen stimulation did not differ between autistic and control groups.

The relative cytokine mRNA expression profiles observed in the autistic enterocolitis cohort relative to ulcerative colitis and LNH patients were greater than those observed in the comparison with Crohn's disease (Table 5.3). Ulcerative colitis unlike Crohn's disease, which commonly affects the terminal ileum, is a disease of the large intestine (Price, 1992). Terminal ileal tissue examined in this study from Crohn's disease patients showed evidence of active inflammation. It was therefore not surprising to find no differences in cytokine mRNA expression profiles in the comparison between ulcerative colitis and LNH (Table 5.5), whereas increased expression of cytokines IL-1β, IL-8, IL-15 and IFNγ was observed in the comparison between Crohn's disease and ulcerative colitis and Crohn's disease and LNH (Table 5.5), reflecting the inflammatory nature of Crohn's disease at this site. There was no evidence of a Th2 response in UC patients as suggested by Fuss *et al.*, (1996), however the tissues examined in this study were from non-inflamed regions of the intestine.

Two other related cytokines TNF-β and lymphotoxin β (LT-β) were upregulated in the terminal ileum tissues from autistic enterocolitis patients compared with IBD and non-IBD controls. Both cytokines belong to the TNF superfamily and there is little information in the literature regarding these cytokines in inflammatory bowel disease. Recent studies have reported expression of LT-β on CD4+ T cells in IBD (Agyekum *et al.*, 2003) and increased expression of TNF-β protein and mRNA have been described in TNBS (Trinitrobenzenesulfonic) acid induced inflammatory bowel disease in rats (Maric *et al.*, 2003).

In summary, this study reports for the first time the cytokine gene expression profiles in the intestine of children with new variant inflammatory bowel disease or "autistic enterocolitis". The results indicate dysregulation of pro-inflammatory cytokines (IL1, IL6 and TNF-α) and the anti-inflammatory cytokine IL-10 in the intestine of these patients, which demonstrates abnormal innate immune responses and intestinal inflammation. This abnormal innate immune response is likely to result in altered adaptive immune responses and contribute to the pathogenesis of autistic enterocolitis. Further analysis of cytokine expression profiles in individual cell types together with investigations into components of the NF-κβ signalling pathway will expand our understanding of this abnormal immune response.

Chapter 6

Genome wide expression profiling in IBD and autistic enterocolitis

6.1 Introduction.

Ulcerative colitis (UC) and Crohn's disease (CD) represent the two main forms of inflammatory bowel disease, although rarer forms do exist. Our group has described an apparently novel form of inflammatory bowel disease (new variant IBD) in children with an autistic spectrum of disorders (Martin *et al.*, 2002; Uhlmann *et al.*, 2002; Wakefield *et al.*, 2000; Wakefield *et al.*, 1998a). The intestinal pathology of this disorder includes ileocolonic lymphonodular hyperplasia and non-specific colitis, which appears to be neither Crohn's disease nor ulcerative colitis (Wakefield *et al.*, 2000). Intestinal symptoms include abdominal pain, bloating, constipation and diarrhoea (often alternating). In Chapter 3 an association between persistent measles virus infection and gut pathology in these children has been described (Martin *et al.*, 2002; Uhlmann *et al.*, 2002) and chapter 5 provides evidence of immunological dysregulation in the terminal ileum of these children.

UC and CD are considered two separate disease entities, although they share many non-characteristic features. The main clinical features common to both CD and UC include abdominal pain, rectal bleeding, diarrhoea, faecal urgency, weight loss and fatigue (Podolsky, 2002; Ferguson, 1994). The inflammatory disease of UC is limited to the superficial mucosal layer and is usually located in the colon or rectum. Conversely, the inflammatory response that occurs in CD is more extensive and can affect the entire gastrointestinal tract but more frequently the terminal ileum (Ferguson, 1994; Price, 1992). The aetiology of IBD remains unknown despite extensive research but it is possibly heterogenous since many factors are believed to be involved in its pathogenesis (Podolsky, 2002; Watts *et al*, 2002; Fiocchi 1998). It is suspected that environmental factors, such as smoking, microbial agents, use of

non-steroidal anti-inflammatory drugs, along with a genetic disposition are major potential causes of IBD (Podolsky, 2002; Fiocchi, 1998; Ferguson, 1994). However, there is increasing evidence of immunological dysregulation in IBD and this is fully discussed in chapter 1.

Mapping studies have suggested a strong inherited component may be associated with susceptibility to IBD. Genome wide scanning and candidate gene analysis have provided strong evidence that this association is likely to be polygenic involving interactions of numerous gene mutations (Ahmad *et al.*, 2001, Watts *et al.*, 2002) and appears to be more important in CD than in UC (Podolsky, 2002). Four regions, which have been widely replicated, known as polymorphic susceptibilty loci have been identified by linkage mapping and are suggested to be significant in the determination of disease susceptibility in CD mainly but occasionally in UC also. They are located on chromosome 16p12-q13 (IBD 1) (Ohmen *et al.*, 1996; Hugot *et al.*, 1996), 12p13.2-q24.1 (IBD 2) (Satsangi *et al.*, 1996), the major histocompatibility complex region on chromosome 6 (IBD 3, the HLA region) (Hampe *et al.*, 1999), chromosome 14q11-12. (IBD 4) (Ahmad *et al.*, 2001), and more recently on chromosome 5q31 (IBD5) (Giallourakis *et al.*, 2003).

Due to the complexity and heterogeneous nature of both UC and CD the majority of research to date on genes implicated in these diseases has focused primarily on inflammation and immune response genes. Conversely, research on new variant inflammatory bowel disease "autistic enterocolitis" is in its infancy and has focused predominantly on histopathological and endoscopic features of the disease. Microarray analysis is an emerging technology in the field of molecular biology and

allows simultaneous analysis of thousands of genes. Microarray expression profiling has only recently been applied to the study of inflammatory bowel disease (Lawrance *et al.,* 2001; Diecgraefe *et al.,* 2000) and these studies have expanded the list of differentially regulated genes known to be involved in inflammatory bowel disease. The use of this technology may also identify disease linked genetic mutations complementing linkage studies and help identify potential new therapeutic targets.

6.2 Study Aims

The aim of this study was to examine the repertoire of genes expressed in new variant inflammatory bowel disease "autistic enterocolitis", ulcerative colitis and Crohn's disease compared with normal patients using the high density Affymetrix oligonucleotide GeneChip arrays.

6.3 Materials and Methods.

6.3.1 Specimens.

For optimisation of RNA extraction protocols, intestinal tissue biopsies were obtained at endoscopy from 8 patients undergoing routine endoscopy for investigation of anaemia, cancer screening, diverticular disease and patients with irritable bowel syndrome. Patients were recruited through the Department of Gastroenterology, Adelaide & Meath Hospital. Biopsies were taken from six sites within the intestine including terminal ileum, caecum, ascending colon, transverse colon, descending colon, sigmoid and rectum. Biopsies were both snap frozen in liquid nitrogen and taken into RNA*later* (Ambion, Inc, 2130 Woodward Street, Austin, Texas 78744, USA) preservative for comparison of tissue collection and RNA extraction procedures.

For the GeneChip Array experiments terminal ileum biopsy material was obtained at endoscopy from the following paediatric patients: 4 histologically confirmed normal controls (median age; 10.5 yrs, range; 10-12 yrs, Males; 2), 7 patients with histologically confirmed autistic enterocolitis (AE) (median age; 7yrs, range; 3-15 yrs, males; 7), 4 patients with histologically confirmed Crohn's disease (CD) (median age; 12yrs, Range; 9-15 yrs, males; 2), and two patients with histologically confirmed ulcerative colitis (UC) (median age; 13.5yrs, range; 12-15 yrs, males; 0). Paediatric tissue samples were obtained from the Department of Gastroenterology, Royal Free Hospital, London. Two biopsies were taken from the same site, one was placed in formalin for routine histology and the other was snap frozen in liquid nitrogen and stored at –80 °C until required.

6.3.2 RNA extraction

For GeneChip analysis total RNA was extracted from each sample using the Qiagen RNeasy protocol for tissue specimens. Tissue biopsies were homogenised in RLT buffer using the Savant BIO 101 FastPrep instrument. RNA extracts were DNAase digested, pooled into groups (AE1, n=3; AE2, n=4; CD, n=4; UC, n=2) and cleaned up using Qiagen RNeasy clean up protocol. Prior to pooling TaqMan RT PCR for GAPDH and measles virus F gene was performed as described in chapter 3, autistic enterocolitis patients were pooled into two groups (AE1 and AE2) based on measles positivity. Pooled RNA was quantified by spectrophotometric analysis and only RNA with an $A_{260/280}$ ratio between 1.9 and 2.1 was used for the Affymetrix GeneChip Analysis. The integrity of the RNA extracts was checked by agarose gel electrophoresis. The Trizol method for extraction of total RNA followed by DNase digestion using the Qiagen DNase set was also evaluated in this study.

6.3.3 RNA labelling and hybridisation to GeneChip Microarrays.

Probe preparation was performed following the protocols in the GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA) and is fully described in section 2.16. Briefly double stranded cDNA was prepared from 5-8 μg of total RNA using the Invitrogen Superscript Choice System. The HPLC purified T7 –(dT)24 primer (Affymetrix, Santa Clara, CA) was used to prime the first strand cDNA synthesis reaction. The following is the sequence of this primer:

T7 –(dT) 24 primer

5' GGCCAGTGAAATTGTAATACGACTCACTATAGGGAGGCGG- (dT)24 3'

The double stranded cDNA was extracted by phenol/chloroform/isoamyl alcohol and ethanol precipitation. *In vitro* transcription and labelling was performed with double stranded cDNA using the BioArray High Yield RNA Transcript Labelling Kit (Enzo Diagnostics, Farmingdale, NY). Biotin labelled cRNA was purified using Qiagen RNeasy clean up protocol (Qiagen, Crawley, West Sussex, UK). cRNA was quantified by UV spectrophotometry. Typical yields of labelled cRNA target were in the range of 23-80μg.

6.3.4 Hybridisation of cRNA to Affymetrix GeneChips.

Twenty micrograms of cRNA was fragmented randomly, by incubating at 94 °C for 35 minutes in 1 X fragmentation buffer (5X Stock: 40 mM Tris acetate (pH 8.1), 125 mM KOAc, 30 mM MgOAc). 15 μg of fragmentated cRNA was added to 300 μl of hybridisation cocktail (1X hybridisation buffer: 10 mM MES, 1M Na^+, 20 mM EDTA, 0.01 % Tween 20, 50pmol of control oligonucleotide B2, 1 X Eukaryotic hybridisation controls, 0.1 mg/ml Herring sperm DNA, 0.5 mg/ml BSA). The cocktail was heated to 99°C for 5 minutes, 45 °C for 5 minutes and hybridised to U133A GeneChip arrays. Hybridisation was performed at 45 °C for 16 hours in a GeneChip rotating hybridisation oven. After hybridisation each chip was washed and stained with streptavidin phycoerythrin, followed by signal amplification with biotin labelled anti-streptavidin phycoerythrin antibodies and re-staining with streptavidin phycoerythrin using the Affymetrix Fluidic station 400. Fluorescent signals were measured on the arrays using the Affymetrix GeneArray laser scanner (Hewlett Packard).

In all cases, cRNA was hybridised to a test array supplied by the manufacturer prior to using the human U133A GeneChip. This was to confirm the RNA was of sufficient quality to detect gene expression in the different tissue samples and to assess the hybridisation quality and array performance.

6.3.5 Analysis of GeneChip Data.

The scanned images were visually inspected using the criteria provided by Affymetrix to assess uniform hybridisation and an acceptable level of noise. Scanned files were initially analysed using MAS 4.0 software. Global scaling was applied to all arrays, which uses all probes to set the average intensity to an arbitary target intensity of 150 as recommended. Each array was analysed using the absolute call analysis software and then by comparative analysis to compare each disease group with the control group as a baseline. The absolute call yielded an average fluoresence difference between the perfect match and mismatch probes for each probe set and an absolute call of absent or present for that transcript. The comparative analysis provided a ratio of the average fluorescence difference of the experimental sample to the control sample and is expressed as a signal log ratio. A signal log ratio of one is equivalent to a two-fold increase in expression of a particular target. In this study a transcript with a fold change than greater than 2 or less than -2 was considered significant.

Acuity 2.0 (Axon Instruments, Inc, USA) was used for further analysis and clustering of the data. The web based Affymetrix Netaffx™ Analysis Centre was used to correlate GeneChip results and gene annotations. Hierarchial clustering was

performed by the method of Eisen *et al.*, (1998). Additional statistical analysis was performed using SPSS software version 11.

6.4 Results

6.4.1 RNA for Gene Expression analysis.

For gene expression analysis the concentration and quality of the extracted RNA is essential for the overall success of the microarray experiment. As part of this study the optimal method for collection and extraction of high quality RNA from intestinal tissue biopsies was determined.

Intestinal pinch biopsies were collected at endoscopy into RNA*later* and an additional biopsy was snap frozen for comparison. Total RNA was extracted using the Qiagen RNeasy and Trizol extraction methods. Total RNA extracted from biopsies taken directly into RNA*later* was of superior quality than those snap frozen in liquid nitrogen (Figure 6.1).

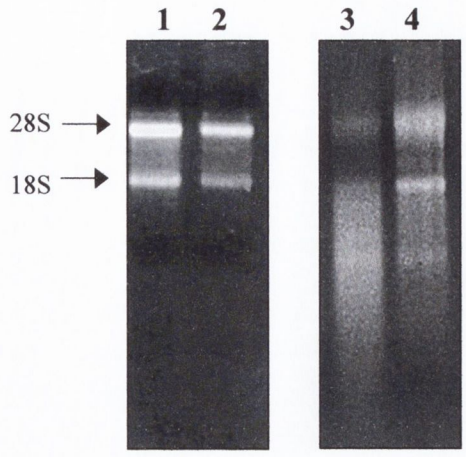


Figure 6.1. Agarose gel electrophoresis of RNA extracted from intestinal tissue biopsies using Qiagen RNeasy after collection in RNA*later* and snap freezing in liquid nitrogen.

Lanes 1 and 2 represent RNA (~1μg) extracted from terminal ileum and caecum tissue biopsies collected into RNA*later* from 2 different patient controls. Lane 3 and 4 represent RNA extracted as above from ileum and transverse tissue biopsies snap frozen at endoscopy from 2 different patient controls. RNA extracted from biopsies taken into RNA*later* showed little or no degradation compared with the smearing observed for snap frozen specimens.

To determine the optimal method for extraction of high quality RNA from intestinal tissue, total RNA was extracted from tissue biopsies collected in RNAlater using two different methods, Qiagen RNeasy (n= 23) and Trizol (n=23), as described in section 2.2. The concentration, purity and integrity of the RNA extracts were determined by spectrophotometry and gel electrophoresis (Figure 6.2A). The Trizol method selects for all RNA species, including 28S, 18S rRNA, 5S rRNA and smaller tRNAs. The Qiagen method selects for the larger rRNA species, 28S and 18S, but often the smaller 5S rRNA and tRNA species are not extracted. The housekeeping gene GAPDH was measured in the total RNA extracts using quantitative TaqMan RT-PCR as described in section 2.8. GAPDH copy numbers obtained using both RNA extraction procedures were compared as shown in figure 6.2B. GAPDH yields were significantly better with RNeasy kit than with Trizol ($p<0.0001$). A range of 0 - $1.2x10^7$ copy numbers of GAPDH were obtained using the Qiagen RNeasy protocol compared to a range of 0 - $5x10^4$ copy numbers of GAPDH for the Trizol protocol.

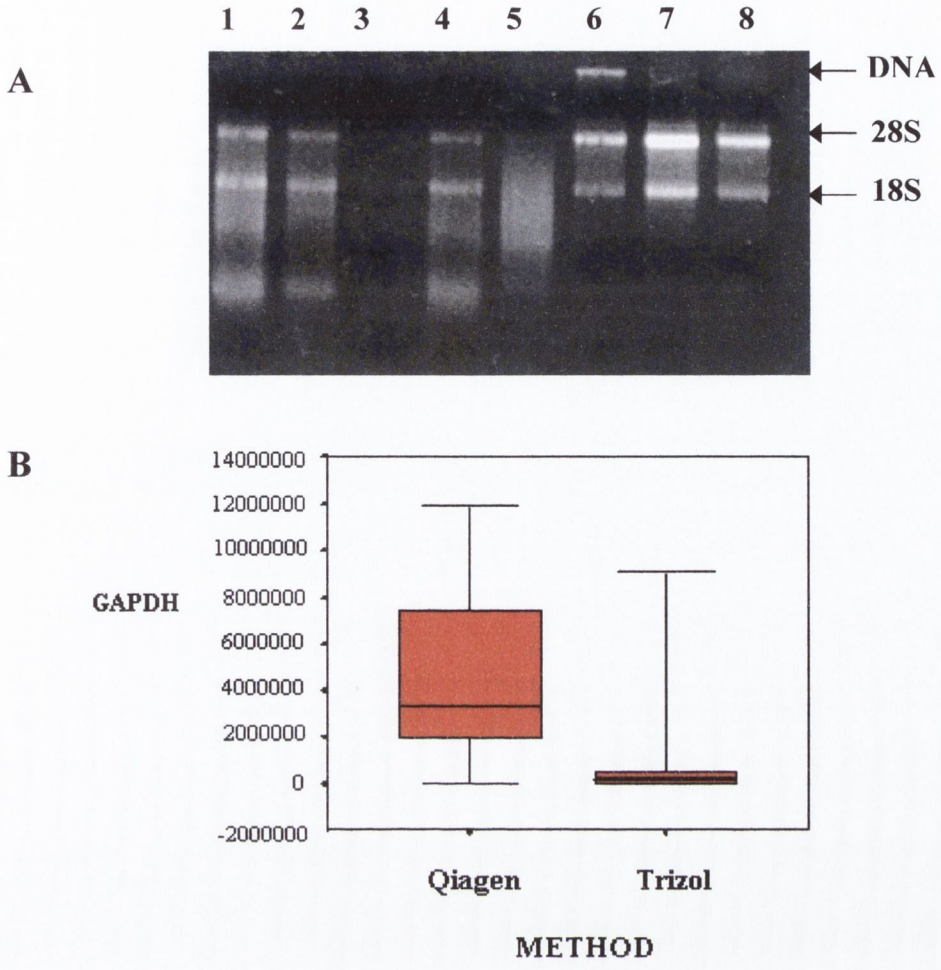


Figure 6.2. Comparison of RNA extracted using QIAGEN and Trizol RNA extraction procedures

A. Agarose gel electrophoresis of total RNA extracted from intestinal tissue biopsies using Qiagen and Trizol extraction protocols.

Lanes 1-4: Total RNA extracts from four patients, and the following biopsy sites (Ascending colon, transverse colon, descending colon and rectum respectively), extracted using Trizol extraction method. No RNA visible in lane 3, spectrophotometry analysis of this sample showed very low yields of RNA.

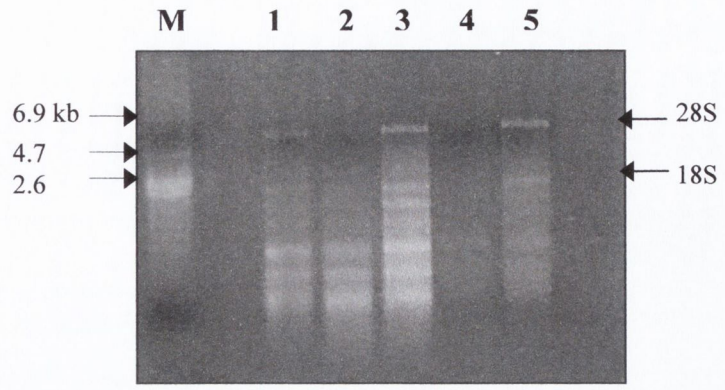
Lanes 5-8: Total RNA extracts from four patients, and four biopsy sites (Descending colon x 2, transverse colon x 2), extracted using Qiagen RNeasy extraction method. Lane 5 RNA is shown to be degraded, the OD 260/280 ratio of this sample was also low, 1.6.

B. A boxplot of GAPDH copy number in RNA extracted from tissue biopsies using two different extraction methods, Qiagen RNeasy and Trizol ($p<0.000$).

For gene expression analysis using Affymetrix GeneChips a minimum of 5μg of total RNA is recommended. For this reason RNA extracted using Qiagen RNeasy protocol from terminal ileum tissue of the paediatric patients, was pooled into disease groups. Pooled RNA was DNAase digested and purified using the Qiagen RNeasy clean up protocol. Prior to pooling the RNA samples, expression levels of the GAPDH housekeeping gene and measles virus F gene were measured by quantitative TaqMan RT PCR. Autistic enterocolitis patients positive for measles virus were pooled into one group (AE2) while samples negative for measles virus F gene were pooled into a separate group (AE1). An aliquot of the pooled RNA was analysed on a 1% agarose gel (Figure 6.3A) and quantified by spectrophotometry. Figure 6.3B shows the concentrations and purity of the pooled RNA samples used in this study. The integrity of the 28S and 18S rRNA bands in the pooled RNA extracts was not ideal. Ribosomal RNA bands were intact in all samples with the exception of AE2, however there was degradation of the RNA represented by the smearing and smaller sized RNAs.

For this study we were limited with the material that was available for analysis. The majority of the terminal ileum biopsies from the paediatric patients had been snap frozen and stored at –80 ^{o}C for up to two years prior to RNA extraction. However, as the $OD_{260/280}$ ratios of the pooled RNAs were within the recommended guidelines, the quality of the samples for GeneChip analyis were tested on the Affymetrix test arrays prior to hybridisation on the Affymetrix high-density arrays (section 6.3.2. below).

A



B

RNA	Concentration	$A_{260/280}$ ratio
AE1	834 ng/µl	1.9
AE2	880 ng/ul	2.1
CD	904 ng/µl	2.0
UC	548 ng/µl	1.9
Normal	500 ng/µl	2.1

Figure 6.3 Pooled Total RNA extracts from terminal ileum tissue biopsies for GeneChip Analysis.

A. Denaturing agarose gel electrophoresis of pooled RNA extracts, (0.5-1µg per lane). Lanes 1-5, correspond to AE1, AE2, CD, UC and Normal. M represents 2 µg of RNA molecular weight marker II (Roche). There is some degradation of RNA as indicated by smearing and smaller sized RNAs. Nothing is visible on this gel for UC, however it was electrophoresed again on a separate gel and was shown to be of similar quality to the other RNA samples.

B. The table shows the concentrations and $OD_{260/280}$ ratios of the pooled RNA extracts. All samples with ratios between 1.9 and 2.1 were taken to be free of contaminating material.

6.3.2 Microarray Quality Control.

Double stranded cDNA synthesised from 5-8 μg of total RNA (AE1, AE2, CD, UC and Normal pooled RNAs) were labelled with biotin UTP in an *in vitro* transcription reaction with T7 RNA polymerase. The cDNA and biotin labelled cRNA were checked by gel electrophoresis and spectrophotometry to estimate the yield and size distribution of labelled transcripts (Figure 6.4). Prior to hybridisation labelled cRNA was fragmented to produce fragments ranging from 50-200 bases (Figure 6.4).

To examine variability in hybridisation quality among the arrays, four control bacterial and phage gene cRNAs BioB, BioC, BioD and Cre were analysed on each microarray. All four genes were present according to the Affymetrix absolute call criteria. The quality of the *in vitro* transcription and cDNA synthesis reactions were assessed on an Affymetrix test array by comparing the 3' and 5' expression ratios of housekeeping genes actin and glyceraldehyde-3-phosphate dehydrogenase for each sample. The Affymetrix test array contains probe sets designed to the 3' end, the middle and the 5'end regions of the GAPDH and actin housekeeping genes (Figure 6.5A). The 3' end probe set signal intensity is compared to the 5' end probe set signal intensity to calculate a 3'/5' ratio and evaluate the efficiency of the transcription reaction. Figure 6.5B shows the ratios for each sample used in this study. Affymetrix recommend ratios below 3 for GAPDH and below 6 for actin. All five cRNA preparations were within the recommended guidelines indicating that RNA was of sufficient quality and integrity for efficient transcription and that hybridisation was optimal.

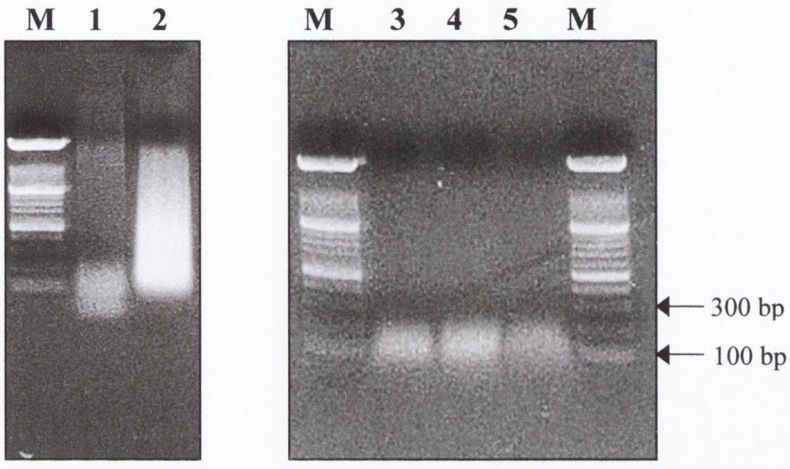
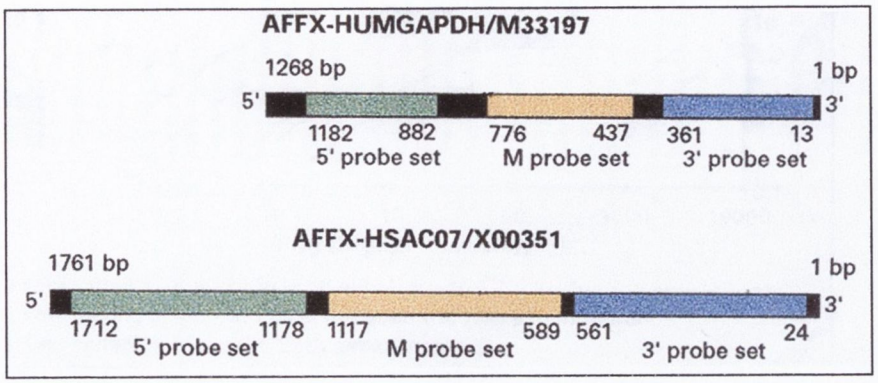


Figure 6.4 Agarose gel (1%) electrophoresis of double stranded cDNA (1µl) synthesised from 5 µg of pooled RNA AE1 (lane 1) and corresponding biotin labelled cRNA (2µg) (lane 2). Lanes 3, 4 and 5 show fragmented cRNA (500ng) prepared from biotin labelled cRNA generated from UC, AE1 and AE2 respectively. M represents 100 bp molecular weight ladder (Roche Diagnostics).

A.



B.

Chip name	Background	Av. Signal	GAPDH	Actin
AE1	55.82	389.3	1.7	2.7
AE2	63.39	508.7	3.2	6.2
Crohns	54.97	347.5	3.0	5.3
Normal	56.54	141.9	1.4	2.0
UC	64.69	409	2.6	5.8

Figure 6.5 GeneChip Test Array Quality Control.

A. Diagram of the 3', middle and 5' probe set positions for Human GAPDH and Actin on the Affymetrix test array, defined by numbered regions starting at the 3' end.

B. The table above provides a summary of the test array quality control metrics. The background values were all within the 20-100 range as recommended. The average signal ranged from 140-508 and data was scaled to normalise for this. The GAPDH 3'/5 probe set ratios were all within the recommended range of 3, with the exception of AE2 which was slightly higher at 3.2. Similarly actin 3'/5' ratios were within the recommended range of 6 with the exception of AE2 which was slightly higher at 6.2.

6.4.3 Gene Expression Results

To identify changes in gene expression patterns among the three IBD groups compared with control tissue we hybridised biotinylated cRNA from the pooled RNA samples (AE1, AE2, UC, CD and Normal) to the Affymetrix U133A GeneChips. The U133A Genechip contains >22,000 probe pairs encoding 18,400 transcripts. The Affymetrix absolute analysis algorithm uses the probe pair intensities to generate a detection p value and an average fluorescence difference for each gene target. The software then assigns a call of absent, present, or marginally present for each transcript level in the target sample (AE1, AE2, CD, UC and Normal) based on this p value as discussed in section 2.16. Marginal calls were considered absent. The total numbers of genes that were present according to the absolute call criteria were as follows: 4618 (AE1), 5121 (AE2), 9059 (CD), 4440 (UC) and 10,147 (Normal). To further analyse the data all probe sets designated as absent by the Microarray Suite software were discarded. To determine the variation in signal intensities of all genes present on each array compared with the baseline (normal) array, the normalised signal intensity of each array was plotted against that of the normal sample (Figure 6.6).

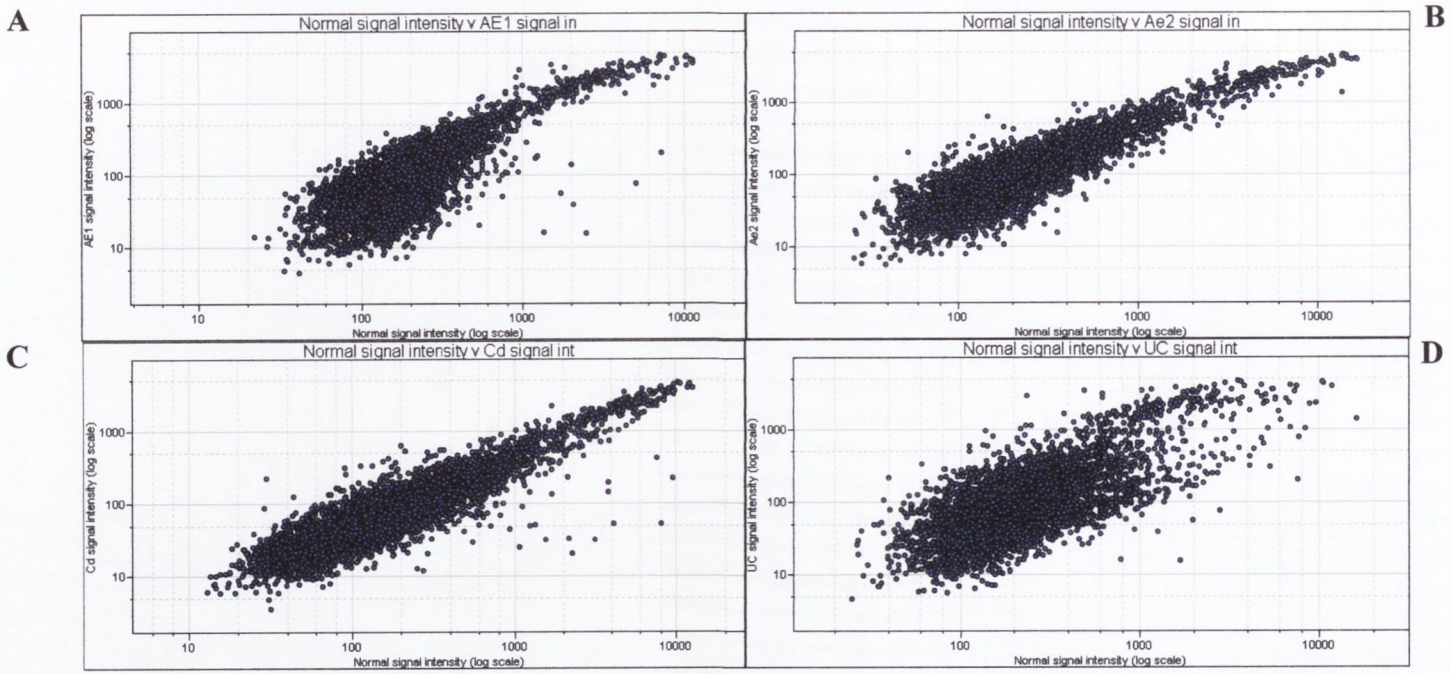


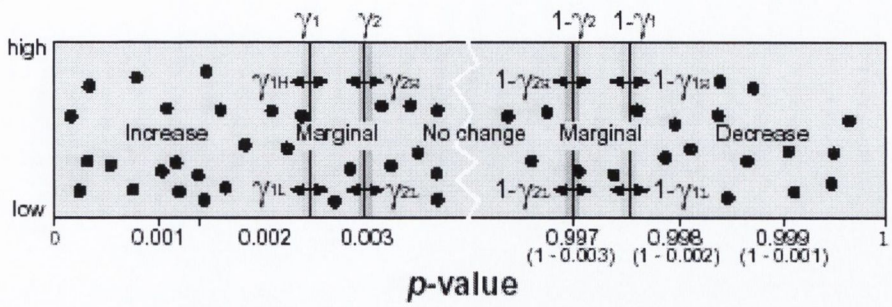
Figure 6.6. Scatter plots of fluorescent signal intensities of genes designated present on each array compared with the normal GeneChip array.

A. AE1 signal intensity plotted against normal signal intensity. **B**. AE2 signal intensity plotted against normal signal intensity. **C**. CD signal intensity plotted against normal signal intensity and **D**. UC signal intensity plotted against normal signal intensity.

6.4.4 Gene Expression Comparative Analysis.

The comparison analysis was performed using MicroArray Suite Software 4.0 (Affymetrix) to determine the relative change in abundance for each transcript between the baseline (Normal) and the experimental samples (AE1, AE2, UC and CD). Comparative data was expressed as a signal log ratio, where a ratio of 1 indicates a 2-fold increase in expression of a particular transcript. This ratio is calculated by comparing the probe pair on the experimental array (AE1, AE2, CD, UC) to the corresponding probe pair on the baseline array (Normal). A change p value is also calculated using the Wilcoxon signed rank test, and indicates a increase, decrease or no change in gene expression. (Figure 6.7A) The numbers of differentially expressed transcripts in each group are shown in figure 6.7B. In total there were 10,061 genes differentially regulated across all four GeneChip microarrays.

A.



B.

	Signal log ratio ≥ 1	Signal log ratio ≤ -1
AE1	680	5
AE2	198	8
CD	950	14
UC	881	83

Figure 6.7. Differentially expressed genes in AE, UC and CD compared with normal baseline GeneChip.

A. Affymetrix comparative analysis change call p values alogorithm. The Y axis is the probe set signal and X axis corresponds to the change p value. Cut off values are called γ1 and γ2 and assigned by the MAS software. Any probe set with a comparison p value <0.002 was considered increased in the experimental sample while any p value > 0.998 was considered decreased. Probe sets with p values in between this range were considered not changed.

B. Number of differentially expressed genes on each GeneChip experiment compared with the same normal GeneChip array as determined by the Affymetrix Change call algorithm.

6.4.5 Clustering of differentially expressed genes.

We attempted to group the differentially regulated genes into clusters using hierarchial clustering methods. Clustering of all the differentially expressed genes (10,061) across all four GeneChips, clustered expression profiles from AE1 and UC together, with AE2 on a sub-branch, while CD was on a separate branch (Figure 6.8). The small number of arrays examined and the wide variation in the expression profiles across each array was problematic for the cluster analysis. Further refinement of the data was performed by selecting transcripts that exhibited a 2 fold or greater change in expression levels.

There were 2161 transcripts differentially regulated greater than 2 fold across all disease groups. Hierachial clustering of these transcripts across all arrays again highlighted the variation in each of the disease groups. Of the 2161 differentially regulated transcripts only 15 were present in all three diseases reflecting the distinctive disease signatures of all three diseases (Table 6.1, figure 6.9). A total of 37 differentially regulated genes were common to AE1 and CD, 310 to AE1 and UC and 95 to CD and UC.

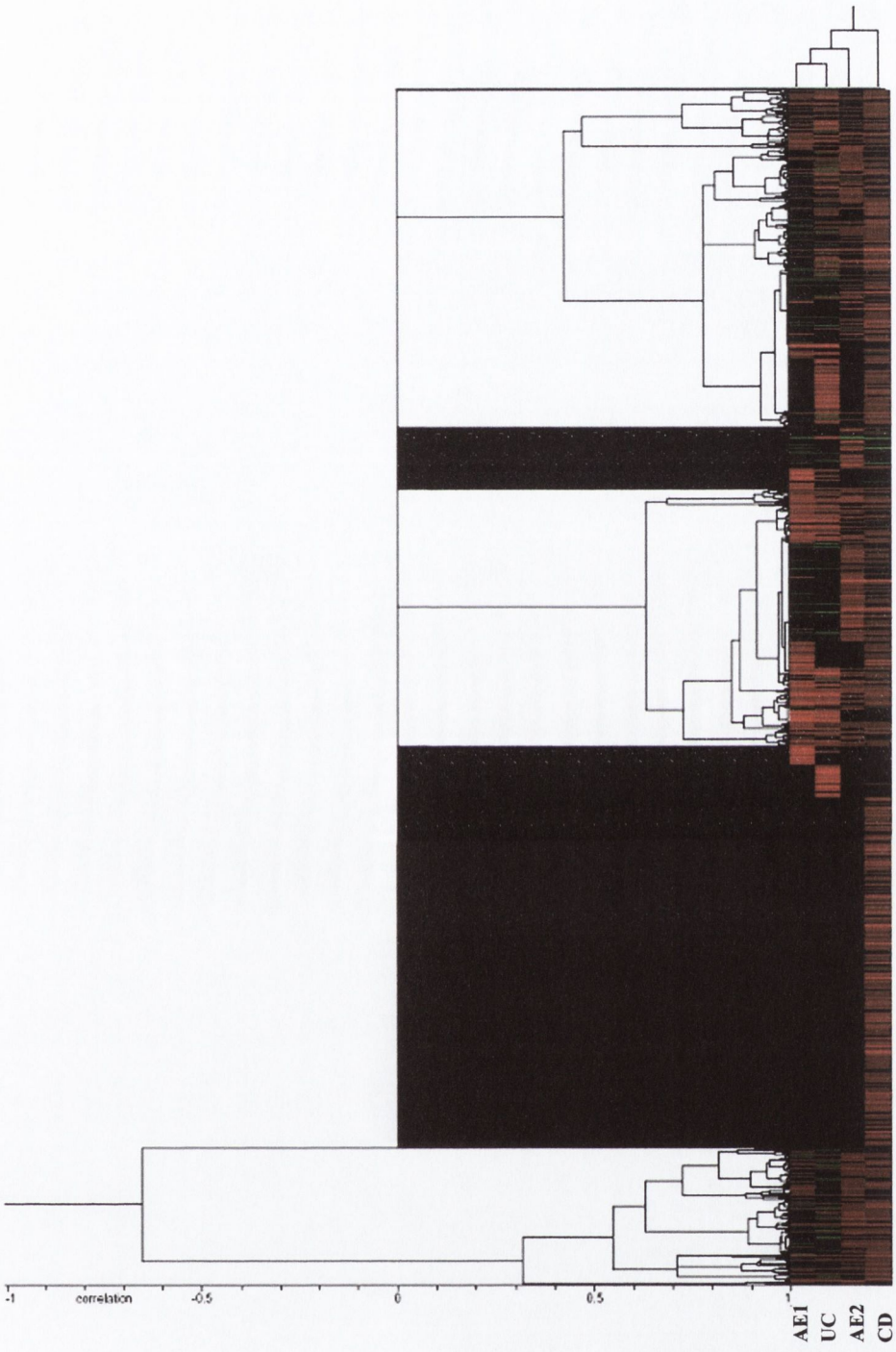


Figure 6.8. Hierarchial clustering of 10,061 transcripts (rows) expressed in different IBD groups (columns). Red shows upregulated transcripts while green shows down regulated transcripts. The large black areas in the dendrogram relate to areas where transcripts have not clustered, in particular in Crohn's disease where a large number of transcripts were unique to this group.

Table 6.1 Differentially expressed transcripts common to across all disease groups.

Probe Set ID	Transcript Name	Map Location	AE1*	CD*	UC *
203963_at	carbonic anhydrase XII	15q22	4.9	4.6	7.0
204256_at	long-chain fatty-acyl elongase	4q25	5.7	7.5	6.1
208250_s_at	deleted in malignant brain tumors 1	10q25.3-q26.1	7.5	90.5	12.1
209537_at	exostoses (multiple)-like 2	1p21	6.5	4.0	4.0
213143_at	hypothetical protein LOC257407	2q37.1	7.0	4.0	10.6
215331_at	KIAA1000 protein	3q13.12	12.1	4.9	7.0
216049_at	Rho-related BTB domain containing 3	5q14.3	34.3	8.0	19.7
216396_s_at	etoposide-induced mRNA	11q24	4.6	4.6	5.3
218211_s_at	melanophilin	2q37.3	5.3	5.3	6.5
219091_s_at	EMILIN-like protein EndoGlyx-1	10q23.2	6.5	3.7	4.3
220048_at	ectodysplasin 1, anhidrotic receptor	2q11-q13	9.8	3.2	8.0
222079_at	ESTs		19.7	10.6	17.1
320_at	peroxisomal biogenesis factor 6	6p21.1	7.0	3.7	5.7
33323_r_at	Stratifin	1p35.3	4.3	9.2	5.3
35666_at	immunoglobulin domain (Ig), short basic domain,secreted, (semaphorin) 3F	3p21.3	6.5	4.9	8.6

*Specific GeneChip data (AE1, CD, UC) is presented as fold change difference compared with the normal sample.

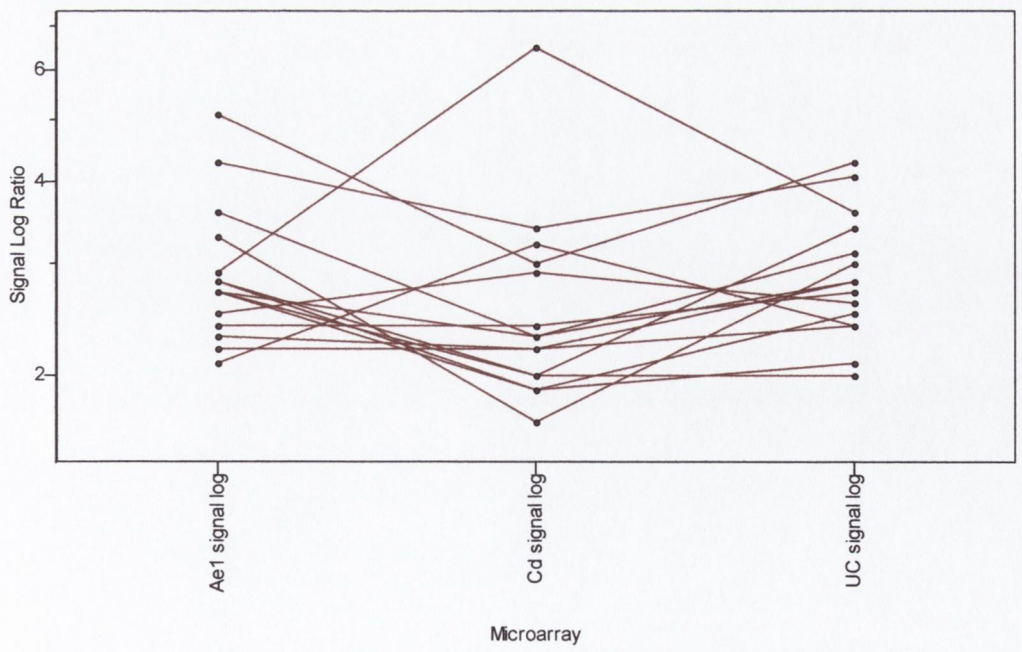


Figure 6.9. Graphical representation of 15 differentially regulated genes common to the three disease groups.

To examine the variability between two different independent experimental samples, two different autistic enterocolitis RNA pools (AE1; n=3, AE2; n=4) were profiled. Patients in the AE1 pool were negative for measles virus F gene by TaqMan RT PCR while patients in the AE2 pool were positive for measles virus F gene. The same control RNA pooled sample (Normal) was profiled each time to generate a baseline for both experiments.

Compared with the control array there were 685 genes differentially expressed at greater than the 2 fold level in the AE1 set compared with 211 genes in the AE2 set. Of these there were 67 transcripts in common across both AE arrays that were differentially expressed at greater than two fold level. The fold change in expression of these 67 genes was compared in both groups (Figure 6.10). A highly significant positive correlation (r=0.661) was obtained indicating that there was a consistent pattern of gene expression of these differentially regulated genes within both AE1 and AE2.

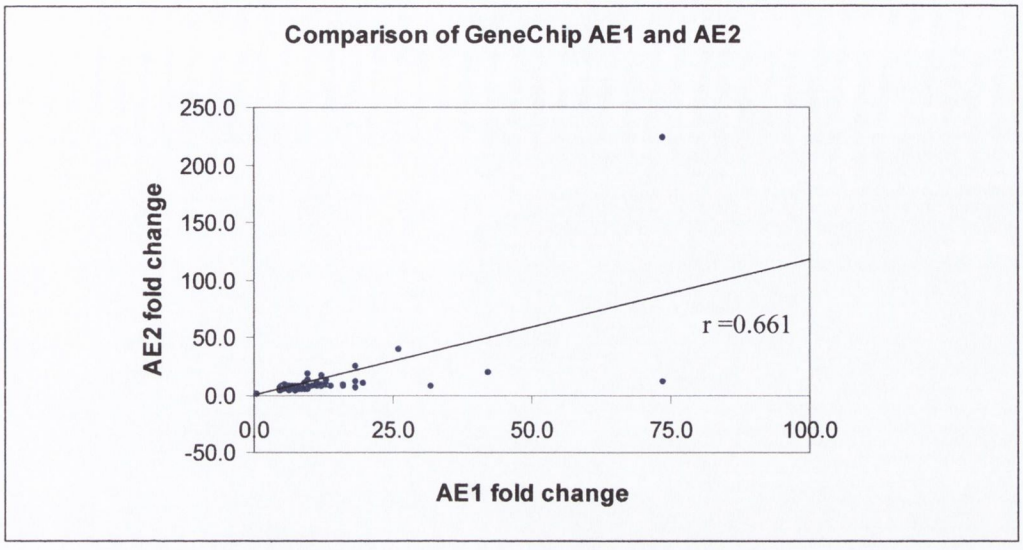


Figure 6.10. Comparison of differentially expressed genes in both the autistic enterocolitis microarrays.

The fold changes of the 67 differentially regulated genes in both AE1 (compared with normal) and AE 2 (compared with normal) were plotted against each other. The Pearson correlation coefficient was calculated using SPSS statistical software. There was a highly significant positive correlation between the common differentially expressed genes on both arrays ($r=0.661$, $p<0.0001$, 2 tailed).

It was not within the scope of this thesis to describe all 2161 significantly differentially regulated transcripts. Guided by the current literature expression of certain immune function genes, chemokines and cytokines, extracellular matrix genes, and cell cycle regulators was examined. Expression of a selection of these genes and their corresponding chromosomal position is shown in table 6.2, some of which will be discussed here. The top 60 differentially regulated genes in each disease group (AE, UC and CD) and their corresponding differential expression (fold change relative to normal) within the other groups are listed in tables 6.3, 6.4 and 6.5.

Table 6.2. Differentially expressed genes.

Probe ID	Gene Name	Map Location	AE	CD	UC
HLA & Immune function Genes					
200905_x_at	Major histocompatibility complex, class I, E	6p21.3	-	3	-
-201137_s_at	Major histocompatibility complex, class II, DP beta 1	6p21.3	-	-	-3
209140_x_at	Major histocompatibility complex, class I, B	6p21.3	-	3	-
209480_at	Major histocompatibility complex, class II, DQ beta 1	6p21.3	-	5	-
211799_x_at	Major histocompatibility complex, class I, C	6p21.3	-	3	-
211990_at	Major histocompatibility complex, class II, DP alpha 1	6p21.3	-	-	-2
206420_at	Immunoglobulin superfamily, member 6 (IGSF6), mRNA		-	8	-
207191_s_at	Immunoglobulin superfamily containing leucine-rich repeat	15q23-q24	-	5	-
221671_x_at	Similar to Human Ig rearranged gamma chain mRNA	2p11.2	-	-	-3
203071_at	Sema domain, immunoglobulin domain (Ig), semaphorin 3B	3p21.3	-	-	12
35666_at	immunoglobulin domain (Ig), short basic domain,secreted, (semaphorin) 3F	3p21.3	7	5	9
210152_at	Leukocyte immunoglobulin-like receptor.	19q13.4	9	-	-
Cytokines and Chemokines & related genes					
202531_at	Interferon regulatory factor 1	5q31.1	-	3	-
202859_x_at	Interleukin 8	4q13-q21	-	15	-
202948_at	Interleukin 1 receptor, type I	2q12	-	5	-
203915_at	Chemokine (C-X-C motif) ligand 9	4q21	-	37	-
206336_at	Chemokine (C-X-C motif) ligand 6	4q21	-	23	-
211122_s_at	Chemokine (C-X-C motif) ligand 11	4q21.2	-	21	-
209774_x_at	Chemokine (C-X-C motif) ligand 2	4q21	-	17	-
204533_at	Chemokine (C-X-C motif) ligand 10	4q21	-	13	-
214038_at	Chemokine (C-C motif) ligand 8	17q11.2	-	13	-
204470_at	Chemokine (C-X-C motif) ligand 1	4q21	-	17	-
206332_s_at	Interferon, gamma-inducible protein 16	1q22	11	-	-3
208436_s_at	Interferon regulatory factor 7	11p15.5	-	3	-
208448_x_at	Interferon, alpha 16	9p22	9	-	-
214569_at	Interferon, alpha 5	9p22	15	-	14
209970_x_at	caspase 1,	11q23	-	6	-
210029_at	interferon, gamma-inducible indoleamine-pyrrole 2,3 dioxygenase	8p12-p11	-	7	-
211366_x_at	Caspase 1, (interleukin 1, beta, convertase)	11q23	-	5	-
211368_s_at	Caspase 1 (interleukin 1, beta, convertase)	11q23	-	7	-
221717_at	Human interferon mRNA,		6	-	7
202411_at	Interferon, alpha-inducible protein 27 (IFI27), mRNA.	14q32	-	6	7
200704_at	Lipopolysaccharide-induced TNF factor	16p13.3-p12	-	4	-
202266_at	TRAF and TNF receptor associated protein	6p22.3-p22.1	-1	-	5
202510_s_at	Tumor necrosis factor, alpha-induced protein 2	14q32	-	3	-
202688_at	Tumor necrosis factor (ligand) superfamily, member 10	3q26	-	4	6
206026_s_at	Tumor necrosis factor, alpha-induced protein 6	2q23.3	-	9	-

Table 6.2 continued.

Probe ID	Gene Name	Map Location	AE	CD	UC
206222_at	Tumor necrosis factor receptor superfamily, member 10c	8p22-p21	16	-	-
206467_x_at	Tumor necrosis factor receptor superfamily, member 6b.	20q13.3	-	5	-
206508_at	Tumor necrosis factor (ligand) superfamily, member 7	19p13	3	-	-
206907_at	Tumor necrosis factor (ligand) superfamily, member 9	19p13.3	7	-	-
207339_s_at	Lmphotoxin beta (TNF superfamily, member 3)	6p21.3	-	-	-3
Protein kinases					
203652_at	Mitogen-activated protein kinase kinase kinase 11	11q13.1-q13.3	-	4	-
204813_at	Mitogen-activated protein kinase 10	4q22.1-q23	6	-	-
205632_s_at	Phosphatidylinositol-4-phosphate 5-kinase, type I, beta	9q13	-	-	6
206139_at	Phosphatidylinositol 4-kinase, catalytic, beta polypeptide	1q21	-	-	7
207187_at	Janus kinase 3 (a protein tyrosine kinase, leukocyte)	19p13.1	-	-	8
207391_s_at	Phosphatidylinositol-4-phosphate 5-kinase, type I, alpha	1q22-q24	-	-	6
209677_at	Protein kinase C,	3q26.3	21.1	-	-
210058_at	Mitogen-activated protein kinase 13	6p21.31	-	-	6
MMP's					
160020_at	Matrix metalloproteinase 14	14q11-q12	8		7
201069_at	Matrix metalloproteinase 2	16q13-q21	-	5	-
203936_s_at	Matrix metalloproteinase 9	20q11.2-q13.1	-	5	-3
204475_at	Matrix metalloproteinase 1	11q22.3	-	91	-
204580_at	Matrix metalloproteinase 12	11q22.3	-	5	-
205828_at	Matrix metalloproteinase 3	11q22.3	-	64	-
Antimicrobial					
207269_at	Defensin, alpha 4, corticostatin	8p23	10	-	-
207529_at	Defensin, alpha 5, Paneth cell-specific	8pter-p21	-	16	9
207814_at	Defensin, alpha 6, Paneth cell-specific	8pter-p21	-	12	8
Other interesting genes					
206323_x_at	Oligophrenin 1	Xq12	32.0	-	32.0
211479.s_at	Serotonin 5HT C receptor	Xq24	8	-	-
207577_at	Serotonin 5HT4 receptor	5q31q33	-	7	-

*Specific GeneChip data (AE, CD, UC) is presented as fold change difference compared with the normal sample.

Table 6.3 Top 60 genes differentially regulated in Autistic Enterocolitis.

Probe ID	Transcript Name	Map Location	AE1*	CD*	UC *
207712_at	B melanoma antigen	21p11.1	181.0	NC	55.7
220138_at	Heart and neural crest derivatives expressed 1	5q33	137.2	NC	NC
207810_at	Coagulation factor XIII, B polypeptide	1q31-q32.1	90.5	NC	NC
209057_x_at	CDC5 cell division cycle 5-like (S. pombe)	6p21	90.5	NC	34.3
216351_x_at	Deleted in azoospermia 4	Yq11	90.5	NC	NC
216247_at	Ribosomal protein S20	8q12	64.0	NC	NC
221154_at	Ring finger protein 18	11p11.12-q12	55.7	NC	NC
206640_x_at	G antigen 7B	Xp11.4-p11.2	48.5	NC	84.4
208281_x_at	Deleted in azoospermia 3	Yq11.23	48.5	NC	NC
207815_at	Platelet factor 4 variant 1	4q12-q21	39.4	NC	NC
214034_at	type 1 tumor necrosis factor receptor shedding Aminopeptidase regulator	5q14.3	36.8	NC	NC
216049_at	Rho-related BTB domain containing 3	5q14.3	34.3	8.0	19.7
220181_x_at	Solute carrier family 30 (zinc transporter), member 5	5q12.1	34.3	NC	NC
204987_at	Inter-alpha (globulin) inhibitor, H2 polypeptide	10p15	32.0	NC	18.4
205075_at	Serine (or cysteine) proteinase inhibitor	17p13	32.0	NC	NC
206323_x_at	Oligophrenin 1	Xq12	32.0	NC	32.0
207466_at	Galanin	11q13.1	32.0	NC	22.6
221394_at	G protein-coupled receptor 58	6q24	32.0	NC	26.0
220405_at	Syntrophin, gamma 1	8q11-q12	27.9	NC	NC
201909_at	Ribosomal protein S4, Y-linked	Yp11.3	26.0	26.0	NC
205264_at	CD3-epsilon-associated protein	19q13.3	26.0	NC	NC
216922_x_at	Deleted in azoospermia	Yq11	26.0	NC	NC
221169_s_at	Histamine receptor H4	18q11.2	24.3	NC	NC
206609_at	Melanoma antigen, family C, 1	Xq26	22.6	NC	NC
207789_s_at	Dipeptidylpeptidase 6	7q36.2	22.6	NC	11.3
220669_at	Hin-1	4q28.1-q28.3	22.6	NC	NC
206165_s_at	Chloride channel.	1p31-p22	21.1	NC	13.9
209646_x_at	Aldehyde dehydrogenase 1 family, member B1	9p11.1	21.1	NC	24.3
209677_at	Protein kinase C,	3q26.3	21.1	NC	NC
206937_at	Spectrin, alpha, erythrocytic 1 (elliptocytosis 2)	1q21	19.7	NC	NC
214205_x_at	Thioredoxin-like 2	6p25.3	19.7	NC	NC
216989_at	Sperm adhesion molecule 1	7q31.3	18.4	NC	13.9
203672_x_at	Thiopurine S-methyltransferase	6p22.3	18.4	NC	16.0
207663_x_at	G antigen 3	Xp11.4-p11.2	18.4	NC	18.4
205357_s_at	Angiotensin II receptor, type 1	3q21-q25	17.1	NC	12.1
205782_at	Fibroblast growth factor 7 (keratinocyte growth factor)	15q15-q21.1	17.1	NC	NC
208302_at	Minor histocompatibility antigen HB-1	5q31.3	17.1	NC	NC
211585_at	Nuclear protein, ataxia-telangiectasia locus	11q22-q23	17.1	NC	NC
214772_at	G2 protein	11p13	17.1	NC	14.9
216809_at	Cylicin, basic protein of sperm head cytoskeleton 1	Xq21.1	17.1	NC	13.9
217133_x_at	Cytochrome P450,	19q13.2	17.1	NC	17.1

Table 6.3. continued

Probe ID	Transcript Name	Map Location	AE1*	CD*	UC *
220195_at	Methyl-CpG binding domain protein 5	2q23.2	17.1	NC	12.1
206222_at	Tumor necrosis factor receptor superfamily, 10c	8p22-p21	16.0	NC	NC
207052_at	Hepatitis A virus cellular receptor 1	5q33.2	16.0	NC	NC
207102_at	Aldo-keto reductase family 1, member D1	7q32-q33	16.0	NC	NC
207687_at	Inhibin, beta C	12q13.1	16.0	NC	16.0
210837_s_at	Phosphodiesterase 4D, cAMP-specific	5q12	16.0	NC	12.1
219372_at	Carnitine deficiency-associated gene	12q24.13	16.0	NC	NC
205609_at	Angiopoietin 1	8q22.3-q23	14.9	NC	NC
205669_at	Neural cell adhesion molecule 2	21q21.1	14.9	NC	NC
206426_at	Melan-A	9p24.1	14.9	NC	NC
207796_x_at	Killer cell lectin-like receptor subfamily D,	12p13	14.9	NC	NC
208410_x_at	Amelogenin	Xp22.31-p22.1	14.9	NC	NC
214569_at	Interferon, alpha 5	9p22	14.9	NC	13.9
201131_s_at	Cadherin 1, type 1, E-cadherin (epithelial)	16q22.1	-2	NC	NC
211430_s_at	Immunoglobulin heavy constant gamma 3	14q32.33	-3.3	NC	-10
215176_x_at	Immunoglobulin kappa constant	2p12	-2.5	NC	NC
217148_x_at	Immunoglobulin lambda joining 3	22q11.1-q11.2	-2.0	NC	NC

*Specific GeneChip data (AE1, CD, UC) is presented as fold change difference compared with the normal sample. NC= no change in expression levels compared with normal sample.

Table 6.4 Top 60 differentially regulated genes in ulcerative colitis.

Probe ID	Transcript Name	Map location	UC	AE	CD
206640_x_at	G antigen 4	Xp11.4-p11.2	84	6	-
220232_at	Stearoyl-CoA desaturase 4	4q21.3	74	6	-
211553_x_at	Apoptotic protease activating factor	12q23	69	-	-
217130_at	HFSE-1 protein	9q22.1	69	-	-
211565_at	SH3-domain GRB2-like 3	15q24	60	6	-
207712_at	B melanoma antigen	21p11.2	56	8	-
209749_s_at	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	17q23	49	-	-
214236_at	Cell division cycle 27	17q12-17q23.2	45	5	-
216217_at	Phospholipase C-like 2	3p25.1-p24.3	37	6	-
206930_at	Glycine-N-acyltransferase	11q12.1	34	-	-
209057_x_at	CDC5 cell division cycle 5-like	6p21	34	7	-
206323_x_at	Oligophrenin 1	Xq12	32	5	-
207739_s_at	G antigen 8	Xp11.4-p11.2	30	3	-
206952_at	Glucose-6-phosphatase	17q21	26	-	-
208383_s_at	Phosphoenolpyruvate carboxykinase 1	20q13.31	26	-	-
221394_at	G protein-coupled receptor 58	6q24	26	5	-
206318_at	Serine protease inhibitor-like,	20q12-q13.2	24	5	-
209646_x_at	Aldehyde dehydrogenase 1 family,	9p11.1	24	4	-
207466_at	Galanin	11q13.1	23	5	-
211567_at	Sialophorin (gpL115, leukosialin, CD43)	16p11.2	21	-	-
220087_at	beta-carotene 15, 15'-dioxygenase	16q21-q23	21	-	-
207638_at	Protease, serine, 7 (enterokinase)	21q21.1	20	-	-
207895_at	N-acetylated alpha-linked acidic dipeptidase-like	11q12	20	-	-
216049_at	Rho-related BTB domain containing 3	5q15	20	5	8
204987_at	Inter-alpha (globulin) inhibitor	10p15	18	5	-
207663_x_at	G antigen 4	Xp11.4-p11.2	18	4	-
221170_at	Histamine receptor H4	18q11.2	18	-	-
206916_x_at	Tyrosine aminotransferase	16q22.1	17	3	-
210445_at	Fatty acid binding protein 6, ileal (gastrotropin)	5q23-q35	17	-	-
211089_s_at	NIMA (never in mitosis gene a)-related kinase 3	13q14.13	17	4	-
215860_at	Synaptotagmin XII	11q13.1	17	3	-
217133_x_at	Cytochrome P450, family 2, polypeptide 6	19q13.2	17	4	-
203672_x_at	Thiopurine S-methyltransferase	6p22.3	16	4	-
205939_at	Cytochrome P450, family 3,polypeptide 7	7q21-q22.1	16	-	-
207501_s_at	Fibroblast growth factor 12	3q28	16	-	-
207687_at	Inhibin, beta C	12q13.1	16	4	-
208235_x_at	G antigen 4	Xp11.4-p11.2	16	3	-
211028_s_at	Ketohexokinase (fructokinase)	2p23.3-p23.2	16	-	-
211349_at	Solute carrier family 15	13q33-q34	16	-	-
212741_at	Monoamine oxidase A	Xp11.4-p11.3	16	-	-
220867_s_at	Solute carrier family 24	9p22-p13	16	-	-
221673_s_at	Casein kinase 1, gamma 1	15q22.1-q22.31	16	4	-

Table 6.4. continued

Probe ID	Transcript Name	Map location	UC	AE	CD
211430_s_at	Immunoglobulin heavy constant gamma 3	14q32.33	-10	-3	-
205242_at	Chemokine (C-X-C motif) ligand 13	4q21	-9	-	-
217028_at	Chemokine (C-X-C motif) receptor 4	2q21	-5	-	-
215121_x_at	Human rearranged immunoglobulin lambda		-5	-	-
203416_at	CD53 antigen	1p13	-4	-	-
201721_s_at	Lysosomal-associated multispanning membrane protein-5	1p34	-3	-	-
203936_s_at	Matrix metalloproteinase 9	20q11.2-q13.1	-3	-	5
208752_x_at	Nucleosome assembly protein 1-like 1	12q21.1	-3	-	-
213911_s_at	H2A histone family, member Z	4q24	-3	-	-
221475_s_at	Ribosomal protein L15	3p24.2	-3	-	-
200036_s_at	Ribosomal protein L10a	6p21.3-p21.2	-3	-	-
201137_s_at	Major histocompatibility complex, class II,	6p21.3	-3	-	-
204606_at	Chemokine (C-C motif) ligand 21	9p13	-3	-	4
203302_at	Deoxycytidine kinase	4q13.3-q21.1	-3	-	-
207339_s_at	Lymphotoxin beta (TNF superfamily, member 3)	6p21.3	-3	-	-
201154_x_at	Ribosomal protein L4	15q22	-3	-	-
206332_s_at	Interferon, gamma-inducible protein 16	1q22	-3	-	-

*Specific GeneChip data (AE1, CD, UC) is presented as fold change difference compared with the normal sample.

Table 6.5 Top 60 differentially regulated genes in Crohn's disease

Probe ID	Gene Name	Map Location	CD	AE	UC
209752_at	Regenerating islet-derived 1 alpha	2p12	147	6	6
217109_at	Mucin 4, tracheobronchial	3q29	97	-	-
204475_at	Matrix metalloproteinase 1	11q22.3	91	-	-
208250_s_at	Deleted in malignant brain tumors 1	10q25.3-q26.1	91	7	12
210873_x_at	Apolipoprotein B mRNA editing enzyme.	22q13.1-q13.2	84	-	-
210084_x_at	Tryptase beta 2	16p13.3	79	-	-
219727_at	Dual oxidase 2	15q15.3	69	-	-
205828_at	Matrix metalloproteinase 3	11q22.3	64	-	-
212531_at	Lipocalin 2 (oncogene 24p3)	9q34	56	-	-
203649_s_at	Phospholipase A2	1p35	52	7	-
209395_at	Chitinase 3-like 1	1q32.1	45	-	-
215382_x_at	Tryptase beta 2	16p13.3	45	-	-
205860_x_at	Folate hydrolase 1	11p11.2	42	-	-
203915_at	Chemokine (C-X-C motif) ligand 9	4q21	37	11	-
202917_s_at	S100 calcium binding protein A8 (calgranulin A	1q21	34	-	-
205000_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3.	Yq11	34	-	-
209969_s_at	Signal transducer and activator of transcription 1.	2q32.2	32	-	-
214974_x_at	Chemokine (C-X-C motif) ligand 5	4q12-q13	30	-	-
214131_at	Chromosome Y open reading frame 15B	Yq11.222	28	37	-
201909_at	Rbosomal protein S4, Y-linked	Yp11.3	26	39	-
208168_s_at	Chitinase 1 (chitotriosidase)	1q31-q32	26	-	-
206239_s_at	Serine protease inhibitor, Kazal type 1	5q32	23	-	9
206336_at	Chemokine (C-X-C motif) ligand 6	4q21	23	-	-
202831_at	Glutathione peroxidase 2 (gastrointestinal)	14q24.1	21	-	-
206262_at	Alcohol dehydrogenase 1C (class I).	4q21-q23	21	-	-
211122_s_at	Chemokine (C-X-C motif) ligand 11	4q21.2	21	-	-
204855_at	Serine (or cysteine) proteinase inhibitor	18q21.3	20	-	-
215223_s_at	Superoxide dismutase 2, mitochondrial	6q25.3	20	-	-
205683_x_at	Tryptase beta 2	16p13.3	18	-	-
203757_s_at	Carcinoembryonic antigen-related cell adhesion molecule 6	19q13.2	17	-	-
204470_at	Chemokine (C-X-C motif) ligand 1	4q21	17	-	-
209774_x_at	Chemokine (C-X-C motif) ligand 2	4q21	17	-	-
204409_s_at	Eukaryotic translation initiation factor 1A.	Yq11.222	16	18	-
205402_x_at	Protease, serine, 2 (trypsin 2)	7q34	16	-	11
207529_at	Defensin, alpha 5, Paneth cell-specific	8pter-p21	16	-	9
210163_at	Chemokine (C-X-C motif) ligand 11	4q21.2	16	-	-
202859_x_at	Interleukin 8	4q13-q21	15	-	-
204136_at	Collagen, type VII, alpha 1	3p21.1	15	-	-
201123_s_at	Eukaryotic translation initiation factor 5A	17p13-p12	14	26	-
204438_at	Mannose receptor, C type 1	10p13	14	-	-
206552_s_at	Tachykinin	7q21-q22	14	-	-
204533_at	Chemokine (C-X-C motif) ligand 10	4q21	13	-	-

Table 6.5 continued

Probe ID	Gene Name	Map Location	CD	AE	UC
205043_at	Cystic fibrosis transmembrane regulator.	7q31.2	13	-	-
207847_s_at	Mucin 1,	1q21	13	-	7
214038_at	Chemokine (C-C motif) ligand 8	17q11.2	13	-	-
200629_at	Tryptophanyl-tRNA synthetase	14q32.31	12	-	-
207076_s_at	Argininosuccinate synthetase	9q34.1	12	-	10
207814_at	Defensin, alpha 6, Paneth cell-specific	8pter-p21	12	-	8
202350_s_at	Matrilin 2	8q22	-2	-	-
204674_at	Lymphoid-restricted membrane protein	12p12.1	-2	-	-
204845_s_at	Glutamyl aminopeptidase	4q25	-3	-	-
205108_s_at	Apolipoprotein B	2p24-p23	-3	-4	6
205927_s_at	Cathepsin E	1q31	-2	-	-
205999_x_at	Cytochrome P450.	7q21.1	-2	-	7
210258_at	Regulator of G-protein signalling 13	1q31.1	-9	-	-
213872_at	Chromosome 6 open reading frame 62	6p22.1	-2	-3	-2
35974_at	Lymphoid-restricted membrane protein	12p12.1	-3	-	-

*Specific GeneChip data (AE1, CD, UC) is presented as fold change difference compared with the normal sample.

6.5 Discussion

The aim of this study was to examine the repertoire of genes expressed in new variant inflammatory bowel, compared with ulcerative colitis and Crohn's disease using Affymetrix high density oligonucleotide GeneChip arrays. In this study a number of genes have been identified, within each of the disease groups, from a range of host cell pathways that show significant changes in expression levels when compared with control samples. These preliminary results identify novel genes that may be implicated in the pathogenesis of IBD and aid in the molecular characterisation of autistic enterocolitis.

The majority of microarray studies to date have centred on cultured cell lines or single cell populations. In this study we used RNA extracted from terminal ileum tissue biopsies, which is comprised of multiple heterogenous cell populations with the specific purpose of gaining an overall insight into all cellular changes associated with the disease pathogenesis. One of the major limitations associated with microarray technology is the high yield and quality of RNA that is required to perform the technique, Affymetrix GeneChip technology requiring between 5-40μg of total RNA. More recently techniques for amplification of small quantities of RNA for microarray expression analysis have been investigated (McClintick *et al.*, 2003; Ohyama *et al.*, 2000). This strategy using two rounds of T7 amplification is currently being pursued in our laboratory. In the present study RNA extracts from individual patients were pooled into patient groups to obtain sufficient RNA for expression analysis. The strategy of pooling samples may lead to problems such as the signal being confounded by mixed cell populations and individual patients. However, recent studies by Lawrance *et al.*, (2001), and Dieckgraefe *et al.*, (2000),

reported gene expression profiling using oligonucleotide arrays on RNA pooled from full thickness surgically resected colonic tissues from Crohn's disease and ulcerative colitis patients.

The quality and integrity of RNA extracted is crucial to the success of any microarray experiment as impurities can affect the probe labelling efficiency and stability (Hedge *et al.,* 2002). Extraction of high quality RNA with intact ribosomal RNA bands was problematic with the tissue samples used in this study. RNA extracted from tissue biopsies collected in RNA*later* was high quality with minimal degradation compared with snap frozen specimens in agreement with that reported by others (Florell *et al.*, 2001, Wang *et al.*, 2001). Unfortunately the paediatric tissue biopsies available for this study were collected retrospectively, snap frozen in liquid nitrogen and stored at –80 °C. However, all of the pooled RNA samples used in this study were hybridised to Affymetrix test arrays, which demonstrated that the RNA was of sufficient quality and integrity to warrant further analysis (Figure 6.5). The practice of collecting biopsies in RNA*later* has since been adopted for gene expression studies.

Hierarchial clustering of differentially expressed genes and disease groups, clustered disease groups into two main clusters. Cluster 1 containing AE1, UC and AE2 while cluster 2 contained the CD group (Figure 6.8), indicating that the autistic enterocolitis and ulcerative colitis groups had similar gene expression profiles. A large number of transcripts were unique to each disease group, in particular in the Crohn's disease cohort, and tended to group together. Further refinement of the

analysis identified 2,161 transcripts that were differentially regulated at greater than the two fold level.

Comparison of the differentially expressed genes in RNA pools AE1 and AE2 revealed, that of all the differentially expressed transcripts in both groups only 8% (67/891) were shared by both groups. Within the AE2 group (positive for measles) there were only 206 differentially regulated genes compared with 685 in the AE1 (negative for measles virus) patient pool. This may be explained by the initial problems encountered with the RNA quality from this pool, which did appear more degraded than the other disease groups. The 3'/5' ratios for GAPDH and actin transcripts on the test arrays were just at the threshold recommended by the manufacturer's. Nevertheless, of the genes that were commonly expressed, there was a significant correlation in expression levels. Thus the variation observed between the two groups may be a reflection of the heterogeneity of this disease entity. Analysis of individual patients is required to confirm this.

Expression profiling of all three groups (AE, CD and UC) revealed only 15 transcripts of all the differentially expressed genes were shared by all three disease groups. The majority of changes were unique to each type of IBD, demonstrating clear distinctions between all three diseases.

It is not within the scope of this chapter to discuss all of the 2161 significantly differentially regulated transcripts (> 2 fold difference in expression). Guided by the current literature a number of transcripts were investigated and these are listed in Table 6.2. As microarray expression analysis is a novel emerging technology,

confirmation of the results by more conventional methods such as RT-PCR and immunohistochemistry, is required to validate the data. The data presented in this chapter is preliminary and requires confirmation.

Significant over expression of HLA class I and class II genes, and immunoglobulin genes were observed, in particular in the Crohn's disease cohort, that corresponded to the IBD3 susceptibility region 6p21.3 on chromosome 6 (Table 6.2). In ulcerative colitis the MHC class II DP gene and a number of immunoglobulin genes were down regulated (Table 6.4). In contrast, GeneChip analysis by Lawrance *et al.*, (2001) demonstrated increased expression of certain HLA genes in colonic mucosa from ulcerative colitis patients and not in Crohn's disease. Comparisons of both studies are difficult as Lawrance *et al.*, (2001) examined full thickness sections of surgically resected tissues from adult cases of IBD, whereas our study examined terminal ileum biopsies from paediatric cases of IBD. However the altered expression of HLA genes observed in both studies reflects abnormal immune responses, which may be important in inflammatory bowel disease pathogenesis.

Various cytokines, chemokines and related genes were also differentially regulated across all disease groups. Chemokines, CXCL1 (GRO-α), CXCL2 (GRO-β), CXCL6 (GCP-2), CXCL9 (Mig), CXCL10 (IP-10), CXCL11 (I-TAC) and IL-8, were among the most highly expressed genes in the Crohn's disease cohort (Table 6.3 and 6.5). This correlates with our findings in chapter 5, where increased IL-8 expression was observed in terminal ileum biopsies from Crohn's disease patients. Increased levels of IL-8 mRNA and protein have been detected in IBD that correlated with histological inflammation (Katsuta *et al.*, 2000; Nielson *et al.*, 1997;

Ina *et al.*, 1997). Expression of various chemokines (MCP-1, MCP-2, MCP-3, MIP-Iα, MIP-1β) and chemokine receptors, (CXCL8, CXCL5, CCL2, CL11, and CXCL10) have been described in intestinal inflammation (D'Ambrosia *et al.*, 2003; Banks *et al.*, 2003). Chemokines act as mediators of intracellular communication between epithelial, immune and inflammatory cells and thus represent important markers of intestinal inflammation. In the colonic epithelium, chemokines may be stimulated by the pro-inflammatory cytokines IL-1 and TNF-α, in response to bacterial and or viral components (Rutgeerts *et al.*, 2003). In addition, blocking IL-1 signalling with an IL-1 receptor agonist has been shown to reduce the amount of IL-8 produced in IBD (Dinarello *et al.*, 1993). IL-1 receptor and IL-1β converting enzyme (Capase 1), required for processing IL-1β precursor into its active form, were upregulated in Crohn's disease compared with normal controls (Table 6.2), however there was no evidence of elevated IL-1 in this experiment. In general the broad upregulation of cytokines, chemokines and related genes in particular within the Crohn's disease cohort is indicative of a dysregulated immune response.

Differential regulation of tumour necrosis factor (TNF), interferon (IFN) and related genes were noted in Crohn's disease, ulcerative colitis and autistic enterocolitis patients (Table 6.2). TNF and members of TNF family of receptors were elevated in autistic enterocolitis, Crohn's disease and ulcerative colitis. This expression pattern would correlate with our previous findings of elevated TNF-α mRNA in autistic enterocolitis patients (chapter 6). The role of TNF-α in IBD and activation of the NF-κβ transcription factor has been fully discussed in chapters 1 and 5. In addition, a number of protein kinases were upregulated across the three disease groups, in

particular in the ulcerative colitis cohort, which are known to play a role in tyrosine mediated signal transduction pathways. In the autistic enterocolitis cohort elevated expression of MAPK 10 and protein kinase C may play a role in activation of the NF-κβ complex and subsequent production of pro-inflammatory cytokines (Santoro *et al.*, 2003). In addition, IFN and IFN inducible genes, were elevated in autistic enterocolitis and ulcerative colitis. IFN induced signalling is regulated by the signal tranducer and activator of transcription (STAT1), which was upregulated in CD, as were the IFN induced genes, IFN regulatory factors 1 and 7.

Compared with ulcerative colitis and autistic enterocolitis patients considerably more matrix metalloproteinase genes (MMP's) were upregulated in the Crohn's disease cohort (table 6.2). Production of metalloproteinase enzymes in the intestine plays a key role in tissue degradation and remodelling. Increased production of MMP-1, MMP-3, MMP-9, MMP-14 and MMP-12 have been reported in IBD (Lawrance *et al.*, 2001; Dieckgraefe *et al.*, 2000; Louis *et al.*, 2000; von Lampe *et al.*, 2000) that correlated with production of proinflammatory cytokines, TNF-α, IL-6, IL-1β and IL-10 (Louis *et al.*, 2000). Elevated MMP-1 and MMP-3 expression has been shown to cause extensive tissue damage in the intestine whereas MMP-14 is thought to be involved in wound healing (von Lampe *et al.*, 2000; Okada *et al.*, 1997). It is therefore likely that elevated expression of MMP-14 mRNA in the autistic enterocolitis and ulcerative colitis cohort reflects wound healing in the terminal ileum.

Antimicrobial genes encoding the α defensin 5 and α defensin 6 antimicrobial peptides were among the most highly upregulated genes in both Crohn's disease and ulcerative colitis patients (Table 6.2, 6.5). α defensin 4 was upregulated in autistic enterocolitis. Similarly, others have recently reported expression of α defensin 5 and α defensin 6 mRNA and protein in both Crohn's disease and ulcerative colitis (Fahlgren *et al.*, 2003; Cunliffe *et al.*, 2001; Lawrance *et al.*, 2001). This is the first study to describe intestinal expression of α defensin 4 (corticostatin), which belongs to the same family of proteins. Expression of these antimicrobial peptides in epithelial cells of the normal intestine is thought to protect normal mucosa against microbial infection. Increased expression of these in IBD may arise as a consequence of an impaired epithelial barrier, which permits adherance and invasion of luminal flora. In support of this α defensins 5 and 6 have been induced in cell culture by proinflammatory cytokines (Wenkamp *et al.*, 2002). In contrast impaired induction of β defensins 2 and 3 has been identified in Crohn's disease compared with normal and ulcerative colitis controls using both immunohistochemistry and real time RT PCR (Fellermann *et al.*, 2003). This may give rise to a defective antimicrobial barrier in the intestine, resulting in infection and inflammation. The mechanisms of activation of defensins are still not understood. One possibility to explain the impaired expression of these peptides in IBD is that altered binding of bacterial components to the defective NOD2/CARD15 protein in Crohn's disease patients alters expression of these antimicrobial peptides.

Among the most highly upregulated genes in the Crohn's disease patient pool was lithostathine Reg1α (Table 6.5). Reg1 α was first discovered upregulated in

pancreatic islet cells where it is believed to inhibit stone formation (Patard *et al.*, 2003). Recent reports have described expression of Reg1α outside the pancreas; Shinozaki *et al.*, (2001) reported upregulation of Reg1α mRNA in the epithelium of inflamed colonic mucosa in IBD. Dieckgraefe *et al.*, (2000) and Lawrance *et al.*, (2001) have also reported over expression of the Reg1α gene by microarray analysis in ulcerative colitis and Crohn's disease colonic tissue. In a subsequent study Dieckgraefe *et al.*, (2002) reported high expression of members of the Reg gene family, namely Reg1α, Reg 1β and Reg III in the setting of tissue injury associated with IBD by microarray analysis, immunohistochemistry and protein sequencing. Previous studies have indicated that PAP1 protein (pancreatitis associated protein 1) a member of the Reg family of proteins confers resistance to apoptosis (Ortiz *et al.*, 1998). The increased expression of Reg1α in this study and those of Dieckgraefe (2002) and Lawrance (2001) may serve to reduce epithelial apoptosis in inflammation.

E-cadherin, a cell adhesion molecule, was down regulated in autistic enterocolitis. E cadherin is the major adhesion molecule in epithelial cells and reduced expression is regarded as one of the main molecular events involved in dysfunction of the cell-cell adhesion system. Cadherin mediated cell-cell adhesion is regulated by the Rho family GTPases (Kaibuchi *et al.*, 1999). When Cdc 42 and Rac 1 are in the GTP bound active forms at cell-cell contact sites they interact with the scaffolding protein IQGAP1, preventing it from interacting with β catenin and stabilising the cadherin-catenin complex. However, activation of these Rho family GTPases by RhoGAP enhances the GTPase activity of the Cdc42 and Rac1 GTPases reconverting them to

the inactive GDP form, thus allowing the scaffolding protein (IQGAP1) to interact with the β catenin and subsequently dissociate and suppress the E-cadherin complex resulting in weakened cell-cell adhesion. Downregulation of E cadherin protein and mRNA have been reported in IBD, and associated with inflammation (Gassler *et al.,* 2001). Similarly, experiments in transgenic mice (Hermiston *et al.*, 1995) and human colitis (Jankowski *et al.,* 1998) have indicated the importance of E cadherin in maintenance of the intestinal barrier, where dysregulation of E-cadherin leads to the development of IBD.

Other genes of interest included the oligophrenin 1 gene, which has not previously been described in inflammatory bowel disease, was strikingly overexpressed in autistic enterocolitis (32 fold) and ulcerative colitis (32 fold) (Table 6.3). The gene encodes for oligophrenin 1 protein, and has been identified as one of seven genes implicated in non-specific X linked mental retardation (Ramakers, 2000; Tentler *et al.*, 1999; Billuart *et al.*, 1998). Frame shift mutations in this gene cause loss of function resulting in abnormal neuronal connectivity and deficient cognitive function (Ramakers, 2000; Billuart *et al.*, 1998). The protein is thought to be involved in cell signalling through RhoGTPases by stimulating the GTPase activity of RhoA, Rac1 and Cdc 42 *in vitro* (Billuart *et al.*, 1998). Rho proteins are signalling molecules that regulate the actin cytoskeleton and various cell adhesion events (Van Aelst *et al.*, 1997). Inactivation of the gene might affect the activity of interacting proteins or cause constitutive activation of potential RhoGTPase targets. Such activation has been shown to affect cell migration and outgrowth of axons and dendrites *in vivo* (Ettienne-Manneville *et al.*, 2002; Zipkin *et al.*, 1997).

The significance of oligophrenin 1 expression in the intestine remains obscure but recent studies have reported expression of oligophrenin 1 in nerve cell bodies in guinea pig and rat intestine (Xiao *et al.*, 2003). Similarly, others have reported strong upregulation of oligophrenin 1 in colorectal tumours (Pinheiro *et al.*, 2001) and in glioblastoma multiform brain tumour tissues using cDNA microarrays and (Ljubimova *et al.*, 2001). It seems therefore that oligophrenin 1 is present not only in central but also in peripheral neurons and may be involved in altering neuronal morphology and connectivity at the peripheral synapses. Physiological changes in enteric neurons have been reported during development of gastrointestinal diseases and after injury. The expression of oligophrenin 1 in enteric ganglia from guinea pig and rat ileum has been suggested in axonal outgrowth and remodelling (Xiao *et al.*, 2003). However, little is known about the molecular mechanisms of enteric neuron remodelling in response to damage. In this study, elevated expression of oligophrenin 1 in both autistic enterocolitis and ulcerative colitis patients may represent abberant Rho signalling associated with axon remodelling and gastrointestinal dysfunction in these diseases.

The serotonin 5'-hydroxytryptamine 2C (5'HT 2C) receptor gene was upregulated 8 fold in both autistic enterocolitis and ulcerative colitis. A number of studies have reported an association between autism and serotonin related genes. Association studies using two common polymorphisms within the serotonin transporter gene (5-HTT) have linked this gene with autism (Mc Dougle *et al.*, 1998; Cook *et al.*, 1997; Klauck *et al.*, 1997) while others reports no significant association of this gene as an independent susceptibility marker for autism (Kim *et al.*, 2002; Maestrini *et al.*, 1999). Mutations in the 5-HT receptor 7 gene, which is abundant in the ileum have

also been linked with autism (Lassig *et al.*, 1999). Serotonin (5-HT) is released in the small intestine from enterochromaffin cells and acts on enteric neurons to initiate motor reflexes and regulate colonic bowel motility. Linden (2003) have recently described altered 5-HT signalling in inflamed intestines of guinea pigs with an induced colitis. This study suggests that colitis alters serotonin signalling by increasing the availability of 5-HT while decreasing the reuptake of 5-HT possibly due to desensitisation of the 5-HT receptor. Reduced immunoreactivity of 5-HT receptors and an increase in enterochromaffin and serotonin cells has been described in the colonic mucosa of patients with colonic inertia (Zhao *et al.*, 2003). Impaired synthesis of 5-HT has also been described in Crohn's and ulcerative colitis patients (Magro *et al.*, 2002). In our study the observation of upregulated serotonin 5-HT 2C receptor gene in the intestine of children with autistic enterocolitis and ulcerative colitis may represent an important finding as to date there is no evidence of the existence of this receptor or its mRNA outside the brain (Fiorica-Howells *et al.*, 2000).

In summary a large number of genes with a range of functions were differentially expressed between all disease groups reflecting the heterogenous nature of inflammatory bowel disease. In this chapter we have discussed some of these findings, however due to the small sample size in each patient pool, it is not possible to interpret the global significance of these findings. Replicate studies on individual patients from a wider range of gastrointestinal diseases including lymphonodular hyperplasia will greatly improve the significance of these findings. This was not previously possible due to the high concentration of RNA required for high-density arrays, however a new technique for analysis of small quantities of RNA is currently

being optimised in our laboratory. Optimal conditions for collection of tissue specimens and extraction of high quality RNA should also improve the reproducibility and the overall expression results. Clustering analysis has grouped autistic enterocolitis and ulcerative colitis based on gene expression profiles. The preliminary results presented here have identified novel differentially expressed genes that may be implicated in the pathogenesis of IBD and aid in the molecular characterisation of autistic enterocolitis.

Chapter 7

General Discussion

7.0 General Discussion.

This thesis describes molecular studies on a new variant of inflammatory bowel disease, provisionally named "autistic enterocolitis". The disease has been described in a cohort of children with developmental disorders (Wakefield *et al.*, 2000; Wakefield *et al.*, 1998a). The intestinal pathology includes ileo-colonic lymphonodular hyperplasia and non-specific colitis, which lacks the specific diagnostic features of Crohn's disease or ulcerative colitis (Wakefield *et al.*, 2000). The aetiology remains unknown, although a speculated association between measles virus infection or vaccination, onset of gastrointestinal symptoms and developmental regression in these children, has caused considerable controversy (Wakefield *et al.*, 1998a). Limited research has been performed on this new variant of inflammatory bowel disease, the majority of studies focussing primarily on the macroscopic and microscopic features of this condition. The overall objective of this thesis was to molecularly characterise this new disease entity, with a view to gaining insight into the pathogenesis of the disease.

This study reports for the first time an association between persistent measles infection and ileocolonic lymphonodular hyperplasia in children with developmental disorders. Measles virus genomes were detected in the intestinal tissues of 74% of affected children compared with 14% of control children using a combination of TaqMan RT PCR and in situ RT PCR. In contrast, detection of measles virus in the peripheral blood was not significantly different between affected children and controls. Measles virus copy number in the intestinal biopsies was generally low but ranged from 1-3 X 10^5 copies/ng of total RNA. There was good correlation between detection of measles virus F and N genes using both techniques. However, 41% of

affected patients with low levels of measles F gene were negative for measles virus H gene in their intestinal tissues as measured by TaqMan RT PCR. This is likely to be related to the low abundance of mRNA of genes encoded at the 5' end of the genome (Oxman, 1997; Sidhu *et al.*, 1994). The origin of the measles virus detected in intestinal tissues from affected cases has been confirmed as vaccine strain in 14 of 23 cases (Sheils *et al.*, unpublished data).

In chapter 4, the TaqMan system for detection of measles virus was further validated in a CD46 transgenic mouse model that is permissive to measles infection. Tissues from the CD46 transgenic animals infected with measles virus, intracerebrally, intraperitoneally and orally expressed measles virus F and H genes at varying concentrations ranging from 10^5 to <10 copies/µl. Detection of measles in spleen, lung, large intestine, kidney, heart and liver tissue correlated with the previous findings of the CD46 receptor in these tissues (Oldstone *et al.*, 1999). High levels of measles virus F and H gene mRNA were detected in brain tissue from the CD46 transgenic animal inoculated with measles virus intracerebrally and intraperitoneally. Oldstone *et al.*, (1999) have previously reported the presence of MV N gene mRNA and protein in multiple sites of the brain in these animals. The problems associated with detection of low levels of virus were also highlighted. In some instances, a positive measles virus TaqMan PCR was not reproduced in all of the replicates. Increasing the number of replicates improved the rates of detection. This intermittent detection is a common finding with low copy viral gene detection and is related to variations in template aliquots.

In terminal ileal biopsies from affected children the virus was localised to follicular dendritic cells and some lymphocytes within reactive follicular hyperplastic centres. The presence of measles virus antigen in follicular dendritic cells may reflect a transient stage in the progression from latent to persistent measles virus infection (Klagge *et al.*, 1999; Tachetti *et al.*, 1997; Pantaleo *et al.*, 1994). Localisation of measles virus in follicular dendritic cells is similar to HIV-1 infection (Klagge *et al.*, 1999). Disruption of cellular immunity, and associated follicular hyperplasia, is associated with expansion of the follicular dendritic cell network and trapping of HIV within germinal centres (Klagge *et al,* 1999, Taccheti *et al.*, 1997). This location for measles virus may favour the induction of immunological tolerance and failure of viral clearance. Suppression of IL-12 production from measles infected dendritic cells is thought to be associated with the inadequate Th1 immune response and profound immunosuppression associated with measles virus (Atabani *et al.*, 2001; Karp *et al.*, 1996). The corresponding shift towards a type-2 immune response, might contribute to establishing persistent infection and delayed immunological events.

Dysregulation of the intestinal immune system is characteristic of intestinal inflammation. The immune response in the mucosa of Crohn's disease patients is dominated by CD4+ lymphocytes, with a type 1 helper T cell phenotype characterised by the production of IFN-γ and IL-2 (Monteleone *et al.*, 1999; Monteleone *et al.*, 1997; Parronchi *et al.*, 1997; Fuss *et al.*, 1996). In contrast the immune response in ulcerative colitis is dominated by type 2 helper T cell response, characterised by production of TGF-β and IL-5 but not IL-4 (Fuss *et al.*, 1996). Little is known about the immune response in lymphonodular hyperplasia and autistic enterocolitis. In chapter 5, the mRNA expression profiles of 24 inflammatory

and pro-inflammatory cytokines were examined in intestinal tissue and peripheral blood from patients with autistic enterocolitis (new variant inflammatory bowel disease) compared with lymphonodular hyperplasia (not associated with autism) and IBD controls. The results indicate a pattern of immune dysregulation in the intestinal tissues of children with new variant inflammatory bowel disease "autistic enterocolitis".

The increased expression of proinflammatory cytokines IL-1, IL-6, TNF-α and the anti-inflammatory cytokine IL-10 observed in the intestinal tissue from autistic enterocolitis children are suggestive of an abnormal innate immune response. Elevated levels of these cytokines (IL-1, IL-6 and TNF-α) have been described in the peripheral blood of autistic patients on elimination diets for gastrointestinal symptoms (Jyonouchi *et al.*, 2001). In addition over expression of these cytokines is thought to be essential for inducing and maintaining intestinal inflammation in inflammatory bowel disease (Brown *et al.*, 2002; Nikolaus *et al.*, 1998; Reimund *et al.*, 1996). Thus the elevated levels of these cytokines in the autistic enterocolitis cohort are indicative of an active inflammatory reaction in the intestine of these children. Regulation of these proinflammatory cytokines is via the NF-κβ transcription factor, elevated levels of which have been reported in lamina propria cells from IBD patients (Schreiber *et al.*, 1998).

The persistence of measles virus in dendritic cells and lymphocytes may contribute to the abnormal innate immune response through disruption of the NFκβ signalling pathway. Interactions of viruses with their cellular receptors, or accumulation of viral proteins or RNA have been shown to activate NF-κβ signalling (Santoro *et al.*,

2003). Recent studies have shown that measles virus fails to phosphorylate the IKK component of the NF-κβ complex in neuronal cells infected with virus (Fang *et al.*, 2002; Fang *et al.*, 2001). This has been suggested as a mechanism for viral persistence in neuronal cells, such as that seen in SSPE.

Alternatively, the elevated levels of pro-inflammatory cytokines may be attributed to an abnormal interaction of intestinal cells with luminal bacteria. Altered signalling of NF-κβ transcription factor and TNF-α in Crohn's disease has been attributed to mutations in the NOD2/CARD15 protein, which is thought to involved in interaction of cells with bacterial components (Ogura *et al.*, 2001a). Supporting this, Hisamatsu *et al.*, (2003) and Rosenstiel *et al.*, (2003) have identified NF-κβ transcription factor as the mediator of TNF-induced CARD15/NOD 2 gene expression in intestinal epithelial cells. Elevated production of TNF-α in the autistic enterocolitis cohort may enhance the intestinal intraepithelial cells responsiveness to invasive bacteria and subsequently continuously activate an innate immune response through the induction of NF-κβ dependant genes, including TNFα, IL-1, and IL-6.

Additional support for an abnormal immune response to enteric bacteria in autistic enterocolitis is provided in chapter 6. Antimicrobial genes encoding α defensin 4, was increased in autistic enterocolitis, while α defensin 5 and α defensin 6 were among the most highly upregulated genes in both Crohn's disease and ulcerative colitis patients. Increased expression of these antimicrobial peptides in the intestine in IBD may arise as a consequence of an impaired epithelial barrier. Alternatively impaired binding of the bacterial components to a defective NOD2/CARD15 protein in Crohn's disease patients may alter expression of these antimicrobial peptides.

Further evidence of an inflammatory reaction and gastrointestinal dysfunction in new variant inflammatory bowel disease and established IBD was determined using Affymetrix high-density microarray technology. The expression profiles of greater than 22,000 transcripts spanning the entire human genome were examined in each of the disease groups. In total 2,161 significantly differentially expressed transcripts (> 2 fold difference in expression) were identified across all disease groups. Only 15 of these were shared by all three disease groups, reflecting the heterogenous nature of each disease entity. The majority of changes were observed in the Crohn's disease cohort.

A number of immune function and related genes were differentially expressed in each disease group. In particular, significant over expression of HLA class I and class II genes, and immunoglobulin genes, in the Crohn's disease cohort, that correspond to the IBD3 susceptibility region 6p21.3 on chromosome 6. Various cytokines, chemokines and related genes were also differentially regulated across all disease groups. IFN, TNF and related genes were increased in the autistic enterocolitis and Crohn's disease cohort, correlating with our findings in chapter 5 of increased TNF-α in autistic enterocolitis. This data provides additional evidence for the role of TNF-α or its signalling counterparts in the pathogenesis of autistic enterocolitis.

Chemokines were among the most highly expressed genes in the Crohn's disease cohort. In intestinal inflammation, chemokines play a role in recruiting immune cells to inflammatory sites and cause inflammation by generating metalloenzymes and reactive oxygen radicals that cause tissue damage. Corroborating this, a number of

matrix metalloproteinases including CXCL1 (GRO-α), CXCL2 (GRO-β), CXCL6 (GCP-2), CXCL9 (Mig), CXCL10 (IP-10), CXCL11 (I-TAC) and IL-8 were elevated in Crohn's disease. MMP-14, which is thought to be play a role in wound healing rather than tissue destruction (von Lampe *et al.*, 2000; Okada *et al.*, 1997) was elevated in the autistic enterocolitis cohort. This data provides evidence of a disrupted intestinal mucosa in autistic enterocolitis. In addition, E-cadherin cell adhesion molecule was down regulated in autistic enterocolitis. This is consistent with previous reports of reduced E-cadherin protein and mRNA in active inflammatory bowel disease (Gassler *et al.*, 2001). Other interesting differentially regulated genes, not previously described in IBD included oligophrenin 1 and the serotonin 5'-hydroxytryptamine 2C (5'HT 2C) receptor gene. The significance of these in the intestine is not known but the may be linked to gastrointestinal dysfunction in IBD.

In summary the findings of this study indicate a novel form of inflammatory bowel disease in children with autism. This statement can be made based on the grouped cytokine analysis and the initial gene expression analysis, where we have identified a number of factors that may play a role in the pathogenesis of new variant inflammatory bowel disease "autistic enterocolitis". In particular, the finding of measles virus in the intestine of children with autism is controversial. This study demonstrates a biological association between the presence of measles virus RNA genomes and the autistic enterocolitis phenotype, however this in no way implies causation. The presence of measles virus RNA genome in biopsies from these children may merely represent a bystander effect, i.e, the persistence of measles virus RNA in the presence of inflammation, or the inability of the inflamed gut to clear

measles virus genome. It is not possible from the data obtained in this study to state that measles virus has caused this inflammation or indeed that it is the underlying pathological agent in this disease process. The presence of measles virus RNA genomes does however indicate that these children have been exposed to measles virus (either through vaccination or natural measles infection). The apparent difference in prevalence between measles virus positivity in autistic enterocolitis and the control "normal" population may represent different individual exposures or differences in measles virus handling by different individuals. The fact that the "normal" control population do not have inflamed intestines may indicate that inflamed mucosa of the small bowel may in some way (as yet unidentified) be unable to clear measles virus genomes. However, no molecular epidemiological study has been performed which examines the persistence and clearance of measles virus from both naturally infected and vaccinated individuals.

7.1 Future Work

Following the work presented in this thesis on children with new variant inflammatory bowel disease, a future objective is to assess the level of gastrointestinal dysfunction in adults and children with autistic spectrum disorders, using the combined approach of endoscopic, immunological and molecular biological examinations. This is of interest to determine if this new variant inflammatory bowel disease is specific to childhood or whether it pervades through to adulthood, and its relationship to conventional IBD.

In this thesis an aberrant innate immune response was observed in the intestine of autistic enterocolitis patients as characterised by elevated expression of IL-1, IL-6 and TNF-α mRNA. These cytokines are regulated by NF-κβ signalling. Further work will be undertaken to examine, in particular, the TNF-α signalling pathway and its components, in autistic enterocolitis and conventional IBD. This will be performed using microarray technology to dissect out the molecular components of TNF-α bioactivity in autistic enterocolitis.

Initial gene expression profiling experiments in new variant of inflammatory bowel disease have identified a number of differentially regulated genes from different host cell pathways. The problems encountered in this thesis with isolation of high quality RNA from intestinal tissue biopsies have been overcome by the use of RNA*later* preservative for collection of biopsy material. A major focus for the future will be to functionally map the mRNA expression profiles in the intestine of children and adults with autistic enterocolitis and conventional IBD. This will define molecular and biochemical pathways *in vivo,* which are implicated in the pathogenesis of these

diseases. These studies will establish the prevalence and pathogenesis of gastrointestinal dysfunction in patients with autistic spectrum disorders

Bibliography

Bibliography

Aaby, P., Bukh, J., Kronborg, D. Delayed excess mortality after exposure to measles during the first six months of life. *American Journal of Epidemiology.* 1990; **132**:211-219.

Afzal, M.A., Osterhaus, A.D., Cosby, S.L., Jin, L., Beeler, J., Takeuchi, K., Kawashima, H. Comparative evaluation of measles virus-specific RT-PCR methods through an international collaborative study. *Journal of Medical Virology.* 2003; **70**:171-176.

Afzal, M.A., Armitage, E., Ghosh, S., Williams, L.C., Minor, P.D., Further evidence of the absence of measles virus genome sequence in full thickness intestinal specimens from patients with Crohn's disease. *Journal of Medical Virology.* 2000; **62**:377-382.

Afzal, M.A., Armitage, E., Begley, J., Bentley, M.L., Minor, P.D., Ghosh, S., Ferguson, A. Absence of detectable measles virus genome sequence in inflammatory bowel disease tissues and peripheral blood lymphocytes. *Journal of Medical Virology.* 1998; **55**:243-249.

Agyekum, S., Church, A., Sohail, M., Krausz, T., Van Noorden, S., Polak, J., Cohen, J. Expression of lymphotoxin-beta (LT-beta) in chronic inflammatory conditions. *Journal of Pathology.* 2003; **199**:115-121.

Ahmad, T., Satsangi, J., McGovern, D., Bunce, M., Jewell, D.P. The genetics of inflammatory bowel disease. *Alimentary Pharmacology and Therapeutics.* 2001; **15**:731-748.

Allen, I.V., McQuaid, S., McMahon, J., Kirk, J., McConnell, R. The significance of measles virus antigen and genome distribution in the CNS in SSPE for mechanisms of viral spread and demyelination. *Journal of Neuropathology and Experimental Neurology.* 1996; **55**:471-480.

American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Health Disorders.* 4th edition, Washington DC: American Psychiatric Association, 1994.

Asadullah, K., Sterry, W., Volk, H.D. Interleukin-10 therapy--review of a new approach. *Pharmacology Review*. 2003; **55**:241-269.

Aschner M., Walker SJ. The neuropathogenesis of mercury toxicity. *Molecular Psychiatry*. 2002; 7:S40-1.

Ashwood, P., Anthony, A., Killalea, A, Montgomery, S.M., O'Leary, J.J., Wakefield, A.J. Detection of measles virus antigen in children with autistic enterocolitis. *Journal of Experimental Biology and Medicine.* (accepted 2003).

Atabani, S.F., Byrnes, A.A., Jaye, A., Kidd, I.M., Magnusen, A.F., Whittle, H., Karp, C.L. Natural measles causes prolonged suppression of interleukin-12 production. *Journal of Infectious Disease*. 2001; **184**:1-9.

Autschbach, F., Braunstein, J., Helmke, B., Zuna, I., Schurmann, G., Niemir, Z.I., Wallich, R., Otto, H.F., Meuer, S.C. In situ expression of interleukin-10 in non-inflamed human gut and in inflammatory bowel disease. *American Journal of Pathology*. 1998; **153**:121-130.

Avota, E., Avots, A., Niewiesk, S., Kane, L.P., Bommhardt, U., Ter Meulen, V., Schneider-Schaulies, S. Disruption of Akt kinase activation is important for immunosuppression induced by measles virus. *Nature Medicine*. 2001; **7**:725-731.

Babyatsky, M.W., Rossiter, G., Podolsky, D.K. Expression of transforming growth factors alpha and beta in colonic mucosa in inflammatory bowel disease. *Gastroenterology*. 1996; **110**:975-984.

Bailey, A., Le Couteur, A., Gottesman, I., Bolton, P., Simonoff, E., Yuzda, E., Rutter, M. Autism as a strongly genetic disorder: evidence from a British twin study. *Psychological Medicine*. 1995; **25**:63-77.

Bairead, E., Harmon, D.L., Curtis, A.M., Kelly, Y., O'Leary, C., Gardner, M.0, Leahy, D.T., Vaughan, P., Keegan, D., O'Morain, C., O'Donoghue, D., Shanahan, F., Parfrey, N.A., Quane, K.A. Association of NOD2 with Crohn's disease in a homogenous Irish population. *European Journal of Human Genetics*. 2003; **11**:237-244.

Banchereau, J., Steinman, R.M. Dendritic cells and the control of immunity. *Nature*. 1998; **392**:245-252.

Banks, C., Bateman, A., Payne, R., Johnson, P., Sheron, N. Chemokine expression in IBD. Mucosal chemokine expression is unselectively increased in both ulcerative colitis and Crohn's disease. *Journal of Pathology*. 2003 ;**199**:28-35.

Barbara, G., Xing, Z., Hogaboam, C.M., Gauldie, J., Collins, S.M. Interleukin 10 gene transfer prevents experimental colitis in rats. *Gut*. 2000;**46**:344-9.

Bartz, R., Firsching, R., Rima, B., Ter Meulen, V., Schneider-Schaulies, J. Differential receptor usage by measles virus strains. *Journal of General Virology*. 1998; **79**:1015-1025.

Bauer, P., Rolfs, A., Regits-Zagrosek, V., Hildebrandt, A., Fleck, E. Use of manganese in RT-PCR eliminates PCR artefacts resulting from DNase I digestion. *Biotechniques*. 1997; **22**:1128-1132.

Bayless, T.M., Tokayer, A.Z., Polito, J.M. 2nd., Quaskey, S.A., Mellits, E.D., Harris, M.L. Crohn's disease: concordance for site and clinical type in affected family members-potential hereditary influences. *Gastroenterology*. 1996; **111**:573-579.

Bell, A.F., Burns, J.B., Fujinami, R.S. Measles virus infection of human T cells modulates cytokine generation and IL-2 receptor alpha chain expression. *Virology*. 1997; **232**:241-247.

Bellanti, J.A., Zeligs, B.J., Malka-Rais, J., Sabra, A. Abnormalities of Th1 function in non-IgE food allergy, celiac disease, and ileal lymphonodular hyperplasia: a new relationship? *Annals of Allergy and Asthma Immunology*. 2003; **90**:84-89.

Berg, D.J., Davidson, N., Kuhn, R., Muller, W., Menon, S., Holland, G., Thompson-Snipes, L., Leach, M.W., Rennick, D. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *Journal of Clinical Investigations*. 1996; **98**:1010-1020.

Bernard, S., Enayati, A., Redwood, L., Roger, H., Binstock, T. Autism: a novel form of mercury poisoning. *Medical Hypotheses*. 2001; **56**:462-471.

Billuart, P., Bienvenu, T., Ronce, N., des Portes, V., Vinet, M.C., Zemni, R., Roest Crollius, H., Carrie, A., Fauchereau, F., Cherry, M., Briault, S., Hamel, B., Fryns, J.P., Beldjord, C., Kahn, A., Moraine, C., Chelly, J. Oligophrenin-1 encodes a rhoGAP protein involved in X-linked mental retardation. *Nature*. 1998; **30**; 923-6.

Bitnun, A., Shannon, P., Durward, A., Rota, P.A., Bellini, W.J., Graham, C., Wang, E., Ford-Jones, E.L., Cox, P., Becker, L., Fearon, M., Petric, M., Tellier, R. Measles inclusion-body encephalitis caused by the vaccine strain of measles virus. *Clinical Infectious Diseases*. 1999; **29**:855-861.

Black, V.C., Kaye, J.A., Jick, H. Relation of childhood gastrointestinal disorders to autism: Nested case control study using data from the UK general practice research database. *British Medical Journal*. 2002; **325**:419-421.

Bobrow, M.N., Harris, T.D., Shaughnessy, K., *et al.* Catalysed reporter deposition, a novel method of signal amplification. Application to immunoassays. *Journal of Immunological Methods*. 1989; **125**:279-285.

Bohme, M., Diener, M., Mestres, P., Rummel, W. Direct and indirect actions of HgCl2 and methyl mercury chloride on permeability and chloride secretion across the rat colonic mucosa. *Toxicology and Applied Pharmacology*. 1992; **114**:285-294.

Bonora, E., Beyer, K.S., Lamb, J.A., Parr, J.R., Klauck, S.M., Benner, A., Paolucci, M., Abbott, A., Ragoussis, I., Poustka, A., Bailey, A.J., Monaco, A.P. Analysis of reelin as a candidate gene for autism. *Molecular Psychiatry*. 2003; **8**:885-892.

Bose, S., Kar, N., Maitra, R., DiDonato, J.A., Banerjee, A.K. Temporal activation of NF-kappaB regulates an interferon-independent innate antiviral response against cytoplasmic RNA viruses. *Proceedings of the National Academy of Science, U S A*. 2003 ;**100**:10890-5.

Bose S, Banerjee AK. Innate immune response against nonsegmented negative strand RNA viruses. *Journal of Interferon and Cytokine Research*. 2003;**23**:401-12.

Boshoff, C., Schulz, T.F., Kennedy, M.M., *et al.* Kaposi's sarcoma-associated herpes virus infects endothelial and spindle cells. *Nature Medicine*. 1995; **1**:1274-1278.

Braegger, C.P., Nicholls, S., Murch, S.H., Stephens, S., MacDonald, T.T. Tumour necrosis factor alpha in stool as a marker of intestinal inflammation. *Lancet*. 1992; **339**:89-91.

Brown, K.A., Back, S.J., Ruchelli, E.D., Markowitz, J., Mascarenhas, M., Verma, R., Piccoli, D.A., Baldassano, R.N. Lamina propria and circulating interleukin-6 in newly diagnosed paediatric inflammatory bowel disease patients. *American Journal of Gastroenterology*. 2002; **97**:2603-8

Buxbaum, J.D., Silverman, J.M., Smith, C.J., Greenberg, D.A., Kilifarski, M., Reichert, J., Cook, E.H. Jr, Fang, Y., Song, C.Y., Vitale, R. Association between a GABRB3 polymorphism and autism. *Molecular Psychiatry*. 2002; **7**:311-6.

Casasnovas, J.M., Larvie, M., Stehle, T. Crystal structure of two CD46 domains reveals an extended measles virus-binding surface. *European Molecular Biology Organisation Journal*. 1999; **18**:2911-2922.

Cattaneo, R., Schmid, A., Spielhofer, P., Kaelin, K., Baczko, K., ter Meulen, V., Pardowitz, J., Flanagan, S., Rima, B.K., Udem, S.A., *et al,* Mutated and hypermutated genes of persistent measles viruses which caused lethal human brain diseases. *Virology*. 1989; **173**:415-425.

Chadwick, N., Bruce, I.J., Schepelmann, S., Pounder, R.E., Wakefield, A.J. Measles virus RNA is not detected in inflammatory bowel disease using hybrid capture and reverse transcription followed by the polymerase chain reaction. *Journal of Medical Virology*. 1998; **55**:305-311.

Chesler, D.A., Shoshkes Reiss, C. The role of IFN-γ in immune responses to viral infections of the central nervous system. *Cytokine and Growth Factor Reviews*. 2002; **13**:441-454.

Chugani, D.C. Role of altered brain serotonin mechanisms in autism. *Molecular Psychiatry*. 2002; 7:S16-17.

Cook, E.H. Jr., Courchesne, R., Lord, C., Cox, N.J., Yan, S., Lincoln, A., Haas, R., Courchesne, E., Leventhal, B.L. Evidence of linkage between the serotonin transporter and autistic disorder. *Molecular Psychiatry*. 1997; **2**:247-250.

Cosnes, J., Beaugerie, L., Carbonnel, F., Gendre, J.P. Smoking cessation and the course of Crohn's disease: an intervention study. *Gastroenterology*. 2001; **120**:1093-1099.

Croonenberghs, J., Bosmans, E., Deboutte, D., Kenis, G., Maes, M. Activation of the inflammatory response system in autism. *Neuropsychobiology*. 2002; **45**:1-6.

Cunliffe, R.N., Rose, F.R., Keyte, J., Abberley, L., Chan, W.C. Mahida, Y.R. Human defensin 5 is stored in precursor form in normal Paneth cells and is expressed by some villous epithelial cells and by metaplastic Paneth cells in the colon in inflammatory bowel disease. *Gut*. 2001; **48**:176-185.

D'Eufemia, P., Ceilli, M., Finocchiaro, R., Pacifico, L., Viozzi, L., Zaccagnini, M., Cardi, E., Giardini, O. Abnormal intestinal permeability in children with autism. *Acta Paediatrica*. 1996; **85**:1076-1079.

Daszak, P., Purcell, M., Lewin, J., Dhillon, A.P., Pounder, R.E., Wakefield, A.J. Detection and comparative analysis of persistent measles virus infection in Crohn's disease by immunogold electron microscopy. *Journal of Clinical Pathology*. 1997; **50**:299-304.

D'Ambrosio, D., Panina-Bordignon, P., Sinigaglia, F. Chemokine receptors in inflammation: an overview. *Journal of Immunological Methods*. 2003 ;**273**:3-13.

DeFelice, M.L., Ruchelli, E.D., Markowitz, J.E., Strogatz, M., Reddy, K.P., Kadivar, K., Mulberg, A.E., Brown, K.A. Intestinal cytokines in children with pervasive developmental disorders. *American Journal of Gastroenterology*. 2003; **98**:1777-1782.

Department of Developmental Services. *Changes in population of persons with autism and pervasive developmental disorders in California's Developmental Services System; 1987 through 1998: a report to the legislature.* Sacramento: California Health and Human Services Agency, March 1999.

D'haens, G., Van Deventer, S., Van Hogezand, R., Chalmers, D., Kothe, C., Baert, F, Braakman, T., Schaible, T., Geboes, K., Rutgeerts, P. Endoscopic and histological healing with infliximab anti-tumor necrosis factor antibodies in Crohn's disease: A European multicenter trial. *Gastroenterology.* 1999; **116**:1029-1034.

Dhossche, D., Applegate, H., Abraham, A., Maertens, P., Bland, L., Bencsath, A., Martinez, J. Elevated plasma gamma-aminobutyric acid (GABA) levels in autistic youngsters: stimulus for a GABA hypothesis of autism. *Medical Science Monitor.* 2002; **8**:1-6.

Dieckgraefe, B.K., Crimmins, D.L., Landt, V., Houchen, C., Anant, S., Porche-Sorbet, R., Ladenson, J.H. Expression of the regenerating gene family in inflammatory bowel disease mucosa: Reg Ialpha upregulation, processing, and antiapoptotic activity. *Journal of Investigative Medicine.* 2002;**50**:421-34.

Dieckgraefe, B.K., Stenson, W.F., Korzenik, J.R., Swanson, P.E., Harrington, C.A. Analysis of mucosal gene expression in inflammatory bowel disease by parallel oligonucleotide arrays. *Physiol Genomics.* 2000;**4**:1-11

Dinarello, C.A., Wolff, S.M. The role of interleukin-1 in disease. *New England Journal of Medicine.* 1993; **328**:106-13.

Dorig, R.E., Marcil, A., Chopra, A., Richardson, C.D. The human CD46 molecule is a receptor for measles virus (Edmonston strain). *Cell.* 1993; **75**:295-305.

Eisen, M.B., Spellman, P.T., Brown, P.O., Botstein, D. Cluster analysis and display of genome-wide expression patterns. *Proceedings of the National Academy of Science.* 1998; **95**:14863-8.

Ekbom, A., Daszak, P., Kraaz, W., Wakefield, A.J. Crohn's disease after in-utero measles virus exposure. *Lancet*. 1996; **348**:515-517.

Ekbom, A., Wakefield, A.J., Zack, M., Adami, H.O. Perinatal measles infection and subsequent Crohn's disease. *Lancet*. 1994; **344**:508-510.

Erlenhofer, C., Duprex, W.P., Rima, B.K., ter Meulen, V., Schneider-Schaulies, J. Analysis of receptor (CD46, CD150) usage by measles virus. *Journal of General Virology*. 2002; **83**:1431-1436.

Erlenhoefer, C., Wurzer, W.J., Loffler, S., Schneider-Schaulies, S., ter Meulen, V., Schneider-Schaulies, J. CD150 (SLAM) is a receptor for measles virus but is not involved in viral contact-mediated proliferation inhibition. *Journal of General Virology*. 2001; **75**:4499-4505.

Etienne-Manneville, S., Hall, A. Rho GTPases in cell biology. *Nature*. 2002 ;**12**: 629-35.

Fahlgren, A., Hammarstrom, S., Danielsson, A., Hammarstrom, M.L. Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. *Clinical Experimental Immunology*. 2003; **131**:90-101.

Fang, Y.Y., Song, Z.M., Wu, T., Raha, A., Kalvakolanu, D.V., Dhib-Jalbut, S. Defective NF-kappaB activation in virus infected neuronal cells is restored by genetic complementation. *Journal of Neurovirology*. 2002; **5**:459-463.

Fang, Y.Y., Song, Z.M., Dhib-Jalbut, S. Mechanism of measles virus failure to activate NF-kappaB in neuronal cells. *Journal of Neurovirology*. 2001; **1**:25-34.

Farmer, R.G., Michener, W.M., Mortimer, E.A. Studies of family history among patients with inflammatory bowel disease. *Clinical Gastroenterology*. 1980; **9**:271-277.

Fatemi, S.H., Stary, J.M., Egan, E.A. Reduced blood levels of reelin as a vulnerability factor in pathophysiology of autistic disorder. *Cellular and Molecular Neurobiology*. 2002; **22**:139-152.

Fellermann, K., Wehkamp, J., Herrlinger, K.R., Stange, E.F. Crohn's disease: a defensin deficiency syndrome? *European Journal of Gastroenterology and Hepatology*. 2003; **15**:627-634.

Ferguson, A. Ulcerative colitis and Crohn's disease. *British Medical Journal.* 1994; 309:355-6.

Fiocchi, C. Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology*. 1998; **115**:182-205.

Fiorica-Howells, E., Maroteaux ,L., Gershon, M.D. Serotonin and the 5-HT(2B) receptor in the development of enteric neurons. *Journal of Neuroscience*. 2000;**20**:294-305.

Fisher, N.C., Yee, L., Nightingale, P., McEwan, R., Gibson, J.A. Measles virus serology in Crohn's disease. *Gut*. 1997; **41**:66-69.

Florell, S. R., Coffin, C. M., Holden, J. A., Zimmermann, J. W., Gerwels, J. W., Summers, B. K., Jones, D. A., Leachman, S. A. Preservation of RNA for Functional Genomics Studies: A Multidisciplinary Tumor Bank Protocol. *Modern Pathology.* 2001;**14**: 116-128.

Folwaczny, C., Jager, G., Schnettler, D., Wiebecke, B., Loeschke, K. Search for mumps virus genome in intestinal biopsy specimens of patients with IBD. *Gastroenterology*. 1999; **117**:1253-1255.

Fombonne, E. Epidemiological trends in rates of autism. *Molecular Psychiatry*. 2002; 7:S4-6.

Fombonne, E., Chakrabarti, S. No evidence for a new variant of measles-mumps-rubella-induced autism. *Pediatrics*. 2001; **108**:E58.

Fudenberg, H.H. Dialysable lymphocyte extract (DLyE) in infantile onset autism: a pilot study. *Biotherapy*. 1996; **9**:143-147.

Furlano, R.I., Anthony, A., Day, R., Brown, A., McGarvey, L., Thomson, M.A., Davies, S.E., Berelowitz, M., Forbes, A., Wakefield, A.J., Walker-Smith, J.A.,

Murch, S.H. Colonic CD8 and gamma delta T-cell infiltration with epithelial damage in children with autism. *Journal of Pediatrics*. 2001; **138**:366-372.

Fuss, I.J., Neurath, M., Boirivant, M., Klein, J.S., De la Motte, C., Strong, S.A., Fiocchi, C., Strober, W. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *Journal of Immunology*. 1996; **157**:1261-1270.

Galbraith, S.E., Tiwari, A., Baron, M.D., Lund, B.T., Barrett, T., Cosby, S.L. Morbillivirus downregulation of CD46. *Journal of Virology*. 1998; **72**:10292-10297.

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. *Journal of Clinical Immunology*. 2000; **20**:362-370.

Gassler, N., Rohr, C., Schneider, A., Kartenbeck, J., Bach, A., Obermuller, N., Otto, H.F., Autschbach, F. Inflammatory bowel disease is associated with changes of enterocytic junctions. *American Journal of Physiology, Gastrointestinal and Liver Physiology*. 2001;**281**:216-28.

Gaulier, A., Sabatier, P., Prevot, S., Fournier, J.G. Do measles early giant cells result from fusion of non-infected cells? An immunohistochemical and in situ hybridization study in a case of morbillous appendicitis. *Virchows Archives of American Pathology Anatomy and Histopathology*. 1991; **419**:245-249.

Giallourakis, C., Stoll, M., Miller, K., Hampe, J., Lander, E.S., Daly, M.J., Schreiber, S., Rioux, J.D. IBD5 is a general risk factor for inflammatory bowel disease: replication of association with Crohn disease and identification of a novel association with ulcerative colitis. *American Journal of Human Genetics*. 2003;**73**:205-11.

Gibson, U., Heid, C.A., Williams, M.P. A novel method for Real Time quantitative RT-PCR. *Genome Research*. 1996; 6:995-1001

Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R., Mathieu, C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods*. 2001; **25**:386-401.

Glasser, A.L , Boudeau, J., Barnich, N., Perruchot, M.H., Colombel, J.F., Darfeuille-Michaud, A. Adherent invasive Escherichia coli strains from patients with Crohn's disease survive and replicate within macrophages without inducing host cell death. *Infection and Immunity*. 2001 ;**69**:5529-37.

Greenstein, R.J. Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Johne's disease. *Lancet Infectious Diseases*. 2003; **3**:507-514.

Griffin, D.E., Ward, B.J. Differential CD4 T cell activation in measles. *Journal of Infectious Diseases*. 1993; **168**:275-281.

Gupta, S., Aggarwal, S., Rashanravan, B., Lee, T. Th1- and Th2-like cytokines in CD4+ and CD8+ T cells in autism. *Journal of Neuroimmunology*. 1998; **85**:106-109.

Gupta, S. Immunology and immunologic treatment of autism. *Proceedings of the National Autism Association, Chicago.* 1996: 455-460.

Haase, A.T., Retzel, F., Staskus, K.A. Amplification and detection of lentiviral DNA inside cells. *Proceedings of the National Academy of Science USA.* 1990; **87**:4971-4975.

Haga, Y., Funakoshi, O., Kuroe, K., Kanazawa, K., Nakajima, H., Saito, H., Murata, Y., Munakata, A., Yoshida, Y. Absence of measles viral genomic sequence in intestinal tissues from Crohn's disease by nested polymerase chain reaction. *Gut*. 1996; **38**:211-215.

Hahm, B., Arbour, N., Naniche, D., Homann, D., Manchester, M., Oldstone, M.B. Measles virus infects and suppresses proliferation of T lymphocytes from transgenic mice bearing human signaling lymphocytic activation molecule. *Journal of Virology*. 2003; **77**:3505-3515.

Halfvarson, J., Bodin, L., Tysk, C., Lindberg, E., Jarnerot, G. Inflammatory bowel disease in a Swedish twin cohort: a long-term follow-up of concordance and clinical characteristics. *Gastroenterology*. 2003; **124**:1767-1773.

Hampe, J., Cuthbert, A., Croucher, P.J., Mirza, M.M., Mascheretti, S., Fisher, S., Frenzel, H., King, K., Hasselmeyer, A., MacPherson, A.J., Bridger, S., Van Deventer, S., Forbes, A., Nikolaus, S., Lennard-Jones, J.E., Foelsch, U.R., Krawczak, M., Lewis, C., Schreiber, S., Mathew, C.G. Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. *Lancet*. 2001; **357**:1925-1928.

Hampe, J., Shaw, S., Saiz, R., Leysen, N., Lantermann, A., Masherretti, S., Lynch, N., MacPherson, A., Bridgere, S., Deventer, S., *et al.* Linkage of inflammatory bowel disease to human chromosome 6p. *American Journal of Human Genetics*. 1999; **65:**1647-1655.

Hedge, P., Qi, R., Abernathy, K., et al., A concise guide to cDNA microarray analysis. *Biotechniques*. 2002; **29**:548-62.

Hermiston, M.L., Gordon, J.I. Inflammatory bowel disease and adenomas in mice expressing a dominant negative N-cadherin. *Science*. 1995; **17**:1203-7.

Herrington, C.S., and O'Leary, J.J. PCR 3: A Practical Approach: PCR. In *Situ Hybridisation*. Oxford University Press, Oxford. 1998; 80-81.

Hisamatsu, T., Suzuki, M., Reinecker, H.C., Nadeau, W.J., McCormick, B.A., Podolsky, D.K. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells**.** *Gastroenterology*. 2003 ;**124**:993-1000.

Holland, P.M., Abramson, R.D., Watson, R., Gelfand, D.H. Detection of specific polymerase chain reaction utilising the 5'-3' exonuclease activity of thermos aquaticus DNA polymerase. *Proceedings of the National Academy of Sciences USA*. 1991; **88**:7276-7280.

Horvath, K., Papadimitriou, J.C., Rabszytn, A., Drachenberg, C., Tildon, J.T. Gastrointestinal abnormalities in children with autistic disorder. *Journal of Paediatrics*. 1999; **135**:599-563.

Hsu, E.C., Sarangi, F., Iorio, C., Sidhu, M.S., Udem, S.A., Dillehay, D.L., Xu, W., Rota, P.A., Bellini, W.J., Richardson, C.D. A single amino acid change in the hemagglutinin protein of measles virus determines its ability to bind CD46 and reveals another receptor on marmoset B cells. *Journal of Virology*. 1998; **72**:2905-2916.

Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, J.P., Belaiche, J., Almer, S., Tysk, C., O'Morain, C.A., Gassull, M., Binder, V., Finkel, Y., Cortot, A., Modigliani, R., Laurent-Puig, P., Gower-Rousseau, C., Macry, J., Colombel, J.F., Sahbatou, M., Thomas, G. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*. 2001; **411**:599-603.

Hugot, J.P., Laurent-Puig, P., Gower-Rousseau, C., Olson, J.M., Lee, J.C., Beaugerie, L., Naom, I., Dupas, J.L., Van Gossumm, A., Orholm, M., Bonaiti-Pellie, C., Weissenbach, J., Mathew, C.G., Lennard-Jones, J.E., Cortot, A., Colombel, J.F., Thomas, G. Mapping of a susceptibility locus for Crohn's disease on chromosome 16. *Nature*. 1996; **379**:821-823.

Huijsdens, X.W., Linskens, R.K., Taspinar, H., Meuwissen, S.G., Vandenbroucke-Grauls, C.M., Savelkoul, P.H. Listeria monocytogenes and inflammatory bowel disease: detection of Listeria species in intestinal mucosal biopsies by real-time PCR. *Scandinavian Journal of Gastroenterology*. 2003;**38**:332-3.

Iizuka, M., Saito, H., Yukawa, M., Itou, H., Shirasaka, T., Chiba, M., Fukushima, T., Watanabe, S. No evidence of persistent mumps virus infection in inflammatory bowel disease. *Gut*. 2001; **48**:637-641.

Iizuka, M., Chiba, M., Yukawa, M., Nakagomi, T., Fukushima, T., Watanabe, S., Nakagomi, O. Immunohistochemical analysis of the distribution of measles related antigen in the intestinal mucosa in inflammatory bowel disease. *Gut*. 2000; **46**:163-169.

Iizuka, M., Masamune, O. Measles vaccination and inflammatory bowel disease. *Lancet*. 1997; **350**:1775.

Iizuka, M., Nakagomi, O., Chiba, M., Ueda, S., Masamune, O. Absence of measles virus in Crohn's disease. *Lancet*. 1995; **345**:199.

Ina, K., Kusugami, K., Yamaguchi, T., Imada, A., Hosokawa, T., Ohsuga, M., Shinoda, M., Ando, T., Ito, K., Yokoyama, Y. Mucosal interleukin-8 is involved in neutrophil migration and binding to extracellular matrix in inflammatory bowel disease. *American Journal of Gastroenterology*. 1997; **92**:1342-1346.

Ishiguro, Y. Mucosal proinflammatory cytokine production correlates with endoscopic activity of ulcerative colitis. *American Journal of Gastroenterology*. 1999; **34**:66-74

Jankowski, J.A., Bedford, F.K., Boulton, R.A., Cruickshank, N., Hall, C., Elder, J., Allan, R., Forbes, A., Kim, Y.S., Wright, N.A., Sanders, D.S. Alterations in classical cadherins associated with progression in ulcerative and Crohn's colitis. *Laboratory Investigations*. 1998; **78**:1155-67.

Jobin, C., Sartor, R.B. The I kappa B/NF-kappa B system: a key determinant of mucosalinflammation and protection. *American Journal of Physiology and Cell Physiology*. 2000; **278**:451-62.

Jones, P., Fine, P., Piracha, S. Crohn's disease and measles. *Lancet*. 1997; **349**:473.

Jyonouchi, H., Sun, S., Itokazu, N. Innate immunity associated with inflammatory responses and cytokine production against common dietary proteins in patients with autism spectrum disorder. *Neuropsychobiology*. 2002; **46**:76-84.

Jyonouchi, H., Sun, S., Le, H. Proinflammatory and regulatory cytokine production associated with innate and adaptive immune responses in children with autism spectrum disorders and developmental regression. *Journal of Neuroimmunology*. 2001; **120**:170-179.

Kaibuchi, K., Kuroda, S., Fukata, M., Nakagawa, M. Regulation of cadherin-mediated cell-cell adhesion by the Rho family GTPases. *Current Opinions in Cell Biology*. 1999;**11**:591-6.

Karlinger, K., Gyorke, T., Mako, E., Mester, A., Tarjan, Z. The epidemiology and the pathogenesis of inflammatory bowel disease. *European Journal of Radiology*. 2000; **35**:154-167.

Karp, C.L., Wysocka, M., Wahl, L.M., Ahearn, J.M., Cuomo, J., Sherry, B., Trinchieri, G., Griffin, D.E. Mechanism of suppression of cell-mediated immunity by measles virus. *Science*. 1996; **273**:228-231.

Katayama, Y., Hirano, A., Wong, T.C. Human receptor for measles virus (CD46) enhances nitric oxide production and restricts virus replication in mouse macrophages by modulating production of alpha/beta interferon. *Journal of Virology*. 2000; **74**:1252-1257.

Katsuta, T., Lim, C., Shimoda, K., Shibuta, K., Mitra, P., Banner, B.F., Mori, M., Barnard, G.F. Interleukin-8 and SDF1-alpha mRNA expression in colonic biopsies from patients with inflammatory bowel disease. *American Journal of Gastroenterology*. 2000 ;**95**: 3157-64.

Kawashima, H., Mori, T., Kashiwagi, Y., Takekuma, K., Hoshika, A., Wakefield A. Detection and sequencing of measles virus from peripheral mononuclear cells from patients with inflammatory bowel disease and autism. *Digestive Disease Science*. 2000; **45**:723-729.

Kaye, J.A., Del Mar Melero-Montes, M., Jick, H. Mumps, measles, and rubella vaccine and the incidence of autism recorded by general practitioners: a time trend analysis. *British Medical Journal*. 2001; **322**:460-463.

Kemper, T.L., Bauman, M.L. Neuropathology of infantile autism. *Molecular Psychiatry*. 2002; **7**:S12-3.

Kim, S.J, Cox, N., Courchesne, R., Lord, C., Corsello, C., Akshoomoff, N., Guter, S., Leventhal, B.L., Courchesne, E., Cook, E.H .Jr. Transmission disequilibrium mapping at the serotonin transporter gene (SLC6A4) region in autistic disorder. *Molecular Psychiatry*. 2002;**7**:278-88.

Klagge, I.M., ter Meulen, V., Schneider-Schaulies, S. Measles virus-induced promotion of dendritic cell maturation by soluble mediators does not overcome the immunosuppressive activity of viral glycoproteins on the cell surface. *European Journal of Immunology*. 2000 ; **30**:2741-2750.

Klagge, I.M., Schneider-Schaulies S. Virus interactions with dendritic cells. *Journal of General Virology*. 1999; **80**:823-833.

Klauck, S.M., Poustka, F., Benner, A., Lesch, K.P., Poustka, A. Serotonin transporter (5-HTT) gene variants associated with autism. *Human Molecular Genetics*. 1997; **6**:2233-2238.

Knivsberg, A.M., Reichelt, K.L., Hoien, T., Nodland, M. A randomised, controlled study of dietary intervention in autistic syndromes. *Nutritional Neuroscience*. 2002; **5**:251-261.

Kokkonen, J., Karttunen, T.J. Lymphonodular hyperplasia on the mucosa of the lower gastrointestinal tract in children: an indication of enhanced immune response? *Journal of Paediatric Gastroenterology and Nutrition*. 2002a; **34**:42-46.

Kokkonen, J., Ruska, T., Karttunen, T.J., Maki, M. Lymphonodular hyperplasia of the terminal ileum associated with colitis shows an increase gamma delta+ t-cell density in children. *American Journal of Gastroenterology*. 2002b; **97**:667-672.

Kokkonen, J., Holm, K., Karttunen, T.J., Maki, M. Children with untreated food allergy express a relative increment in the density of duodenal gamma delta+ T cells. *Scandinavian Journal of Gastroenterology*. 2000; **35**:1137-1142.

Kokkonen J. Lymphonodular hyperplasia on the duodenal bulb indicates food allergy in children. *Endoscopy*. 1999; **31**:464-467.

Komatsu, M., Kobayashi, D., Saito, K., Furuya, D., Yagihashi, A., Araake, H., Tsuji, N., Sakamaki, S., Niitsu, Y., Watanabe, N. Tumor necrosis factor-alpha in serum of patients with inflammatory bowel disease as measured by a highly sensitive immuno-PCR. *Clinical Chemistry*. 2001; **47**:1297-1301.

Krebs, M.O., Betancur, C., Leroy, S., Bourdel, M.C., Gillberg, C., Leboyer, M. Absence of association between a polymorphic GGC repeat in the 5' untranslated region of the reelin gene and autism. *Molecular Psychiatry*. 2002; **7**:801-804.

Kromer, W. Endogenous and exogenous opioids in the control of gastrointestinal motility and secretion. *Pharmacology Review*. 1988; **40**:121-162.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., Muller, W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*. 1993; **75**:263-274.

Kusugami, K., Fukatsu, A., Tanimoto, M., Shinoda, M., Haruta, J., Kuroiwa, A., Ina, K., Kanayama, K., Ando, T., Matsuura, T., *et al.* Elevation of interleukin-6 in inflammatory bowel disease is macrophage- and epithelial cell-dependent. *Digestive Disease Science*. 1995; **40**:949-959.

Kutyavin, I.V., Afonina, I.A., Mills, A., Gorn, V.V., Lukhtanov, E.A., Belousov, E.S., Singer, M.J., Walburger, D.K., Lokhov, S.G., Gall, A.A., Dempcy, R., Reed, M.W., Meyer, R.B., Hedgpeth, J. 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Research*. 2000; **28**:655-661.

Lassig, J.P., Vachirasomtoon, K., Hartzell, K., Leventhal, M., Courchesne, E., Courchesne, R., Lord, C., Leventhal, B.L., Cook, E.H. Jr. Physical mapping of the serotonin 5-HT(7) receptor gene (HTR7) to chromosome 10 and pseudogene (HTR7P) to chromosome 12, and testing of linkage disequilibrium between HTR7 and autistic disorder. *American Journal of Medical Genetics*. 1999; **88**:472-5.

Lawrence, D.M., Vaughn, M.M., Belman, A.R., Cole, J.S, Rall, G.F. Immune response-mediated protection of adult but not neonatal mice from neuron-restricted measles virus infection and central nervous system disease. *Journal of Virology*. 1999; **73**:1795-1801.

Lawrance, I.C., Fiocchi, C., Chakravarti, S. Ulcerative colitis and Crohn's disease: distinctive gene expression profiles and novel susceptibility candidate genes. *Human Molecular Genetics*. 2001; **10**:445-456.

Lecouturier, V., Fayolle, J., Caballero, M., Carabana, J., Celma, M.L., Fernandez-Munoz, R., Wild, T.F., Buckland, R. Identification of two amino acids in the hemagglutinin glycoprotein of measles virus (MV) that govern hemadsorption, HeLa cell fusion, and CD46 downregulation: phenotypic markers that differentiate vaccine and wild-type MV strains. *Journal of Virology*. 1996; **70**:4200-4204.

Lee, L.G., Connell, C.R., Bloch, W. Allelic discrimination by nick-translation PCR with fluorogenic probes. *Nucleic Acids Research*. 1993; **21**:3761-3766.

Lewin, J., Dhillon, A.P., Sim, R., Mazure, G., Pounder, R.E., Wakefield, A.J. Persistent measles virus infection of the intestine: confirmation by immunogold electron microscopy. *Gut*. 1995; **36**:564-569.

Linden, D.R., Chen, J.X., Gershon, M.D., Sharkey, K.A., Mawe, G.M. Serotonin availability is increased in mucosa of guinea pigs with TNBS-induced colitis. *American Journal of Physiology Gastrointestinal and Liver Physiology*. 2003; **285**:207-16.

Liu, Y., van Kruiningen, H.J., West, A.B., Cartun, R.W., Cortot, A., Colombel, J.F. Immunocytochemical evidence of Listeria, Escherichia coli, and Streptococcus antigens in Crohn's disease. *Gastroenterology*. 1995; **108**:1396-1404.

Livak, K.J., Flood, S.J.A., Marmaro, J., Giusti, W., Deetz, K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridisation. *PCR methods and applications*. 1995; **4**:357-362.

Livak, K.J., Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; **25**:402-408.

Ljubimova, J.Y., Khazenzon, N.M., Chen, Z., Neyman, Y.I., Turner, L., Riedinger, M.S., Black, K.L. Gene expression abnormalities in human glial tumours identified by gene array. *International Journal of Oncology*. 2001;**18**:287-95.

Louis, E., Ribbens, C., Godon, A., Franchimont, D., De Groote, D., Hardy, N., Boniver, J., Belaiche, J., Malaise, M. Increased production of matrix metalloproteinase-3 and tissue inhibitor of metalloproteinase-1 by inflamed mucosa in inflammatory bowel disease. *Clinical and Experimental Immunology*. 2000 ;**120** :241-6.

Louis, E., Satsangi, J., Roussomoustakaki, M., Parkes, M., Fanning, G., Welsh, K., Jewell, D. Cytokine gene polymorphisms in inflammatory bowel disease. *Gut*. 1996; **39**:705-710.

Lucarelli, S., Frediani, T., Zingoni, A.M., Ferruzzi, F., Giardini, O., Quintieri, F., Barbato, M., D'Eufemia, P., Cardi, E. Food allergy and infantile autism. *Panminerva Med*. 1995; **37**:137-141.

Madsen, K.M., Lauritsen, M.B., Pedersen, C.B., Thorsen, P., Plesner, A.M., Andersen, P.H., Mortensen, P.B. Thimerosal and the occurrence of autism: negative ecological evidence from Danish population-based data. *Pediatrics*. 2003; **112**:604-606.

Madsen, K.M., Hviid , A., Vestergaard, M., Schendel, D., Wohlfahrt, J., Thorsen, P., Olsen, J., Melbye, M. A population-based study of measles, mumps, and rubella vaccination and autism. *New England Journal of Medicine*. 2002; **347**:1477-1482.

Maestrini, E., Lai, C., Marlow, A., Matthews, N., Wallace, S., Bailey, A., Cook, E.H., Weeks, D.E., Monaco, A.P. Serotonin transporter (5-HTT) and gamma-aminobutyric acid receptor subunit beta3 (GABRB3) gene polymorphisms are not associated with autism in the IMGSA families. The International Molecular Genetic Study of Autism Consortium. *American Journal Medical Genetics*. 1999; 88:492-6

Magro, F., Vieira-Coelho, M.A., Fraga, S., Serrao, M.P., Veloso, F.T., Ribeiro, T., Soares-da-Silva, P. Impaired synthesis or cellular storage of norepinephrine, dopamine, and 5-hydroxytryptamine in human inflammatory bowel disease. *Digestive Disease Science*. 2002;**47**:216-24.

Mahida, Y.R., Kurlac, L., Gallagher, A., Hawkey, C.J. High circulating concentrations of interleukin-6 in active Crohn's disease but not ulcerative colitis. *Gut*. 1991; **32**:1531-1534.

Manchester, M., Liszewski, M.K., Atkinson, J.P., Oldstone, M.B. Multiple isoforms of CD46 (membrane cofactor protein) serve as receptors for measles virus. *Proceedings of the National Academy of Science, USA*. 1994; **91**:2161-2165.

Maric, I., Poljak, L., Zoricic, S., Bobinac, D., Bosukonda, D., Sampath, K.T., Vukicevic, S. Bone morphogenetic protein-7 reduces the severity of colon tissue damage and accelerates the healing of inflammatory bowel disease in rats. *Journal of Cell Physiology*. 2003; **196**:258-264.

Martin, C.M., Uhlmann, V., Killalea, A., Sheils, O., O'Leary, J.J. Detection of measles virus in children with ileo-colonic lymphoid nodular hyperplasia, enterocolitis and developmental disorder. *Molecular Psychiatry*. 2002; **7**:S47-48.

Martin, E.R., Menold, M.M., Wolpert, C.M., Bass, M.P., Donnelly, S.L., Ravan, S.A., Zimmerman, A., Gilbert, J.R., Vance, J.M., Maddox, L.O.,Wright, H.H., Abramson, R.K., DeLong, G.R., Cuccaro, M.L., Pericak-Vance, M.A. Analysis of linkage disequilibrium in gamma-aminobutyric acid receptor subunit genes in autistic disorder. *American Journal Medical Genetics*. 2000; **96**:43-48.

McClintick, J.N., Jerome, R.E., Nicholson, C.R., Crabb, N.W., Edenberg, H.J. Reproducibility of oligonucleotide arrays using small samples. *BMC genomics*. 2003; **4**:4

McDougle, C.J., Epperson, C.N., Price, L.H., Gelernter, J. Evidence for linkage disequilibrium between serotonin transporter protein gene (SLC6A4) and obsessive compulsive disorder. *Molecular Psychiatry*. 1998; **3**:270-3.

McDougle, C.J., Naylor, S.T., Cohen, D.J., Volkmar, F.R., Heninger, G.R., Price, L.H. A double-blind, placebo-controlled study of fluvoxamine in adults with autistic disorder. *Archives of Genetic Psychiatry*. 1996; **53**:1001-1008.

Melgar, S., Yeung, M.M., Bas, A., Forsberg, G., Suhr, O., Oberg, A., Hammarstrom, S., Danielsson, A., Hammarstrom, M.L. Over-expression of interleukin 10 in mucosal T cells of patients with active ulcerative colitis. *Clinical and Experimental Immunology*. 2003; **134**:127-137.

Melmed, R., Schneider, C., Fabes, R., Phillips, J., Reichelt, K. *Journal of Paediatric Gastroenterology and Nutrition*. 2000; **31**:S31-S32.

Meresse, B., Rutgeerts, P., Malchow, H., Dubucquoi, S., Dessaint, J.P., Cohard, M., Colombel, J.F., Desreumaux, P. Low ileal interleukin 10 concentrations are predictive of endoscopic recurrence in patients with Crohn's disease. *Gut*. 2002; **50**:25-28.

Mishina, D., Katsel, P., Brown, S.T., Gilberts, E.C., Greenstein, R.J. On the etiology of Crohn disease. *Proceedings of the National Academy of Science*, U S A. 1996 **93**:9816-20.

Miyamoto, H., Tanaka, T., Kitamoto, N., Fukuda, Y., Shimoyama, T. Detection of immunoreactive antigen, with a monoclonal antibody to measles virus, in tissue from a patient with Crohn's disease. *Journal of Gastroenterology*. 1995; **30**:28-33.

Molloy, C.A., Manning-Courtney, P. Prevalence of chronic gastrointestinal symptoms in children with autism and autistic spectrum disorders. *Autism*. 2003; **7**:165-171.

Monteleone, G., Mann, J., Monteleone, I., Vavassori, P., Bremner, R., Fantini, M., Del Vecchio, Blanco, G., Tersigni, R., Alessandroni, L., Mann, D., Pallone, F., MacDonald, T.T. A failure of TGFbeta 1 negative regulation maintains sustained NF-KB activation in gut inflammation. *Journal of Biological Chemistry*. 2003. Epub ahead of print.

Monteleone, G., Kumberova, A., Croft, N.M., McKenzie, C., Steer, H.W., MacDonald, T.T. Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease. *Journal of Clinical Investigations*. 2001; **108**:601-609.

Monteleone, G., Trapasso, F., Parrello, T., Biancone, L., Stella, A., Iuliano, R., Luzza, F., Fusco, A., Pallone, F. Bioactive IL-18 expression is up-regulated in Crohn's disease. *Journal of Immunology*. 1999; **163**:143-147.

Monteleone, G., Biancone, L., Marasco, R., Morrone, G., Marasco, O., Luzza, F., Pallone, F. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology*. 1997; **112**:1169-1178.

Montgomery, S.M., Morris, D.L., Pounder, R.E., Wakefield, A.J. Paramyxovirus infections in childhood and subsequent inflammatory bowel disease. *Gastroenterology*. 1999; **116**:796-803.

Morris, D.L., Montgomery, S.M., Thompson, N.P., Ebrahim, S., Pounder, R.E., Wakefield AJ. Measles vaccination and inflammatory bowel disease: a national British Cohort Study. *American Journal of Gastroenterology*. 2000; **95**:3507-3512.

Moss, M.T., Sanderson, J.D., Tizard, M.L., Hermon-Taylor, J., el-Zaatari, F.A., Markesich, D.C., Graham, D.Y.Polymerase chain reaction detection of Mycobacterium paratuberculosis and Mycobacterium avium subsp silvaticum in long term cultures from Crohn's disease and control tissues. *Gut*. 1992 ;**33**:1209-13.

Moss, M.T., Green, E.P., Tizard, M.L., Malik, Z.P., Hermon-Taylor, J. Specific detection of Mycobacterium paratuberculosis by DNA hybridisation with a fragment of the insertion element IS900. *Gut*. 1991;**32**:395-8.

Murch, S.H., MacDonald, T.T., Walker-Smith, J.A., Levin, M., Lionetti, P., Klein, N.J.. Disruption of sulphated glycosaminoglycans in intestinal inflammation. *Lancet*. 1993a; **341**:711-714.

Murch, S.H., Braegger, C.P., Walker-Smith, J.A., MacDonald, T.T. Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease. *Gut*. 1993b; **34**:1705-1709.

Naniche, D., Varior-Krishnan, G., Cervoni, F., Wild, T.F., Rossi, B., Rabourdin-Combe, C., Gerlier, D. Human membrane cofactor protein (CD46) acts as a cellular receptor for measles virus. *Journal of Virology*. 1993; **67**:6025-6032.

Nelson, K.B., Bauman, M.L. Thimerosal and autism? *Pediatrics*. 2003; **111**:674-679.

Nielsen, O.H., Rudiger, N., Gaustadnes, M., Horn, T. Intestinal interleukin-8 concentration and gene expression in inflammatory bowel disease. *Scandinavian Journal of Gastroenterology*. 1997; **32**:1028-1034.

Nielsen, O.H., Vainer, B., Madsen, S.M., Seidelin, J.B., Heegaard, N.H. Established and emerging biological activity markers of inflammatory bowel disease. *American Journal of Gastroenterology*. 2000; **95**:359-367.

Nikolaus, S., Bauditz, J., Gionchetti, P., Witt, C., Lochs, H., Schreiber, S. Increased secretion of pro-inflammatory cytokines by circulating polymorphonuclear neutrophils and regulation by interleukin 10 during intestinal inflammation. *Gut*. 1998; **42**:470-476.

Nussbaum, O., Broder, C.C., Moss, B., Stern, L.B., Rozenblatt, S., Berger, E.A. Functional and structural interactions between measles virus hemagglutinin and CD46. *Journal of Virology*. 1995; **69**:3341-3349.

Nyberg, F., Lieberman, H., Lindstrom, L.H., Lyrenas, S., Koch, G., Terenius, L. Immunoreactive beta-casomorphin-8 in cerebrospinal fluid from pregnant and lactating women: correlation with plasma levels. *Journal of Clinical Endocrinology Metabolism*. 1989; **68**:283-289.

Ogura, Y., Bonen, D.K., Inohara, N., Nicolae, D.L., Chen, F.F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R.H., Achkar, J.P., Brant, S.R., Bayless, T.M., Kirschner, B.S., Hanauer, S.B., Nunez, G., Cho, J.H. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature*. 2001a; **411**:603-606.

Ogura, Y., Inohara, N., Benito, A., Chen, F.F., Yamaoka, S., Nunez, G. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *Journal of Biological Chemistry*. 2001b; **276**:4812-4818.

Ohmen, J.D., Yang, H.Y., Yamamoto, K.K., Zhao, H.Y., Ma, Y., Bentley, L.G., Huang, Z., Gerwehr, S., Pressman, S., McElree, C., Targan, S., Rotter, J.I., Fischel-Ghodsian, N. Susceptibility locus for inflammatory bowel disease on chromosome 16 has a role in Crohn's disease, but not in ulcerative colitis. *Human Molecular Genetics*. 1996;5:1679-83.

Ohyama, H., Zhang, X., Kohno, Y., Alevizos, I., Posner, M., Wong, D.T., Todd R. Laser capture microdissection-generated target sample for high-density oligonucleotide array hybridization. *Biotechniques*. 2000; **29**:530-6.

Okada, A., Tomasetto, C., Lutz, Y., Bellocq, J.P., Rio, M.C., Basset, P. Expression of matrix metalloproteinases during rat skin wound healing: evidence that membrane type-1 matrix metalloproteinase is a stromal activator of pro-gelatinase A. *Journal of Cell Biology*. 1997; **137**:67-77.

Oldstone, M.B., Lewicki, H., Thomas, D., Tishon, A., Dales, S., Patterson, J., Manchester, M., Homann, D., Naniche, D., Holz, A. Measles virus infection in a transgenic model: virus-induced immunosuppression and central nervous system disease. *Cell*. 1999; **98**:629-640.

Orholm, M., Binder, V., Sorenson, T., Kyvik, K. Inflammatory bowel disease in a Danish twin register. *Gut*. 1996; **39**:A187.

Orlando, C., Pinzani, P., Pazzagli, M. Developments in quantitative PCR. *Clinical Chemistry Laboratory Medicine*. 1998; **36**:255-69.

Ortiz, E.M., Dusetti, N.J., Vasseur, S., Malka, D., Bodeker, H., Dagorn, J.C., Iovanna, J.L. The pancreatitis-associated protein is induced by free radicals in AR4-2J cells and confers cell resistance to apoptosis. *Gastroenterology*. 1998; **114:**808-16.

Oxman, M.N. Measles. In *Clinical Virology*. Edited by Richman, D.D., White, R.J., and Hayden F.G. Churchill Livingstone Press. 1997; 821-853.

Pantaleo, G., Graziosi, C., Demarest, J.F., *et al.* Role of lymphoid organs in the pathogenesis of human immunodeficiency virus (HIV) infection. *Immunological Reviews*. 1994; **140**:105-130.

Pardi, D.,S., Tremaine, W.J., Sandborn, W.J., Loftus, E.V. Jr., Poland, G.A., Harmsen, W.S., Zinsmeister, A.R., Melton, L.J. 3rd. Early measles virus infection is associated with the development of inflammatory bowel disease. *American Journal of Gastroenterology*. 2000; **95**:1480-1485.

Pardi, D.S., Tremaine, W.J., Sandborn, W.J., Loftus, E.V. Jr., Poland, G.A., Melton, L.J. 3rd. Perinatal exposure to measles virus is not associated with the development of inflammatory bowel disease. *Inflammatory Bowel Disease*. 1999; **5**:104-106.

Parkes, M., Jewell, D. Ulcerative colitis and Crohn's disease; molecular genetis and clinical implications. *Expert Reviews in Molecular Medicine*. 2001; accession (01)00391-Xa.

Parks, C.L., Lerch, R.A., Walpita, P., Wang. H.P., Sidhu, M.S., Udem, S.A. Comparison of predicted amino acid sequences of measles virus strains in the Edmonston vaccine lineage. *Journal of Virology*. 2001; **75**:910-920.

Parronchi, P., Romagnani, P., Annunziato, F., Sampognaro, S., Becchio, A., Giannarini, L., Maggi, E., Pupilli, C., Tonelli, F, Romagnani, S. Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. *American Journal of Pathology*. 1997; **150**:823-832.

Patard, L., Lallemand, J.Y., Stoven, V. An insight into the role of human pancreatic lithostathine. *Journal of the Pancreas*. 2003;**4**:92-103.

Patterson, J.B., Manchester, M., Oldstone, M.B. Disease model: dissecting the pathogenesis of the measles virus. *Trends in Molecular Medicine*. 2001;**7**:85-8.

Pease, A.C., Solas, D., Sullivan, E.J. *et al.* Light generated oligonucleotide arrays for rapid DNA sequence analysis. *Proceedings of the National Academy of Science, USA*. 1994; **91**:5022-5026.

Persico, A.M., D'Agruma, L., Maiorano, N., Totaro, A., Militerni, R., Bravaccio, C., Wassink, T.H., Schneider, C., Melmed, R., Trillo, S., Montecchi, F., Palermo, M., Pascucci, T., Puglisi-Allegra, S., Reichelt, K.L., Conciatori, M., Marino, R., Quattrocchi, C.C., Baldi, A., Zelante, L., Gasparini, P., Keller, F. Reelin gene alleles and haplotypes as a factor predisposing to autistic disorder. *Molecular Psychiatry*. 2001; **6**:150-159.

Pinheiro, N.A., Caballero, O.L., Soares, F., Reis, L.F., Simpson, A.J. Significant overexpression of oligophrenin-1 in colorectal tumors detected by cDNA microarray analysis. *Cancer Letters*. 2001; **172**:67-73.

Piven, J., Tsai, G.C., Nehme, E., Coyle, J.T., Chase, G.A., Folstein, S.E. Platelet serotonin, a possible marker for familial autism. *Journal of Autism and Development Disorders*. 1991; **21**:51-59.

Podolsky, D.K. Inflammatory bowel disease. *New England Journal of Medicine*. 2002; **347**:417-429.

Pounder, R.E. Measles virus and Crohn's disease: research from the Royal Free Hospital in London. *Journal of Gastroenterology*. 1995; **30**:48-51.

Price, A. Inflammatory Bowel Disease. In *Oxford Textbook of Pathology, Pathology of Systems*. Edited by Mc Gee, J., O'D., Isaacson, P.G., and Wright, N.A. 1992; **2**:1234-1254.

Rall, G.F., Manchester, M., Daniels, L.R., Callahan, E.M., Belman, A.R., Oldstone, M.B. A transgenic mouse model for measles virus infection of the brain. *Proceedings of the National Academy of Science, USA*. 1997; **94**:4659-4663.

Ramakers, G.J. Rho proteins and the cellular mechanisms of mental retardation. *American Journal of Medical Genetic*. 2000; **94**:367-71.

Rapin, I. Autism. *New England Journal of Medicine*. 1997; **337**:97-104.

Rath, H.C., Schultz, M., Freitag, R., Dieleman, L.A., Li, F., Linde, H.J., Scholmerich, J., Sartor, R.B. Different subsets of enteric bacteria induce and perpetuate experimental colitis in rats and mice. *Infection and Immunity*. 2001; **69**:2277-2285.

Ray, R., Cooper, P.J., Sim, R., Chadwick, N., Earle, P., Dhillon, A.P., Pounder, R.E., Wakefield, A.J. Direct in situ reverse transcriptase polymerase chain reaction for detection of measles virus. *Journal of Virology Methods*. 1996; **60**:1-17.

Raymond, I., Saati, T.A., Tkaczuk, J., Chittai, S., Delsol, G. CNA.42, a new monoclonal antibody directed against a fixative resistant antigen of follicular dendritic reticulum cells. *American Journal of Pathology*. 1997; **151**:1577-1585.

Reichelt, K.L., Knivsberg, A.M. Can the pathophysiology of autism be explained by the nature of the discovered urine peptides? *Nutritional Neuroscience*. 2003; **6**:19-28.

Reichelt, K.L., Hole, K., Hamberger, A., Saelid, G., Edminson, P.D., Braestrup, C.B., Lingjaerde, O., Ledaal, P., Orbeck, H. Biologically active peptide-containing fractions in schizophrenia and childhood autism. *Advanced Biochemical Psychopharmacology*. 1981; **28**:627-643.

Reimund, J.M., Wittersheim, C., Dumont, S., Muller, C.D., Kenney, J.S., Baumann, R., Poindron, P., Duclos, B. Increased production of tumour necrosis factor-alpha interleukin-1 beta, and interleukin-6 by morphologically normal intestinal biopsies from patients with Crohn's disease. *Gut*. 1996; **39**:684-689.

Rima, B.K., Earle, J.A., Baczko, K., Rota, P.A., Bellini, W.J. Measles virus strain variations. *Current Topics in Microbiology and Immunology*. 1995; **191**:65-83.

Roitt, I., Brosthoff, J., Male, D. Cytokines and Cytokine Receptors. In *Immunology*. 6th Edition. Mosby, Press. 2001.

Romagnani, S. Biology of human TH1 and TH2 cells. *Clinical Immunology*. 1995; **15**:121-129.

Rosenstiel, P., Fantini, M., Brautigam, K., Kuhbacher, T., Waetzig, G.H., Seegert, D., Schreiber, S. TNF-alpha and IFN-gamma regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. *Gastroenterology*. 2003; **124**:1001-1009.

Rubin, G.P., Hungin, A.P., Kelly, P.J., Ling, J. Inflammatory bowel disease: epidemiology and management in an English general practice population. *Alimentary Pharmacology and Therapeutics*. 2000; **14**:1553-1559.

Rutgeerts, P., Van Deventer, S., Schreiber, S. Review article: the expanding role of biological agents in the treatment of inflammatory bowel disease - focus on selective adhesion molecule inhibition. *Alimentary Pharmacological Therapy*. 2003; **17**:1435-50.

Rutgeerts P, Goboes K, Peeters M, Hiele M, Penninckx F, Aerts R, Kerremans R, Vantrappen G. Effect of faecal stream diversion on recurrence of Crohn's disease in the neoterminal ileum. *Lancet*. 1991; **338**:771-4.

Ruseler-van Embden, J.G., Schouten, W.R., van Lieshout, L.M. Pouchitis: result of microbial imbalance? *Gut*. 1994 ;**35**:658-64.

Ryan, P., Bennett, M.W., Aarons, S., Lee, G., Collins, J.K., O'Sullivan, G.C., O'Connell, J., Shanahan, F. PCR detection of Mycobacterium paratuberculosis in Crohn's disease granulomas isolated by laser capture microdissection. *Gut*. 2002; **51**:665-70.

Sabra A., Bellanti, J.A., Colon, A.R. Ileal-lymphoid-nodular hyperplasia, non-specific colitis, and pervasive developmental disorder in children. *Lancet*. 1998; **352**:234-235.

Saiki, R.K., Scharf, S., Faloona, F., *et al.* Enzymatic amplification of beta globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. *Science*. 1985; **230**:1350-1354.

Santoro, M.G., Rossi, A., Amici, C. NF-κβ and virus infection: who controls whom. *The EMBO Journal*. 2003; **22**:2552-2560.

Satsangi, J., Grootscholten, C., Holt, H., Jewell, D.P. Clinical patterns of familial inflammatory bowel disease. *Gut*. 1996; **38**:738-41.

Satsangi, J., Parkes, M., Louis, E., Hashimoto, L., Kato, N., Welsh, K., Terwilliger, J.D., Lathrop, G.M., Bell, J.I., Jewell, D.P. Two stage genome-wide search in inflammatory bowel disease provides evidence for susceptibility loci on chromosomes 3, 7 and 12. *Nature Genetics*. 1996;**14**:199-202.

Sayos, J., Wu, C., Morra, M., Wang, N., Zhang, X., Allen, D., van Schaik, S., Notarangelo, L., Geha, R., Roncarolo, M.G., Oettgen, H., De Vries, J.E., Aversa, G., Terhorst, C. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature*. 1998; **395**:462-469.

Schmid, A., Spielhofer, P., Cattaneo, R., Baczko, K., ter Meulen,V., Billeter, M.A.. Subacute sclerosing panencephalitis is typically characterized by alterations in the fusion protein cytoplasmic domain of the persisting measles virus. *Virology*. 1992; **188**:910-915.

Schmittgen, T.D., Zakrajsek, B.A. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *Journal Biochemical and Biophysical Methods*. 2000; **46**:69-81.

Schneider-Schaulies, J., ter Meulen, V., Schneider-Schaulies, S. Measles infection of the central nervous system. *Journal of Neurovirology*. 2003; **9**:247-252.

Schneider-Schaulies, S., ter Meulen, V. Measles virus and immunomodulation: molecular bases and perspectives. *Expert Reviews in Molecular Medicine*. 2002a; **4**:1-18.

Schneider-Schaulies, S., Schneider-Schaulies, J., Niewiesk, S., Ter Meulen, V. Measles virus: immunomodulation and cell tropism as pathogenicity determinants. *Medical Microbiology and Immunology*. 2002b ; 191:83-87.

Schreiber, S., Nikolaus, S., Hampe, J. Activation of nuclear factor kappa β inflammatory bowel disease. *Gut*. 1998; **42**:477-484.

Servet-Delprat, C., Vidalain, P.O., Bausinger, H., Manie, S., Le Deist, D. Measles virus induces abnormal differentiation of CD40 ligand-activated human dendritic cells. *Journal of Immunology*. 2000; **164**:1753-1760.

Sheils, O., Smyth, P., Martin, C., O'Leary, J.J. Development of an allelic discrimination type assay to differentiate between strain origins of measles virus detected in intestinal tissue of children with ileocolonic lymphonodular hyperplasia and concomitant developmental disorder. *Journal of Pathology*. 2002; **198**:1A-58A.

Shinozaki, S., Nakamura, T., Iimura, M., Kato, Y., Iizuka, B., Kobayashi, M., Hayashi, N.Upregulation of Reg 1alpha and GW112 in the epithelium of inflamed colonic mucosa. *Gut*. 2001; **48**:623-9.

Sidhu, M.S., Crowley, J., Lowenthal, A., Karcher, D., Menonna, J., Cook, S., Udem, S., Dowling, P. Defective measles virus in human subacute sclerosing panencephalitis brain. *Virology*. 1994; **202**:631-641.

Singh, V.K., Jensen, R.L. Elevated levels of measles antibodies in children with autism. *Pediatric Neurology*. 2003; **28**:292-294.

Singh, V.K., Lin, S.X., Newell, E., Nelson, C. Abnormal measles-mumps-rubella antibodies and CNS autoimmunity in children with autism. *Journal of Biomedical Science*. 2002; **9**:359-364.

Singh,V.K., Lin, S.X., Yang, V.C. Serological association of measles virus and human herpesvirus-6 with brain autoantibodies in autism. *Clinical Immunology Immunopathology*. 1998; **89**:105-108.

Singh, V.K., Singh, E.A., Warren, R.P. Hyperserotoninemia and serotonin receptor antibodies in children with autism but not mental retardation. *Biological Psychiatry*. 1997; **41**:753-755.

Singh, V.K. Plasma increase of interleukin-12 and interferon-gamma. Pathological significance in autism. *Journal of Neuroimmunology*. 1996; **66**:143-145.

Singh, V.K., Warren, R.P., Odell, J.D., Warren, W.L., Cole, P. Antibodies to myelin basic protein in children with autistic behavior. *Brain Behaviour Immunology*. 1993; **7**:97-103.

Smith, M.W., Phillips, A.D. Abnormal expression of dipeptidylpeptidase IV activity in enterocyte brush-border membranes of children suffering from coeliac disease. *Experimental Physiology*. 1990; **75**:613-616.

Sonoda, S., Kitahara, M., Nakayama, T. Detection of measles virus genome in bone-marrow aspirates from adults. *Journal of General Virology*. 2002; **83**:2485-2488.

Sonoda, S., Nakayama, T. Detection of measles virus genome in lymphocytes from asymptomatic healthy children. *Journal of General Virology*. 2001; **65**:381-387.

Southern, E.M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology*. 1975; **97**:503-517.

Spitzer, W.O. Measles, mumps, and rubella vaccination and autism. *New England Journal of Medicine*. 2003; **348**:951-4.

Spitzer, W.O., Aitken, K.J., Dell'Aniello, S., Davis, M.W. The natural history of autistic syndrome in British children exposed to MMR. *Adverse Drug Reaction Toxicology Reviews*. 2001; **20**:160-163.

Sun, Z., Cade, J.R., Fregly, M.J. Privette, R.M. β-caseomorphin induces Fos like immunoreactivity in discrete brain regions relevant to schizophrenia and autism. *Autism*. 1999; **3**:67-83.

Suzuki, Y., Saito, H., Kasanuki, J., Kishimoto, T., Tamura, Y., Yoshida, S. Significant increase of interleukin 6 production in blood mononuclear leukocytes obtained from patients with active inflammatory bowel disease. *Life Science*. 1990; **47**:2193-2197.

Tacchetti, C., Favre, A., Moresco, L., Meszaros, P., Luzzi, P., Truini, M., Rizzo, F., Grossi, C.E., Ciccone, E., Tacchetti, C., Favre, A., Moresco, L., Meszaros, P., Luzzi, P., Truini, M., Rizzo, F., Grossi, C.E., Ciccone, E. HIV is trapped and masked in the cytoplasm of lymph node follicular dendritic cells. *American Journal of Pathology.* 1997; **150**:533-542.

Tatsuo, H., Ono, N., Tanaka, K., Yanagi, Y. SLAM (CDw150) is a cellular receptor for measles virus. *Nature.* 2000; **406**:893-897.

Taylor, B., Miller, E., Lingam, R., Andrews, N., Simmons, A., Stowe, J. Measles, mumps, and rubella vaccination and bowel problems or developmental regression in children with autism: population study. *British Medical Journal.* 2002; **324**:393-396.

Taylor, B., Miller, E., Farrington, C.P., Petropoulos, M.C., Favot-Mayaud, I., Li, J., Waight, P.A. Autism and measles, mumps, and rubella vaccine: no epidemiological evidence for a causal association. *Lancet.* 1999; **353**:2026-2029.

Tentler, D., Gustavsson, P., Leisti, J., Schueler, M., Chelly, J., Timonen, E., Anneren, G., Willard, H.F., Dahl, N. Deletion including the oligophrenin-1 gene associated with enlarged cerebral ventricles, cerebellar hypoplasia, seizures and ataxia. *European Journal of Human Genetics.* 1999; **7**:541-8.

Thompson, N.P., Driscoll, R., Pounder, R.E., Wakefield, A.J. Genetics versus environment in inflammatory bowel disease: results of a British twin study. *British Medical Journal.* 1996; **312**:95-96.

Thompson, N.P., Montgomery, S.M., Pounder, R.E., Wakefield, A.J. Is measles vaccination a risk factor for inflammatory bowel disease? *Lancet.* 1995; **345**:1071-1074.

Torrente, F., Ashwood, P., Day, R., Machado, N., Furlano, R.I., Anthony, A., Davies, S.E., Wakefield, A.J., Thomson, M.A., Walker-Smith, J.A., Murch, S.H. Small intestinal enteropathy with epithelial IgG and complement deposition in children with regressive autism. *Molecular Psychiatry.* 2002; **7**:334, 375-382.

Truelove, S.C., Witts, L.J. Cortisone and corticotrophin in ulcerative colitis. *British Medical Journal.* 1959; **14**:387-94.

Tsujimura, A., Shida, K., Kitamura, M., Nomura, M., Takeda, J., Tanaka, H., Matsumoto, M., Matsumiya, K., Okuyama, A., Nishimune, Y., Okabe, M., Seya, T. Molecular cloning of a murine homologue of membrane cofactor protein (CD46): preferential expression in testicular germ cells. *Biochemical Journal*. 1998; **330**:163-168.

Tysk, C., Lindberg, E., Jarnerot, G., Floderus-Myrhed, B. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. *Gut*. 1988; **29**:990-996.

Uhlmann, V., Martin, C.M., Sheils, O., Pilkington, L., Silva, I., Killalea, A., Murch, S.B., Walker-Smith, J., Thomson, M., Wakefield, A.J., O'Leary, J.J. Potential viral pathogenic mechanism for new variant inflammatory bowel disease. *Molecular Pathology*. 2002; **55**:84-90.

Uhlmann, V., Rolfs, A., Mix, E. *et al.* A novel, rapid in-cell RNA amplification technique for the detection of low copy mRNA transcripts. *Journal of Clinical Pathology & Molecular Pathology*. 1998; **51**:160-163.

Van Aelst, L., D'Souza-Schorey, C. Rho GTPases and signaling networks. *Genes and Development*. 1997;**11**:2295-322.

van Heel, D.A., Udalova, I.A., De Silva, A.P., McGovern, D.P., Kinouchi, Y., Hull, J., Lench, N.J., Cardon, L.R., Carey, A.H., Jewell, D.P., Kwiatkowski, D. Inflammatory bowel disease is associated with a TNF polymorphism that affects an interaction between the OCT1 and NF(-kappa)B transcription factors. *Human Molecular Genetics*. 2002; **11**:1281-1289.

Van Kruiningen, H.J., Mayo, D.R., Vanopdenbosch, E., Gower-Rousseau, C., Cortot, A., Colombel, J.F. Virus serology in familial Crohn disease. *Scandinavian Journal of Gastroenterology*. 2000; **35**:403-407.

van Montfrans, C., Peppelenbosch, M., te Velde, A.A., van Deventer, S. Inflammatory signal transduction in Crohn's disease and novel therapeutic approaches. *Biochemical Pharmacology*. 2002; **64**:789-795.

Vavassori, P., Borgiani, P., D'Apice, M.R., De Negris, F., Del Vecchio, Blanco, G., Monteleone, I., Biancone, L., Novelli, G., Pallone, E. 3020insC mutation within the NOD2 gene in Crohn's disease: frequency and association with clinical pattern in an Italian population. *Digestive and Liver Disease*. 2002; **34**:153.

Veillette, A., Latour, S. The SLAM family of immune-cell receptors. *Current Opinion on Immunology*. 2003; **15**:277-285.

Volkmar, F.R., Pauls, D. Autism. *Lancet*. 2003; **362**:1133-1141.

von Lampe, B., Barthel, B., Coupland, S.E., Riecken, E.O., Rosewicz, S. Differential expression of matrix metalloproteinases and their tissue inhibitors in colon mucosa of patients with inflammatory bowel disease. *Gut*. 2000 ;**47** :63-73.

Wakefield, A.J., Puleston, J.M., Montgomery, S.M., Anthony, A., O'Leary, J.J., Murch, S.H. Review article: the concept of entero-colonic encephalopathy, autism and opioid receptor ligands. *Alimentary Pharmacology and Therapeutics*. 2002; **16**:663-674.

Wakefield, A.J., Anthony, A, Murch, S.H., Thomson, M.A. Montgomery, S.M., Davies, S., O'Leary, J.J., Berelowitz, M., Walker-Smith, J.A. Enterocolitis in Children with developmental disorders. *American Journal of Gastroenterology*, 2000; 95:2285-2295.

Wakefield, A.J., Murch, S.H., Anthony, A., *et al.* Ileal-lymphoid-nodular hyperplasia, non-specific colitis, and pervasive developmental disorder in children. *Lancet*. 1998a; **351**:637-641.

Wakefield, A.J., Anthony, A., Schelpelmann, S., Murch, S., Montgomery, S.M., Walker–Smith, J.A. Persistent measles virus infection and immunodeficiency in children with autism, ileocolonic lymphonodular hyperplasia and non-specific colitis. *Gut*. 1998b; **42**:A86.

Wakefield, A.J., Sim, R., Akbar, A.N., Pounder, R.E., Dhillon, A.P. In situ immune responses in Crohn's disease: a comparison with acute and persistent measles virus infection. *Journal of Medical Virology*. 1997; **51**:90-100.

Wakefield, A.J., Ekbom, A., Dhillon, A.P., Pittilo, R.M., Pounder, R.E. Crohn's disease: pathogenesis and persistent measles virus infection. *Gastroenterology.* 1995; **108**:911-916.

Wakefield., A.J., Pittilo, R.M., Sim, R., Cosby, S.L., Stephenson, J.R., Dhillon, A.P., Pounder, R.E. Evidence of persistent measles virus infection in Crohn's disease. *Journal of Medical Virology.* 1993; **39**:345-353.

Wall, S., Kunze, Z.M., Saboor, S., Soufleri, I., Seechurn, P., Chiodini, R., McFadden, J.J. Identification of spheroplast-like agents isolated from tissues of patients with Crohn's disease and control tissues by polymerase chain reaction. *Journal of Clinical Microbiology.* 1993;31:1241-5.

Wang, G., Liszewski, M.K., Chan, A.C., Atkinson, J.P. Membrane cofactor protein (MCP; CD46): isoform-specific tyrosine phosphorylation. *Journal of Immunology.* 2000; **164**:1839-1846.

Wang, P., Wu, P., Anthes, J.C., Siegel, M.I., Egan, R.W., Billah, M.M. Interleukin-10 inhibits interleukin-8 production in human neutrophils. *Blood.* 1994; **83**:2678-2683.

Wang, W-H., McNatt, L. G., Shepard, A. R., Jacobson, N., Nishimura, D. Y., Stone, E. M., Sheffield, V. C., Clark, A. F. Optimal procedure for extracting RNA from human ocular tissues and expression profiling of the congenital glaucoma gene *FOXC1* using quantitative PCR. *Molecular Vision.* 2001; **7**: 89-94.

Ward, B.J., Griffin, D.E. Changes in cytokine production after measles virus vaccination: predominant production of IL-4 suggests induction of a Th2 response. *Clinical Immunology and Immunopathology.* 1993; **67**:171-177.

Watts, D.A., Satsangi, J. The genetic jigsaw of inflammatory bowel disease. *Gut.* 2002; **50**:III31-36.

Watzl, B., Abrahamse, S.L., Treptow-van Lishaut, S., Neudecker, C., Hansch, G.M., Rechkemmer, G., Pool-Zobel, B.L. Enhancement of ovalbumin-induced antibody production and mucosal mast cell response by mercury. *Food and Chemical Toxicology.* 1999; **37**:627-637.

Wehkamp, J., Schwind, B., Herrlinger, K.R., Baxmann, S., Schmidt, K., Duchrow, M., Wohlschlager, C., Feller, A.C., Stange, E.F., Fellermann, K. Innate immunity and colonic inflammation: enhanced expression of epithelial alpha-defensins. *Digestive Disease Science*. 2002 ;**47**:1349-55.

Xiao, J., Neylon, C.B., Hunne, B., Furness, J.B. Oligophrenin-1, a Rho GTPase-activating protein (RhoGAP) involved in X-linked mental retardation, is expressed in the enteric nervous system. *The Anatomical Record.* 2003;**273A**:671-6.

Yang, H., McElree, C., Roth, M.P., Shanahan, F., Targan, S.R., Rotter, J.I.. Familial empirical risks for inflammatory bowel disease: differences between Jews and non-Jews. *Gut*. 1993; **34**:517-524.

Zhao, R.H., Baig, M.K., Thaler, K.J., Mack, J., Abramson, S., Woodhouse, S., Tamir, H., Wexner, S.D. Reduced expression of serotonin receptor(s) in the left colon of patients with colonic inertia. *Diseases of the Colon and Rectum.* 2003 ;**46:**81-6.

Zipkin, I.D., Kindt, R.M., Kenyon, C.J. Role of a new Rho family member in cell migration and axon guidance in *C. elegans*. *Cell*. 1997; 90:883-94.