

Inflammasome Involvement in Barrett's and Oesophageal Adenocarcinoma



**Thesis submitted to the University of Dublin, Trinity College
for the Degree of Doctor of Philosophy**

By

Gillian Louise Barber

May 2020

Supervised by

Dr Emma Creagh,

School of Biochemistry and Immunology

Prof. Jacintha O'Sullivan,

Department of Surgery, School of Medicine

I. Declaration of Authorship

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

I consent to the examiner retaining a copy of the thesis beyond the examining period, should they so wish.

Gillian Barber

Date submitted

II. Abstract

Barrett's oesophagus is an inflammatory condition and a neoplastic precursor to oesophageal adenocarcinoma (OAC). Inflammasome signalling pathways contribute to acute and chronic inflammation, and can be enhanced by obesity. All inflammasome signalling results in caspase-1 activation, leading to the secretion of IL-1 β and IL-18, and inflammatory cell death (pyroptosis). A full understanding of inflammasome involvement during Barrett's and OAC is yet to be achieved. However, increased IL-1 β levels are reported in Barrett's, indicting a role for these pathways in disease progression.

We hypothesized that inflammasome-mediated inflammation has an important role during oesophageal pathogenesis. Caspase-1 expression was profiled in three models of disease progression (Normal-Barrett's-OAC): a cell line model; a transgenic Barrett's mouse model; and resected tissue from Barrett's-associated OAC patients. In all three models, caspase-1 is shown to be highly upregulated during Barrett's ($p<0.01$), which is not maintained in later stages of disease. In the mouse model, results show that stromal caspase-1 correlates with histological scores for inflammation during disease progression ($p<0.05$). Similarly, the inflammasome component AIM2 is elevated during Barrett's, *in vitro* ($p<0.05$) and in the Barrett's mouse model ($p<0.01$). Where epithelial ($p<0.01$) and stromal ($p<0.05$) scores correlate with disease progression. *Ex vivo* culture of Barrett's biopsies and Barrett's murine organoids in the presence of a caspase-1 inhibitor limits cytokine secretion, suggesting that targeting caspase-1 activity may represent a strategy for reducing inflammation.

A high fat diet (HFD) and obesity are known risk factors for Barrett's and OAC. In this study we demonstrate involvement of the inflammasome pathway during associated inflammation. Inflammasome components are upregulated in response to OAC patient adipose conditioned media in an oesophageal cell line model, particularly in the normal cell line. To support this, we show that TLR2 ($p<0.05$), caspase-1 ($p<0.05$) and AIM2 ($p<0.01$) protein expression are elevated and IL-18 secretion ($p<0.05$) is reduced in normal murine oesophageal tissue from HFD-fed mice. Oesophageal AIM2 ($p<0.05$) and caspase-1 ($p<0.05$) expression levels are also upregulated in response to a high fat diet

in Barrett's pL2-IL-1 β mice. However, no differences are observed for TLR2 expression during a HFD.

An additional finding of this study demonstrates that TLR2 activity mediates secretion of IL-8 orthologs, MIP-2 ($p<0.001$) and CXCL1 ($p<0.01$) in Barrett's organoids taken from the pL2-IL-1 β mouse model. This signalling is inhibited using an inhibitor of TLR2 ($p<0.001$, $p<0.05$), suggesting a therapeutic advantage during Barrett's. Additionally, we observe TLR2 expression is upregulated in a Barrett's cell line ($p<0.05$), supporting previous preliminary studies implicating TLR2 and IL-8 during oesophageal disease. However, no differences in oesophageal TLR2 expression are observed during disease progression in the Barrett's mouse model when immunohistochemistry analysis is performed. Further studies are required to fully elucidate the role of TLR2 during oesophageal pathogenesis.

Collectively, in this study we identify the inflammasome pathway and TLR2 signalling as potential mediators of inflammation during disease progression to Barrett's and OAC. Further, obesity enhances inflammation in oesophageal tissue, which may be further promoting disease. We speculate the therapeutic potential of targeting these pathways for prevention of oesophageal disease.

III. Acknowledgements

Firstly, I would like to give my sincere thanks and appreciation to my supervisor, Emma Creagh whose constant support, encouragement and mentorship throughout my PhD has been invaluable. Thank you for all the patience and time you have given me over the last three years. My grateful thanks to my co-supervisor, Jacintha O'Sullivan for your continued support and advice during my PhD. Your constructive feedback and direction were invaluable in guiding me through my research.

I would also like to thank the past and present members of the Creagh lab, Brian, Joan, Sinead, Ewelina and Ciara. I feel fortunate to have worked with such a supportive group. In particular, I would like to thank Ewelina and Ciara for their friendship, you made coming into work every day so much more enjoyable and I appreciate all the laughs we've had together. I also want to thank all the people in the 6th floor reading room for your wonderful company. Thank you Marilena for being a great desk buddy, and Rebecca for being someone to talk to. Thanks also to all those in the Department of Biochemistry for their help. Also, to Karen for making college a fun experience.

I want to thank everyone in the Department of Surgery in James's for being so friendly and inclusive over the years, you were great company. In particular, to Aisling Heeran and Niamh Clarke for their help with my research and answering all my questions!

My appreciation to all the patients in St. James's Hospital who consented to partake in this research, and to all those working in the hospital. Thanks to the John Scott Fellowship and the School of Medicine for their funding and support during this project. A big thank you to Michael Quante, Akanksha Anand and all those in the Quante lab in Munich for their collaboration, feedback and help with the mouse model. Thanks also to Lydia Lynch and Roisin Loftus for their help with the high fat diet mice.

To my family, I want to give my most grateful thanks for always supporting and believing in me and all I do. To my parents, Tom and Josephine for your constant love and reassurance, and to my siblings for always being there for me.

Lastly, to my boyfriend Glen thank you for your continuous loving support throughout the last 3 years. Your positivity and encouragement got me to the finish line, and I will always be grateful.

IV. Thesis outputs

Publications

First author:

- Characterizing caspase-1 involvement during esophageal disease progression. Barber G, Anand A, Oficjalska K, Phelan JJ, Heeran AB, Flis E, Clarke NE, Watson JA, Strangmann J, Flood B, O'Neill H, O'Toole H, MacCarthy F, Ravi N, Reynolds JV, Kay EW, Quante M, O'Sullivan J, Creagh EM, *Cancer Immunology, Immunotherapy*. In print, 2020.

Contributing author:

- Caspase-11 mediated cell death contributes to the pathogenesis of Imiquimod-induced psoriasis. Kenealy S, Manils J, Raverdeau M, Munoz-Wolf N, Barber G, Liddicoat A, Lavelle EC, Creagh EM, *Journal of Investigative Dermatology*, 2019.
- Caspase-11 regulates the tumour suppressor function of STAT1 in a murine model of colitis-associated carcinogenesis. Flood B, Manils J, Nulty C, Flis E, Kenealy S, Barber G, Fay J, Mills KHG, Kay EW, Creagh EM, *Oncogene*, 2018.
- Evaluating host responses to *Helicobacter pylori* using ELISA and Western blot. *Methods in Molecular Biology*, Springer Publisher. In review.

In preparation:

- Involvement of the AIM2 inflammasome during a high fat diet and disease progression to Barrett's oesophagus. Barber G, Anand A, Phelan J, Strangmann J, Quante M, O'Sullivan JJ, Creagh EM.
- HMGB1-mediates TLR2 inflammatory pathways during oesophageal adenocarcinoma. Flis E, Barber G, Keogh B, Anand A, Quante M, O'Sullivan J, Creagh EM.

Poster presentations

- “Examining the role of caspase-1 during progression to Barrett’s oesophagus and oesophageal adenocarcinoma.” Irish Association for Cancer Research (IACR), Dublin, Ireland, February 2018.
 - “Identifying a role for caspase-1 during progression to Barrett’s oesophagus and oesophageal adenocarcinoma.” Irish Association for Cancer Research (IACR), Belfast, Ireland, February 2019.
 - “Identifying a role for caspase-1 during progression to Barrett’s oesophagus and oesophageal adenocarcinoma.” Oesophageal Cancer Symposium, CRUK, London, April 2019.
 - “Caspase-1 associated inflammation is important during disease progression to Barrett’s oesophagus and oesophageal adenocarcinoma.” TBSI Research Symposium, Dublin, September 2019.
 - “Involvement of caspase-1 during progression to Barrett’s oesophagus and oesophageal adenocarcinoma.” Irish Society of Immunology (ISI), Dublin, September 2019.
 - “Involvement of caspase-1 during progression to Barrett’s oesophagus and oesophageal adenocarcinoma.” 11th International Cancer Conference, TTMI, Dublin, September 2019.
- Poster presentation winner.

Oral presentations

- “Inflammatory caspase involvement in Barrett’s and oesophageal adenocarcinoma.” Tipton presentation, School of Biochemistry and Immunology, TBSI, Trinity College Dublin, October 2018.
- “Inflammatory caspase involvement in Barrett’s and oesophageal adenocarcinoma.” Immunology Research Forum, TBSI, Trinity College Dublin, May 2018.
- “Characterising the role of the inflammasome in the progression to OAC.” Technische Universität München (TUM), Munich, Germany, July 2017.

Courses and modules:

- General Data Protection Regulations (GDPR), Tier 1, training workshop, Trinity College Dublin, St. James's Hospital, Dublin, Ireland, April 2019.
- Health Research Regulations (HRR), Tier 1, training workshop, Trinity College Dublin, St. James's Hospital, Dublin, Ireland, April 2019.
- LAST-Ireland animal handling training programme (small animals), Ireland, 2017
- Certificate of Good Clinical Practice, Clinical Research Facility, St. James's Hospital, Dublin, Ireland, 2017.
- Cancer and metabolism, Translational oncology MSc, School of Medicine, Trinity College Dublin, February-March 2018, **5 credits**.
- Science education MSc, School of Education, Trinity College Dublin, **15 credits**.
 - Module 1: Science education, September-December 2017.
 - Module 2: Communicating science, January-May 2018.

Other:

- Biomedical practical demonstrator, School of Biochemistry and Immunology, TBSI, Trinity College Dublin, 2016-2019.
- Science communication tutor, School of Education, Trinity College Dublin, 2018-2020.

V. Table of contents

I. Declaration of Authorship.....	i
II. Abstract	ii
III. Acknowledgements	iv
IV. Thesis outputs	v
V. Table of contents.....	viii
VI. List of Schematic Figures	xiii
VII. List of Tables	xiv
VIII. Abbreviations	xv

Chapter 1: General introduction

1.1 Innate immunity.....	2
1.1.1 Innate immunity and inflammation	2
1.1.2 Pattern recognition receptors (PRRs)	3
1.1.3 Toll-like receptors (TLRs) and signalling.....	3
1.1.4 Nod-like receptors (NLRs).....	7
1.1.5 AIM2-like receptors (ALRs)	9
1.2 Inflammasome signalling	10
1.2.1 Caspases	10
1.2.2 Caspase-1 and the canonical inflammasome	12
1.2.3 NLRP3 and the canonical inflammasome	15
1.2.4 The AIM2 inflammasome	19
1.2.5 The non-canonical inflammasome pathway	22
1.2.6 The inflammasome and inflammation-induced cancer.....	25
1.3 Barrett's-associated oesophageal adenocarcinoma.....	28
1.4 Barrett's metaplasia (Barrett's oesophagus).....	32
1.4.1 Aetiology of Barrett's oesophagus	32
1.4.2 Barrett's diagnosis	34
1.4.3 Dysplasia and Barrett's oesophagus	36
1.4.4 Barrett's metaplasia, cell of origin	39

1.4.5 Management of Barrett's oesophagus.....	40
1.4.6 Mouse models of Barrett's and OAC	42
1.4.7 pL2-IL-1 β Barrett's mouse model	43
1.4.8 Inflammation in disease progression to Barrett's and OAC	44
1.5 Obesity	48
1.5.1 Obesity, inflammation and cancer.....	49
1.5.2 Obesity-associated Barrett's and OAC.	50
1.5.3 Barrett's, obesity and inflammation.....	50
1.6 Project hypothesis and aims	54

Chapter 2: Materials and Methods

Materials	56
Methods.....	59
2.1 <i>In vitro</i> studies	59
2.1.1 Cell line culture	60
2.1.2 Cell counting	62
2.1.3 Cryopreservation	62
2.1.4 Thawing cells from stocks	62
2.1.5 Mycoplasma detection in cell cultures.....	63
2.1.6 DNA gel electrophoresis.....	65
2.2 Western Blot analysis.....	65
2.2.1 Preparation of lysates	65
2.2.2 Determination of protein concentration	65
2.2.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)	66
2.2.4 Wet Transfer	67
2.2.5 Blocking, immunoblotting and visualisation of protein.....	67
2.2.6 Densitometric analysis of protein expression	69
2.3 Enzyme-linked immunosorbent assay (ELISA)	69
2.4 Data set analysis	71
2.5 Human studies	72
2.5.1 Adipose tissue retrieval and processing.....	72
2.5.2 Horse-Radish Peroxidase (HRP) conjugation to primary antibody	72

2.5.2.1 Antibody clean-up	72
2.5.2.2 HRP conjugation	73
2.5.3 <i>Ex vivo</i> human Barrett's explants	73
2.5.4 Quantification of Barrett's explant secretion	74
2.5.5 MSD Multiplex analysis	75
2.5.6 LDH assay.....	75
2.6 High fat diet mouse model.....	76
2.7 Barrett's oesophagus mouse model	77
2.7.1 Mice phenotypes	78
2.7.2 Histology	82
2.7.2.1 Tissue processing	82
2.7.2.2 Sectioning	83
2.7.2.3 Haematoxylin and Eosin (H&E) staining.....	83
2.7.2.4 Immunohistochemistry.....	86
2.7.2.5 Immunohistochemistry analysis.....	87
2.8 Statistical analysis	88
2.9 Supplementary methods.....	89
2.9.1 Human resected tissue from OAC patients	90
2.9.1.1 Ethics, patient consent and oesophageal biopsy acquisition	90
2.9.1.2 Immunohistochemistry.....	90
2.9.1.3 Ki-67 IHC staining	91
2.9.2 Murine three-dimensional organoid culture.....	92

Chapter 3

3.1 Introduction	99
3.2 Results.....	103
3.2.1 Patient oesophageal tissue displays elevated epithelial caspase-1 expression in areas of metaplasia.	103
3.2.2 Analysis of public data sets reveal altered inflammatory caspase mRNA levels in Barrett's metaplasia, compared to adjacent normal tissue.	106
3.2.3 Caspase-1 is strongly expressed in Barrett's metaplastic and dysplastic cell lines and is poorly expressed in OAC cell lines.	107

3.2.4 Caspase-1 is elevated in epithelial cells in the Barrett's region of the squamocolumnar junction in twelve-month-old pL2-IL-1 β mice.....	108
3.2.5 Enhanced epithelial caspase-1 expression levels in the Barrett's region from high fat diet-fed pL2-IL-1 β mice.	110
3.2.6 pL2-IL-1 β FXR ^{-/-} mice have upregulated epithelial caspase-1 and reduced levels of stromal capase-1 in metaplastic tissue, compared to pL2-IL-1 β mice.	112
3.2.7 Caspase-1 expression levels are reduced in pL2-LKR mice, compared to pL2-IL-1 β mice, at 12 months.	113
3.2.8 Time course of caspase-1 expression in pL2-IL-1 β mice at three, six, nine and twelve months.	114
3.2.9 Summary of main findings of Chapter 3.....	117
3.3 Discussion	118

Chapter 4

4.1 Introduction.....	153
4.2 Results	156
4.2.1 Biopsies from Barrett's regions secrete higher levels of inflammatory mediators than adjacent normal biopsies.	156
4.2.2 Caspase-1 inhibitor, WEHD.CHO optimally inhibits IL-1 β secretion from THP-1 cells at 10 μ M.	157
4.2.3 Caspase-1 inhibition results in reduced inflammatory cytokine secretion in biopsies from obese Barrett's patients.....	159
4.2.4 TLR2 and caspase-1 mediate inflammation in Barrett's organoids from pL2-IL-1 β mice.	162
4.2.5 Inflammasome proteins NLRP3 and AIM2 are strongly expressed in oesophageal tissue and cell lines.	165
4.2.6 Inflammasome-related genes are upregulated in OE33 tumour cells when co-cultured with adipose tissue explants from obese cancer patients.....	166
4.2.7 OE33 cell line culture with adipose conditioned media (ACM) results in upregulation of caspase-1 and IL-1 β	168
4.2.8 Cross-reactivity of secondary anti-rabbit antibody with heavy chain immunoglobulins present in ACM produces non-specific bands in caspase-1 Western blots.	169
4.2.9 Upregulation of inflammasome proteins when oesophageal cell lines are stimulated with ACM from obese OAC patients.	171

4.2.10 Summary of the main findings of Chapter 4.....	175
4.3 Discussion.....	176

Chapter 5

5.1 Introduction	228
5.2 Results.....	232
5.2.1 Priming of inflammatory components in normal oesophageal tissue during a high fat diet.....	232
5.2.2 Histological comparison of disease development in pL2-IL-1 β mice fed a SFD and HFD.....	235
5.2.3 Optimisation of AIM2 and TLR2 antibodies for IHC analysis in murine Barrett's tissue sections.....	236
5.2.4 AIM2 expression was upregulated at the squamocolumnar junction (SCJ) during a HFD in Barrett's pL2-IL-1 β mice.	237
5.2.5 No differences in TLR2 expression levels at the SCJ during disease progression in pL2-IL-1 β mice fed a SFD and HFD.	239
5.2.6 Summary of the main findings of Chapter 5.....	241
5.3 Discussion.....	242

Chapter 6: General discussion and future directions

6.1 General discussion	276
6.2 Future directions.	286

References	289

VI. List of Schematic Figures

1. Introduction

Figure 1.1: Schematic of TLR signalling.	5
Figure 1.2: NOD-like receptor classification and protein structure.	9
Figure 1.3: Schematic representation of ALR PYHIN domains present in IFI16 and AIM2.	10
Figure 1.4: Schematic representing the structure and function of inflammatory and apoptotic caspases in humans and mice.	12
Figure 1.5: Inflammasome activation leads to caspase-1 mediated inflammation.	15
Figure 1.6: Signal 1, NLRP3 canonical inflammasome priming.	16
Figure 1.7: Signal 2, NLRP3 canonical inflammasome activation.	18
Figure 1.8: AIM2 inflammasome regulation and activation.	21
Figure 1.9: Caspase-4/ -5/ -11 non-canonical inflammasome pathway activated by intracellular LPS.	24
Figure 1.10: Incidence of oesophageal carcinoma in Ireland, 1994-2009.	28
Figure 1.11: Endoscopic image of Barrett's oesophagus.	35
Figure 1.12: Sequence of pathogenesis.	37
Figure 1.13: Histological features of metaplasia and dysplasia using haematoxylin and eosin (H&E) staining.	38
Figure 1.14: Proposed theories for the origin of Barrett's metaplasia.	40
Figure 1.15: Inflammation during disease progression to OAC.	47
Figure 1.16: Immune cell infiltrate in the tumour microenvironment of obese patients.	52

2. Materials and methods

Figure 2.1: DNA gel reveals no mycoplasma is present in oesophageal and THP-1 cell lines.	64
Figure 2.2: Epstein-Barr virus (EBV)-IL-1 β construct containing mature form of human IL-1 β cDNA downstream of the promoter.	79
Figure 2.3: Depiction of Lgr5 ⁺ cell specific excision of target genes in pL2-IL-1 β mice using the Cre-LoxP recombination system.	81
Figure 2.4: Diagram depicting the physiology of the human and mouse stomach.	83
Figure 2.5: Schematic showing organoid set-up and culture.	96
Figure 2.6: Representative images of Barrett's organoids at various stages of growth.	97

6. General discussion and future directions

Figure 6.1: Schematic representing the main findings of this study.	284

VII. List of Tables

Introduction

Table 1.1: T staging of depth of invasion, TMN system.	29
Table 1.2: Anatomical stage groupings, TMN system.	30

Materials and methods

Table 2.1: Materials.	56
Table 2.2: Buffers and Solutions	57
Table 2.3: Cell lines	59
Table 2.4: Mycoplasma primers for PCR	64
Table 2.5: PCR reaction components	64
Table 2.6: PCR reaction conditions	64
Table 2.7: Formulations for preparing polyacrylamide gels	67
Table 2.8: Primary antibodies for immunoblot analysis	68
Table 2.9: Secondary antibody for immunoblot analysis.	69
Table 2.10: Commercial human ELISA kits	70
Table 2.11: Commercial mouse ELISA kits.	70
Table 2.12: Non-Kit murine ELISA, IL-18.	71
Table 2.13: MSD human multiplex cytokines	75
Table 2.14: MSD mouse multiplex cytokines	75
Table 2.15: Formulation for rodent diet with 60 kcal% fat.	77
Table 2.16: Standard chow verses high fat chow	79
Table 2.17: Genotyping primers	82
Table 2.18: Scoring method for H&E staining	85
Table 2.19: Dysplasia scoring system for mice histology	85
Table 2.20: Antibodies for IHC murine studies.	87
Table 2.21: Intensity scoring system for IHC analysis	88
Table 2.22: Supplementary materials	89
Table 2.23: Scoring system for patient oesophageal IHC analysis.	91

Chapter 3

Table 3.1: Characteristics of OAC patients that were IHC stained for caspase-1, 4 and -5 expression.	132

Chapter 4

Table 4.1: Characteristics of Barrett's patients.	191
Table 4.2: Characterisation of the OAC patient cohort used to generate ACM.	216

Chapter 6

Table 6.1: Summarised results of key findings	285

VIII. Abbreviations

5-FU	5-Fluorouracil
ACM	Adipose conditioned media
AIM2	Absent in melanoma 2
ALR	AIM2-like receptor
ANOVA	Analysis of variance
AOM	Azoxymethane
AP-1	Activator protein-1
APPR	Acute phase protein response
ASC	Apoptosis associated speck-like protein containing a CARD
ATP	Adenosine tri-phosphate
BCA	Bicinchoninic acid
BIR	Baculoviral inhibitory repeat
BMDM	Bone marrow derived macrophage
BMI	Body mass index
BSA	Bovine Serum Albumin
CA	Cholic acid
CAC	Colitis associated CRC (colorectal associated cancer)
CANTOS	Canakinumab Anti-inflammatory Thrombosis Outcome Study
CAPS	Cryopyrin associated periodic syndromes
CARD	Caspase activation and recruitment domain
cDNA	Complementary DNA
CD	Cluster of differentiation
CDCA	Chenodeoxycholic acid
CDX2	Caudal-type homeobox protein 2
cGAMP	Cyclic guanosine monophosphate-adenosine monophosphate
cGAS	cGAMP synthase
Cis	Cisplatin
CLR	C-type lectin receptors
CML	Chronic myelogenous leukaemia
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CRC	Colorectal cancer
CROSS	Chemoradiotherapy for Oesophageal Cancer followed by Surgery Study
CRP	C-reactive protein
CXCL	Chemokine (C-X-C motif) ligand
DAMP	Damage associated molecular pattern
DC	Dendritic cell

DCA	Deoxycholic acid
DED	Death effector domain
DISC	Death-induced signalling complex
DMEM	Dulbecco's modified eagle media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
mtDNA	Mitochondrial DNA
DSS	Dextran sodium sulphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethyleneglycoltetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMR	Endoscopic mucosal resection
FA	Fatty acid
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GBP	Guanylate binding protein
GC	Goblet cell
GEMM	Genetically engineered mouse models
GOJ	Gastro-oesophageal junction
GORD	Gastro-oesophageal reflux disease
GTP	guanine tri-phosphate
H&E	Haematoxylin and Eosin
HEK	Human embryonic Kidney
HFD	High fat diet
HGD	High-grade dysplasia
HMGB1	High mobility group box one
HRP	Horse radish peroxidase
HSP	Heat shock protein
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFI16	IFN-inducible protein-16
IFN	Interferon
IgG	Immunoglobulin G
IHC	Immunohistochemistry

IκB	Inhibitor of kappa B
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	IL-1R-associated protein kinases
IRF	Interferon regulatory factor
IxP	Intensity multiplied by positivity
LBP	LPS-binding protein
LDH	Lactose dehydrogenase
LDL	Low density lipoprotein
LGD	Low-grade dysplasia
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAGIC	Medical Research Council Adjuvant Gastric Infusional Chemotherapy
MAL	MyD88 adaptor like
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MD2	Myeloid differentiation factor-2
MDP	Muramyl dipeptide
MIP-2	Macrophage inflammatory protein 2
mRNA	messenger RNA
mtDNA	mitochondrial DNA
MW	Molecular weight
MyD88	Myeloid differentiation factor 88
NAIP	Neuronal apoptosis inhibitory protein
NASH	Non-alcoholic steatohepatitis
NAT	Neoadjuvant treatment
NBD	Nucleotide binding domain
NF-κB	Nuclear factor-κB
NK	Natural killer
NKT	Natural killer T
NLR	Nod like receptor
NLRP	Nod like receptor protein
NO	Nitric oxide
NSAID	Nonsteroidal anti-inflammatory drugs
OAC	Oesophageal adenocarcinoma
OMV	Outer membrane vesicle
Pam2 CSK4	Dipalmitoylated lipopeptide CysSerLys4
Pam3 CSK4	Tripalmitoylated lipopeptide CysSerLys4
PAMP	Pathogen associated molecular pattern

PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDX	Patient derived xenograph
PMA	Phorbol 12-myristate 13-acetate
PPI	Proton pump inhibitor
PRR	Pattern recognition receptor
PYD	Pyrin domain
RAGE	Receptors for advanced glycation end-products
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
RCT	randomised controlled trial
RFA	Radio frequency ablation
RIG-I	Retinoic acid inducible gene-I
RIP	Receptor interacting serine/threonine protein
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SAT	Subcutaneous adipose tissue
SCC	Squamous cell carcinoma
SCJ	Squamocolumnar junction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEAP	Secreted embryonic alkaline phosphatase
SFD	Standard fat diet
ssRNA	Single stranded ribonucleic acid
STAT	Signal transducer and activator of transcription
TAB	TAK1-binding protein
TAK	Transforming growth factor-beta-activated kinase
TCD	Trinity College Dublin
TGF	Transforming growth factor
TIR	Toll IL-receptor
TLR	Toll like receptor
TNFα	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor
TNM	Tumour/ lymph nodes/ metastasis
TRAF	TNFR-associated factor
TRAM	TRIF related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFN- β
TTMI	Trinity Translational Medicine Institute

TUM	Technische Universität München
UV	Ultraviolet
V	Volts
VAT	Visceral adipose tissue
VEGF	Vascular endothelial growth factor
VFA	Visceral fat area
WC	Waist circumference
WEHD.CHO	Trp-Glu-His-Asp-aldehyde
WHO	World Health Organisation
WT	Wild-type
YVAD.fmk	Z-Tyr-Val-Ala-Asp (OMe)-fluoromethylketone

Chapter 1: General Introduction

1.1 Innate immunity

1.1.1 Innate immunity and inflammation

The immune system is a complex system in the body that comprises the natural defence that protects against disease. In humans, it consists of two arms; the innate and the adaptive immune systems. The innate system is evolutionary highly conserved and is generally regarded as the first line of host defence. It was traditionally considered to provide 'non-specific' immunity, however research has since come to recognise the essential and equally important role of innate immunity compared to the adaptive response. The second line of defence is known as the adaptive immune system which evolved in early vertebrates to generate stronger and more specific responses using immunological memory. B and T cells are activated and respond with antigen specific recognition to ensure pathogen clearance (1, 2).

Innate defence mechanisms in mammals are found in all tissues of the body but are particularly important as barriers such as the skin and mucosa of the lungs and gastrointestinal tract. The epithelium acts as a physical barrier which produces secretions such as lysozyme, mucous and oils to help prevent pathogen entry. It also aids detection of microorganisms that is fundamental for the surveillance of potential threats but also in helping to train and maintain immune homeostasis (3). All microorganisms generate pathogen-associated molecular pattern (PAMP) molecules that can be recognised by various pathogen recognition receptors (PRRs) present on the surface of cells. Commonly encountered PAMPs include lipopolysaccharide (LPS), lipoproteins, peptidoglycans, flagellin and nucleic acids from bacteria, fungus and viruses (4). PRRs also recognise endogenous 'danger' signals or danger-associated molecular patterns (DAMPs). DAMPs are host derived molecules that are hidden under normal physiological conditions. During conditions of cellular stress or injury, these molecules are released and subsequently detected by PRRs. Common examples of DAMPs include reactive oxygen species (ROS), deoxyribonucleic acids (DNA) and adenosine tri-phosphate (ATP) (5).

If a PAMP or DAMP is considered abnormal or threatening, the host will rapidly respond by upregulating an inflammatory response to eliminate the invasion. Pro-inflammatory cytokines and chemokines mediate the recruitment and activation of immune cells to the site of damage or infection. While a robust immune response is considered crucial for resolution of an infection, over-activation and dysregulation of responses can result in perpetual inflammation. This may lead to the development of auto-immune or auto-inflammatory diseases, tissue injury or inflammation-driven carcinogenesis (6, 7).

1.1.2 Pattern recognition receptors (PRRs)

PRRs recognise DAMPs and PAMPS which initiate downstream signalling pathways and promote specific inflammatory responses. There are five main classes of PRRs that can be subdivided into two general groups; transmembrane and cytosolic receptors (8). Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are transmembrane spanning and are found on the cell surface or bound to intracellular organelles (9, 10). Cytosolic receptors include nucleotide-binding domain and leucine-rich repeat containing receptors (NOD-like receptors, NLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) and absent in melanoma 2 protein (AIM2)-like receptors (ALRs). Other more recently discovered PRRs include receptors for advanced glycation end-products (RAGE) and cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase (cGAS) (1, 5). For the purpose of this study, TLRs, NLRs and ALRs will be further discussed due to their involvement in signalling pathways associated with inflammatory caspase upregulation and activation

1.1.3 Toll-like receptors (TLRs) and signalling

Toll-like receptors (TLRs) are PRRs that recognise conserved structures in DAMPs and PAMPS triggering the activation of pro-inflammatory signalling pathways critical for host defence (11). TLRs are type I transmembrane proteins that consists of three major domains. The ecto-domain is comprised of leucine-rich repeats (LRRs) that mediate pathogen recognition, the transmembrane domain and the intracellular

Toll/interleukin-1 (IL-1) receptor (TIR) domain required for initiation of signal transduction and downstream signalling (12). To date, there are 10 known TLRs in humans and 12 in mice, of which TLR1-9 are conserved in both species (9, 13, 14). Each TLR has a specific LRR structure which compliments binding of conserved PAMPs or 'ligands' derived from bacteria, viruses and fungi (12). TLR expression is almost ubiquitous on immune cells of the myeloid and lymphoid lineage, including monocytes, macrophages, dendritic cells (DCs), neutrophils, T cells and B cells. Many 'non-immune' cells also express TLRs, including astrocytes, fibroblasts and epithelial cells. The most studied examples of these are TLR4 and TLR2 expression on mucosal epithelium of the gut, airways and oral cavity (15).

TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are located on the cell surface and carry out surveillance for extracellular ligands or PAMPs and DAMPs. While TLR3, TLR7, TLR8 and TLR9 are expressed intracellularly and localise to the membranes of cytosolic endosomes and lysosomes (16). All TLRs undergo receptor dimerization following ligand binding to the LRR domain, which results in a conformational change of the intracellular TIR domain. This mediates recruitment of adaptor proteins and triggers downstream signalling. Currently identified adaptor proteins include myeloid differentiation factor 88 (MyD88) (17), MyD88 adaptor-like (Mal, TIRAP) (18), TIR domain-containing adaptor protein including interferon (IFN)- β (TRIF) (19) and TRIF-related adaptor molecule (TRAM) (20). There are two main signalling pathways associated with TLR signalling; MyD88-dependent and MyD88-independent (or TRIF dependent) (**Figure 1.1**) (21).

With exception of TLR3, MyD88 triggers TLR signal transduction as well as the IL-1 receptor (IL-1R) family. TLR2 and TLR4 also require MAL recruitment. Subsequently, MyD88 recruits IL-1R-associated protein kinases (IRAKs) IRAK4, IRAK1 and IRAK2 which then activates tumour necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) to form the myddosome complex (22). TRAF6 then triggers TGF- β -activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), TAB2 and TAB3 activation leading to TAK1/TAB complex formation (23). This then activates mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF- κ B) signalling pathways resulting in upregulation of inflammatory cytokines, chemokines, type II IFNs, mucins and defensins (15). TLR2, TLR3 and TLR4 can also upregulate the MyD88 independent pathway (24, 25). TLR activation

is instead mediated by TRIF and TRAF3 leading to activation of interferon regulatory factor (IRF)3 through IKKs, TBK1 and IKK ϵ pathways (19, 26, 27). This leads to transcriptional upregulation of type I IFNs and IL-10. TLR3-TRAF signalling can also drive MyD88-dependent TRAF6 and RIP-1 leading to NF- κ B activation (28). Activated TLR4 may endocytose and signal from endosomes *via* TRIF and TRAM recruitment, to activate downstream signalling pathways TRAF6/RIP1 and TRAF3/IRF3 (**Figure 1.1**) (16).

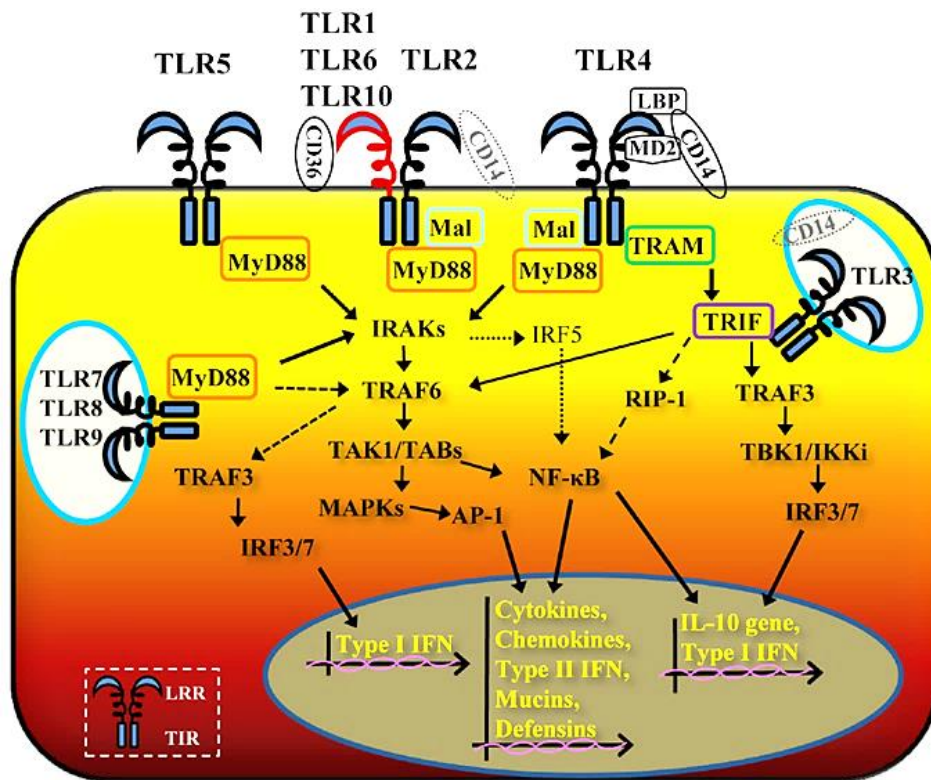


Figure 1.1: Schematic of TLR signalling.

TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are located on the cell surface, while TLR3, TLR7, TLR8 and TLR9 are expressed intracellularly. TLR1, TLR2, TLR4-9 activates MyD88-dependent pathways. Activation of TLR2/TLR1, TLR2/TLR6, TLR4, -5, -7, -8 and -9 triggers transduction through MyD88, MAL (only TLR2 and -4), IRAKs and TRAF6 leading to activation of NF- κ B and MAPK and upregulation of inflammatory cytokines, chemokines, IFNs, mucins and defensins. TLR7, -8 and -9 also signal through TRAF3. TLR3, TLR4 and TLR2 can activate TRIF-dependent pathways, leading to NF- κ B, MAPKs and IRF pathway activation. Figure taken from McClure and Massari, *Frontiers in Immunology*, 2014 (15).

TLR2 and TLR4 are the best characterised TLRs involved in mucosal and oesophageal immunity. This is likely due to their ability to become activated in response to a large array of ligands (29). They also appear to play an important role in immune tolerance and regulating inflammatory responses in the gut (30-32). For the purpose of this study, research surrounding their ligand binding and activation will be focused on in this introduction. TLR4 recognises a wide range of ligands in humans and mice derived from viruses, bacteria and fungus, including LPS, mannan, envelope F protein, glycosylphosphatidylinositol, flavolipin, Taxol, fusion protein, heat shock protein (HSP)60 and HSP70, oligosaccharides of hyaluronic acid, polysaccharide fragments of heparan sulphate and fibrinogen (16). TLR4 has a unique structure and is best characterised by its direct binding to LPS, which has been demonstrated through crystal structure studies (33). LPS is a component of the outer membrane of gram negative bacteria which binds TLR4 when the receptor is in a heterodimer complex with myeloid differentiation protein 2 (MD-2) (34). At binding, conformational changes occur which trigger signal transduction through either the MyD88-dependent or -independent pathways. Additionally, LPS-binding protein (LBP) and CD14 help guide LPS binding to TLR4. LBP is a soluble plasma protein that directly binds LPS, which is then recognised by CD14 and delivered to the TLR4-MD2 complex (35).

TLR2 becomes activated in response to a wide variety of PAMPs due to its ability to form heterodimers with other TLRs. Such ligands include lipopeptides, peptidoglycan, lipoteichoic acid, lipoarabinomannan, porin, a phenol soluble modulin, phospholipomannan, glycosylphosphatidylinositol anchor, glycolipids, zymosans, HSP70, hyaluronan, hemagglutinin (16). TLR2 predominantly forms heterodimers with TLR1 or TLR6. Specific recognition of a ligand is dependent on the acyl group. The TLR2-TLR1 heterodimer recognises triacylated lipopeptides from gram negative bacteria and mycoplasma. While the TLR2-TLR6 heterodimer recognises diacylated lipopeptides from gram-positive bacteria (36, 37). Furthermore, studies have shown that the TLR2 heterodimer complexes also interact with accessory molecules and co-receptors CD14, LBP and CD36 to initiate signalling cascades (38). TLR2 heterodimeric clusters are thought to occur in lipid raft microdomains, which facilitate weakly associated multimer formation in the absence of ligands that rearrange upon ligand binding (37, 39).

Although a wide range of ligands have been proposed as activators of TLR2 and TLR4 through direct binding to the receptors. An insightful paper by Clett Erridge warns that many of these molecules may not actually be TLR-stimulating and rather the stimulatory effect observed is derived from reagent contamination (40). The article highlights that many of the proposed ligands failed to demonstrate stimulating properties when highly purified reagents were used. This included investigative studies of HSP60/70, HMGB1, β -amyloid and hyaluronan (41-47). However some of these molecules, such as HMGB1 may function as mediators or facilitators of TLR ligand binding in an analogous manner similar to LPS-binding protein to enhance pro-inflammatory responses (43).

Activation of TLRs leads to upregulation of inflammatory cytokines and specific proteins which enable the cell to subsequently mount more robust immune responses, such as IL-1 secretion. Examples of proteins which are robustly upregulated following LPS-TLR4 activation include pro-IL-1 β , the NLR sensor protein, NLRP3, or the ALR sensor protein, AIM2 (48-50). Transcriptional upregulation of these proteins is referred to as 'priming' and they make up the building blocks for inflammasome activation mediated by NLRs and ALRs. Therefore, the relationship between TLRs (priming) and NLRs/ALRs (activation) is crucial for effective inflammasome-mediated inflammation (51).

1.1.4 Nod-like receptors (NLRs)

NLRs are a class of highly conserved intracellular PRRs. There are currently 22 known NLRs in humans and 34 in mice. All NLRs are characterised by the presence of a central nucleotide binding domain (NBD; NOD; NACHT) thought to be required for oligomerisation, and a COOH-terminal LRR domain responsible for ligand sensing (52). The NBD domain is also referred to as NACHT (NAIP, CIITA, HET-E and TP1) and can bind to GTP and ATP (53).

NLRs can be classified into four main subfamilies based on the NH₂-terminal effector domains. 1) NLRA, the acidic transactivation domain which includes only one member, MHC-II transactivator (CIITA). 2) NLRB, the baculoviral inhibitory repeat (BIR)-like domain which also has only one member, NAIP. 3) NLRC, the caspase-1 activation and

recruitment domain (CARD) which has six known members; NOD1 (NLRC1), NOD2 (NLRC2), NLRC3, NLRC4, NLRC5 and NLRX1. 4) NLRP, the pyrin domain, consisting of 14 members NLRP1-14. NLRP10 does not have a LRR domain (**Figure 1.2**) (54, 55).

NLRs, NOD1 and NOD2 are primarily involved in transcriptional upregulation following recognition of PAMPs and have been reported to activate the transcription factor signalling pathways, NF- κ B and MAPK resulting in transcription of pro-inflammatory cytokines (56-58). NOD2 has additionally been reported to sense viral ssRNA triggering activation of IRF3 and an IFN anti-viral response (59). Furthermore, several of the NLRs containing pyrin domains and NLRC4 are known to oligomerise and form an inflammasome complex after sensing pathogen or sterile activators. The pyrin domain contains a protein-protein interaction motif that allows for NLRs to homotypically interact with the pyrin domain present on apoptosis speck-like protein containing a CARD (ASC). ASC is an adaptor protein that is recruited by certain NLRs, including NLRP3, to form the activated inflammasome complex. ASC can subsequently recruit pro-caspase-1 directly to the complex *via* CARD-CARD domain interactions. Pro-caspase-1 is then cleaved into p10 and p20 subunits and secreted from the cell. The cleaved subunits of caspase-1 are thought to form a tetramer with active cysteine protease abilities, which leads to the processing and maturation of the inflammatory cytokines IL-1 β and IL-18 (55). Additionally, active caspase-1 mediates pyroptosis, an inflammatory form of cell death, through cleavage and activation of the pore forming protein, gasdermin D (60).

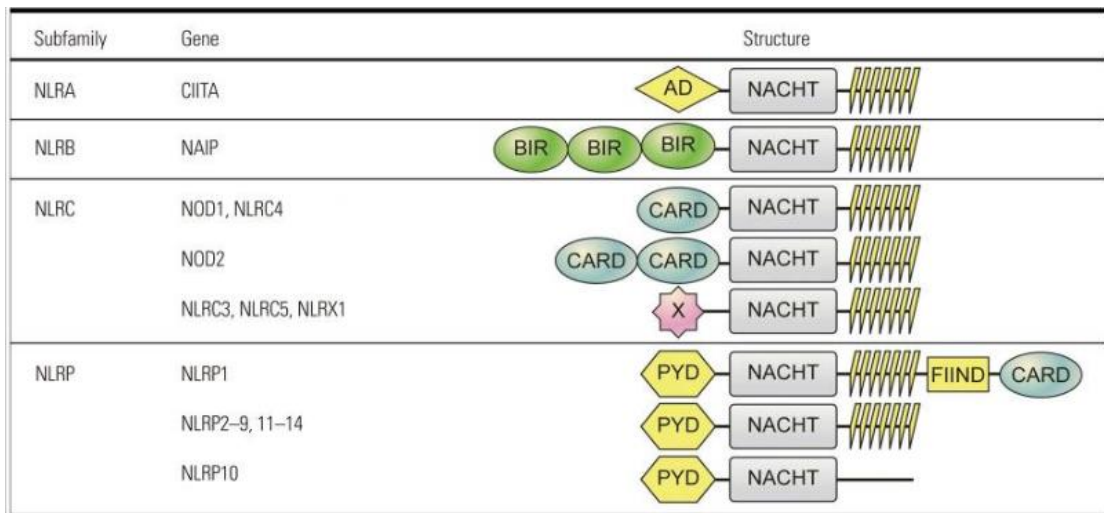


Figure 1.2: NOD-like receptor classification and protein structure.

Schematic displaying all known members of the NLR family. All NLRs express the defining central NACHT (NOD) domain flanked by a C-terminal LRR domain and an effector domain at the N-terminal. Members are divided into four subfamilies based on N-terminal domain; NLRA, NLRB, NLRC and NLRP. CIITA is the sole member of the NLRA subfamily and acts as a transcription factor. Similarly, NAIP is the only known member of the NLRB subfamily and expresses a BIR domain. NLRC/X subfamily is determined by the presence of a CARD domain or an unidentified X recruitment domain. Lastly, NLRP subfamily represents the largest group and are categorised by the presence of a pyrin domain. Figure is taken from Kim *et al.*, *Yonsei Medical Journal*, 2016 (55).

1.1.5 AIM2-like receptors (ALRs)

AIM2 and IFN-inducible protein 16 (IFI16) make up an inflammasome family called ALRs. Structured differently to NLRs, ALRs are part of a larger group of proteins called PYHINs. These proteins have a characteristic make up of a PYRIN (PYD) domain at the N-terminal and a DNA binding hematopoietic interferon-inducible nuclear antigen (HIN) domain at the C-terminal (**Figure 1.3**)(61). The PYD domain consists of a protein-protein interaction motif that can homotypically interact with other PYHINs or other proteins containing a PYD domain. The HIN domain contains 200 amino acid repeat sequences that act as major DNA recognition sites, three subgroups exist; A, B and C (62, 63). IFI16 expresses an N-terminal localisation factor that causes predominant localisation to the nucleus. Conversely, AIM2 is primarily found in the cytosol, although can also be found in the nucleus (62, 64).

IFI16 acts as a DNA sensor that can upregulate anti-viral signalling pathways leading to inflammatory responses such as robust IFN- β production (65). Studies have shown that IFI16 co-localises with transfected vaccinia virus (VACV) dsDNA in the cytoplasm of THP-1 monocytes (66) and also with Kaposi's sarcoma-associated herpesvirus (KSHV) DNA in the nucleus (67). IFI16 inflammasome formation has been reported, however further evidence is required to fully confirm these observations (67, 68).

Much like IFI16, AIM2 acts as a DNA sensor, sensing viral, bacterial and self-derived DNA such as cytomegalovirus (CMV), *Francisella tularensis* and *Listeria* (69-71). Activation of the AIM2 inflammasome will be discussed in more detail later in this chapter.

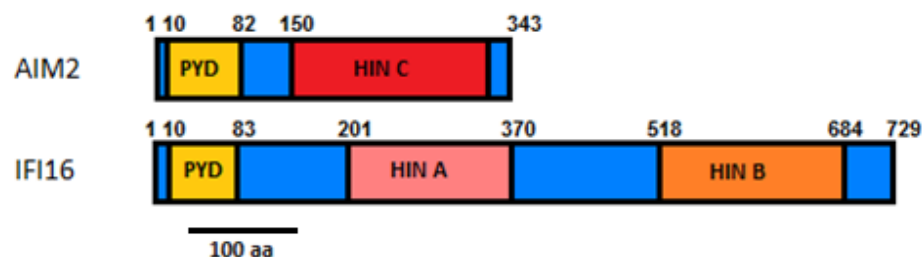


Figure 1.3: Schematic representation of ALR PYHIN domains present in IFI16 and AIM2.

The PYD domain at the N-terminal is capable of protein-protein interactions. At the C-terminal a DNA-binding HIN domain is important for sensing DNA. Figure taken and adapted from Connolly & Bowie, *Biochem Pharmacol.*, 2014 (61).

1.2 Inflammasome signalling

1.2.1 Caspases

Caspases (cysteine-aspartic proteases) are a family of proteases consisting of 12 members. They are almost ubiquitously expressed in all cell types as inactive zymogens (pro-caspases). The caspases have been functionally sub-divided into two groups; inflammation and cell death (apoptosis). Human caspase-2, -3, -6, -7, -8, -9 and -10 are apoptotic, while caspase-1, -4, -5, and -12 are inflammatory. In mice, caspases-4 and -5

are not present and caspase-11 is thought to act as a functional ortholog (72). Apoptotic caspases are further sub-divided into groups based on the structure of the N-terminal pro-domain. Initiator caspases express a protein interaction domain such as a death effector domain (DED) (caspase-8 and -10) or a CARD domain (caspase-2, -9). While executioner (effector) caspases (caspase-3, -6 and -7) lack a protein interaction domain (73). The inflammatory caspases all share a similar structure, all possess a CARD prodomain at the N-terminal and a protease domain at the C-terminal consisting of a large (~p20) and small (~p10) subunit containing a catalytic active site (**Figure 1.4**) (74).

In order for caspases to become activated and carry out protease activities, they must first be cleaved and heterodimerise. Protein-interaction domains accommodate recruitment of caspases to multimeric activation complexes, such as the inflammasome, the apoptosome and death-induced signalling complex (DISC). Once activated, caspases tend to cleave their substrates after tetrapeptide sequences containing aspartic acid (Asp) in the P1 position. This can lead to downstream activation of effector proteins such as IL-1 β and gasdermin D (74). Although caspases have traditionally been divided into inflammatory and apoptotic arms, studies have shown functional specificity of the caspases often overlaps between the two groups. For example, caspase-8 is an important initiator caspase of the extrinsic apoptotic pathway, however recent studies suggest that it also has an important role in inflammation and inflammasome signalling (75, 76). Moreover, the inflammatory caspases -1 and -11 have been implicated in the inflammatory form of cell death, pyroptosis which results in pore formation and osmotic lysis of the cell. These examples highlight the close interaction and relationship between cell death and inflammation in molecular biology (60, 77).

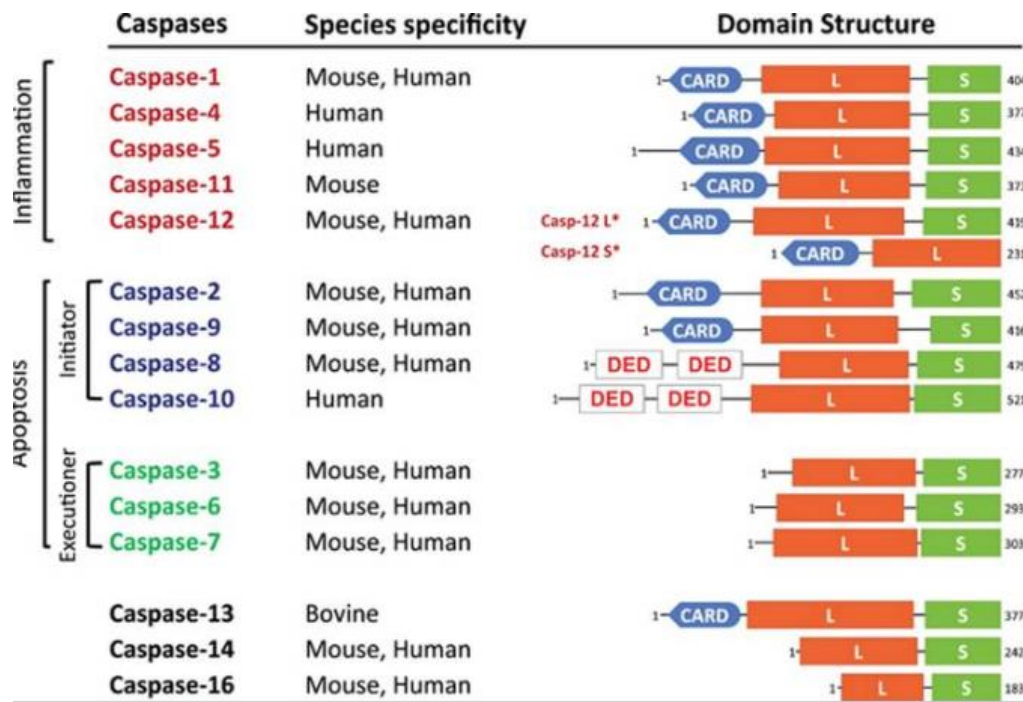


Figure 1.4: Schematic representing the structure and function of inflammatory and apoptotic caspases in humans and mice.

Inflammatory caspases include caspases -1, -4, -5 and -12 in humans and caspases -1, -11 and -12 in mice. The structure is characterised by a protein-interaction domain, CARD, located at the N-terminal and a C-terminal protease domain comprised of a large and small subunit. Apoptotic proteins are sub-categorised into initiator caspases -2, -9, -8 and -10, and executioner (effector) caspases; -3, -6 and -7. All initiator caspases are comprised of a protein-interaction domain of either CARD or DED at the N-terminal and a protease domain similar to the inflammatory caspases at the C-terminal. Executioner caspases are lacking a protein-interaction domain at the N-terminal. Key: L, large subunit; S, small subunit; S*, short form; L*, long form. Figure taken from Shalini *et al. Cell Death & Differentiation*, 2015 (72).

1.2.2 Caspase-1 and the canonical inflammasome

Caspase-1, also known as IL-1 β -converting enzyme (ICE), was first identified in 1989 as a cysteine protease responsible for cleavage and maturation of the potent pro-inflammatory cytokine IL-1 β in monocytes (78, 79). Studies have since revealed that active caspase-1 forms a crucial part of the inflammasome signalling pathway leading to

activation of IL-1 β and IL-18, promoting inflammation and also cleaving gasdermin D, leading to pyroptosis.

Activation of caspase-1 is tightly regulated, due to its potent inflammatory effects upon activation. Activation occurs *via* a two-step process. Signal 1 or 'priming' is responsible for upregulation of pro-IL-1 β and inflammasome components, such as NLRP3. This occurs following the extracellular detection of PAMPs or DAMPs by PRRs (eg. LPS-TLR4 activation), resulting in activation of NF- κ B. 'Signal 2' or 'activation' involves the subsequent activation and assembly of the inflammasome complex. Inflammasomes are multimeric complexes that oligomerise following intracellular recognition of PAMPs or DAMPs by cytoplasmic sensors or PRRs, such as NLRP3 and AIM2 (80-82).

Upon assembly of the inflammasome complex, NLRP1 and NLRC4 can homotypically bind and activate caspase-1 *via* a CARD domain located at the N-terminus of both proteins (83, 84). Contrary to this, AIM2 and NLRP3 recruit ASC *via* pyrin domain protein-protein interactions to form a multimeric complex referred to as a 'speck'. ASC also contains a CARD domain which can recruit caspase-1 to the assembled inflammasome (**Figure 1.5**) (83, 85, 86). Upon recruitment of multiple caspase-1 proteins to an inflammasome complex, caspase-1 has traditionally been thought to undergo subsequent proximity-induced auto-processing (87). This occurs when caspase-1 proteins are in close proximity to one another on the inflammasome platform. The product of this reaction is a catalytically active tetramer consisting of two p20 and p10 subunits. Activated caspase-1 subsequently cleaves pro-IL-1 β , pro-IL-18 or gasdermin D to generate their mature and active forms (88-90).

Notably, a recent paper published by Boucher *et al.* has challenged the assumption that the tetramer forms active caspase-1. They argue that in bone marrow-derived macrophages (BMDMs) self-cleavage of caspase-1 is a limiting mechanism used to terminate inflammasome activity. In contrast, they suggest that the active forms of caspase-1 are in fact full-length p46 and the transient species p33/p10. This model incorporates an intrinsic self-limiting mechanism where caspase-1 activity is terminated upon dissociation from the inflammasome (91). Indeed, another study by Walsh *et al.* remarked upon the exceptionally short half-life of processed caspase-1 of approximately 9 minutes in comparison to processed caspase-3 (8 hours) and -8 (11

hours), indicating its rapid degradation and resolution following dissociation (90). It is clear that there remains much to resolve regarding the mechanisms of inflammasome activation, however, ultimately the result is the maturation of IL-1 β , IL-18 and gasdermin D.

As previously mentioned, IL-1 β and IL-18 are potent pro-inflammatory cytokines. They are members of the same IL-1 family which plays a central role in mediating immune responses of both the innate and adaptive system. IL-1 β influences various biological activities such as fever, vasodilation, haematopoiesis, leukocyte attraction, angiogenesis and acute-phase response (92). IL-1 β has also been associated with chronic inflammation contributing to carcinogenesis such as inflammatory bowel disease (IBD) and colorectal cancer, and Barrett's oesophagus and oesophageal adenocarcinoma (OAC) (93-95). IL-18 is primarily involved in Th1 cell responses by promoting upregulation of IFN- γ production, Th1 cell proliferation and NK cell activation (96). Despite these apparent anti-tumorigenic roles, IL-18 is often considered a double-edged sword in cancer, as activation can often lead to induction of tumour progression and angiogenesis (97). It has been hypothesized that IL-18 has contrasting roles depending on the site of its expression – protective in intestinal epithelial cells (IECs), but inflammatory at the level of the lamina propria (98). Studies have shown that IL-18 plays a crucial role in epithelial healing, repair and proliferation (92, 99, 100).

Recently, gasdermin D has been identified as the mediator of pyroptosis, which contributes to the release of mature IL-1 β and IL-18 from the cell. Pyroptosis is distinguished from apoptosis for a few key reasons. Firstly, inflammatory caspases are required for pyroptosis but not apoptotic caspases. Secondly, pyroptosis is extremely inflammatory while apoptosis is considered immunologically silent. Lastly, pyroptosis involves osmotic swelling and lysis of the cell as opposed to cell shrinkage observed during apoptosis. This is caused by pores formed by the N-terminal domain of gasdermin D following cleavage by caspase-1 (60, 101). Release of cellular contents, termed alarmins, such as ATP, high motility group box one (HMGB1), DNA fragments and pro-IL-1 α into the tissue mediates rapid activation and migration of other immune cells to the site of infection (102).

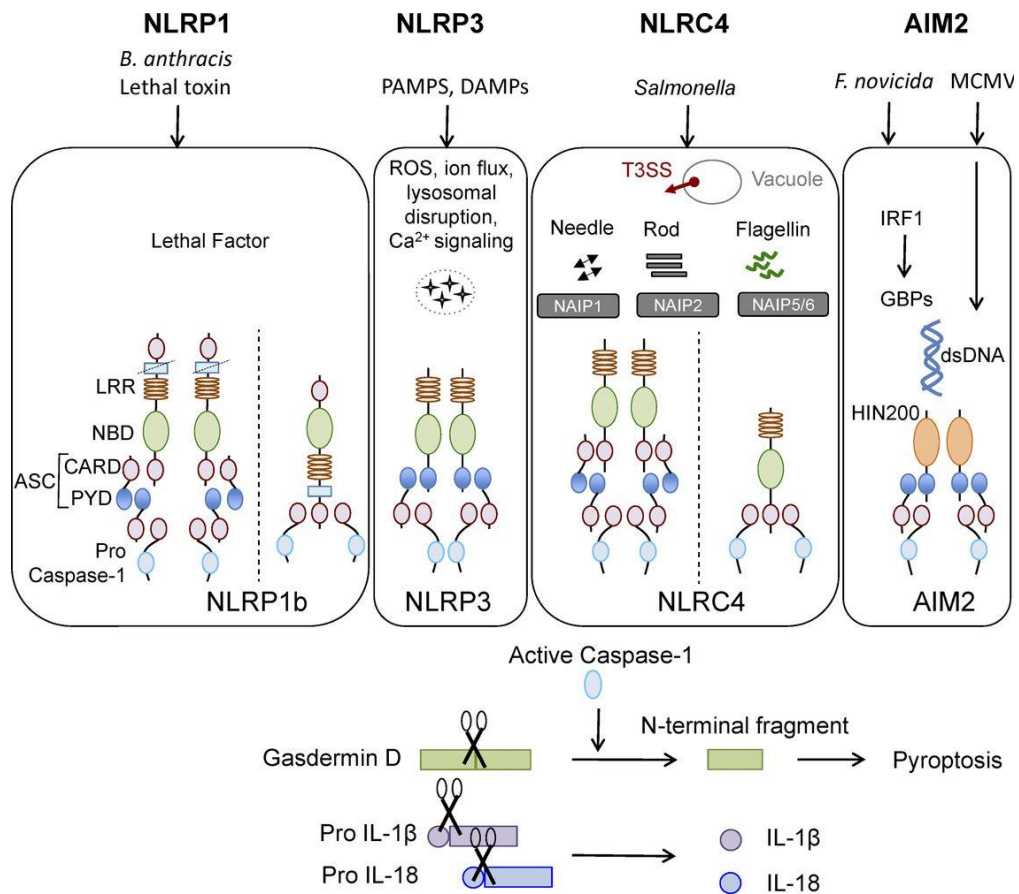


Figure 1.5: Inflammasome activation leads to caspase-1 mediated inflammation.

Inflammasome proteins are activated by DAMPs and PAMPs leading to the formation of the inflammasome complex. NLRP3 and AIM2 oligomerise with ASC upon activation. NLRP1 and NLRC4 activation can occur dependent or independent of ASC. All canonical inflammasome activation leads to caspase-1 cleavage and activation. Subsequently leading to cleavage of IL-1 β , IL-18 and gasdermin D. Image taken and adapted from Sharma and Kanneganti, *Journal of Cell Biology*, 2016 (103).

1.2.3 NLRP3 and the canonical inflammasome

As mentioned, there are two signals required for NLRP3 inflammasome activation in macrophages. Signal 1, priming involves upregulated gene transcription of inflammasome components through pathways that signal through MyD88, TRIF or other NF- κ B such as TNF receptor (TNFR), TLRs, IL-1R and NOD2 (**Figure 1.6**). Priming and protein translation is thought to occur rapidly and involves various post-translational modifications such as NLRP3 and ASC ubiquitination and phosphorylation. Additionally,

NLRP3 and caspase-1 have recently been shown to independently associate with the mitochondria during priming in response to ROS in BMDMs (104-106).

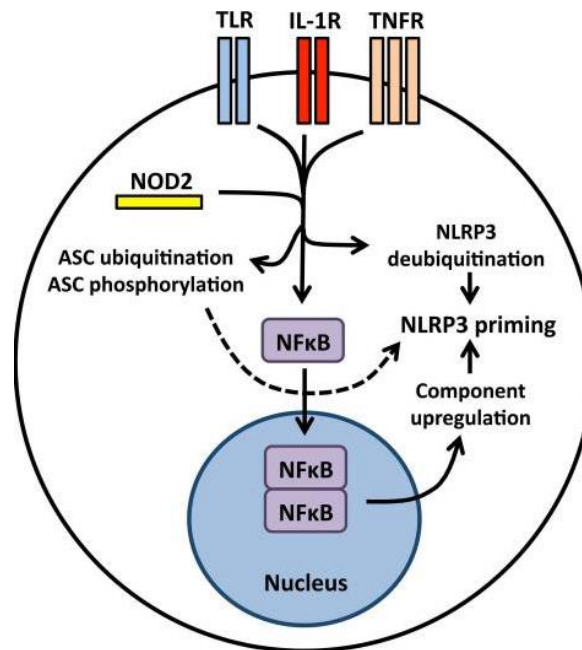


Figure 1.6: Signal 1, NLRP3 canonical inflammasome priming.

Schematic representing priming (signal 1) of the NLRP3 inflammasome. The priming signal is received through various receptors including TLR, IL-1R, TNFR and NOD2 to activate NF-κB and induce upregulation of gene transcription of inflammasome components such as NLRP3 and ASC. Post-translational modifications occur prior to activation, such as NLRP3 de-ubiquitination and ASC ubiquitination and phosphorylation. Figure taken from Elliot *et al. Immunology Reviews*, 2015 (105).

Signal 2 or activation involves NLRP3 sensing of danger signals (**Figure 1.7**). NLRP3 is the best characterised of all the inflammasomes and this is partly due to its ability to sense a diverse range of ligands. Such ligands include PAMPs (e.g. *Streptococcus pneumoniae* and *Staphylococcus aureus*), endogenous DAMPS (such as monosodium urate crystals, islet amyloid peptide and ATP) and even environmental stimulants (e.g. alum and silica). Due to this wide spectrum of ligands and a failure to prove any directly bind to NLRP3, it is speculated that the inflammasome becomes activated through more indirect pathways related to cellular stress. Examples include ion fluxes such as K^+ / Na^+ ,

phagosomal and lysosomal destabilisation, calcium signalling (Ca^{2+}) and mitochondria ROS production (80, 105). Following NLRP3 activation, ASC is recruited to the mitochondrial membrane to assemble before recruiting caspase-1 to form the inflammasome complex (104). ASC recruitment and oligomerisation of NLRP3 is an absolute prerequisite for inflammasome activation, failure to do so results in no inflammasome assembly (107). An assembled complex then activates caspase-1, leading to IL-1 β and IL-18 release. Recently, SARM (Sterile α and HEAT Armadillo motif-containing protein) has been reported to negatively regulate IL-1 β release by directly restraining the NLRP3 inflammasome (108).

To date, the mechanism of NLRP3 activation remains elusive. The most convincing argument was reported by Munoz-Planillo *et al.*, who proposed a unifying model of K^+ efflux. Where a drop in cytosolic K^+ leads to NLRP3 activation (109). Additionally, the newly identified NLRP3 inflammasome component, NEK7 (NIMA-related kinase 7), which is required for NLRP3 activation has also been shown to require K^+ efflux (110, 111). However, this model has since been challenged by studies showing K^+ efflux is not required for peptidoglycan-N-acetylglucosamine-induced hexokinase activation of the NLRP3 inflammasome in BMDMs following release from the mitochondrial outer membrane, or during caspase-8 mediated activation of the NLRP3 inflammasome in human monocytes (112, 113). Whether these pathways occur independent of Nek7 is unclear. Subsequently, K^+ efflux has been identified as important but not always necessary for NLRP3 inflammasome signalling.

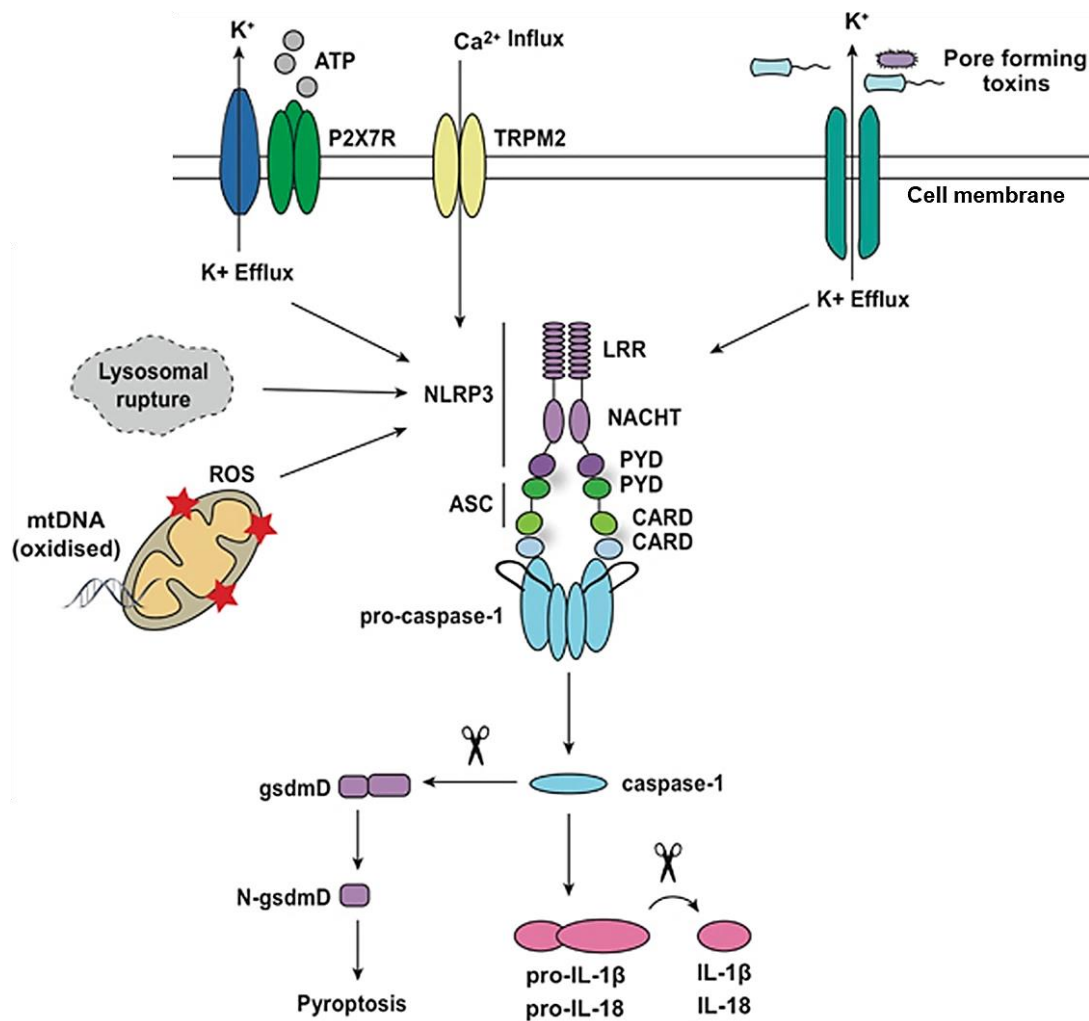


Figure 1.7: Signal 2, NLRP3 canonical inflammasome activation.

Schematic representing models of NLRP3 canonical inflammasome activation. Such mechanisms include K⁺ efflux, Ca²⁺ influx and pore forming channels at the cell membrane, mitochondrial-derived ROS and DNA and phagolysosomal degradation. Following recognition of a DAMP or PAMP, NLRP3 oligomerises and associates with ASC *via* pyrin (PYD) domain protein-protein interactions to form a 'speck'. ASC can then recruit caspase-1 *via* the CARD domain located at the C-terminus, facilitating proximity-induced self-cleavage and subsequent activation. Active caspase-1 then cleaves pro-IL-1β and IL-18 to initiate an inflammatory response or cleaves gasdermin D leading to pyroptosis. Figure taken and adapted from Feltham *et al. Clinical and Translational Immunology*, 2017 (81).

1.2.4 The AIM2 inflammasome

AIM2 has specificity for the recognition of cytosolic double stranded DNA leading to inflammasome activation (114). This DNA can be derived from altered or damaged DNA that is mislocalised within the cell (115). Aberrant DNA may be self-derived from the nucleus or mitochondria. For example, if the integrity of the nuclear envelope is compromised and causes genomic DNA leakage into the cytosol (116). To avoid inappropriate activation, cells have evolved methods to compartmentalise DNA to the nucleus or degrade mislocalised self-DNA by deoxyribonucleases (117). AIM2 is also essential for the detection of pathogens, including bacteria, viruses, fungus and parasites (118). When foreign DNA accumulates in the cell, it is sensed by AIM2 to initiate an inflammatory response. Detection of DNA occurs in a sequence-independent manner, but rather is sensed based on molecule length (minimum 70 base pairs) for activation (86, 119). In this way, AIM2 activation does not differentiate between self-derived and foreign DNA.

AIM2 mRNA expression was shown to be mostly expressed in the spleen, small intestine and in peripheral blood leukocytes (119), however upregulation of expression can be rapidly induced with stimulus, particularly with type I IFN signalling (120). There are a number of ligands that can cause transcriptional upregulation of AIM2 that have been reported in the literature, including LPS, type I and II IFNs, TNF α , poly(dAdT) (dsDNA), HMGB1, low density lipoprotein (50, 115, 121, 122). Upon sensing dsDNA, AIM2 can interact with ASC by PYD-PYD domain interaction leading to the formation of the AIM2 inflammasome which then recruits and activates caspase-1, causing pro-IL-1 β maturation (114, 123, 124). Murine AIM2 has also been shown to act as a DNA sensor in a similar way to human AIM2 and was shown to interact with ASC to form the inflammasome (123).

AIM2 plays an important role in mediating inflammation during diseases involving tissue injury and fibrosis. Komada *et al.* observed that during fibrosis in chronic kidney disease, macrophages scavenge necrotic cellular debris including DNA which drives AIM2 activation and inflammasome signalling (125). This has similarly been observed in an atherosclerosis model in mice (126). In patients, AIM2 expression was concentrated

around the necrotic or calcified areas of the atherosclerotic lesions (121). Additionally, significant AIM2 activity was detected in primary keratinocytes found in the psoriatic lesions of patients (115).

AIM2 predominantly functions in the cytosol but can also traffic into the nucleus and become activated (114, 124, 127). Mechanistically still poorly understood, Hu *et al.* demonstrated that irradiated primary macrophages resulted in AIM2 activation and co-localisation with ASC to form inflammasome 'specks' in the nucleus. This indicated that AIM2 could sense radiation-induced dsDNA damage in mice (127). Indeed, mice deficient in AIM2 or caspase-1 were completely protected from epithelial cell death and injury, and rescued the mice from radiation-induced lethality (127). This is supported by another study that showed AIM2 expression localised in the nucleus of IFN- γ stimulated cancer cell lines (128).

The AIM2 inflammasome pathway can also be activated indirectly through an additional DNA sensing signalling pathway, cyclic-GMP-AMP synthase (cGAS)- stimulator of interferon genes (STING). Cytosolic sensor, cGAS detects and binds low concentrations of dsDNA in a length-dependent manner and undergoes conformational changes resulting in active conversion of ATP and GTP into cyclic GMP-AMP (cGAMP) (129-131). Subsequently, cGAMP binds to STING located on the endoplasmic reticulum (ER) membrane, which becomes activated and induces transcriptional upregulation of type I IFN genes *via* IRF3, or pro-inflammatory cytokines *via* NF- κ B (132-135). Type I IFNs are secreted and bind the IFN receptor, leading to activation of IFN-stimulated gene factor 3 (ISGF3), which is made up of STAT1/2 and IRF9 (136, 137). These drive gene expression of IRF1 and IFN-inducible GTPases, such as guanylate-binding proteins (GBPs). GBPs, in particular GBP2 and GBP5 facilitate bacterial DNA exposure. They do this through disruption of the bacteria-containing vacuole and induction of bacteriolysis in the cytosol, allowing for AIM2 recognition and inflammasome activation (**Figure 1.8**) (138, 139).

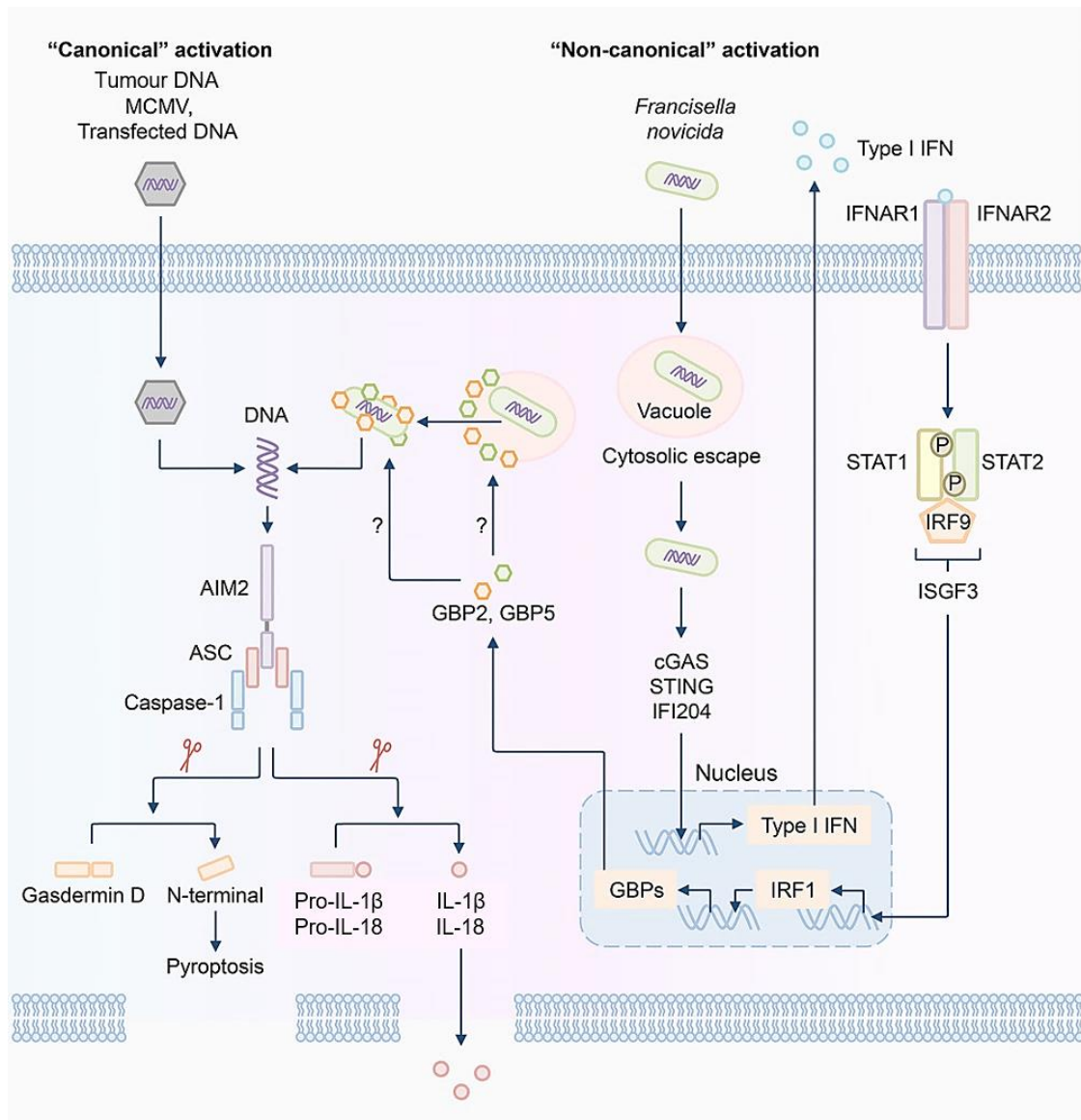


Figure 1.8: AIM2 inflammasome regulation and activation.

AIM2 is activated by sensing dsDNA derived from bacteria, viruses and self. Activation of AIM2 *via* the canonical pathway involves direct recognition of DNA by AIM2, causing oligomerisation with ASC, caspase-1 cleavage and subsequent activation of IL-1 β , IL-18 and gasdermin D. Non-canonical AIM2 activation can also occur *via* the STING-cGAS pathway, which requires type I IFNs. Intracellular infection, such as *F. novicida* causes release of DNA into the cytosol where it is sensed by cGAS, which then activates STING. This drives type I IFN transcription and secretion, causing a feed-back loop *via* ISGF3 induction. Transcription factor IRF1 mediates upregulation of IFN-inducible GTPases, such as GBP2 and GBP5. These GBPs directly target bacterial structures resulting in bacteria rupture and DNA release, which are recognised by AIM2. Figure taken and adapted from Man *et al.*, *European Journal of Immunology*, 2017 (135).

1.2.5 The non-canonical inflammasome pathway

In addition to the canonical inflammasome pathway mentioned, non-canonical inflammasome pathways have also been reported involving inflammatory caspases-4 and -5 in humans and caspase-11 in mice (**Figure 1.9**). Gene sequencing suggests that caspases-4 and -5 are derived from a murine caspase-11 gene duplication event and are therefore most homologous to caspase-11 (140).

Caspase-11 is termed the murine functional ortholog of caspase-4 and -5 and can be activated as a direct response to gram-negative bacteria, but not gram positive (141). Active caspase-11 carries out two known functions: pyroptosis and inflammasome-mediated inflammation. This occurs either through direct cleavage of gasdermin D, bypassing the inflammasome to pyroptosis or by activation of caspase-1 through the inflammasome complex (60, 142). Caspase-11 can also indirectly activate the NLRP3 inflammasome by cleaving gasdermin D, which induces pore formation and consequently K⁺ efflux (143). Multiple studies have shown that caspase-11^{-/-} mice are more resistant to lethal doses of LPS compared to wild-types (WT), suggesting a crucial role for caspase-11 in mediating inflammatory responses and endotoxic shock in mice (142, 144). Moreover, caspase-11 has been reported to bind intracellular LPS directly following LPS release from vacuoles mediated by GBPs in murine macrophages (145). This binding occurs through direct interaction of the caspase-11 CARD domain with the lipid A moiety present on LPS (146). Indeed, studies have previously shown that caspase-11 can be activated independent of ASC, which suggested direct binding of LPS. The binding affinity between LPS and caspase-11 has since been reported to be comparable to TLR4/MD2 complex binding (140, 147), strongly supporting the argument that caspase-11 is an integral cytosolic sensor.

Despite promising research identifying caspase-11 as an intracellular receptor of PAMPs, much remains to be elucidated regarding the cellular pathways and mechanisms of caspase-11-mediated inflammasome activation. Studies published by two independent research groups have reported that caspase-11 activation and pyroptosis are mediated by endogenous type I IFN signalling in gram negative bacterial-infected BMDMs (148, 149). During gram-negative infections, Rathinam *et al.* observed

that caspase-11 induction and auto-activation was driven by type I IFN signalling regulated *via* a TRIF-dependent pathway. Subsequently, caspase-11 can then activate the 'non-canonical NLRP3 inflammasome pathway' by synergising with NLRP3 to promote caspase-1-dependent IL-1 β and IL-18 maturation. This study identified TRIF as an important regulator of caspase-11 activity and TLRs as important master regulators (149). However, work from our laboratory has shown that caspase-11 upregulation in intestinal epithelial cells is independent of type I IFN and TRIF signalling, and requires type II IFN signalling (150), suggesting that differential regulatory mechanisms govern caspase-11 expression in macrophages and IECs. Indeed, other research in BMDMs identified L-adrenaline-induced cyclic adenosine monophosphate (cAMP) as a regulator of the caspase-11 inflammasome activated by cytosolic LPS, therefore identifying potential metabolic modulation (151). Together, these studies identify caspase-11 as a crucial mediator of inflammatory responses during gram negative bacterial infections and septic shock.

Despite a lack of a direct murine model, studies are emerging that provide evidence that caspase-4 and -5 are involved in a human non-canonical inflammasome pathway similar to what has been reported regarding murine caspase-11 (152). The mechanism of activation and assembly of the non-canonical inflammasome in human myeloid cells was found to be almost directly comparable to the mechanism observed in mice BMDMs. Similar to caspase-11, studies have shown pro-caspase-4 and -5 appear to also directly bind cytosolic LPS *via* interaction with the caspase CARD motif and lipid A moiety present in LPS. Upon binding, caspase-4/ -5 oligomerise to form an active non-canonical inflammasome complex. This complex may then induce pyroptosis *via* direct gasdermin D cleavage or it may activate the inflammasome pathway, leading to IL-1 β and IL-18 secretion (**Figure 1.9**) (140, 152-154).

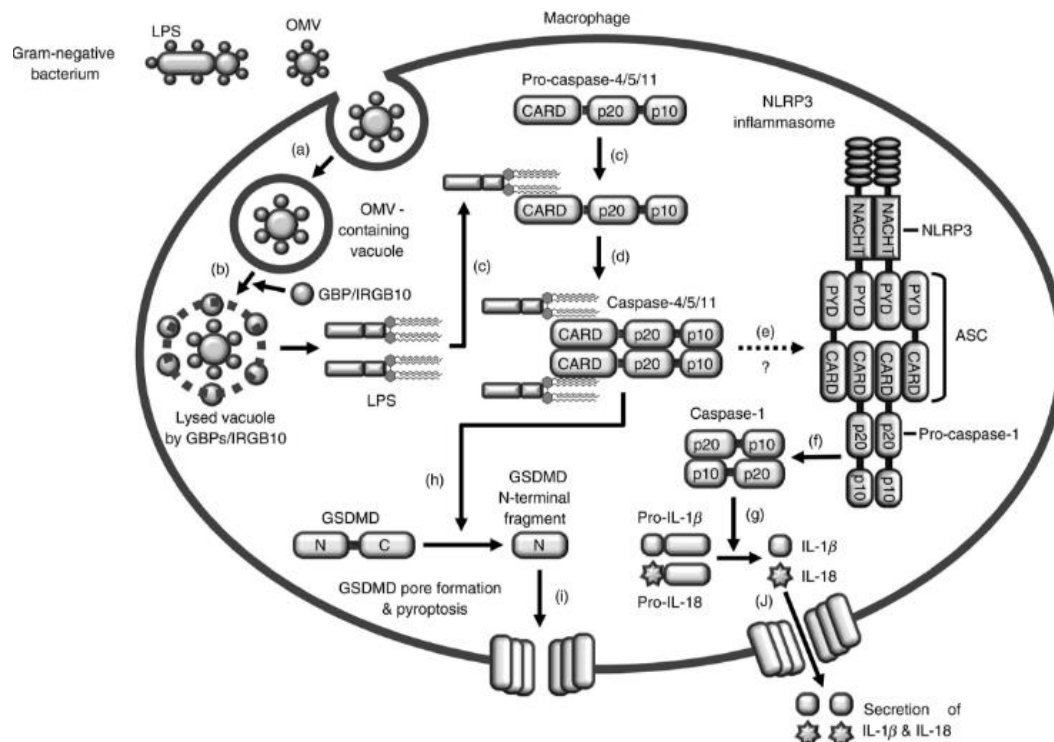


Figure 1.9: Caspase-4/ -5/ -11 non-canonical inflammasome pathway activated by intracellular LPS.

A) Outer membrane vesicles (OMVs) containing LPS are internalised into macrophages to form intracellular vacuoles. **B)** LPS is released from the vacuoles into the cytoplasm by guanylate binding proteins (GBPs). **C)** The lipid A moiety on LPS is recognised by the CARD motifs present on pro-caspase-4/-5/-11. **D)** Upon binding, oligomerisation of caspase-4/ -5/ -11 occurs. **E)** Active caspase-4/ -5/ -11 activates the NLRP3 inflammasome and assembly through an unknown mechanism. **F)** Caspase-1 is then cleaved and activated. **G)** Which in turn cleaves and activates the pro-inflammatory cytokines IL-1 β and IL-18. **H)** The active non-canonical inflammasome also cleaves the linker loop of gasdermin D, generating the C- and N-terminal fragments. **I)** The N-terminal gasdermin D fragment migrates to the cell membrane and oligomerises before forming pores causing osmotic swelling and pyroptosis. **J)** Mature IL-1 β and IL-18 are secreted from the cell. Image taken from YS Yi *et al. Immunology*, 2017 (141).

One study reported that caspase-4 is required for UVB-induced activation of the NLRP3 inflammasome in skin-derived human keratinocytes and is necessary for the maturation of pro-IL-1 β and pro-IL-18. Furthermore, this study also showed that caspase-4 was essential in activation of caspase-1 in both the NLRP3 and AIM2 inflammasome in THP-

1 macrophages (155). Analogous to murine caspase-11, this research demonstrates an important role for human caspase-4 in innate immune responses.

Caspase-5 is thought to be recruited to the NLRP1 inflammasome during activation. NLRP1 is activated by the bacterial peptidoglycan constituent, muramyl dipeptide (MDP), however the exact mechanism of how this happens remains unclear. Upon activation, the CARD domain of NLRP1 directly binds the CARD domain of pro-caspase-5, while the pyrin domain binds to ASC. In turn, ASC subsequently recruits caspase-1 to complete the assembly. It is thought that the close proximity of caspase-1 and caspase-5 enables proximity-induced activation of both caspases (85, 156).

Collectively, caspase-4 and -5 appear to function similarly to caspase-11 as cytosolic sensors of LPS and are important inducers of pyroptosis and inflammation as part of the non-canonical inflammasome pathway. Despite this, much more research is required to elucidate the regulatory mechanisms of this pathway in humans.

1.2.6 The inflammasome and inflammation-induced cancer.

Chronic inflammation has long been associated with facilitating the processes of neoplasia, proliferation, migration and survival. Indeed, it is now considered an emerging 'hallmark of cancer', which is often thought of as a wound that fails to heal (157, 158). During wound healing, proliferation of cells is enhanced. This is further accommodated by migration of immune cells and production of signalling molecules such as cytokines, chemokines and growth factors to facilitate regeneration. After tissue reparation, resolution of inflammation rapidly occurs to complete the healing process. In a neoplastic setting which may be driven by DNA damage or spontaneous mutation, inflammation does not subside and proliferation is sustained. This is further promoted by a microenvironment enriched with growth and survival factors that are continually secreted due to failed resolution (159).

Cancer is a genetic disease and develops from dysregulation of familial or sporadic gene mutations. This process is enhanced by important environmental influences such as inflammation. A well-studied example of this is colorectal cancer (CRC), which arises due

to genetic and environmental factors (160, 161). IBD patients are at increased risk of developing CRC due to chronic intestinal inflammation (162, 163). Inflammatory production of ROS and reactive nitrogen species (RNS) can induce DNA damage and mutations (164, 165). Similarly, inducible nitric oxide synthase (iNOS) expression induces cellular stress and oxidative DNA damage, further promoting tumorigenesis (166).

There are many diseases associated with inflammasome dysregulation including autoimmune diseases such as psoriasis and auto-inflammatory diseases such as cryopyrin-associated periodic syndromes (CAPS) (167, 168). In a similar manner, inflammasome dysregulation has also been associated with aiding carcinogenesis. Particularly in cancers associated with chronic inflammation, such as breast cancer, fibrosarcoma, gastric and oesophageal adenocarcinoma, CRC and lung metastasis (169). Much remains uncharacterised about the inflammasome in tumour development, however studies in recent years are starting to elucidate its processes, revealing contrasting mechanisms of tumour promotion or protection specific to the cancer type.

Many studies have reported a protective role for the inflammasome in DSS-induced colitis in mice. Harrison *et al.* observed that IL-18 derived from epithelial cells directly interacted with CD4⁺ T cells to reduce Th17 cell differentiation, partially through antagonising IL-1R signalling. They also noted that IL-18 appeared to promote Treg cell function, helping to regulate colitis and IBD (170). Colitis-associated CRC (CAC) can arise from severe IBD and inflammation (171). Using a murine model of CAC, involving treatment with AOM and two/ three rounds of DSS, NLRP3 and caspase-1 knockout mice were found to have an increased susceptibility to colorectal tumorigenesis (172, 173). Similarly, our lab showed caspase-11 deficient mice were more susceptible to AOM/DSS-induced CAC (150, 174). These studies associated disease development with a decrease in IL-18 secretion and an increase in chronic inflammation. This indicates a protective role of the NLRP3 inflammasome in developing CAC (150, 172, 175). Our lab also showed that during established CAC in mice, IL-1 β activates caspase-11 and STAT1 in macrophages and epithelial cells to provide anti-tumour immunity (174). Other studies reported a role for NLRC4 in antitumor responses. NLRC4-deficient mice that underwent treatment using the AOM/DSS model were found to have increased tumour

load, increased epithelial proliferation and reduced apoptosis (175). These studies argue there is a protective functional role of the inflammasome in colon cancer.

In contrast to IBD and CAC, inflammasome-associated inflammation has been associated with skin cancer and melanoma development. Using an inflammation-induced skin cancer model involving 7,12-dimethylbenz(a)anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA) treatments, IL-1R- and caspase-1-deficient mice developed late-onset cancer accompanied by reduced papilloma lesion load, compared to WT controls (176). A different study using the same model reported reduced tumour incidence in NLRP3 knockout mice, when compared to WT mice (177). Taken together, these studies suggest that inflammasome components are associated in driving tumour development in this skin cancer mouse model, which suggests inflammasome involvement in human skin cancer development. Kolb *et al.* demonstrated that the NLRC4 inflammasome was induced and activated during obesity-associated breast cancer progression in a caspase-1 dependent manner. In caspase-1 knockout mice, there was significant reduction of tumour growth in obese mice only. Caspase-1 knockout mice fed on a normal diet did not appear to differ in tumour growth. The mechanism of how NLRC4 is specifically activated by obesity remains unclear, however this study highlights a causal relationship between cancer, the inflammasome and obesity (178).

Inflammasomes are also associated with gastric carcinogenesis. Elevated levels of IL-1 β and IL-18 are related to *H. pylori* infections (179-182). This bacterium often causes persistent unresolved infections which result in chronic inflammation. In response to infection, IL-1 β can suppress gastric acid secretion leading to a hypo-acidic environment in the stomach. Over time, this can lead to loss of parietal cells, gastric atrophy, metaplasia and potentially gastric carcinoma (179, 181, 183, 184). Moreover, development of Barrett's-associated OAC has been linked to chronic inflammation and IL-1 β (185, 186). Studies have reported elevated levels of IL-1 β secretion from oesophagitis, Barrett's metaplasia and OAC patient tissue (93). Another study observed elevated NLRP3 in a Barrett's metaplastic cell line (48). OAC is considered an inflammation-induced cancer (185), therefore we speculate an important role for the

inflammasome in promoting inflammation. This will be discussed in greater detail later in this introduction.

1.3 Barrett's-associated oesophageal adenocarcinoma

Despite rapid advances in the field of cancer research, oesophageal cancer prognosis remains dismal with an overall 5-year survival of 15-25% (187). It is now the sixth most common cause of cancer-related death, which is predominately (95%) caused by squamous cell carcinomas (SCC) and adenocarcinomas. Other subtypes include small cell carcinoma, sarcoma and lymphoma. Cases of OAC have increased exponentially in Western countries, while incidence of SCC is decreasing (**Figure 1.10**) (188, 189). SCC has been highly associated with alcohol and smoking. However, there has been approximately 50% reduction in smokers since the 1980s in England, which may provide reasons for the predicted decreasing trends in SCC incidence (190-192).

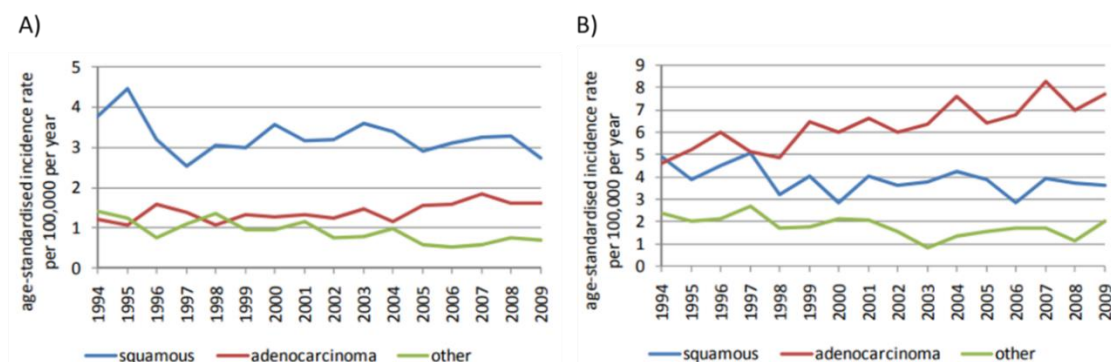


Figure 1.10: Incidence of oesophageal carcinoma in Ireland, 1994-2009.

Graphs represent incidence of oesophageal cancers from 1994 to 2009 in **A)** females and **B)** males. Blue line: squamous cell carcinoma, annual incidence rate fell by 0.9% for females and 1.4% for males. Red line: Oesophageal adenocarcinoma, annual incidence rate increased by 2.2% for females and 3% for males. The cause of this rise has been attributed to a 'Western' lifestyle. Green line: other oesophageal cancers. Graphs taken from *Cancer Trends No 8. Cancers of the oesophagus and stomach*, National Cancer Registry Ireland, 2011 (193).

OAC is associated with high body mass index (BMI), gender (male), age, gastro-oesophageal reflux disease (GORD) and Barrett's. Reasons for higher incidence in males compared to females remains unknown (X6 times higher in European population) (194). Differences may be due to sex hormones and their associated functions, such as those relating to breastfeeding (195, 196). However, a full understanding of these gender disparities remains unclear and more research is required.

At the time of presentation in the clinic, OAC disease can often be advanced resulting in poor prognosis, with over half of patients presenting with late stage (stages III and IV). This is often accompanied by metastasis, typically localised in the peri-oesophageal lymph nodes, lungs and liver (189). However recent advances in standard of care means those patients presenting with localised disease have an improved chance of survival of approximately 35-50% when treated with curative intent (197).

Prior to treatment, clinical staging of OAC is undertaken using the widely accepted TNM classification system (tumour/lymph nodes/metastasis) which was developed by the American Joint Committee on Cancer (AJCC). The AJCC 7th edition guidelines are the current staging criteria used at St. James's Hospital, Dublin (197, 198). The tumour (T) classifies primary tumour depth and extent of invasion. If the tumour has invaded as far as the surrounding mediastinal structures, the patient would likely no longer be considered for surgery (**Table 1.1**). 'M' identifies whether distant metastasis has occurred or not. 'N' identifies regional lymph node metastasis. Treatment the patient receives is based on the TMN stage (**Table 1.2**) (199, 200).

Table 1.1: T staging of depth of invasion, TMN system.

Category	Description
Tis	Carcinoma in situ/ high-grade dysplasia
T1	T1a: Lamina propria T1b: Submucosa
T2	Muscularis propria
T3	Adventitia
T4	Adjacent structures T4a: pleura, pericardium, diaphragm or adjacent peritoneum T4b: other adjacent structures eg. aorta, vertebral body, trachea

Table 1.2: Anatomical stage groupings, TMN system.

Stage	Tumour	Node	Metastasis
0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T2	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T1, T2	N1	M0
Stage IIIA	T4a	N0	M0
	T3	N1	M0
	T1, T2	N2	M0
Stage IIIB	T3	N2	M0
Stage IIIC	T4a	N1, N2	M0
	T4b	Any N	M0
	Any T	N3	M0
Stage IV	Any T	Any N	M1

Information taken from: Oesophagus 7th edition. TMN definitions according to the Union for International Cancer Control (UICC) (200).

In recent years, a series of randomised controlled trials (RCTs) for oesophageal cancer have established that treatment with neoadjuvant and adjuvant therapies is superior compared to surgical resection alone. One such important trial, the Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) trial, recruited 503 gastric, junctional and oesophageal adenocarcinoma patients to compare surgery alone to a combined treatment of 3 cycles of epirubicin, cisplatin and 5-Fluorouracil (5-FU) administered pre- and post-operatively. These drugs work by targeting DNA and the cell cycle, which inhibits protein and RNA synthesis and causes cell death (201-203). The 5-year survival was 23% for surgery alone, while the multimodality treatment was 36% ($p=0.009$) (204). This trial provided convincing evidence that combination chemotherapy could significantly improve survival compared to surgery alone.

The CROSS trial (Chemoradiotherapy for Oesophageal Cancer followed by Surgery Study) was the first convincing trial that compared surgery alone to preoperative combination therapy of both chemotherapy and radiation treatments before surgery (paclitaxel, carboplatin and 41.4 Gy/23 fractions). Similar to the drugs used in the MAGIC regimen, these carboplatin and radiation therapies target cellular DNA (205, 206). While paclitaxel is believed to cause chromosome mis-segregation and multipolar divisions (207). The median overall survival was 24 months for surgery alone compared to 49

months for the multimodal therapy. While 5-year survival was 34% for surgery alone and 47% for the multimodality arm (208, 209). This study showed strong evidence that use of chemoradiotherapy may be a suitable alternative to surgery alone for OAC treatment.

A trial currently underway in the Department of Surgery, in St. James's Hospital is the Neo-AEGIS trial (NEOadjuvant trial in Adenocarcinoma of the oEsophagus and oesophagoGastric junction International Study). This trial was set up to compare two increasingly used standards of care; the MAGIC regimen of pre- and postoperative chemotherapy, with the CROSS regimen of combined chemotherapy with radiation for treatment of adenocarcinoma of the oesophagus or junction (T2-3, N0-3, M0). It is hoped that upon completion of this trial, a superior method of treatment for OAC patients will be identified (197).

Due to continued poor survival rates relative to many other cancers, ongoing research for OAC disease prevention is also underway in St. James's hospital. As mentioned, Barrett's is a risk factor for OAC and is currently the only known neoplastic precursor. A recently published Swedish study reported that the incidence of OAC in Barrett's patients was 0.15% among 7932 participants. Furthermore, 97% of OAC cases that arose were in patients with no previous history of Barrett's (210). Another study reported that the annual risk of progression to OAC from Barrett's was 0.4% (211). This highlights that the vast majority of OAC cases are discovered outside of endoscopic surveillance programs related to Barrett's. Interestingly, the Swedish study also revealed that OAC is primarily diagnosed in the first months following initial Barrett's diagnosis, with 69% of progressors to OAC diagnosed within one year. The authors suggest that it may be beneficial to carry out more regular endoscopic check-ups during the initial months following Barrett's diagnosis (210). Despite the relatively low percentage of Barrett's patients developing OAC, Barrett's is the only known predictor of OAC and therefore remains one of the best predictors of cancer development. Taking into consideration OAC prognosis in patients without diagnosed Barrett's is extremely poor, with a 5-year survival of <15% (212), earlier detection and diagnosis is crucial for better patient prognosis.

1.4 Barrett's metaplasia (Barrett's oesophagus)

1.4.1 Aetiology of Barrett's oesophagus

Barrett's metaplasia arises in the distal oesophagus and is characterised by the replacement of normal stratified squamous epithelium with columnar epithelium with specialised intestinal metaplasia (213).

Epidemiology studies reveal that Barrett's incidence occurs predominantly in patient's aged between 55-65 years old. Men are two-three times more likely than women to develop the condition, while white males represent more than 80% of patients (214). Evaluating prevalence of Barrett's in the general population has proven difficult as incidence is thought to range considerably between populations and studies (0.9-10%). Differences in studies are often caused by controversial histological definitions and a lack of consensus for Barrett's diagnosis. Despite this, it is clear that Barrett's incidence is on the rise (215). A Swedish study published by Ronkainen *et al.* is thought to be the best designed study available. This study estimates prevalence within the general Swedish population at approximately 1.6% (216). Another well-recognised Italian study estimated the general Italian population prevalence was 1.3% for Barrett's (217). Both studies confirmed Barrett's by the presence of specialised intestinal metaplasia.

Barrett's is thought to arise as a complication of oesophagitis or 'GORD' (217). Tissue injury is induced by prolonged bile acid exposure. Such acids include cholic acid (CA), chenodeoxycholic acid (CDCA) and the most potent acid, deoxycholic acid (DCA) (218). Prolonged exposure in the distal oesophagus can erode the mucosa, promote chronic inflammation, immune cell infiltration and epithelial necrosis. The switch between cell types in the oesophagus is thought to occur as an adaptive response to prolonged adverse conditions, starting with the spontaneous presentation of cardiac mucosa at the squamocolumnar junction (SCJ) (219, 220). Reflux-damaged squamous cells are replaced by columnar cells. Frequently, the intestinal-like columnar epithelium will present later in disease with all four intestinal cell types, including absorptive cells (enterocytes) and secretory cells (enteroendocrine, Paneth and goblet cells), confirming Barrett's diagnosis (221, 222).

Bile acid exposure of Barrett's cell lines and patient biopsies was reported to increase ROS production leading to DNA damage and activation of the NF- κ B pathway (223). Bile acids have also been shown to induce cytokine-mediated tissue damage and upregulate expression of cyclooxygenase 2 (COX-2) and MUC2, which are associated with intestinal metaplasia development (213, 224-226). Bile acids bind to and are regulated by the farnesoid X receptor (FXR). This nuclear receptor controls bile acid synthesis and homeostasis. Its activation has been shown to have anti-inflammatory functions in mice, suggesting FXR could potentially be a therapeutic target during Barrett's (227, 228). As approximately 46% of Barrett's report no reflux symptoms, it is likely that other risk factors, such as diet are playing a role in inducing Barrett's development (217). One theory proposes that dietary nitrates are contributing to Barrett's development. Nitrates are present in our food due to its frequent use in packaging and fertilisers. When nitrates contact acid in the stomach they are predisposed to forming nitrites and nitric oxide which are carcinogenic and may cause tissue damage (229). Another study reported that epidermal growth factor receptor (EGFR) was induced in oesophageal cells *in vitro* when stimulated with nitrate (230), which may have disease promoting effects.

Other risk factors include age, race, sex and the presence of hiatal hernia. Barrett's is more likely to occur in older, Caucasian males (231). Andrici *et al.* observed that up to 90% of Barrett's patients have an associated hiatal hernia, which can contribute to gastric reflux through weakening of the lower oesophageal sphincter muscle (232, 233). Other lifestyle factors have been identified, such as smoking and alcohol for developing both Barrett's and OAC (216, 234). Additionally, the bacterial infection *H. Pylori* has been identified as a major contributor to gastric carcinogenesis (235). However, its role in Barrett's development is not as well established. One study found that it may play a protective role against development of intestinal metaplasia due to reduced gastric acidity (236). Obesity is also known as a major risk factor for Barrett's and OAC development (237). This will be discussed further later in this introduction.

1.4.2 Barrett's diagnosis

Diagnosis of Barrett's is undertaken using endoscopic surveillance of the distal oesophagus and requires proximal displacement of the SCJ (238). This is visually identified by the presence of columnar mucosa extending above the gastroesophageal junction (GOJ). Columnar mucosa is a dark salmon colour in contrast to the paler squamous epithelium (**Figure 1.11**). Diagnosis at St. James's Hospital Dublin is histologically confirmed using oesophageal biopsy specimens to confirm columnar metaplasia with specialised intestinal metaplasia (goblet cells) (222, 239). Historically, the extent of columnar metaplasia migration up the distal oesophagus was used as a basis for a Barrett's classification system, thereby separating Barrett's into two groups based on segment length; long-segment or short segment (≥ 3 cm or < 3 cm, respectively) (240). A study has since reported that long segment Barrett's was linked to a higher risk of progression to neoplasia (241). The use of the Prague classification system has since been incorporated into the standardised nomenclature of Barrett's. Also called the circumferential and maximum (C and M) classification, this system is thought to be more accurate than calculating segment length, due to it being easier to learn and more accurately reproduced by endoscopists (242). Additionally, the Vienna classification is used for histological diagnosis. This assigns a score to patients based on pathological assessment of biopsy tissue. A number from 1-4 is assigned for the following: (1) no dysplasia, (2) indefinite for dysplasia, (3) LGD, (4) HGD and invasive epithelial neoplasia (243, 244).



Figure 1.11: Endoscopic image of Barrett's oesophagus.

Darker, salmon pink colour represents columnar-lined mucosa in the distal oesophagus found proximal to the gastroesophageal junction. Further up the oesophagus, tissue colour visibly changes to a paler, glossier pink which represents the normal stratified squamous epithelium. Image taken from Amadi & Gatenby, *World Journal of Gastroenterology*, 2017 (245).

The definition of what constitutes as a Barrett's diagnosis remains controversial and varies between countries. In the USA, periodic acid-Schiff (PAS) positive staining for the presence of goblet cells (intestinal metaplasia) is the required histological criteria for Barrett's diagnosis, as laid out by the U.S. gastroenterology societies (246, 247). Other societies, such as the British Society of Gastroenterology, additionally consider mucous-secreting columnar epithelium without goblet cells (cardiac mucosa) as a Barrett's diagnosis (248). Like the US, in Ireland the presence of specialised intestinal metaplasia is required (249). The premise underlying this disagreement is whether the presence of goblet cells acts as an indicator of increased risk of progression to OAC. Initial studies showed that Barrett's accompanied by the presence of goblet cells contained DNA abnormalities, however, later studies showed that columnar-lined epithelium without goblets cells showed similar or significantly more DNA abnormality content (250, 251). This suggested that goblet and non-goblet cell phenotypes had equal potential for

neoplastic progression. A more recent study has since observed that high goblet cell count is inversely related to ploidy abnormalities and risk of disease progression to OAC. The authors of the study speculated that increased numbers of goblet cells represented a successful adaptive response against the adverse effects of bile and acid reflux by producing thick mucus barriers along the distal oesophagus (252).

1.4.3 Dysplasia and Barrett's oesophagus

Barrett's oesophagus is a condition that is part of a larger sequence of events that predisposes to OAC (**Figure 1.12**) (185). With time and disease progression, Barrett's may begin to develop features of dysplasia, first low-grade dysplasia (LGD) then high-grade dysplasia (HGD). While metaplasia can be defined as a phenotypic switch between cell types as a result of tissue injury. Dysplasia, in contrast, is defined by abnormal epithelial cell development, growth or differentiation (253). Dysplasia is not malignant, however is it considered a pre-malignant condition with an increased risk of developing cancer. Histologically, LGD maintains a relative preservation of glandular structure with some cellular abnormalities. LGD is confirmed by the presence of closely packed overlapping basal nuclei with elongation and hyperchromasia, basal stratification of nuclei, reduced mucous and goblet cell count (**Figure 1.13**) (253). Despite general guidelines for pathological confirmation of LGD, diagnosis is difficult as conditions are highly variable between patients and tissue segments (253). A Danish study found that patients with a baseline diagnosis of LGD had a relative 4.8% increased risk of developing OAC compared to Barrett's patients without dysplasia (254).

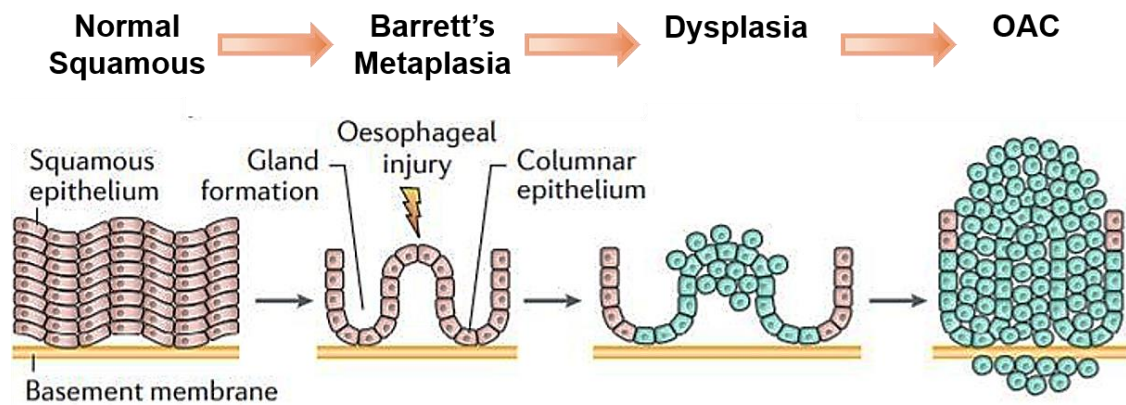


Figure 1.12: Sequence of pathogenesis.

Barrett's metaplasia arises as a consequence of oesophageal injury in the distal oesophagus. It is defined by a phenotypic switch of cell types, from normal squamous epithelium to columnar epithelium with intestinal metaplasia (goblet cells). Disease progression results in the subsequent development of pre-neoplastic low- and high-grade dysplasia, which are characterised by less defined crypt structures. In some patients, dysplasia may progress to oesophageal adenocarcinoma where regulation of cellular proliferation and crypt structure is lost. Figure taken and adapted from Peters *et al.*, *Nature Reviews Disease Primers*, 2019 (255).

Following incidence of LGD, a patient's risk of developing HGD increases (253). Patients who develop HGD have a 30-40% chance of developing OAC within five years (256). The degree of dysplasia is determined by histology by identifying cytology (nuclear and cytoplasmic features such as nuclear alignment), tissue architecture, structure and degree of surface maturation (comparing nucleus size within crypts to size at the mucosa surface) and lastly by examining the degree of overall inflammation in the surrounding tissue. Histological HGD dysplasia will present signs of mild to significant tissue architectural distortion, enlarged nuclei, loss of nuclear polarity or nuclear misalignment, crowded glands and loss of lamina propria (**Figure 1.13**) (243).

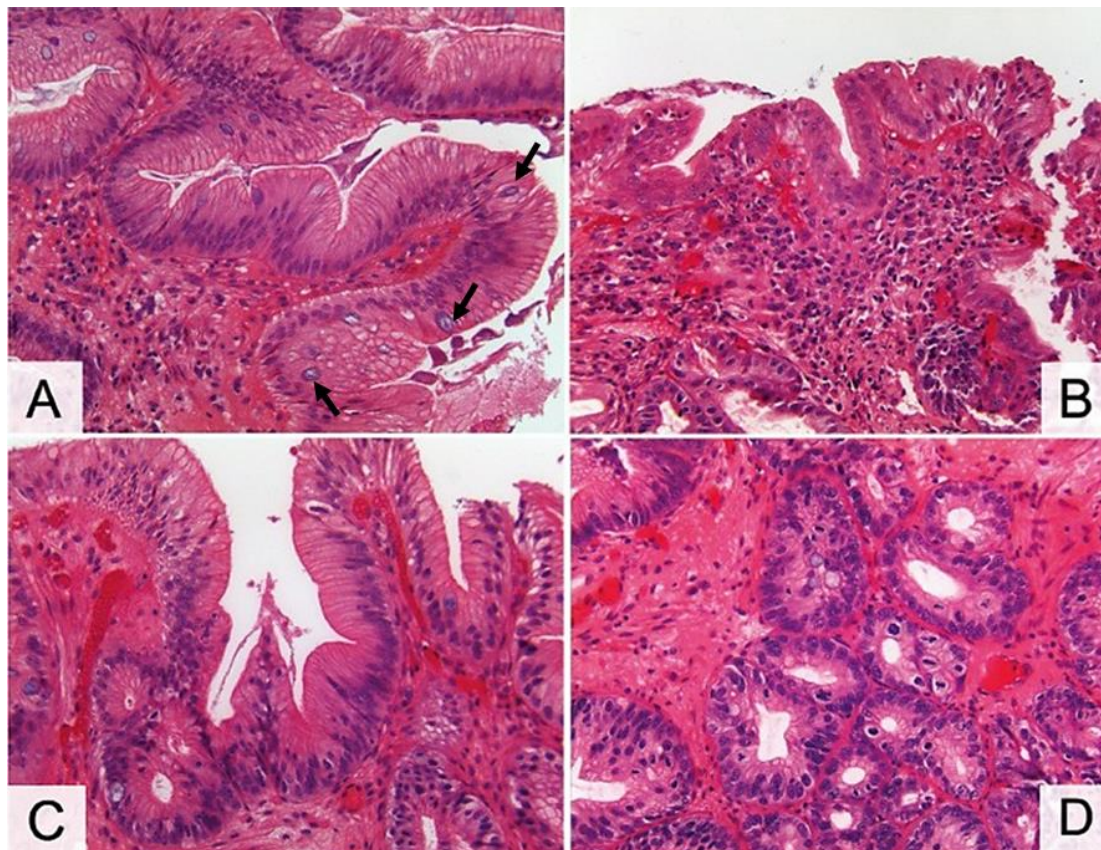


Figure 1.13: Histological features of metaplasia and dysplasia using haematoxylin and eosin (H&E) staining.

Metaplastic columnar epithelium with mucin-filled goblet cells are (tinted blue, arrows). No dysplasia is present. Tissue structure is preserved with regular lamina propria and basally aligned nuclei in the columnar epithelium. **B)** Indefinite for dysplasia. A term used to describe a biopsy that cannot be definitively confirmed as dysplastic or neoplastic. Image shows increased inflammation, nuclei enlargement and mild crowding of glands. Tissue structure is mostly normal. **C)** Low-grade dysplasia. Nuclear atypia present with hyperchromasia, nuclear overlap, increased pleomorphism and increased gland crowding. **D)** High-grade dysplasia. Distinct cytosolic changes with nuclear enlargement, pleomorphism, hyperchromasia and overlap. Glands are crowding and lamina propria are almost absent. Image taken from Booth and Thompson, *Journal of Gastrointestinal Oncology*, 2012 (243).

1.4.4 Barrett's metaplasia, cell of origin

The cell of origin for Barrett's is still under considerable debate and has proven difficult to resolve. This is due to the heterogeneous nature of Barrett's metaplasia. It arises proximal to the GOJ and is comprised of gastric- and intestinal-like columnar epithelium with mucus-producing goblet cells (257). Several theories have been proposed (**Figure 1.14**). The first suggests that squamous cells directly transdifferentiate into columnar cells. The premise for this theory is based on embryonic development studies in mice where squamous cells arise by direct conversion from columnar cells found in the basal compartment (258). However, it seems unlikely that transdifferentiation would result in the various gastric- and intestinal-like cell types seen in Barrett's (213, 259). Additionally, squamous epithelium replaces Barrett's columnar epithelium following endoscopic ablation, which argues against the theory of transdifferentiation (260).

Another theory for the cell of origin is transcommitment of pluripotent stem cells responsible for replenishing the squamous epithelium into intestinal-like cells. This is a popular theory and there are several suggestions as to where immature progenitor cells originate from, including the basal layer of the squamous epithelium, the immature cells that line the ducts of sub-mucosal glands or the gastric cardia (213).

Historically, Hamilton and Yardley proposed that Barrett's originates from upward migration of cells from the gastric cardia (a transitional region of unique cells) at the GOJ caused by gastric refluxate (261). This theory was largely discounted for many years due to the publication of several animal studies that argued the origin was intrinsic to the oesophagus (262). However, more recent studies have been published to support this original idea. Quante *et al.* have shown using the Barrett's mouse model (pL2-IL-1 β) and lineage studies that progenitor cells from the gastric cardia migrate up the oesophagus (263). Furthermore, Wang *et al.* showed that embryonic-type cells at the GOJ migrated up the oesophagus to repair damaged squamous epithelium (264). In contrast, another study which used a rat model of gastric reflux showed that Barrett's developed from circulating stem cells that were originally derived from the bone marrow. They located to the site of tissue injury in the oesophagus and differentiated into columnar cells (265). Clearly all of these studies offer quite different theories of cellular origin, however its

seems likely that Barrett's does arise through transcommitment of immature progenitor cells, as they hold the capacity to differentiate into multiple cell types (213).

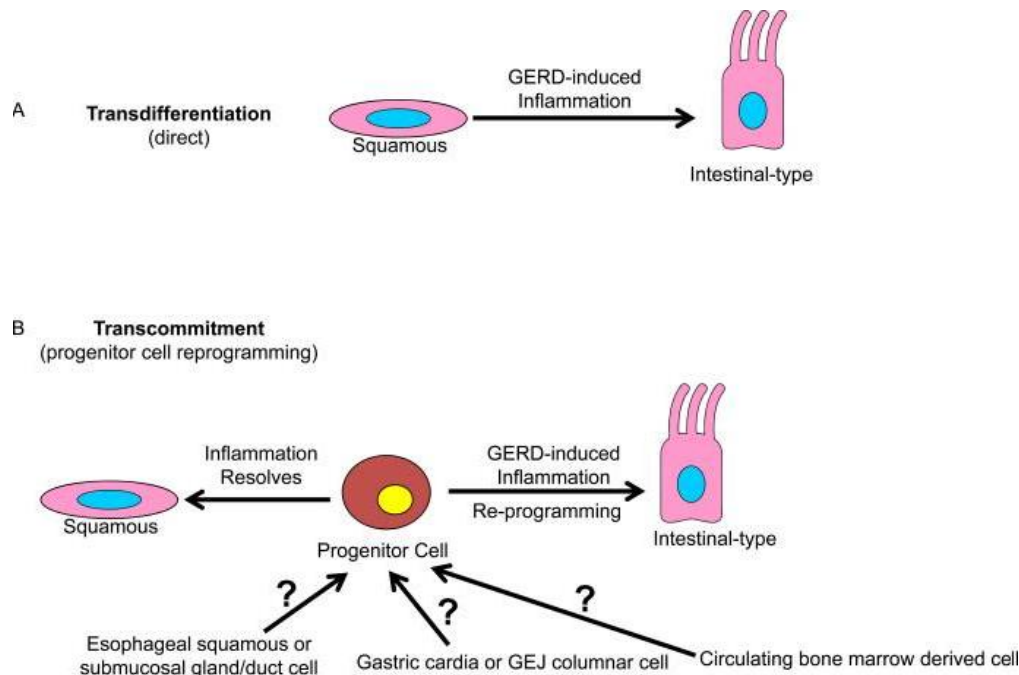


Figure 1.14: Proposed theories for the origin of Barrett's metaplasia.

A) Transdifferentiation: an adult (fully differentiated) squamous cell changes directly into an intestinal-like columnar cell when induced by chronic gastric reflux. **B)** Transcommitment: immature progenitor cells are reprogrammed during GORD-induced inflammation to differentiate into intestinal-like metaplasia. Progenitor cells may arise from the basal squamous epithelium or the submucosal gland ducts, the gastric cardia, the GOJ or bone marrow derived cells. Image taken from R.F. Souza, *Digestive Diseases*, 2016 (213).

1.4.5 Management of Barrett's oesophagus

Diagnosed Barrett's oesophagus patients will undergo regular endoscopic surveillance for OAC to facilitate early detection of the tumour. These are carried out typically every one to five years depending on the patient's status and the centre (256, 266-269).

During Barrett's metaplasia, gastric acid reflux can cause chronic inflammation, DNA abnormality and increased proliferation (270). To treat these symptoms, Barrett's patients are administered proton-pump inhibitors (PPIs) such as esomeprazole and

lansoprazole to target H⁺K⁺ ATPase. PPIs are reasonably effective at reducing reflux oesophagitis, however poorly reduce GORD symptoms such as heart burn (271). The long-term benefits of PPI use are generally disagreed upon despite being regularly prescribed to patients. Singh *et al.* observed a protective effect of PPI use and decreased risk of OAC progression (272). While a meta-analysis performed by Hu *et al.* reported that PPI use does not reduce cancer progression in Barrett's patients (273). Controversially, a recent large-scale Swedish study has reported that the long-term use of PPIs consistently led to an increased risk of oesophageal cancer. They remarked that long-term PPI use should be prescribed with caution (274).

Interestingly, another recent study involving a large cohort of participants (121,700 female registered nurses), showed that regular aspirin use was associated with a 15% lower risk of developing Barrett's (275). This study suggests that aspirin could be used as a relatively safe and cheap alternative therapy against development of Barrett's. Recently, the results of the anticipated AspECT trial have been published (276). The objective of this study was to determine the benefits of PPI and aspirin use in a large, well-controlled randomised trial which followed 2557 Barrett's patients. Results showed that high-dose PPI (esomeprazole) treatment was superior to low-dose, and was protective against all-cause mortality, OAC and HGD. High dose PPI use in combination with aspirin had the most significant benefit. The study suggests that combination use for a minimum of 9 years improves survival and provides sufficient evidence to recommend combination treatment to patients (276). Notably, long term use of aspirin has been associated with gastric ulcer complications (277). However, PPI treatment in combination with regular aspirin or nonsteroidal anti-inflammatory drug (NSAID) use has been shown to reduce risk of ulcer bleeding (278-280).

For treatment of HGD and sometimes LGD, endoscopic mucosal resection (EMR) and ablative therapy is often offered to the patient as a more aggressive form of treatment (281). EMR involves excision of mucosa or submucosa tissue (2-3 cm), often down to the interface with the muscularis propria. Tissue is removed intact and can be assessed for histology (282). Radiofrequency ablation (RFA) involves a balloon catheter with a bipolar electrode array. This electrode delivers thermal energy *via* radio waves directly onto oesophageal tissue, eradicating all the Barrett's mucosa (233). In the Department

of Surgery in St. James's hospital, patients with HGD or intramucosal cancer undergo EMR $\pm$ Halo[®] ablation (RFA) followed by frequent endoscopic check-ups (283, 284). The Halo[®] system is a technique that minimises tissue invasion by using ultra-short pulse radiofrequency, that preserves the submucosa (285). The Department has found that EMR $\pm$ RFA offers an acceptable alternative to traditional resection surgery following publication of a recent trial (283). This study reported patient status (n=96) after a median follow up of 13 months. Results showed there was 100% patient disease control and 72% had no endoscopic or histological evidence of Barrett's (283). This research proves RFA to be a successful new treatment.

1.4.6 Mouse models of Barrett's and OAC

Mouse models play an important role during the development of successful new treatments. To date, there are relatively few mouse models of Barrett's and OAC available, none of which are well established. Examples of available models include xenografts (patient derived, subcutaneous and orthotopic), chemical- or diet-induced models or genetically engineered mouse models (GEMMs) (286, 287).

One such GEMM model is the p63-deficient neonatal mouse (264). P63 functions as an important regulator of proliferation in stratified stem cells and p63 deficient mice lose the ability to form stratified epithelium (288, 289). The model is characterised by 'well developed columnar epithelium' that migrates proximal of the SCJ and Alcian blue-PAS positive staining. This model provides insights into the development and cell of origin of Barrett's however is severely limited by an inability of the mice to survive into adulthood due to the lethal p63^{-/-} phenotype (264).

Another animal model for Barrett's can be generated by a technically challenging oesophagojejunostomy in rats, which causes gastric duodenal reflux. This inflammatory assault on oesophageal tissue induces the development of oesophagitis and columnar epithelium lining the distal oesophagus. Subsequently, almost half of treated rats will develop OAC 16 weeks post-operatively (290).

Xenograft models have also been studied extensively and can provide valuable insights into OAC and Barrett's pathology, however they have many limitations. For example, the patient derived xenograft (PDX) model, which involves implanting a biopsy directly into a mouse following surgery, requires an immunodeficient host (291), making this model impractical for studying the tumour microenvironment and immune response. Similarly, transplantation of cultured oesophageal cells, either subcutaneously or orthotopically into the oesophageal wall have been used with limited success. The subcutaneous model requires a large injection of cells into an immunodeficient host and fails to mirror the same tumour development heterogeneity observed in OAC (292, 293). Conversely, an orthotopic procedure involving the implantation of cultured cells or tumour tissue directly into the oesophagus can be technically challenging and time consuming due to the small size of the mouse oesophagus (293). Despite this, one successful study using OAC OE-19 and PT1590 cells implanted into NMR/nu mice showed a highly aggressive metastatic spread to liver, lungs and lymph. This model could prove useful for evaluating therapeutic effects and responses in advanced OAC (294, 295).

1.4.7 pL2-IL-1 β Barrett's mouse model

Quante *et al.* developed the pL2-IL-1 β mouse model for Barrett's oesophagus (263). This model is currently the only established transgenic mouse model available for Barrett's and is the most convincing model yet created. The histopathology and genetic signatures of this model closely resemble those observed in human Barrett's tissue, including upregulation of gene signatures such as TFF2, Bmp2, Cdx2, Notch1 and IL-6 (263).

This is a model of chronic inflammation and its use is uniquely beneficial in its capacity to study Barrett's development and progression as the mice age. The model involves the insertion of modified human IL-1 β cDNA downstream of the Epstein-Barr virus ED-L2 promoter which directly targets all stratified squamous epithelium, particularly of the oral cavity, oesophagus and squamous forestomach mucosa of mice (see Section 2.7.1 for further genetic information). Mice constitutively express mature hIL-1 β in the

oesophagus and forestomach resulting in chronic inflammation, mimicking symptoms of oesophagitis observed in Barrett's development (181, 263, 296). Histologically, at three months old these mice show initial inflammation in the oesophagus. At six months, the mice show moderate inflammation and profound columnar metaplasia originating at the SCJ. When the mice reach 12 months, a Barrett's phenotype is well-established, characterised by severe columnar metaplasia and inflammation and the presence of goblet-like cells (Muc5ac⁺, PAS⁺). At 20 months of age, the mice develop features of high-grade dysplasia or intramucosal OAC (263).

Studies using this model observed that treatment with bile acid alone in WT mice was not enough to induce a Barrett's phenotype, however the addition of bile acids alongside the pL2-IL-1 β phenotype accelerated disease progression (186, 263). This suggests that bile acids may promote disease progression by mediating inflammation, rather than acting directly on epithelial cells.

1.4.8 Inflammation in disease progression to Barrett's and OAC

OAC is considered an inflammation-derived cancer, therefore studying the inflammatory mechanisms during disease development may prove insightful for future therapies. During the sequence of pathogenesis, normal-GORD-Barrett's-OAC, differential expression of inflammatory cytokines and immune cell populations occurs (185, 270).

A study by Fitzgerald *et al.* revealed an inflammatory gradient exists throughout the Barrett's segment, where inflammation was maximal at the new SCJ in patients. There was a 2-fold and 3.8-fold increase in pro-inflammatory cytokines IL-8 and IL-1 β expression in the proximal segment respectively, in comparison to the distal segment. In contrast, Barrett's biopsies that were located distally expressed higher anti-inflammatory IL-10 (93). Indeed, a study published by Nadatani *et al.* observed upregulation and activation of the NLRP3 inflammasome pathway and IL-1 β release in LPS-stimulated Barrett's cell lines (48). Similarly, Souza *et al.* showed that exposure of bile acids to squamous oesophageal cells induced significant upregulation of IL-8 and IL-

1 β *in vitro* (297). While Fitzgerald *et al.* demonstrated that pulsatile bile acid exposure induces IL-1 β in Barrett's metaplasia (93). These findings illustrate that normal and Barrett's cells respond to stimulus in an inflammasome-associated manner which may be driving disease.

In support of this, in the Barrett's mouse model IL-1 β is overexpressed in the oral cavity, oesophagus and forestomach of mice. These mice have chronic oesophagitis leading to spontaneous development of Barrett's-like features, eventually causing severe HGD and intramucosal cancer. This chronic inflammation is associated with IL-6 signalling, as IL-6 deficiency in these mice was shown to strongly inhibit the IL-1 β -induced phenotype (263). The pro-inflammatory relationship between IL-1 β and IL-6 signalling is well established, and has been linked with carcinogenesis (93, 95, 298). IL-6 was elevated during Barrett's and further elevated in OAC tissue conditioned media (95). Zhang *et al.* showed that IL-6-dependent STAT3 activation promotes inflammation and apoptosis resistance in Barrett's cell lines, therefore contributing to cellular transformation (298, 299). A role for IL-6/STAT3 signalling during Barrett's has been similarly proposed by Dvorak *et al.* (299, 300). Moreover, IL-6 has been proposed as a more superior diagnostic biomarker of oesophageal cancer than current classic tumour biomarkers (301).

Other inflammatory factors that are upregulated by bile acids include COX-2, nitric oxide (NO), IL-17 and intracellular ROS (239, 302). Apart from the ability of ROS to cause DNA double stranded breaks, ROS can activate the NLRP3 inflammasome and therefore may be mediating IL-1 β -associated inflammation (105, 303).

Following gastric reflux, T cells infiltrate the squamous mucosa and cause chronic mucosal inflammation characterised by a Th1 cell response (185, 304). It is thought that high levels of pro-inflammatory cytokines such as IL-6, IFN- γ , IL-8 and IL-1 β in oesophagitis drive progression to Barrett's (305, 306). Additionally, in a small subset of patients, high eosinophil counts have been observed (307). Once Barrett's is established, a humoral and Th2 cell type response predominately exists (95, 306, 308). This is associated with increased expression of IL-6, IL-1 β , TNF α and NF- κ B (95, 185). Significantly increased numbers of macrophages, mast cells and plasma cells are present which promote inflammation (308). Additionally, large populations of immature

myeloid cells and neutrophils have been reported in oesophageal tissue taken from Barrett's pL2-IL-1 β mice. IL-8 secretion (and CXCL1 in mice) has been implicated in neutrophil recruitment, combined with reductions in NK cell populations which may advance disease (32). Elevated levels of dendritic cells have also been reported during Barrett's high-grade dysplasia, which increase further during OAC (309, 310).

There are also increased levels of IL-10- and IL-4-producing T cells in Barrett's tissue (185). IL-4-producing Th2 cells can induce MUC2 expression which promotes differentiation of intestinal epithelial cells into goblet cells in mice (311). This suggests IL-4-induced MUC2 expression may promote intestinal metaplasia in Barrett's (312).

Additionally, circulating mucosal-associated invariant T (MAIT) cells were shown to decrease in the blood, while increased infiltration was observed in oesophageal tissue of Barrett's and OAC patients, when compared to healthy controls. However, this increase was accompanied by diminished effector functions including reduced IFN- γ and TNF α secretion, which is suggestive of impaired anti-tumour immunity (313). This is supported by Kavanagh *et al.* who showed increased IFN- γ -producing T cells in OAC tissue, while less activated CD4⁺ T cells overall were observed compared to normal, oesophagitis and Barrett's tissue (95). Furthermore, an increase in Treg cells has been reported in oesophageal cancers, which has been correlated with poorer prognosis (314). See **Figure 1.15**.

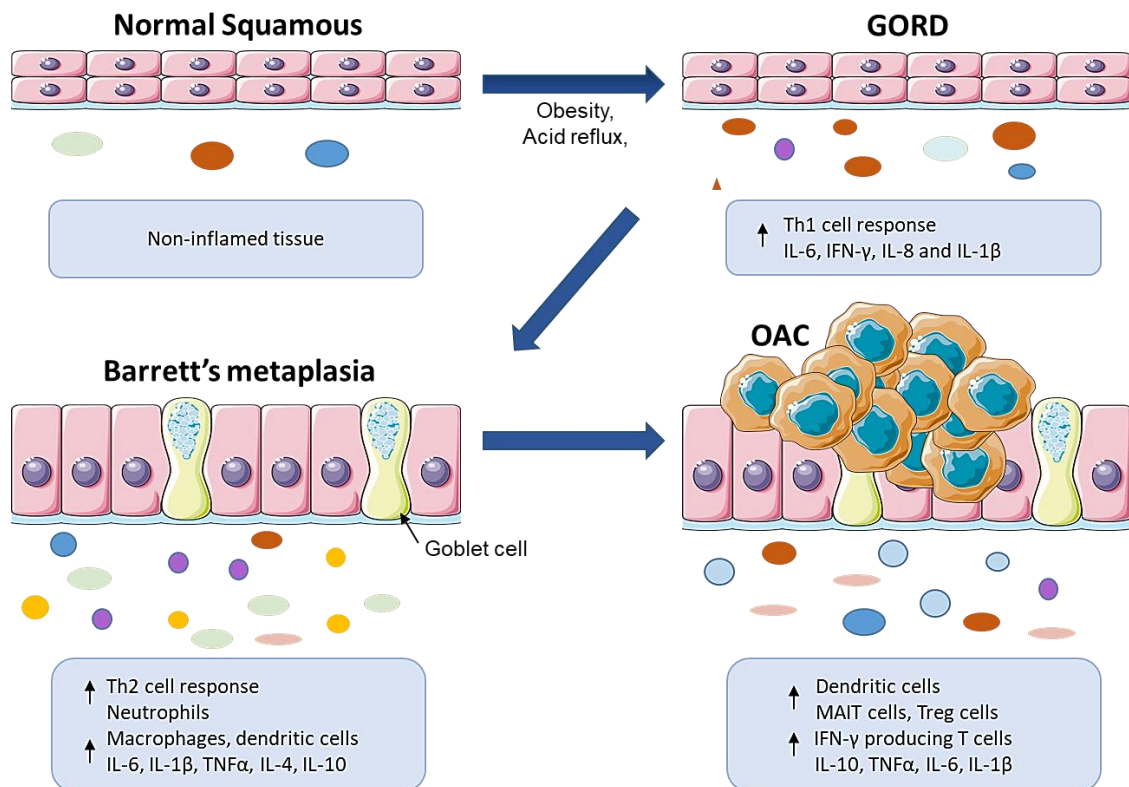


Figure 1.15: Inflammation during disease progression to OAC.

During normal conditions immune homeostasis occurs. Risk factors such as obesity and acid reflux cause chronic inflammation leading to GORD, characterised by a Th1 pro-inflammatory response. Spontaneous development of Barrett's metaplasia occurs, and a humoral response becomes dominant, however tissue is still highly pro-inflammatory with elevated levels of neutrophils. Disease progression leads to tumour development which has increased levels of Th1 and Treg cells. Circles: Red, Th1 cells; purple, macrophages; light blue, Treg cells; green, Th2 cells; yellow, neutrophils; pink, dendritic cells.

TLRs are important in mediating inflammation in disease, predominantly through transcription factors AP-1, NF- κ B and IRF signalling (315). In disease progression to OAC, TLR1, -2, -4 and -6 expression in oesophageal patient tissue was found to increase stepwise from normal squamous epithelium towards HGD (316). In OAC tumour tissue expression varied between patients. TLR4 expression correlated with distant metastasis and poor prognosis (316), indicating a role in promoting cancer. To support this, Kohtz *et al.* confirmed that TLR4 expression increased in OAC cells when compared to normal cells. TLR4 activation in oesophageal cells resulted in increased proliferation that was

dependent on the MyD88-TRAF6-NF- κ B signalling pathway (317). Other research determined that TLR2 mRNA and protein expression in oesophageal biopsies increased stepwise from normal to oesophagitis to Barrett's to OAC tissue (318). Additionally, TLR2 activation in a Barrett's cell line resulted in increased TLR2 expression and IL-8 secretion. This observation may be due to a reported switch in the microbiome phenotype from a more gram positive phenotype in normal tissue to a more gram positive colonisation during reflux and Barrett's (319, 320). Further studies by Huhta *et al.* showed that TLR2 expression was microbiome dependent by comparing expression levels in germ free and normal mice (31).

1.5 Obesity

Obesity in Ireland is a major public health concern. Incidence has been exponentially rising and by the year 2030 Ireland is predicted to be the most obese country in Europe. According to the World Health Organisation (WHO), more than 60% of Irish adults are currently overweight and more than 25% are obese (321).

The WHO measures obesity using the BMI, where a BMI score of greater than or equal to 30 kg/m² is considered obese (322, 323). However, studies have shown that this method of measurement is sub-optimal for use in clinic because it does not take into consideration composition of fat and its distribution around the body (324). Other measurements are also now used as a means of measuring obesity. Waist circumference (WC) predominantly measures abdominal (central) obesity. Cut-offs as determined by the International Diabetes Federation for European patients are >80 cm for females and >94 cm for males (325). Additionally, computed tomography has been established as the gold standard for abdominal fat quantification in clinic, whereby visceral fat area (VFA) and subcutaneous fat area (SFA) can be determined following an abdominal CT scan. VFA cut-off values for gastrointestinal cancers have been determined as 163.8 cm² in males and 80.1 cm² in females (326).

1.5.1 Obesity, inflammation and cancer

Many studies have been carried out implicating obesity-associated inflammation with carcinogenesis. Stattin *et al.* observed that colon cancer incidence correlated with increased leptin and IL-6 expression (327). Similarly, plasma leptin levels were associated with breast cancer risk in patients (328). Additionally, Kim *et al.* showed that increased circulating plasma IL-6, TNF α and C-reactive protein (CRP) that were associated with obesity, may be involved in early development of colorectal neoplasia in patients (329).

Studies are arising linking obesity-mediated inflammasome activation with carcinogenesis. One study implicates NLRC4 inflammasome activation and associated IL-1 β signalling in driving breast cancer development. Activation of IL-1 β was reported to mediate disease progression through adipocyte-mediated vascular endothelial growth factor A (VEGFA) in mice (178). Another study found that the NLRP3 inflammasome was highly activated in non-alcoholic steatohepatitis (NASH) in mice, contributing to associated liver fibrosis, a pre-cancerous liver condition highly associated with obesity (330, 331). AIM2 has also been implicated in liver disease. Using AIM2 deficient mice, Gong *et al.* showed that AIM2 is an important regulator of hepatic fat. The study identifies AIM2 as a potential therapeutic target for non-alcoholic fatty liver disease (NAFLD), and could subsequently prevent cancer (332).

Additionally, IL-1 β is known to be a key mediator of obesity-associated inflammation and subsequent pancreatic β -cell dysfunction and insulin resistance. Yun-Hee Youm *et al.* showed that the NLRP3 inflammasome mediated this IL-1 β inflammation and therefore contributed to pancreatic islet fibrosis in chronically obese mice. Pancreatic fibrosis is the scarring of tissue caused by tissue injury often associated with chronic inflammation and is known to increase risk of pancreatic cancer. Therefore, this study suggests that the NLRP3 inflammasome may be contributing to obesity-promoting inflammation that in turn may be driving development of pancreatic cancer (333). Collectively, these studies suggest that inflammasome signalling may be contributing to neoplasia in pre-neoplastic inflammatory conditions and subsequently driving progression to obesity-associated cancers.

1.5.2 Obesity-associated Barrett's and OAC.

Obesity is thought to be one of the leading risk factors attributed to the development of Barrett's (231). However, studies linking the relationship between BMI and Barrett's have been somewhat inconsistent. Some studies have shown there is no association between BMI and Barrett's (334-336), others have shown an association only in women (337, 338), while others found there was a positive association (339-342). A meta-analysis study by Cook *et al.* reported that there was no association of BMI with Barrett's (343), but there was a twofold risk increase associated with WC (344). Other studies have supported the finding that WC, but not BMI, is an independent risk factor for Barrett's (345). In contrast, BMI has been linked with OAC development by many large studies. A meta-analysis of 22 of these studies observed that the relative risk overall was three-fold higher for obese participants compared to those with a normal BMI score (346). Moreover, WC was also shown to be associated with increased risk in cancer development, similar to Barrett's (347, 348).

Taken together, these studies outline that obesity, in particular central obesity is associated with Barrett's and OAC development. Indeed, Barrett's and OAC incidence is prevalent in white males, a demographic cohort that is largely susceptible to central obesity compared to their female counterparts (349).

1.5.3 Barrett's, obesity and inflammation

Taking the previously mentioned studies into consideration, there is little doubt that abdominal obesity is a risk factor for Barrett's and OAC development (324). Use of WC is limited by its unspecific measurement of both subcutaneous and visceral fat located around the abdomen (350). One study compared visceral adipose tissue (VAT) with subcutaneous adipose tissue (SAT) using CT scans from Barrett's patients and found that visceral obesity rather than total central obesity, more specifically contributes to Barrett's risk (236). This was further supported by other smaller studies (351).

It is important to differentiate fat depots when discussing inflammatory and metabolic processes, due to the significant functional differences between subcutaneous and

visceral fat (352). Subcutaneous adipose tissue (SAT) is found beneath the skin and may contribute to mechanical pressure on the stomach and oesophagus, although this is still controversial (353). Interestingly, studies on peripheral subcutaneous fat have reported protective effects on metabolic and energy homeostasis (354). Conversely, visceral adipose tissue (VAT) is located primarily around internal organs in the abdomen and is known to be very metabolically active and is associated with development of metabolic syndrome diseases such as cancer, insulin resistance, type 2 diabetes, atherosclerosis, hypertension and liver disorders (355, 356).

Adipose tissue was once regarded as an inert organ of energy storage, however in recent years its role as an important endocrine organ has become apparent (357). Adipose tissue is now known to secrete significant amounts of adipokines and adipocytokines which act as important mediators of metabolism, such factors include leptin, adiponectin, resistin and omentin (324). Obesity is associated with a systemic state of chronic, sterile low-grade inflammation, also known as 'meta-inflammation', characterised by abnormal cytokine production, immune cell activation and signalling (358). In circulation during obesity, increased levels of inflammatory cytokines are reported, including elevated leptin, IL-6, IFN- γ , CRP, CCL1 and CCL5. This coincides with changes in circulating immune cell populations such as decreased CD8⁺ T cells, Treg cells and V δ 2 T cells, and increased numbers of M1 macrophages, CD4 T cells, B cells and NK cells (359). In addition, studies have shown that obesity can impair function of certain circulating dendritic cells, NK cells and T cells, limiting anti-tumour responses (360-364). These changes demonstrate the systemic effect obesity has on the entire body and are indicative of homeostatic disruption (**Figure 1.16**).

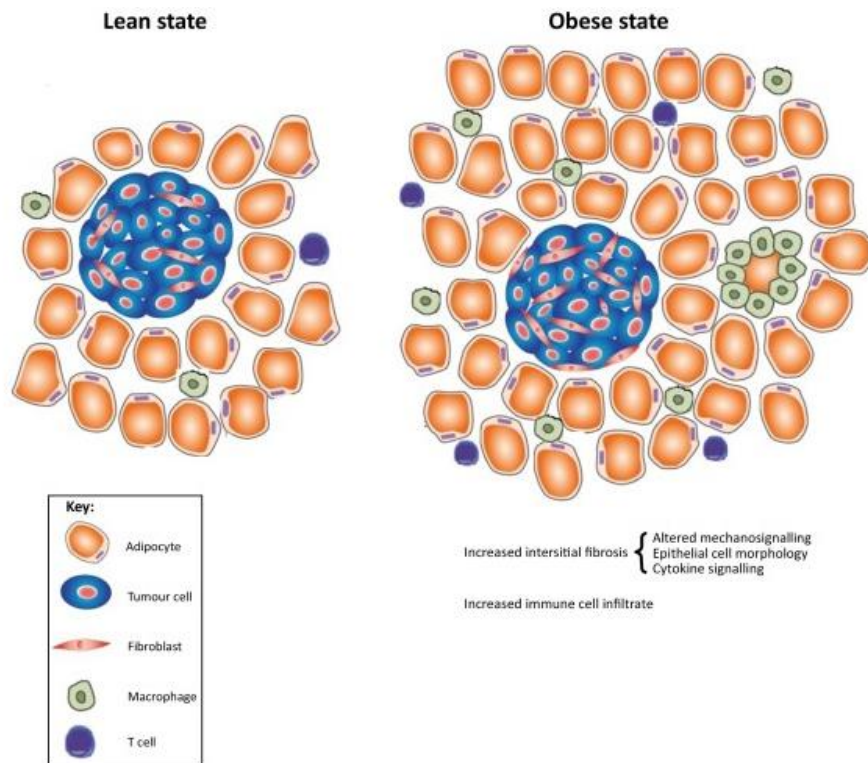


Figure 1.16: Immune cell infiltrate in the tumour microenvironment of obese patients.

In obese conditions, the peritumoral fat consists of a unique immune cell infiltrate repertoire. Adipose tissue macrophages, with an M1 phenotype, increase in number mediated through monocyte chemoattractant protein-1 (MCP-1) and TNF α signalling. This is associated with higher secretion levels of IL-6 and TNF α . Additionally, T lymphocytes and NK cells populations migrate and expand. Obesity can also assist interstitial fibrosis by inducing alterations in the extracellular matrix (ECM), which effects tissue structure, cellular morphology and promotes tumour progression. Image taken from C.L. Donohoe *et al.*, *Trends in Endocrinology and Metabolism*, 2017 (365).

Chronic inflammation is pronounced in obese visceral tissue, with increased expression levels of IL-17, IL-22, TNF α , IL-6 and MCP-1 (129, 366). It is probable that this chronic inflammation is a key link between obesity and cancer (367). When visceral fat area was measured in reflux oesophagitis patients, highly significant correlations between abdominal visceral fat and cytokine levels of IL-1 β , IL-6 and IL-8 were observed. The study suggested higher levels of visceral fat increased susceptibility of developing reflux oesophagitis (305). Similarly, VAT taken from obese OAC patients has been shown to secrete significant amounts of inflammatory mediators such VEGF and IL-6, creating

low-grade systemic inflammation in the body (368). Additionally, TNF α expression levels in subcutaneous tissue were directly proportional with adipose tissue volume and correlated with BMI (369). Although it is expressed in relatively low levels in normal squamous epithelium, it has been shown to increase in expression as disease progresses to carcinoma (370). Interestingly, pL2-IL-1 β Barrett's mice progress to Barrett's significantly faster when fed a high fat diet (HFD) compared to normal chow (364). This study proposed that the IL-8 homolog CXCL1 was elevated at the SCJ in HFD-fed mice. This resulted in altered neutrophil/ NK cell ratios that they suggested was mediated by TLR2 dependent microbiome signalling (364). The Barrett's phenotype in this mouse model is induced by IL-1 β -mediated chronic inflammation, therefore these findings may indicate that a fatty diet exacerbates inflammation in the Barrett's model, thus accelerating disease progression (263, 364).

1.6 Project hypothesis and aims

Overall hypothesis

Inflammasome-mediated inflammation is involved in disease progression to Barrett's and OAC, and is enhanced by obesity.

Specifically, the main aims of this project are:

1. To determine inflammatory caspase expression levels during Barrett's and OAC.

Using:

- Resected human OAC tissue.
- *In vitro* cell lines as a model of progression.
- Mouse model of Barrett's.

2. To investigate inflammasome-mediated inflammation during disease progression.

Using:

- *Ex vivo* Barrett's patient biopsies.
- Barrett's organoids taken from the pL2-IL-1 β mouse model.
- Publicly available datasets of patient tissue mRNA expression.
- Culture of oesophageal cell lines with adipose-conditioned media from obese OAC patients.

3. To examine the relationship between a high fat diet and inflammasome pathways during oesophageal disease.

Using:

- Oesophageal explants taken from high fat diet-fed mice.
- Oesophageal tissue sections taken from high fat diet-fed pL2-IL-1 β Barrett's mice.

Chapter 2: Materials and Methods

Materials

Table 2.1: Materials

Product	Source
Acetone (grade)	TCD Biohazard Stores
Agarose (Electrophoresis grade)	Invitrogen
Accutase	Millipore
Ammonium persulfate (APS)	Sigma Aldrich
Aprotinin	Sigma Aldrich
Bicinchoninic acid (BCA) assay	Thermo Scientific
Bovine Serum Albumin (BSA)	Sigma Aldrich
BEGM BulletKit	Lonza
Bromophenol Blue	Sigma Aldrich
Cell culture plastics	Cruinn Ltd.
Chemiluminescent	Millipore
Collagen Bovine type I, Purecol®	Advanced Biomatrix
Dimethyl Sulfoxide (DMSO)	Sigma Aldrich
Dithiothreitol (DTT)	Sigma Aldrich
DNA Ladder, Quick-Load 2-log	Brennan & Co.
Dulbecco's Modified Eagles Medium (DMEM)	Biosciences
Ethanol	TCD Biohazard Stores
Foetal Bovine Serum (FBS) – heat inactivated	Labtech
Human fibronectin	Millipore
Glycerol	Sigma Aldrich
Glycine	Sigma Aldrich
GoTaq Hot start green master mix	Promega
High binding- 96-well plate	Cruinn Diagnostics
HEPES	Sigma Aldrich
Hydrochloric Acid (HCl)	Sigma Aldrich
Human IFN-γ	PeproTech
Isoflurane	CP Pharma
Isopropanol	TCD Hazardous Materials
Quick Load® 2-Log DNA Ladder	New England BioLabs
Leupeptin	Sigma Aldrich
Lipofectamine 2000	Invitrogen
Lipopolysaccharide (LPS)	Sigma Aldrich
BD Matrigel Basement Membrane Matrix	BD Biosciences
Medium 199	Biosciences
Methanol	TCD Biohazard Stores
Nitrocellulose membrane 0.45 um	Sigma Aldrich
NP-40	Sigma Aldrich
Opti-MEM	Biosciences

OmmEdge pen,	Vector laboratories
Pam3 CSK – synthetic triacylated lipoprotein	Invivogen
Penicillin Streptomycin	Invitrogen
Phenylmethylsulphonyl fluoride (PMSF)	Sigma Aldrich
Phosphate Buffered Saline (PBS)	Biosciences
Poly(dA:dT)	Sigma Aldrich
Potassium Chloride	Sigma Aldrich
Protein ladder, SeeBlue Plus2 pre-stained	Biosciences
ProtoGel – Acrylamide	Analab
RPMI 1640 GlutaMAX	Biosciences
Sodium chloride (NaCl)	Sigma Aldrich
Sodium deoxycholate	Sigma Aldrich
Sodium dodecyl sulphate	Sigma Aldrich
Sodium phosphate monobasic (NaH ₂ PO ₄)	Sigma Aldrich
Sodium phosphate dibasic (NaHPO ₄)	Sigma Aldrich
SYBR Safe DNA stain	Biosciences
TEMED	MWG
Tris-HCl	Sigma Aldrich
Trizma base	Sigma Aldrich
TrypLE Express	Biosciences
VectorMount Mounting Medium	Vector laboratories
Versene	Biosciences
Virkon tablets 5g	VWR
Water, DNase/RNase free	Biosciences
YVAD.fmk	Bachem

Table 2.2: Buffers and Solutions

Name	Composition
10X Phosphate-Buffered Saline (PBS)	• 1.45 M NaCl • 39 mM NaH₂PO₄ (Sigma-Aldrich) • 227 mM NaHPO₄ (Sigma-Aldrich) • pH 7.2
10X Tris-Buffered Saline (TBS)	• 10 mM Trizma Base (Tris) • 150 mM NaCl • pH 8.0
10X WB transfer buffer	• 0.25 M Trizma Base • 1.9 M Glycine • 35 mM SDS
10X WB running buffer	• 0.25 M Trizma base • 1.9 M Glycine • 35 mM SDS
Wash buffer (PBST)	• 0.1% (v/v) Tween-20/ PBS (1X)
Resolving gel	• 1.5 M Trizma base

	• pH 8.8
Stacking gel	• 0.5 M Trizma base • pH 6.8
1X Laemmli (Laemmli was used as a 2X and 5X stock)	• 10% (v/v) Glycerol • 10% (w/v) SDS • 0.1% (w/v) Bromophenol Blue (Sigma-Aldrich) • 62.5 mM Tris-HCl • 150 mM DTT • pH 6.8
1X TAE buffer (Tris-acetate-EDTA)	• 40 mM Tris-acetate • 1 mM EDTA
DNA agarose gel	• 2% w/v agarose • TAE buffer (Tris-acetate-EDTA)
IP lysis buffer	• 150 mM NaCl • 50 mM Tris-HCl pH8 • 1% NP-40 Supplemented with: • 1 mM PMSF • 1 µg/µL leupeptin • 1 µg/µL aprotinin
RIPA buffer	• 50 mM Tris pH8 • 150 mM NaCl • 0.1% w/v SDS • 0.5% w/v sodium deoxycholate • 1% v/v NP-40 Supplemented with: • 1 mM PMSF • 1 µg/µL leupeptin • 1 µg/µL aprotinin

Methods

2.1 *In vitro* studies

Table 2.3: Cell lines

Name	Description	Media	Source
Het-1A	- Human, normal oesophageal squamous epithelium, male - Non-tumorigenic - SV-40 T antigen immortalised	BEGM + supplied supplements (minus 'red' gentamycin supplement)	American Type Culture Collection (ATCC)
QH	- Human, oesophageal columnar epithelium - Barrett's non-dysplastic metaplasia, male - Also known as CP-A or KR-42421 - hTERT immortalised	- BEGM + supplied supplements 10% (v/v) FBS 1% (v/v) Pen-Strep	American Type Culture Collection (ATCC)
GO	- Human, oesophageal columnar epithelium - High-grade dysplasia, male - Also known as CP-B - hTERT immortalised	- BEGM + supplied supplements 10% (v/v) FBS 1% (v/v) Pen-Strep	American Type Culture Collection (ATCC)
OE33	- Human Oesophageal adenocarcinoma (OAC) - Poorly differentiated - Taken from a 73-y/o female patient, stage IIA - Established from patient progressed from Barrett's metaplasia	- RPMI 1640 GlutaMAX™ 10% (v/v) FBS 1% (v/v) Pen-Strep	European Collection of Authenticated Cell Cultures (ECACC)

SKGT4	• Human, Oesophageal adenocarcinoma • Well differentiated • Established from a primary adenocarcinoma arising in a male with dysplastic Barrett's metaplasia. Stage IIB.	• RPMI 1640 GlutaMAX™ • 10% (v/v) FBS • 1% (v/v) Pen-Strep	• European Collection of Authenticated Cell Cultures (ECACC)
THP-1	• Human Monocyte cell line • Derived from acute monocytic leukaemia patient	• RPMI 1640 GlutaMAX™ • 10% (v/v) FBS • 1% (v/v) Pen-Strep	• American Type Culture Collection (ATCC)

2.1.1 Cell line culture

All cell culture was carried out using a strict aseptic technique in a grade II laminar air flow cabinet, sterilised daily with a UV-C germicidal lamp and cleaned weekly with Virkon and ethanol. All equipment, reagents and the flow cabinet were sprayed and wiped with 70% (v/v) ethanol before and after use.

Cell lines were cultured in media as detailed in **Table 2.3**. All cell line media, with the exception of Het-1A cell line media, was conditioned with 10% foetal calf serum (FBS) and penicillin-streptomycin (Pen-Strep, 100 U/mL and 100 µg/mL, respectively). OE33, SKGT4 and THP-1 cell lines were cultured in Roswell Park Memorial Institution (RPMI) 1640 GlutaMAX™. Het-1A, QH and GO were cultured in BEGM™ Bronchial Epithelial Cell Growth Medium with supplements (2 mL BPE, 0.5 mL hydrocortisone, 0.5 mL hEGF, 0.5 mL epinephrine, 0.5 mL transferrin, 0.5 mL insulin, 0.5 mL retinoic Acid, 0.5 mL triiodothyronine, 0.5 mL GA-1000 per 500 mL media). Cultured cells were grown in sterile T75 or T175 flasks in an incubator maintained at 37 °C, 5% CO₂. Cells were monitored daily under a bright light microscope (Leica DMIL) to observe cell morphology and check for any visible signs of infection. All oesophageal cell lines were maintained in the log stage of growth and were not cultured beyond a passage number of 40.

OE33, SKGT4 and GO cell lines were passaged two-three times weekly (approximately 80% confluency). Media was removed from the flasks before washing in 5 mL sterile

PBS. Cells were detached by incubating in TrypLE Express (2 mL, 37 °C, 5 mins). TrypLE Express was neutralised in 4 mL media and cells were transferred to a 50 mL falcon tube for centrifugation (400 x g, 5 mins). The supernatant was discarded, and cells were sub-cultured at the appropriate seeding density; OE33 and SKGT4 cells ($1\text{--}2 \times 10^4$ cells/cm² (1:5 ratio)) and GO cells (3×10^4 cells/cm² (1:4 ratio)).

QH (Barrett's metaplasia) cell line was grown in conditioned BEGM. QH cells were passaged one to two times per week when cells reached approximately 90% confluency. This cell line is very adherent and difficult to detach from the flask. Care was taken to ensure that QH cells were not incubated in TrypLE Express longer than 10 minutes. Incubations >10 mins resulted in cells being unable to recover sufficiently for re-attachment. To expedite cell detachment, media was removed from the flask and cells were washed twice with PBS (5 mL) prior to the addition of Versene (3 mL, Gibco) for a 2 min incubation at RT. Versene is a solution of EDTA (0.48 mM) dissolved in PBS that is used for gentle non-enzymatic cell dissociation. Versene was removed and replaced with 4 mL TrypLE Express for further incubation (7 mins, 37 °C, 5% CO₂). Cell detachment was checked under a light microscope. Gentle tapping of the flask against a work surface was required to fully detach the cells. TrypLE Express was neutralised with BEGM (4 mL), before transferring into a 50 mL falcon for centrifugation (5 mins, 400 x g). Supernatant was discarded and the QH cells were re-suspended in fresh BEGM at approximately 3×10^4 cells/cm² (approx. 1:3 ratio).

The Het-1A cell line was grown in serum free BEGM without antibiotic. Cells were passaged once per week, when cells reached approximately 90% confluency. Media was changed twice weekly to ensure nutrient replenishment. Media was removed from the flask and cells were washed with PBS, before addition of 2 mL Accutase (1:3 reagent dilution in PBS, Accutase enzymes, 0.5 mM EDTA-4Na and 3 mg/L Phenol Red). Cells were incubated (37 °C, 5% CO₂) for 7 minutes until detachment was observed. Accutase was diluted with 6 mL PBS and transferred into a 50 mL falcon for centrifugation (400 x g, 5 mins). The supernatant was discarded, and the cells were sub-cultured in a pre-coated flask at a seeding density of 3.5×10^4 cells/cm² (1:2-3 ratio). The flask was pre-coated overnight at 4 °C with 2 mL of a sterile mix containing 0.03 mg/mL bovine

collagen II, 0.01 mg/mL BSA (0.22 micron sterile filtered), 0.01 mg/mL human fibronectin.

THP-1 cells are a suspension, human monocytic cell line that was used a positive control during *in vitro* experimentation. Cells were considered confluent and were passaged at approximately 8×10^5 cells/mL. THP-1 cells were centrifuged (400 x g, 5 mins) and re-suspended at 3×10^5 cells/mL.

2.1.2 Cell counting

Cell counting from a cell suspension was carried out using a bright light haemocytometer. The haemocytometer was cleaned before and after use with ethanol. A coverslip was affixed to the haemocytometer to cover the 9 squares engraved on it, creating a small chamber that holds a defined volume of 1×10^{-4} mL per square (Dimensions: 1 x 1 x 0.1 mm). Cell suspension (10 μ L) was pipetted under the coverslip into the chamber. The haemocytometer was placed under a bright light microscope and focused at the 10X objective before beginning to count the cells. The number of cells were counted in the 4 corner squares. This count was averaged, and the value multiplied by 10^4 to give the number of cells per millilitre.

2.1.3 Cryopreservation

Stocks of all cell lines at low passage number were cryopreserved in liquid nitrogen (-180°C). Cell pellets were obtained by centrifugation (400 x g, 5 mins) in a Sorvall centrifuge. The supernatants were discarded, and the cells were re-suspended at a concentration of 1×10^7 cells/mL in freezing media (10% (v/v) sterile dimethylsulfoxide (DMSO), 50% (v/v) FBS, 40% appropriate growth media). With exception to Het-1A cell line which is inhibited by FBS. Het-1A cells were frozen in 10% FBS and 90% relevant media. Resuspended cells (1 mL) were then aliquoted into 1.5 mL cryopreservation vials before placing into a Mr. Frosty™ freezing container containing isopropanol. This was then placed in the -80°C freezer. The Mr. Frosty™ freezing container was used to ensure the cells cooled at a rate of $1^\circ\text{C}/\text{min}$, minimising hypothermal shock to cells. After 24 hours, the cells were transferred to liquid nitrogen for long term storage.

2.1.4 Thawing cells from stocks

Frozen stocks were removed from liquid nitrogen and defrosted immediately in a water bath (37°C). Cells were then transferred to 10 mL pre-warmed media before

centrifugation (400 x g, 5 mins). Cell pellets were re-suspended in 10 mL media, before repeating centrifugation. Cells were then re-suspended in 10 mL media and transferred to a T25 cell culture flask and placed in an incubator (37 °C, 5% CO₂) until confluent. Cells were monitored and when judged as confluent were passaged. Cell lines were passaged at least twice before experimentation.

2.1.5 Mycoplasma detection in cell cultures

Continuous cell culture is liable to mycoplasma contamination. These infections are not visible by microscope but can have adverse effects on cell characteristics. These include decreased rate of cell proliferation, failure of adherent cells to reattach efficiently, cell aggregation and morphological changes. Such characteristics were carefully monitored for during this project. Additionally, all cell lines were tested for mycoplasma every 3-4 months and before new cell stocks were frozen in liquid nitrogen by performing polymerase chain reaction (PCR) as described by Young *et al.* (**Table 2.4**) (371).

Media (1 mL) was taken from a confluent flask of cells, transferred into an Eppendorf and centrifuged (1 min, 400 x g, RT) to pellet any cellular debris. A PCR reaction mix was made up as described in **Table 2.5**. DNA for each cell line was present in the flask supernatants derived from dead cells, 0.5 µL supernatant was added directly into the PCR mix for amplification. Water was used as a negative control and supernatant previously known to contain mycoplasma was used as a positive control. The PCR reaction was performed in a PTC-200 Peltier Thermal Cycler (Bio-Rad, **Table 2.6**). Amplified DNA products were run on a 2% agarose gel by DNA electrophoresis. DNA bands were visualised and imaged using a GelDoc-it System (UVP, LLC, CA, USA) under ultra-violet light (**Figure 2.1**).

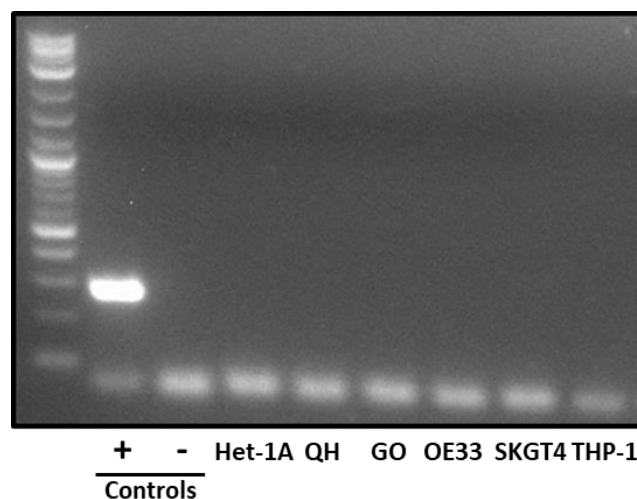


Figure 2.1: DNA gel reveals no mycoplasma is present in oesophageal and THP-1 cell lines.

Table 2.4: Mycoplasma primers for PCR

Primer	Sequence
Forward	5'-TGCACCATCTGTCACTCTGTAAACCTC-3'
Reverse	5'-GGGAGCAAACAGGATTAGATACCCT-3'

Table 2.5: PCR reaction components

Component	Volume (μL)
DNA (taken from cell culture supernatant)	0.5
Forward primer (10 μM stock)	0.5
Reverse primer (10 μM stock)	0.5
GoTaq® Hot Start Master Mix (2X)	12.5
Nuclease free water	11
Total reaction volume	25

Table 2.6: PCR reaction conditions

Temperature (°C)	Time (cycles)
95	5 minutes (1)
94	30 seconds (40)
55	30 seconds (40)
72	1 minute (40)
72	10 minutes (1)
4	∞

2.1.6 DNA gel electrophoresis

2% (w/v) agarose gels were prepared by dissolving agarose in 1X TAE buffer by heating. Once dissolved, SYBR Safe DNA stain (1:10,000 dilution) was added, before pouring into a sealed cassette, taking care to avoid bubbles. The gel was left to set out of direct light for approximately 1.5 hours. The solidified gel was transferred to a Horizon 11.14 Gel Electrophoresis Apparatus rig (Life Technologies) and immersed in 1X TAE buffer. All PCR samples were loaded into wells and Quick-Load 2-Log DNA ladder (0.1 kb-10 kb, Brennen and Co.) was loaded as a DNA size standard. The gel was run away from direct sunlight (120 V, 40 mins). DNA bands were visualised and imaged using a GelDoc-it System (UVP, LLC, CA, USA) under ultra-violet light.

2.2 Western Blot analysis

2.2.1 Preparation of lysates

Cells for lysis were taken from the incubator and placed directly on ice. Supernatants were removed, and cells were washed with ice cold PBS and lysed with lysis buffer (10 mins, $\sim 1 \times 10^7$ cells/mL). IP lysis buffer was used as a low stringency buffer (generating cytoplasmic lysates). RIPA buffer was used as a high stringency lysis buffer (generating total cell lysates). Lysates were transferred to 1.5 mL tubes and centrifuged (20,000 x g, 10 mins, 4 °C) in an Eppendorf 5417R refrigerated centrifuge. The pellet containing cellular debris was discarded and the lysates were transferred to a labelled tube and stored at -20 °C or -80 °C.

2.2.2 Determination of protein concentration

The Bicinchoninic acid (BCA) assay was used to determine total protein concentration of lysates to ensure equalised protein loading on a SDS-PAGE gel. The BCA assay is a colorimetric technique that is primarily dependant on two reactions.

This method combines the reduction of Cu^{+2} to Cu^{+1} by protein present in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid. The purple reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water soluble complex exhibits a strong

absorbance at 562 nm that is nearly linear with increasing protein concentration over a broad working range (20-2000 µg/mL) (372).

A standard curve between 2.5-320 µg/mL of bovine serum albumin (BSA) was created by two-fold serial dilution in PBS. Sample (50 µL) was added in duplicate to a 96 well plate, followed by 50 µL Micro BCA™ Working Reagent (25 parts Reagent MA, 24 parts Reagent MB and one-part Reagent MC). The plate was then incubated (37 °C, 30 mins) in the dark. Absorbance was acquired by reading the plate at 562 nm using the SpectraMax PLUS³⁴⁰ Microplate Reader (Molecular Devices). Unknown protein concentration was determined relevant to the BSA standard curve. Normalised concentrations of lysates were then prepared with PBS. Following protein normalisation, sample lysates were denatured by boiling (70 °C, 10 mins) in Laemmli sample buffer, before running on a gel.

2.2.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method used to separate proteins based on molecular mass. The anionic detergent, SDS, confers a net negative charge on denatured proteins causing them to be attracted to the anode when an electrical charge is applied to the gel. In polyacrylamide gels, smaller proteins migrate faster than larger proteins towards the anode, resulting in size gradient separation of proteins.

Polyacrylamide gels were made as detailed in **Table 2.7**. Clean glass plates were wiped with ethanol and assembled. Resolving gels were made (**Table 2.7**) in a fume hood. Immediately following addition of TEMED, the solution was mixed and poured into the gap between the glass plates, leaving enough space for the stacking gel. H₂O was added gently over the gel mix using a P1000 pipette, to eliminate air bubbles and ensure a level distribution of the gel. After 20 minutes the gel was set, the water was removed, and the gel dried with filter paper. The prepared stacking gel was poured onto the resolving gel and a comb was immediately inserted.

Gels were placed upright in a Biorad gel electrophoresis rig, placed in a tank and filled with 1X running buffer. Combs were removed, and wells were loaded with lysate samples (>20 µg protein/well, <30 µL). SeeBlue Plus2 pre-stained protein ladder (6 µL)

was loaded as a molecular weight reference. A current of 90 V was applied to the gel until the dye moved into the resolving gel, before increasing voltage to 120 V.

Table 2.7: Formulations for preparing polyacrylamide gels

Reagent	15% Resolving	12% Resolving	10% Resolving	5% Stacking
dH ₂ O	2.4 mL	3.4 mL	4.1 mL	3.4 mL
1.5 M Tris-HCl (pH 8.8)	2.5 mL	2.5 mL	2.5 mL	-
1 M Tris-HCl (pH 6.8)	-	-	-	1.5 mL
Protogel (30% acrylamide, 0.8% bisacrylamide)	5 mL	4 mL	3.3 mL	1 ml
10% SDS	100 µL	100 µL	100 µL	60 µL
10% APS	50 µL	50 µL	50 µL	60 µL
TEMED	5 µL	5 µL	5 µL	6 µL

2.2.4 Wet Transfer

The gel was removed from the glass plates and the stacking gel disposed. A Biorad wet transfer system was used to transfer protein onto a nitrocellulose membrane. The filter paper and sponges were soaked in 1X transfer buffer, while the nitrocellulose was activated in H₂O for 10 minutes prior to transfer set-up. The transfer sandwich was set up in a cassette as follows: sponge, two filter papers, nitrocellulose membrane, gel, two filter papers and sponge. Taking care to avoid any air bubbles, the sandwich was sealed inside the cassette, assembled into the transfer rig and submerged in a container filled with 1X transfer buffer, alongside one ice pack. The protein in the gel was transferred to the nitrocellulose membrane for 40-80 minutes (depending on protein size) at 200 mA.

2.2.5 Blocking, immunoblotting and visualisation of protein

Following protein transfer to nitrocellulose membrane, non-specific binding sites on the membrane were blocked by incubation in 5% NFDm (5% non-fat dried milk (Marvel) in 1X TBST, 1 hour, RT).

Following blocking, the membrane was probed for the protein of interest by incubating with relevant primary antibody diluted in 5% NFDm overnight at 4 °C (**Table 2.8**). The following day, the membrane was washed (3x10 mins TBST), before incubation with relevant secondary antibody conjugated to horse-radish peroxidase (HRP) (1 hour, RT) (**Table 2.9**). The membrane was washed (3x10 mins TBST) before using an enhanced

chemiluminescent (ECL) substrate to detect antibody-bound proteins on the membrane. The ECL substrate (Millipore) consists of a luminol reagent and peroxide buffer. In the presence of HRP, the luminol oxidises and forms an excited-state product that emits light as it decays to the ground state. This appears as a luminescent band on the membrane. The ECL was added to the membrane and incubated for 30 seconds before placing in a BioRad Gel Doc™XR+System to develop using the BioRad ChemiDoc™ MP Imaging System for analysis.

Table 2.8: Primary antibodies for immunoblot analysis

Name (anti-)	Features	Dilution	Source
hCaspase-1 p10 (sc-515)	- Rabbit polyclonal - Clone: M-20	1:500	Santa Cruz
mCaspase-1 p10 (sc-514)	- Rabbit polyclonal - Clone: M-20	1:500	Santa Cruz
TLR2	- Goat polyclonal - AF2616	1:400	R&D Systems
hCaspase-1 (sc-622)	- Rabbit polyclonal - Clone: A19	1:500	Santa Cruz
Caspase-4/ Tx (M029-3)	- Mouse monoclonal - Clone: 4BD	1:1000	MBL
Caspase-5 (M060-3)	- Mouse monoclonal - Clone: 4F7	1:1000	MBL
mCaspase-11 (C1354)	- Rat monoclonal - Clone: 17D9	1:200	Sigma
CDX2 (12306)	- Rabbit polyclonal - Clone: D11D10	1:500	Cell Signaling
Villin (2369)	- Rabbit polyclonal - Clone: R814	1:1000	Cell Signaling
IL-1beta (SC-7884)	- Rabbit polyclonal - Clone: H-153	1:500	Santa Cruz
IL-1beta	- Goat polyclonal - Clone: AF-401	1:700	R&D Systems
hAIM2 (AG-20B-0040)	- Mouse monoclonal - Clone: 3D10	1:1000	Adipogen
mAIM2	- Rabbit polyclonal 14-6008-93	1:1000	Biosciences
hNLRP3 (13158)	- Rabbit polyclonal - Clone: D2P5E	1:1000	Cell Signaling
mNLRP3 (15101)	Rabbit polyclonal	1:1000	Cell Signaling

	• Clone: D4D8T		
β -Actin (A3854)	• Mouse monoclonal • Peroxidase conjugate • Clone: AC-15	1:10,000	Sigma Aldrich
GAPDH	• Mouse monoclonal • Clone 6C5	1:10,000	Calbiochem

Table 2.9: Secondary antibody for immunoblot analysis

Name	Dilution	Source
HRP conjugated goat anti-mouse IgG	1:3000	Jackson Immunolabs
HRP conjugated goat anti-rabbit IgG	1:3000	Jackson Immunolabs
HRP conjugated donkey anti-goat IgG	1:3000	Jackson Immunolabs
HRP conjugated goat anti-rat IgG	1:3000	Jackson Immunolabs

2.2.6 Densitometric analysis of protein expression

Densitometric analysis of Western blots was performed using Biorad Image Lab software. The relative density of each band of interest was calculated against a reference band (unstimulated control) to calculate fold change. The relative density of the loading control was similarly calculated. Values were then normalised to the density of the relative loading control.

2.3 Enzyme-linked immunosorbent assay (ELISA)

ELISA kits were used to quantify specific cytokine levels in supernatants, as specified. The protocol was followed as issued by the manufacturer and kits (**Table 2.10, 11, 12**). All ELISAs were performed in high binding 96 well plates (Greiner MICROLON®).

Briefly, ELISA plates were coated with capture antibody (50 μ L per well) at 4 °C overnight. Wells were washed x4 times with PBST before blocking with 200 μ L assay diluent (1% BSA in PBS, RT, 1 hour) to reduce non-specific binding. PBST washes were repeated before samples/standards were added to the wells in the appropriate dilutions. Standards were serially diluted (1/2) 7 times in assay diluent before applying 50 μ L per well. The plate was incubated at RT for 3 hours or overnight at 4 °C. Plates

were washed again before adding 50 µL per well of biotinylated detection antibody diluted in assay diluent. Washing was repeated and 50 µL HRP-conjugated Streptavidin was applied to the plate and incubated (30 mins, RT) away from direct sunlight. The plates were washed (x5) with 30 second incubations to reduce background. Substrate solution (50 µL) was added to each well and incubated for 15-30 mins at RT, in the dark. By visual determination, the reaction was stopped at the most appropriate time by adding 50 µL stop solution (1 M H₂SO₄).

The optical density (OD) values were determined by measuring absorbance values at 450 nm and 570 nm using a Spectamax Microplate Reader (Molecular Devices). Values at 570 nm were deducted from the 450 nm reading for wavelength correction. Unknown cytokine concentrations were determined by plotting a standard curve of the known concentrations for each ELISA plate.

Table 2.10: Commercial human ELISA kits

Cytokine	Supplier	Top working standard
IL-1β	Biolegend/MSC	125 pg/mL
IL-1α	Biolegend/MSC	250 pg/mL
TNF-α	Biolegend/MSC	500 pg/mL
IL-18	R&D Biosystems	750 pg/mL
IL-6	Biolegend/MSC	500 pg/mL
IFN-γ	Biolegend/MSC	500 pg/mL
IL-8	Biolegend/MSC	500 pg/mL

Table 2.11: Commercial mouse ELISA kits

Cytokine	Supplier	Top working standard
IL-1β	Biolegend/MSC	2000 pg/mL
IL-1α	Biolegend/MSC	125 pg/mL
TNF-α	Biolegend/MSC	500 pg/mL
IL-6	Biolegend/MSC	500 pg/mL
IFN-γ	Biolegend/MSC	500 pg/mL
CXCL1	R&D Biosystems	500 pg/mL
IL-10	Biolegend/MSC	2000 pg/mL
MIP-2	R&D Biosystems	500 pg/mL

Table 2.12: Non-Kit murine ELISA, IL-18

Step	Reagent	Supplier
Capture	Monoclonal rat anti-mouse IL-18	MBL
Detection	Monoclonal rat anti-mouse IL-18	MBL
Standard	Recombinant mouse IL-18	MBL
Avidin-HRP	Avidin-HRP	Biolegend/MSC

2.4 Data set analysis

Analysis of publicly available datasets was carried out using online Geo DataSet records on the National Centre for Biotechnology Information (NCBI) website. In this study two data sets containing gene expression levels in patient oesophageal tissue were analysed [GDS4350], [GDS1321] (373, 374).

Data sets were analysed as follows: starting from the main NCBI webpage, Geo DataSets was selected from the 'All Databases' dropdown menu. 'Barrett's oesophagus' was entered into the search bar. A relevant study was selected from the list of studies relating to Barrett's. 'Analyze with GEO2R' was then selected from the bottom of the study's webpage. This causes the main analysis webpage to appear for the study.

In order to analyse, each sample must first be assigned to a group. Group names were created by the user (eg. Normal, BO) and entered into 'Define groups', before assigning names to each sample.

Next, using the functions in the box at the bottom of the screen, the 'value distribution' tab was selected and 'viewed'. This function gives the user an idea whether the data is median-centered for all the samples, and if they can be cross-compared. The line of boxes should be reasonably aligned for analysis.

In the GEO2R tab, the 'Top 250' genes were analysed. If the gene of interest was not present in the top 250 genes, then the 'profile graph' tab was selected and the 'view data for [dataset no.]' .txt file was downloaded. This file was opened with Windows NotePad and copied and pasted (special) into a Microsoft Excel file. The 'control + find' function in Excel was used to find the gene of interest. The gene ID was subsequently pasted into the 'Profile graph' search bar online and 'set'. The gene value results were

copied into an excel file for records and the results were graphed and analysed in GraphPad Prism.

2.5 Human studies

2.5.1 Adipose tissue retrieval and processing

Omental adipose tissue was excised from OAC patients undergoing oesophageal resection surgery at St. James's Hospital (SJH). The excised visceral adipose tissue was placed immediately in sterile adipose transport buffer (PBS, 5.5 mM glucose, 50 µg/mL gentamicin) when collected in surgery. Ethical approval was granted by the St. James's Hospital/AMNCH Review Board. All participating patients provided informed consent to participate in the adipose tissue biobank (est. 2007).

Adipose tissue was processed for the generation of adipose conditioned media (ACM) under sterile conditions within 1 hour of tissue removal from the patient, using a protocol based on that described by Fried *et al.* (375). In a laminar flow hood, tissue was placed in a sterile dish, quickly cut into 20-50 mg pieces and washed in sterile PBS to remove excess blood. Adipose tissue was incubated in M199 media (Medium 199, with Earle's salts (Gibco) supplemented with 50 µg/mL gentamicin) (10 mL M199 media/5 g adipose tissue) for 72 hours (37 °C, 5% CO₂). Following incubation, the conditioned media was passed through a 70 µm polypropylene filter to remove tissue and debris. ACM was aliquoted into 1.5 mL cryogenic vials and stored at -80 °C.

2.5.2 Horse-Radish Peroxidase (HRP) conjugation to primary antibody

2.5.2.1 Antibody clean-up

Primary antibody clean-up of caspase-1 SC-622 (Santa Cruz) was performed using the AbSelect BSA removal kit (Expedeon).

This kit was used prior to HRP conjugation to:

- Increase the concentration of antibody in solution.
- Remove proteins such as BSA from the buffer that may interfere with HRP conjugation.
- To exchange antibody buffer for more favourable conjugation conditions.

BSA removal buffer was pre-warmed in a water bath (36 °C, 10 mins) before shaking to completely re-dissolve all components in the buffer. Once dissolved the buffer was maintained at 22 °C to prevent crystal formation. 250 µL caspase-1 SC-622 (200 µg/mL) was added to 200 µL BSA removal buffer. This was mixed, incubated (5 mins, RT) and centrifuged (5 mins, RT, 13,000 x g).

The tube was placed on ice and the supernatant removed, taking care to not displace the pellet containing the antibody. The pellet was then re-suspended in 10 µL re-suspension buffer.

2.5.2.2 HRP conjugation

HRP conjugation to primary antibody was performed using the Lightning-Link HRP conjugation kit (Expedeon). Briefly, 1 µL LL-modifier was added to 10 µL purified antibody, before transferring directly onto the lyophilised Lightning-Link reagent and mixed gently by withdrawing and re-dispensing the mix twice using a pipette. The cap was placed back on the vial and the mix was left to incubate at RT (22 °C-25 °C) for 3 hours. LL-quencher reagent (1 µL) was added to the mix to complete HRP conjugation.

The HRP-conjugated antibody was stored at 4 °C.

2.5.3 *Ex vivo* human Barrett's explants

All participating patients gave informed written consent to partake in this research. Ethical approval was granted by the St. James's Hospital/ Tallaght University Hospital Review Board. All patients included in this study were histologically confirmed to have Barrett's by a trained pathologist. Barrett's patients were recruited prospectively at St. James's Hospital national referral centre for upper GI malignancy, as part of Ireland's National Registry & Biobank for Barrett's Oesophagus Patients. During endoscopy, the Barrett's segment was assessed using both the Prague and the Vienna classification system. Eight fresh biopsies were surgically removed from each patient (n=10), 4 Barrett's biopsies were taken from identified areas of metaplasia and 4 matched adjacent squamous biopsies were taken from microscopically identified non-inflamed tissue that was ≥5 cm from the proximal border of the Barrett's region. The biopsy tissue was immediately transferred into 1 mL supplemented M199 (10% FBS, 1% Pen/Strep, 1 mg/mL insulin) (Gibco).

Before treating explants, caspase-1 inhibitor optimisation experiments were carried out in a THP-1 cell line. Firstly, caspase-1 tetrapeptide inhibitors (1 μ M) were compared for efficacy by measuring IL-1 β secretion; YVAD.fmk (Z-Tyr-Val-Ala-Asp (OMe)-fluoromethylketone) dissolved in DMSO and WEHD.CHO (Trp-Glu-His-Asp-aldehyde). Following a decision to use WEHD.CHO, inhibitor concentration was assessed to achieve maximal efficacy.

Explants were incubated (37 °C, 5% CO₂) for 16 hours in 500 μ L M199 (10% FBS, 1% Pen/Strep, 1 mg/mL insulin) supplemented with or without 10 μ M WEHD.CHO (Bachem), dissolved in H₂O. Supernatants were collected and stored at -80 °C. Biopsies were snap frozen in liquid nitrogen before storage at -80 °C for later analysis. Two matched biopsies were used per group, to account for biopsy heterogeneity, which were then averaged for each patient. Biopsies were not split in two for inhibitor treatment due to the small size of the biopsies (approximately 2.5 mm diameter).

Biopsy protein was isolated using the AllPrep RNA/Protein Kit (Qiagen) as per manufacturer's instructions. The resulting protein lysates were incompatible with SDS-PAGE electrophoresis; therefore, protein was subsequently precipitated using acetone. Four volumes of ice-cold acetone were added to protein lysate, incubated on ice (15 mins) and centrifuged (12,000 x g, 4 °C). The supernatant was discarded and the pellet air dried, before re-suspending in RIPA buffer.

2.5.4 Quantification of Barrett's explant secretion

Enzyme linked-immunosorbent assays (ELISAs) were used to profile and quantify specific cytokine secretion levels (pg/mL) in Barrett's conditioned media. Cytokines IL-1 β , IL-6 and TNF α were quantified using MesoScale Diagnostics (MSD) multiplex as per manufacturers protocol. Cytokine kits IL-8, IL-1 α and IFN- γ were sourced from BioLegend®, CA, USA and IL-18 from R&D Systems, Inc. MN, USA. All singleplex ELISAs were performed in high binding 96 well plates (Greiner MICROLON®) using the protocol issued by the manufacturer (see **Section 2.3**). All cytokine levels were normalised to the total protein content of each biopsy, which were determined using the BCA assay method.

2.5.5 MSD Multiplex analysis

Quantification of the cytokine secretome in Barrett's conditioned media and in mouse organoid conditioned media was performed using MSD multiplex analysis. The protocol was performed as issued by the manufacturer and in the presence of a company representative. Human work was performed using the V-PLEX Human Proinflammatory Panel II (4-PLEX, K15053D-1, **Table 2.13**). Adjacent normal supernatants were diluted 1:2, while Barrett's supernatants were diluted 1:10 to ensure all signals for all 3 cytokines were kept within the dynamic range of the assay. IL-8 was not included on the plate due to expected high levels of cytokine.

Table 2.13: MSD human multiplex cytokines

Cytokine	Standard curve range (pg/mL)
IL-1 β	587 - 0.143
IL-6	742 - 0.181
TNF α	378 - 0.0923

Mouse organoid supernatants were analysed using the V-PLEX Proinflammatory Panel I (2-PLEX, custom made, **Table 2.14**). Organoid secretion levels for IL-1 β and IL-6 were very low and could not be analysed using the standard singleplex kits from Biolegend.

Table 2.14: MSD mouse multiplex cytokines

Cytokine	Standard curve range (pg/mL)
IL-1 β	1480 - 0.361
IL-6	4660 - 1.14

2.5.6 LDH assay

Cellular cytotoxicity was assessed by measuring release of lactate dehydrogenase (LDH, CytoTox 96®, Promega) from damaged cells. This colorimetric assay was carried out by the enzymatic conversion of tetrazolium salt (INT) into a red formazan product in the presence of LDH.

Supernatant (50 μ L) and CytoTox 96 Reagent (50 μ L) were added to each well of a 96 well plate. Relevant culture media (50 μ L) was used as a negative control. The plate was incubated at RT on a shaker and in the dark for 30 mins. Stop solution (50 μ L) was added

and absorbance was read at 490 nm using the SpectraMax Microplate Reader (Molecular Devices). Differences were analysed by calculating fold change.

2.6 High fat diet mouse model

This work was performed in collaboration with Prof. Lydia Lynch's research group, TBSI, Dublin. All mice used in this study were the C57BL/6j OlaHsd substrain, male and 15 weeks old when culled. Mice were fed either a standard chow diet (LabDiet 5053) or a specialised 60 kcal% lard diet (D12492, Research Diets Inc., **Table 2.15**) for 9 weeks. This study was conducted as part of a larger study performed by the Lynch lab and mice used were made up of the control groups for this larger study. For this reason, some conditions the mice were exposed to were recognised as sub-optimal, therefore results should be considered preliminary observations. Such suboptimal conditions include exposure to small amounts of DMSO and stimulation with poly I:C. These factors likely influenced a particular inflammatory phenotype in both WT and HFD-fed mice and may have had an effect on observations.

Seven days prior to harvest (8 weeks HFD), mice received daily intraperitoneal (IP) injections (100 μ L 2:1 PBS/DMSO solution). On harvest day, all mice were administered 100 μ g poly I:C via IP injection (2:1 PBS/H₂O) 4 hours before culling. Mice were sacrificed by CO₂ inhalation, followed by cervical dislocation. The oesophagi were resected, and a segment of the distal oesophagus meeting the stomach was formalin fixed (10%, 48 h). The tissue samples were placed in 70% (v/v) ethanol for storage until they were ready for tissue processing (dehydration, clarification, paraffin infiltration) using the TP1020 Automatic Tissue Processor (Leica, Microsystems Ireland). Samples were then individually paraffin embedded in cassettes using the EG1150H Heated Paraffin Embedding Module (Leica Microsystems).

The remaining oesophagi were cut longitudinally to expose the squamous mucosa, each oesophagus was cut into 4 small biopsies of similar size and placed into 200 μ L supplemented DMEM. The 4 biopsies were treated in the following manner: unstimulated, Pam2 CSK4 (500 ng/mL), anti-TLR2 blocking antibody (1 μ g/mL) and NLRP3 inhibitor MCC950 (10 μ M). Biopsies were cultured for 18 hours (37 °C, 5% CO₂),

before supernatants were collected and frozen (-80 °C). Biopsies were placed into 220 µL RIPA buffer on ice before homogenisation using a bench-top rotor-stator homogeniser (Ultra-Turrax, Janke & Kunkel). Oesophageal homogenates were centrifuged (20,000 x g, 10 mins, 4 °C), the pellet was discarded, and the lysates were stored at -80 °C.

Quantification of biopsy cytokine secretion was performed by ELISA and normalised to total protein content of explant tissue using the BCA assay method.

Table 2.15: Formulation for rodent diet with 60 kcal% fat

Class description	Ingredient	Grams
Protein	Casein, lactic, 30 mesh	200
Protein	Cystine, L	3
Carbohydrate	Lodex 10	125
Carbohydrate	Sucrose, fine granulated	72.8
Fibre	Solka Floc, FCC200	50
Fat	Lard	245
Fat	Soybean oil, USP	25
Mineral	S10026B	50
Vitamin	Choline Bitartrate	2
Vitamin	V10001C	1
Dye	Dye, blue FD&C #1, alum. lake 35-42%	0.05
Caloric information		
<u>Composition</u>	<u>HFD feed</u>	<u>SFD feed</u>
Protein	20%	25%
Fat	60%	13%
Carbohydrate	20%	62%
Energy density	5.21 kcal/g	4.07 kcal/g

2.7 Barrett's oesophagus mouse model

All Barrett's-related murine research studies were performed in collaboration with Dr. Michael Quante and his research lab, AG Quante, in the Technische Universität München (TUM), Germany. All animal experiments were carried out in accordance with German Animal Welfare and Ethical Guidelines and were ethically approved by the District Government of Bavaria.

2.7.1 Mice phenotypes

1. pL2-IL-1 β

This mouse model was originally described by Quante *et al.*, 2012, as a novel, transgenic model for the study of Barrett's and OAC (263). This model was established using the Epstein-Barr virus (EBV) ED-L2 transgene that specifically targets stratified epithelium of mice (296, 376). The specificity of the EBV construct to only stratified squamous epithelial (keratinocytes) and not non-squamous cells is due to the presence of a CACCC box-like cis-regulatory element located on the ED-L2 promoter (between -218 to -187). A transcription factor that uniquely functions in keratinocytes, called keratinocyte specific factor (KSF) binds the CACCC box element, causing transcriptional upregulation of the transgene (377). Aside from the Barrett's model, this keratinocyte specificity has been exploited to target oncogenes in other transgenic models, including oral-oesophageal, gastric and skin (181, 296, 378-380).

In the Barrett's mouse model, the transgene contains a human IL-1 β cDNA insert which is fused with a signal sequence from the structurally similar IL-1RA, to aid extracellular release (381). This was sub-cloned alongside a hGH/polyadenylation sequence into a pB-KS(+) vector, downstream of the Epstein-Barr lytic cycle promoter; ED-L2 (**Figure 2.2**). The cDNA encodes for the mature form of human IL-1 β (17 kDa), meaning it does not require caspase-1 cleavage and is constitutively expressed (181, 382). Founder mice were first developed by inserting the construct via pronuclear injection into fertilised oocytes before injecting into C57BL/6 x SJL F2 mice (263). All Barrett's transgenic mice originated from a C57/B6 line purchased from Jackson laboratories.

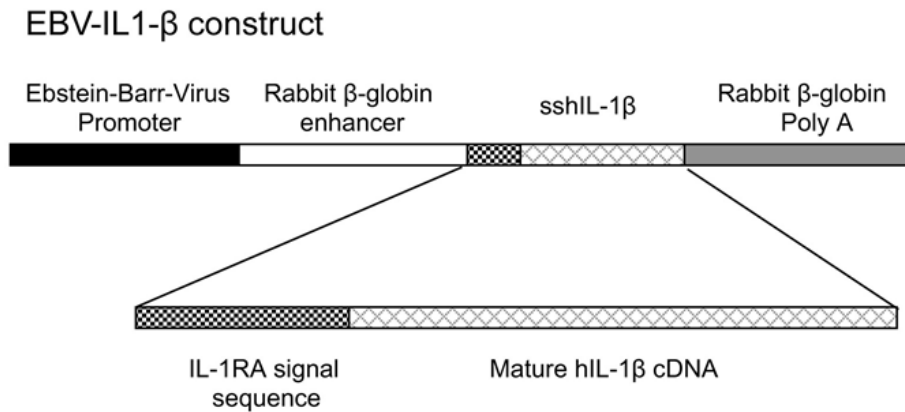


Figure 2.2: Epstein-Barr virus (EBV)-IL-1 β construct containing mature form of human IL-1 β cDNA downstream of the promoter.

Figure from Quante *et al.*, 2012. *Cancer cell* (263).

2. High Fat Diet pL2-IL-1 β

HFD-fed pL2-IL-1 β weigh less than their standard fat diet (SFD) pL2-IL-1 β mice counterparts at 9 and 12 months (364). These mice develop a characteristic oily coat because of their high palm oil diet. HFD-fed mice are from the Freiseng facility in TUM, Munich. Following weaning at approximately 6-8 weeks the mice were fed with a special high fat chow (Ssniff spezialdiäten), (**Table 2.16**). HFD-fed pL2-IL-1 β mice were fed a 48% palm oil diet, compared to 12% fat in SFD-fed mice. HFDs have a metabolizable energy of 19.7 MJ/kg, compared to SFDs at 14 MJ/kg. Therefore, there is a 30% energy increase between diets.

Table 2.16: Standard chow versus high fat chow

Dietary components	SFD	HFD Palm oil	Crude Nutrients (%)	SFD	HFD Palm oil
Gross energy (GE)	16.7 MJ/kg	21.9 MJ/kg	Protein	22	21.2
Metabolizable energy (ME)	14.0 MJ/kg	19.7 MJ/kg	Fat	4.5	25.1
Protein [kJ%]	27	18	Fibre	3.9	5.0
Fat [kJ%]	12	48	Ash	6.2	5.3
Carbohydrate [kJ%]	61	34	Starch	34.2	26.7
			Sugar	5.1	6.1

Amino Acids [%]				N free extracts	51.2	37.7
Lysine	1.5	1.8				
Methionine	0.5	0.8		Vitamins [IU/kg]		
Met + Cys	0.4	1.1		Vitamin A	25,000	18,000
Threonine	0.9	0.9		Vitamin D ₃	1,500	1,800
Tryptophan	0.3	0.3		Vitamin E	135	180
Fatty acids [%]				Minerals [%]		
C12:0	-	0.01		Calcium	1.0	0.9
C14:0	0.01	0.21		Phosphorus	0.7	0.7
C16:0	0.54	9.18		Sodium	0.2	0.2
C18:0	0.14	1.11		Magnesium	0.2	0.2
C20:0	0.02	0.1				
C16:1	0.02	0.1				
C18:1	1.03	9.19				
C18:2	2.42	4.67				
C18:3	0.28	0.35				

*Table taken and adapted from Munch *et al.*, *Gastroenterology*, 2019 (32).

3. pL2-IL-1 β -Lgr5CreTM/KRas^{G12D}/Rb1^{fl/fl} (pL2-LKR) mice

In order to understand the importance of genetic alterations in stem cells at the gastro-oesophageal junction (GOJ), the pL2-IL-1 β mouse model was crossed with Lgr5CreTM mice followed by combination with the KRas^{G12D} and Rb1^{fl/fl} mice to generate pL2-LKR (pL2-IL-1 β /Lgr5CreTM/KRas^{G12D}/Rb1^{fl/fl}) mice, an accelerated model of OAC. This model is not yet published by Quante's lab (Akanksha Anand).

Kras and Rb expression were exclusively targeted in Lgr5/GPR49⁺ cells using the Cre-Lox recombination system with tamoxifen (CreTM). Lgr5 is a validated intestinal stem cell marker found on the columnar epithelial-lined stomach and gastric cardia of mice, but not in the normal squamous epithelium of the oesophagus and forestomach (263, 383). Gene sequences are 'floxed' between two LoxP sites which become excised at Cre-recombinase expression.

In the case of KRAS gene knock-in, the KRAS^{G12D} stop codon was 'floxed' with two LoxP flanks. Excision of the stop codon during Cre-mediated recombination leads to localised

oncogenic expression of Kras. Conversely for the RB1 gene knock-out, a functional part of the sequence was ‘floxed’ and Rb functionality was removed at recombination (**Figure 2.3**).

Activation of Cre-recombinase is considered conditional gene targeting, as it allows for the removal of a gene at a specific time and tissue. All mice of this phenotype used in this chapter were administered Tamoxifen via oral gavage at 4 months old to activate the expression of Cre-recombinase in a *Lgr5*⁺ specific manner. Five pL2-LKR mice were sacrificed, and the Barrett’s tissue was IHC stained for caspase-1 expression for comparison with the pL2-IL-1 β mice.

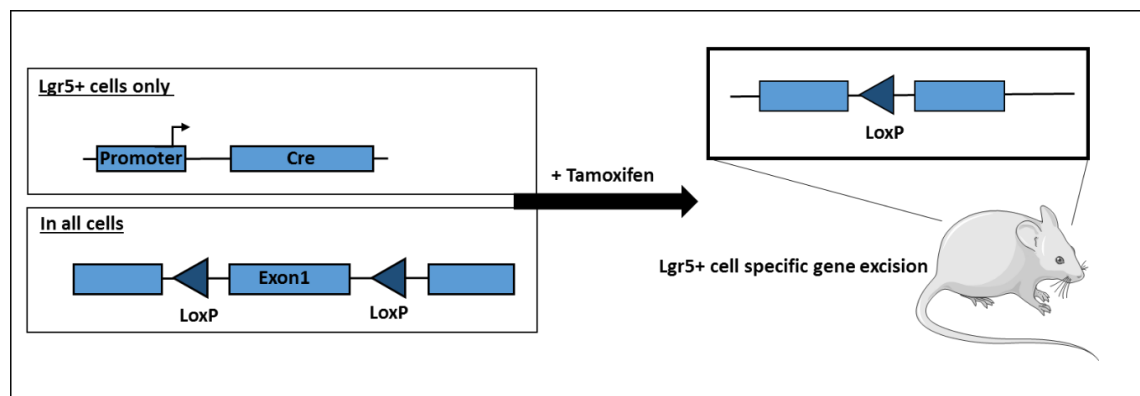


Figure 2.3: Depiction of *Lgr5*⁺ cell specific excision of target genes in pL2-IL-1 β mice using the Cre-LoxP recombination system.

1. pL2-IL-1 β -FXR^{-/-}

Farnesoid X receptor (FXR) is a bile acid receptor that has been shown to have anti-inflammatory functions in mice (227, 228). The pL2-IL-1 β -FXR^{-/-} phenotype was created by crossing the pL2-IL-1 β phenotype with a FXR^{-/-} mouse model containing a whole body, germline knock-out. From previous research carried out in the Quante lab, increased inflammation and accelerated progression has been observed in this phenotype in the progression to Barrett’s and OAC (Quante lab, unpublished). FXR^{-/-} mice were constructed as previously described by Sinal *et al*, 2000 (384). Briefly, transgenic mice expressing the P1 Cre recombinase bacteriophage under the adenovirus promoter, EIIA were crossed with homozygous mice for the FXR target allele. The ligand binding domain (AA 406-484) and the poly(A) signal sequence (AA 1228-1779) were the deleted FXR gene exons. DNA and protein analysis of liver tissue confirmed the FXR null

mice (384). FXR^{-/-} mice were subsequently crossed with pL2-IL-1 β mice and the genotype was confirmed (Quante, unpublished).

Genotyping

DNA was isolated from the tail tips taken at approximately 3-4 weeks and digested using 200 μ L 1x PCR Tail lysis buffer with Proteinase K (overnight, 55 °C), isolated and analysed via PCR analysis before running on a 2% (w/v) TAE agarose gel. Performed by Quante lab.

Table 2.17: Genotyping primers

Gene	Primer sequence (5'-3')	
	Forward	Reverse
pL2-IL-1 β	CTT CCT GTT CCA TTC AGA GAC GAT	CTC CAG CTG TAG AGT GGG CTT ATC
FXR (WT)	TCT CTT TAA GTG ATG ACG GGA ATC T	GCA TGC TCT GTT CAT AAA CGC CAT
FXR forward (Null)	GCT CTA AGG AGA GTC ACT TGT GCA	

2.7.2 Histology

2.7.2.1 Tissue processing

All mice were sacrificed by isoflurane inhalation. The mice were placed in a chamber containing tissue soaked in isoflurane for approximately 10 mins. The mice were checked for signs of death by pinching the back feet and end of the tail for signs of reflex. The mice were dissected followed by resection of the stomach and oesophagus together. A small opening present where the duodenum was cut from the stomach provided the starting point where the stomach was cut open along the greater curvature. It was cut from the antrum of the stomach around towards the distal oesophagus before being cut upwards to open the oesophagus longitudinally. This was then spread out on a surface (see **Figure 2.4**) The organs were subsequently fixed either in 4% paraffin formaldehyde (PFA) for 24 hours (phenotypes; wild-type, pL2-IL-1 β , -Kras) or in zinc formalin fixative for 1 week (phenotypes: pL2-IL-1 β -FXR^{-/-}, - HFD). Zinc formalin was used in FXR^{-/-} and HFD phenotypes instead of PFA following previous lab optimisations showing better tissue structure visualisation and antigen retrieval in these

inflammatory and fatty tissues. Following fixation, the tissue was dehydrated with ethanol and xylene in the Leica ASP 300S. The tissue was then stored at 60 °C before embedding was carried out in the Leica EG 1150H, completing the formalin-fixed, paraffin-embedded (FFPE) blocks.

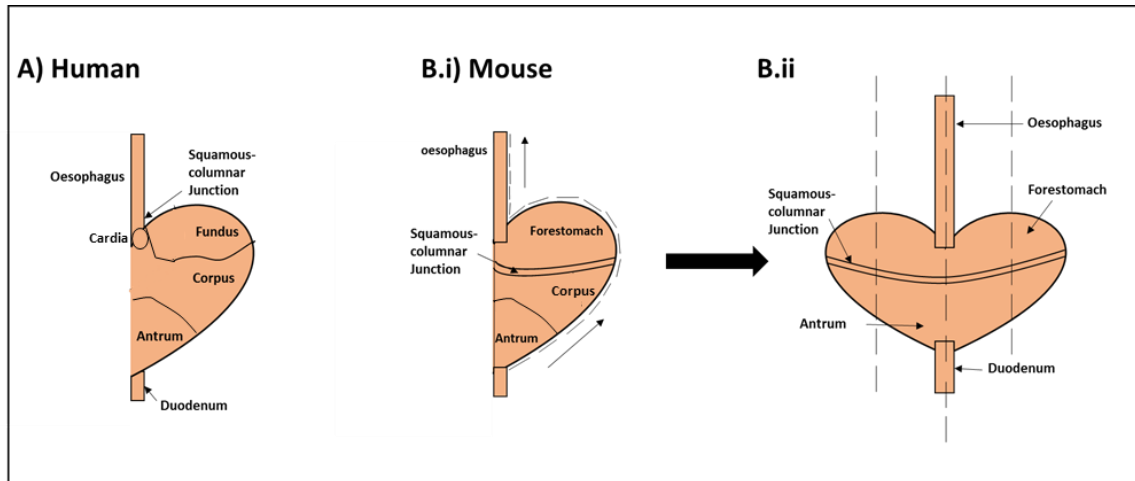


Figure 2.4: Diagram depicting the physiology of the human and mouse stomach.

The squamocolumnar junction (SCJ) marks the region where stratified squamous epithelium is replaced by columnar epithelium. **A)** Physiology of the human stomach. The junction in humans is less defined than in mice. **B.i)** Physiology of the mouse stomach. Resection of stomach starts at the opening where the duodenum joins to the oesophagus, cutting then continues upwards around the anterior of the stomach reaching the distal oesophagus. **B.ii)** Physiology of the stomach when opened out. The SCJ separates the forestomach from the stomach and is where Barrett's and tumours are located. Tissue was cut longitudinally (represented by broken lines) before embedding.

2.7.2.2 Sectioning

All FFPE blocks were stored at RT. Prior to sectioning, the blocks were placed at -20 °C for 1 hour to cool. Sections (3-4 µm) were cut using a microtome (Microm HM 355S, Thermo Scientific) and carefully displaced to a water bath (45 °C) (Kunz instruments HIR-3), before placing on a glass slide (Super Frost Plus, Thermo Scientific) and dried overnight (37 °C). The slides were then heated (1 hour, 60 °C, Heraeus Instruments Function Line incubator) and stored at RT.

2.7.2.3 Haematoxylin and Eosin (H&E) staining

The standard H&E stain is a two-dye stain. The first is Haematoxylin, a dark purple dye which stains the chromatin within the nucleus a dark purple/blue colour. The second is

Eosin, a dark orange/red dye which stains the cytoplasmic features of the cell in various shades of red, pink and orange.

Slides were deparaffinised in xylene (10 mins) before progressive rehydration in 100%, 96%, 70% (v/v) ethanol for 10 mins, and rehydration in dH₂O for 5 mins. Slides were dyed in Haematoxylin (2 mins), rinsed under running tap water (3 mins) and counterstained with eosin (2 mins) before rinsing again under running tap water (3 mins). Progressive dehydration in 100%, 96%, 70% (v/v) ethanol before xylene (10 mins) was performed. Slides were mounted (VectorMount Mounting Medium) and dried overnight in a fume hood. H&E was scored by Dr. Michael Quante using an adopted scoring method from a scoring system previously described by Fox *et al.*, 2000 (385),

Table 2.18.

Histological inflammation represents a score of all immune cells within a defined area of tissue around the SCJ, predominantly made up of neutrophil myeloid cells. Metaplasia was assessed through identification of mucous producing cells per gland, and the number of glands with mucous producing cells in the Barrett's area. Dysplasia was assessed by evaluating cellular atypia in the presence of low- and high-grade dysplasia within each gland. Goblet cell ratio was determined as a percentage of goblet cell positivity within each crypt in the metaplastic Barrett's region, previously described by Schellnegger *et al.*, 2017 (386).

Table 2.18: Scoring method for H&E staining

H&E score	Inflammation	Metaplasia	Dysplasia
0	No inflammation No immune cell influx	No metaplasia	No dysplasia
1	Mild inflammation Up to 10 immune cells.	Rare mucous cells	Superficial epithelial atypia
2	Moderate inflammation Up to 30 immune cells	Single metaplastic glands	Atypia in granular complexity
3	Severe inflammation More than 30 immune cells	Multiple metaplastic glands	Low-grade dysplasia
4	-	-	High-grade dysplasia

Adapted from Munch, S. *et al. Gastroenterology*, 2019 (32).

Table 2.19: Dysplasia scoring system for mice histology

Score	Dysplasia	Description
0	No neoplastic progression	Crypt structure preserved with well-spaced glands Minimal atypia Regular nuclei, situated basally and aligned, smooth membranes Abundant lamina propria between glands Mild distortion in the presence of evident inflammation.
1	Superficial epithelial atypia	Crypt structure maintained Contains mucous producing cells Selected single cells in individual glands have cellular and nuclear atypia such as; mild nuclear enlargement and elongation, basally located
2	Atypia in granular complexity	Relatively preserved crypt structure with some alterations Contains mucous-producing cells Atypia spanning several cells in multiple glands such as; slight nuclei enlargement and elongation with some overlap and stratification, basally located. Mild crowding

3	Low grade dysplasia	Some alterations in crypt structure May have reduced lamina propria and mucous-producing cell number Slight nuclear enlargement and misalignment, with some loss of polarity Hyperchromatic nuclei with mild crowding and stratification Some increased mitosis
4	High grade dysplasia	Distorted architecture, crypt structure and size irregularity Budding and crowding (back-to-back) crypts Severely reduced lamina propria Loss of nuclear polarity, markedly enlarged nuclei with overlapping, pleomorphism, hyperchromatism Increased mitosis Dysplastic epithelium on mucosal surface Loss of some mucous producing cells

2.7.2.4 Immunohistochemistry

Sections were deparaffinised in xylene (10 mins) before progressive rehydration in 100%, 96%, 70% (v/v) ethanol (10 mins) followed by dH₂O (5 mins).

Heated antigen retrieval was carried out in 10 mM citrate buffer, pH 6.0 in a microwave (5 min, 900 W, followed by 10 min, 400 W), and cooled (20 mins, RT).

Sections were washed with PBST (10 mins), drawn around with a hydrophobic pen (OmmEdge pen) and placed in a wet chamber. Endogenous peroxidases were quenched with 3% H₂O₂ PBS (10 mins), washed in PBST (5 mins) and blocked for 1 hour at RT in blocking solution (3% BSA/PBS, 5% normal goat serum, 4 drops Streptavidin/1 mL blocking solution (Vector laboratories)). All slides were washed again in PBST (5 mins) before incubation with primary antibody (RT, 30 mins) (**Table 2.20**) in 3% BSA/PBS and 4 drops Biotin/1 mL (Vector Laboratories).

Slides were washed in PBST (3x5 mins) before incubation with relevant secondary (30 mins), before washing again (PBST, 3x5 mins). Negative controls were included during each staining day (omission of primary antibody). Incubation of tissue sections with

secondary antibody alone determined whether any unspecific binding of secondary antibody was occurring within the tissue and therefore causing false positive results.

HRP staining was carried using ABC solution (ABC-kit Elite Vectastain, Vector laboratories) for 30 mins, RT, followed by washing (PBST, 3x5 mins).

DAB (3,3-diaminobenzidine) chromogen was used as the substrate for the HRP reaction producing a dark brown colour for positivity (DAB-kit, Vector laboratories). The solution was made up directly before use (1 mL dH₂O, 20 µL buffer, 20 µL H₂O₂ and 40 µL DAB). DAB (100 µL) was applied to each slide for 2 mins and washed in dH₂O.

Slides were counterstained in haematoxylin (30 secs) and washed under warm running tap water. Following this, slides were dehydrated in 70%, 96%, 100% ethanol, before incubation in xylene (10 min). Slides were mounted (VectorMount Mounting Medium) and dried overnight in the fume hood.

Table 2.20: Antibodies for IHC murine studies

Name (anti-)	Features	Dilution	Source
Caspase-1	• Anti-rabbit • ab74279	1:50	Abcam
Ki-67	• Anti-rabbit • ab15580	1:1000	Abcam
TLR2	• Anti-mouse • OPN-301	1:300	Opsona Therapeutics
AIM2	• Anti-rabbit • 14-6008-93	1:1000	Biosciences

2.7.2.5 Immunohistochemistry analysis

Quantification and imaging were performed using either NDP.view2 image viewing software (Hamamatsu) scanned in the Pathology department, St. James's Hospital, Dublin or using Aperio ImageScope viewing software, version 12.3.2.8013 (Leica Biosystems) scanned in Rechts der Isar Hospital, TUM, Munich. Scoring was carried out using a validated semi-quantitative scoring system by 2 blinded reviewers. Barrett's columnar epithelium was scored for percentage (%) positivity within the Barrett's region. If no metaplasia was present, an automatic score of zero was given. Stroma was assessed by scoring percentage (%) positivity of the supportive tissue directly surrounding the Barrett's columnar epithelium at the SCJ. IHC performed in Munich was

assessed for positivity (%) alone. IHC performed in Dublin was carried out on the same day and was assessed for both positivity (%) and intensity (**Table 2.21**). An IxP score was calculated by multiplying positivity and intensity to give an overall score.

Table 2.21: Intensity scoring system for IHC analysis

Intensity score	Staining intensity
0	Negative
1	Mild
2	Moderate
3	Strong

2.8 Statistical analysis

Data was analysed using Excel Microsoft Office 365 ProPlus and Prism software, version 5 (GraphPad). Error bars indicate standard error of the mean (SEM). For both *in vitro* and *in vivo* studies, statistical analysis between the means of two groups was analysed using the unpaired or paired Student's *t* test. When three or more variables were present, the one-way analysis of variance (ANOVA) was used. The Tukey honest significant difference (HSD) multiple comparison test was then applied as a post hoc test to determine which means differ significantly. For human clinical data, differences in parameters between two groups were determined using the unpaired Mann Whitney *U*-test for unmatched groups, while matched patient groups were analysed using the Wilcoxon signed rank test. Correlation analysis was undertaken using the Spearman's correlation coefficient. $p < 0.05$ was considered statistically significant.

Sample sizes varied between models. All *in vitro* cell line work had little variation; therefore, experiments were repeated three times to ensure the results were reproducible. OAC data showed large patient-to-patient variability, therefore sample size was approximately 30 patients per group to reduce SEM and increase the accuracy of results. A sample size of 10 Barrett's patient's explants was determined as an acceptable preliminary representation of findings based on statistics from previous studies in the Department of Surgery, St. James's Hospital. Patient sample size numbers were also limited by access to patient samples. Mouse trials consisted of 4 or 5 mice per experimental group. These numbers were used based on pre-trial sample size and

power calculations. The HFD mouse trial in collaboration with Lynch's lab was carried out twice to ensure results could be reproduced.

2.9 Supplementary methods

The following methods were performed by another individual and analysis was carried out by Gillian Barber. Therefore, these methods have been included in a supplementary section.

Table 2.22: Supplementary materials

Product	Source
Accutase	Life Technologies
Anti-caspase-1 antibody	Santa Cruz
Anti-caspase-4 antibody	MBL
Anti-caspase-5 antibody	MBL
Anti-Ki-67 antibody	DAKO
B27	Life Technologies
Bond Epitope Retrieval Solution I	Leica Biosystems
Bond Primary Antibody Diluent	Leica Biosystems
Bond Polymer Refine Detection Kit	Leica Biosystems
70 µm cell strainer	BD Biosciences
DMEM/F12	Life Technologies
FBS	Life Technologies
Glutamax-I supplement	Life Technologies
HEPES	Life Technologies
LPS	Sigma Aldrich
L-WRN cell line	ATCC
Matrigel	BD Biosciences
N2 supplement	Life Technologies
N-acetylcysteine	Sigma Aldrich
Nigericin	Invivogen
OPN-301 blocking antibody	Gift from Opsona Therapeutics
Pam2 CSK4	Invivogen
PBS	Life Technologies
Pen/Strep	Life Technologies
Recombinant epithelial growth factor	Peprtech
Serum-free B27	Life Technologies
WEHD.CHO inhibitor	Bachem

2.9.1 Human resected tissue from OAC patients

Staining was carried out in Beaumont Hospital. Kasia Oficjalska and James Phelan scored caspase IHC. Gillian Barber and Hazel O'Neill scored Ki-67 IHC. Gillian Barber carried out analysis.

2.9.1.1 Ethics, patient consent and oesophageal biopsy acquisition

All patients gave informed written consent for use of their tissue in this study. Ethical approval was granted by the St. James's Hospital/ Tallaght University Hospital Review Board. All patients considered for inclusion were histologically confirmed with Barrett's or Barrett's associated-OAC and were prospectively recruited as part of the National Barrett's Registry and the National referral centre for upper GI malignancy, Ireland. Exclusion criteria for this study were patients with previous history of any malignancy of any type, including any form of OAC, and any ablative therapies. Tissue from OAC patients was taken from both treatment naïve (n=19) and neoadjuvant treated (n=13) patients undergoing surgical oesophagectomy. Areas of normal, metaplasia and OAC were identified by a trained pathologist (Prof. Elaine Kay, RCSI, Ireland). Three cores were sampled from each area of tissue to account for heterogeneity.

2.9.1.2 Immunohistochemistry

Human OAC tissue was formalin-fixed, paraffin-embedded and tissue micro-arrays (TMAs) were constructed. IHC staining of 4 µm tissue sections was performed using the Leica Bond-III fully automated tissue stainer (Leica Biosystems, Newcastle upon Tyne, UK). Slides were dewaxed before pre-treatment with Bond Epitope Retrieval Solution I. Primary antibody (anti-caspase-1, Santa Cruz, 1:250 dilution; anti-caspase-4, MBL, 1:400 dilution; anti-caspase-5, MBL, 1:100 dilution) was diluted in Bond Primary Antibody Diluent. Detection and visualisation of stained cells was achieved using the Bond Polymer Refine Detection Kit, using DAB as the chromogen. Counter-staining was achieved with light haematoxylin staining. Negative controls were included (secondary antibody alone). Caspase expression was assessed in the stroma and the epithelium by two blinded reviewers (Kasia Oficjalska, James Phelan) using a validated semi-quantitative scoring method, where both intensity and positivity were scored and multiplied (IxP) (**Table 2.23**), as described previously by J.J. Phelan *et al.*, 2014 (387). This scoring system was developed by the Department of Surgery in St. James's Hospital

as an effective method of determining and analysing IHC results. The scoring methodology has been discussed with the respected R. Fitzgerald's oesophageal research group in Cambridge and has been further validated through the peer-review process by publication in scientific journals (387-390). Statistical analysis of scores were performed by Gillian Barber.

Table 2.23: Scoring system for patient oesophageal IHC analysis.

Intensity						
Negative (0)	Mild (1)		Moderate (2)		Strong (3)	
Positivity %						
0	10	25	50	75	90	100
Intensity multiplied by positivity (IxP)			Min score: 0		Max score: 300	

2.9.1.3 Ki-67 IHC staining

Cellular proliferation was assessed by Ki-67 staining (Dako).

Similar to above, patient tissue samples were sourced from prospectively recruited and consented patients in the National Barrett's Registry and the National referral centre for upper GI malignancy. Resected tissue was derived from patients undergoing surgical oesophagectomy for OAC treatment. Tissue areas of normal adjacent squamous tissue, metaplastic tissue and OAC tumour were identified by pathologist Prof. Elaine Kay (RSCI, Ireland). Immunostaining was performed as described above, where paraffin-embedded slides were dewaxed and stained using Leica Bond-III fully automated tissue stainer (Leica Biosystems, Newcastle upon Tyne, UK). DAB was used as the chromogen, and tissue was counter-stained with haematoxylin. All slides were scanned using a Hamamatsu NanoZoomer-XR Digital slide scanner in the Department of Histopathology, St. James's Hospital. Viewing software (Hamamatsu NDP.view2) was used in the visualisation and analysis of slides. Immunoreactivity was assessed digitally under 40X magnification in a semi-quantitative manner by two blinded reviewers (Gillian Barber, Hazel O'Neill). Epithelial cells were evaluated and graded based on staining positivity (Scale: 0, 10, 25, 50, 75, 90, 100%). Three tissue samples were taken per disease area

for each patient, which were stained and assessed to reduce inpatient sampling variability. These scores were then averaged to give a representative value.

2.9.2 Murine three-dimensional organoid culture

For the purpose of this study, tissue was taken from the SCJ of pL2-IL-1 β mice and processed for organoid culture as previously described and characterised by Quante's research team (391). All culture of organoids was carried out at TUM, Munich (A. Anand). Experimental design, sample processing and analysis was performed in TBSI, Dublin (G. Barber).

Long term culture of 3D *ex vivo* organoid tissue was first described by the Hans Clever research group and represented a breakthrough model system that could be used for enhanced biomedical research (392). Self-renewing stem cells are located within the bottom of intestinal crypts and can rapidly proliferate and differentiate into a multitude of intestinal cells. They identified specific Lgr5⁺ cycling crypt base columnar (CBC) cells that can be cultured long term *in vitro*, which grow and maintain intestinal crypt-like physiology (392, 393). The CBC stem cells can produce enterocytes, enteroendocrine cells and goblet cells which migrate up the crypt-villus axis. Paneth cells remain at the crypt bottom and aids differentiation. Non-epithelial cells are absent (392, 393). The growth of cardia organoids used in this study was dependent on specifically tailored supplemented media which resulted in the expansion of Lgr5⁺ intestinal metaplasia epithelial cells present at the SCJ. Wnt signalling, mediated by R-spondin 1, is essential for crypt proliferation (394-396). Epidermal growth factor (EGF) is required for proliferation and long term expansion (397), and Noggin is necessary for crypt expansion, and is required for successful passaging of organoids (392, 398). Growth of gastric and cardia organoids specifically and strictly requires Wnt3a for Wnt signalling in local stem cells (383, 399, 400). Other supplements such as N2, B27 and N-acetylcysteine are commonly used during organoid culture and were used in this experiment. N2 is a formulation based on Bottenstein's original N2 formula and is used in serum free media for growing post-mitotic cells (401, 402). B27 is a supplement formulation originally optimised by Brewer *et al.* for growth of neuronal cell is important

in organoid culture for growth and increasing spheroid forming efficiency (403-405). N-acetylcysteine is a ROS scavenging antioxidant, and provides a source of cysteine for glutathione generation (406). Gastrin and fibroblast growth factor (FGF)10 are required for the growth of murine gastric organoids but were not required in this culture (383, 407).

Isolation

Mice (pL2-IL-1 β) were raised in the TUM campus facility (Zentrum für Präklinische Forschung, mouserroom 3, Building 543, 45.0.41) and were sacrificed by isoflurane inhalation at 12 months old. The stomachs of the pL2-IL-1 β mice were resected and opened along the outer curvature to expose the epithelium. To isolate tissue from the SCJ, the stomach was flattened on a hard surface and the cardia region was excised. The tissue was washed thoroughly with PBS to remove any food particles and was subsequently chopped into small pieces. To aid tissue disruption, the tissue was transferred to a 1.5 mL tube containing 200-300 μ L cell detachment reagent, Accutase, and incubated on the shaker (15 mins, RT). The tissue was transferred into a 20 mL cold dissociation mix (sterile PBS with EDTA (2 mM, ethylenediaminetetraacetic acid) and EGTA (2 mM, ethyleneglycoltetraacetic acid)) and incubated on a shaker on ice (45 mins) to further detach and separate individual tissue layers and crypts.

Moving to a sterile tissue culture hood, the tissue was allowed to sediment in a 50 mL falcon tube before the supernatant was carefully removed and replaced with 10 mL cold PBS/10% FBS. To avoid tissue sticking to the inside of pipette surfaces, pipette pre-wetting was carried out with PBS/FBS. The tissue suspension was pipetted up and down 5-6 times to further break down the tissue layers. The tissue pieces were allowed to settle before collecting the supernatant containing dissociated crypts and filtered through a 70 μ m strainer. Oesophageal crypts are small and pass through the strainer, while small tissue fragments stay in the strainer.

A further 10 mL PBS/10% FBS was added to the tissue pieces and the suspension was mixed and the supernatant strained 3 more times to give a total volume of 40 mL. Following this, the tissue pieces were discarded and the remaining 40 mL suspension was centrifuged for 10 mins (500 x g, 4 °C). The supernatant was discarded, and the

pellet was resuspended in 300 μ L Matrigel (BD Biosciences). Drops of Matrigel (50 μ L) were gently placed into the middle of the wells of a prewarmed 24-well plate, avoiding air bubbles. This was allowed to solidify at 37 °C (2-3 mins) before adding 500 μ L conditioned L-WRN media (details described below) and left to grow in an incubator at 37 °C, 5% CO₂.

Maintenance

To provide a source of Wnt3a for Wnt signalling, the L-WRN cell line (ATCC) was cultured for 24 hours (37 °C, 5% CO₂, 100% confluency) in advanced DMEM/F12 (Life Technologies), HEPES (10 μ M, Life Technologies), Pen/Strep (500 μ g/mL, Life Technologies) and Glutamax-I supplement (1:100, Life Technologies) (408). Culture of the L-WRN cell line also provided a source of essential proteins, R-spondin and Noggin required for organoid growth and maintaining crypt structure (391, 392).

Conditioned L-WRN media was collected, centrifuged (2000 x g, 5 mins) to remove debris before freezing in 50 mL aliquots until required for culture. When needed, the L-WRN media was defrosted and diluted by 50% with fresh supplemented DMEM/F12 media (as described above), before supplementing with recombinant murine EGF (50 ng/mL, Peprotech), N-acetylcysteine (50 mM, Sigma), serum free B27 (1:50, Life Technologies) and N2 (1:100, Life Technologies) for use with organoid culture.

As organoids grow, they begin to bud and mature epithelial cells shed into the lumen. They are ready for passaging when they grow larger, have a dark central lumen when viewed under the microscope and have multiple crypt domains. Organoids were passaged once a week for 3 weeks to break up the organoids. To passage, the media was removed and 1 mL cold PBS/10% FBS was added to each well, incubated on ice before pipetting several times to disrupt the Matrigel. The crypt Matrigel suspension was transferred to one 50 mL tube on ice. PBS/ 10% FBS was added to the tube to give a total volume of 40 mL and pipetted 8-10 times, before centrifuging (10 mins, 500 x g, 4 °C). The supernatant was discarded, and the pellet resuspended in 600 μ L Matrigel to seed double the number of wells present before passaging occurred (50 μ L/well, splitting ratio 1:2).

During week 4, the media was replaced daily. Organoid treatments were performed in triplicate wells as technical repeats, and four mice were used as biological repeats to provide statistical significance. Organoids were pre-treated for 1 hour with inhibitors WEHD.CHO [50 μ M] or OPN-301 [10 μ g/mL] and stimulated with LPS [1 μ g/mL] or Pam2 CSK4 [40 ng/mL] in 400 μ L supplemented media (2x24 hour treatments). On the morning before harvesting the organoids, appropriate WEHD.CHO/LPS-treated wells were re-treated with WEHD.CHO (1 hour, 50 μ M) and stimulated with Nigericin (1 hour, 5 μ M) to activate the inflammasome. Supernatants were then carefully taken from the organoid cultures without disturbing the Matrigel, centrifuged (10 mins, 500 x g, 4 °C) to remove cellular debris and aliquoted for future analysis of excretion factors. Organoid protein lysates were extracted from the Matrigel by disruption in cold PBS/FBS (1 mL) and centrifuging (5 mins, 500 x g, 4 °C) to pellet the organoids. Protein was lysed in cold RIPA buffer (50 μ L, 10 mins), no homogenisation was required. All samples were immediately shipped to Dublin and were stored at -80 °C until required. Cytokine secretion levels were normalised to total organoid protein per well (pg/ μ g), as determined using the BCA method.

Growth curve analysis:

Organoids were assessed under a light microscope (Zeiss, Axiovert 200M) and analysed using ImageJ software (Fiji). A minimum of 20 organoids were randomly selected per well. The diameter (μ m) of each of these organoids was assessed three times and an average taken, before calculating the average organoid diameter per well. Diameters were assessed at 0 hours (before treatment begins), 24 hours (during treatment) and 48 hours (before harvest).

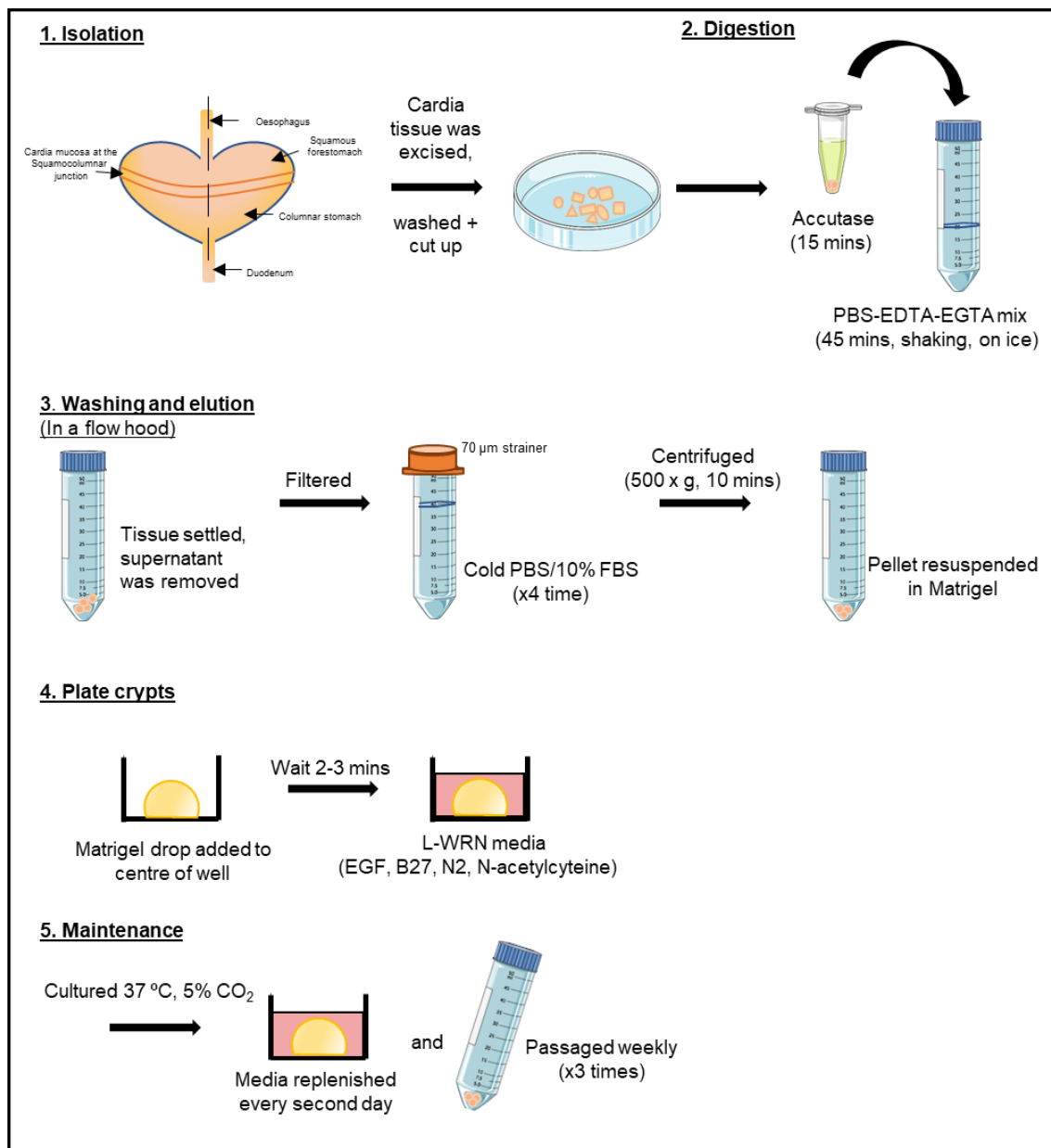


Figure 2.5: Schematic showing organoid set-up and culture.

Organoids were expanded from the SCJ at the cardia region of the stomach in pL2-IL-1 β mice. Tissue was washed and cut up before digestion in cell dissociation reagents. Crypts were isolated through washing and filtration before resuspending in Matrigel. One drop of Matrigel containing crypts was added per well and cultured in supplemented conditioned L-WRN media. Organoids were cultured continuously for 3 weeks, changing media every second day and passaging weekly. Organoids were broken up during passaging and re-seeded. During week 4, organoids were treated and stimulated for experimental investigation.

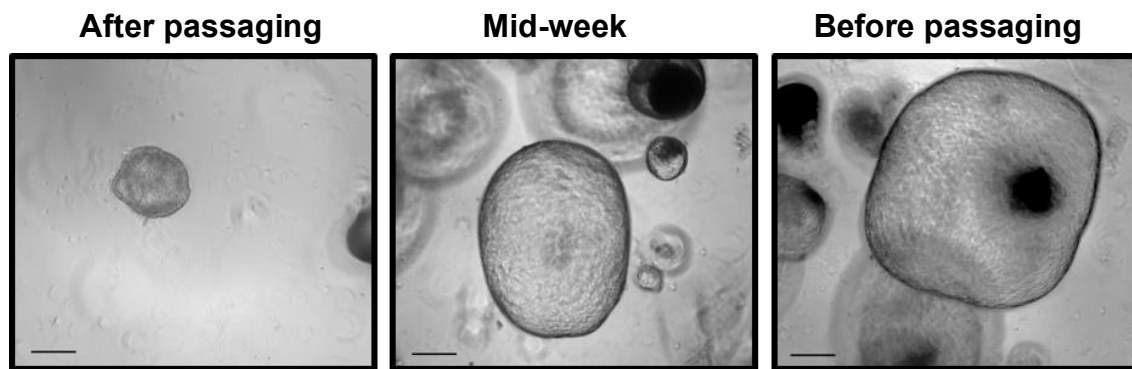


Figure 2.6: Representative images of Barrett's organoids at various stages of growth.

Images were taken under a light microscope (magnification 5X, scale bars: 200 μm), and are representative of organoid morphology: after passaging, growth during mid-week culture and before they were re-passaged. Organoids form spheroids which consist of a monolayer of columnar epithelium and contain mucous producing cells which secrete mucous into the lumen of the Barrett's organoid (dark spots), as characterised by Pastula *et al.* (391).

Chapter 3

3.1 Introduction

Oesophageal cancer is the eighth most common cancer in the world, with a low five-year survival of 15-25% (187). Barrett's oesophagus is an inflammatory, pre-neoplastic condition, and overtime chronic inflammation associated with this disease can predispose to oesophageal adenocarcinoma (OAC). Barrett's is pathologically defined by the replacement of normal squamous epithelium with specialised intestinal metaplasia in the distal oesophagus. It is thought to arise as an adaptive response to chronic oesophagitis or gastroesophageal reflux disease (GORD) (222). With disease progression, Barrett's patients may develop features of low- and high-grade dysplasia leading to an increased risk of neoplasia (254). Barrett's incidence is common, at approximately 1-2% of the European population (216, 254). However, in contrast, the annual risk of developing Barrett's-associated OAC is relatively low at 0.12%. Barrett's patients currently undergo invasive surveillance endoscopies, despite the low rates of progression (222). A better understanding of the molecular drivers of disease is required in order to identify biomarkers to predict disease progression, and new targets for preventive therapies. This in turn would allow surveillance to be concentrated on those high-risk patients and reduce the progression to high-grade dysplasia and cancer.

GORD and Barrett's arise through tissue injury incurred directly by gastric refluxate and indirectly through immune responses to these acids (409). Prolonged acid exposure in the distal oesophagus can erode the mucosa, promote chronic inflammation, immune cell infiltration and epithelial necrosis, therefore driving disease (246). Other risk factors for Barrett's include cigarette smoking and obesity (234, 343). Barrett's and OAC are considered to have immune-active microenvironments, with high numbers of T cells, dendritic cells and macrophages reported (95, 270, 410). These populations are expressed differentially across the disease sequence of pathogenesis and are mediated by heterogeneous expression of inflammatory cytokines. In Barrett's, increased levels of IL-6, IL-10 and in particular IL-1 β have been reported, caused by increased local populations of Th2 cells and macrophages (48, 93, 95, 308). While in circulation, increased levels of IL-12p70, IL-6 and IL-8 have been observed, with reduced expression of anti-inflammatory cytokines (411). Indeed, the transgenic Barrett's mouse model,

pL2-IL-1 β , overexpresses IL-1 β in the normal mucosal of the oesophagus (185). The resulting inflammation alone is enough to drive the spontaneous development of metaplasia, emphasizing a role for inflammasome-mediated inflammation. To support this, Nadatani *et al.* has shown significant priming of the NLRP3 inflammasome in Barrett's cell lines, which becomes activated and secretes IL-1 β in response to LPS (48). With exception to these studies, limited information exists regarding the mechanistic role of caspase-1 and the inflammasome pathway in Barrett's and OAC.

Caspase-1 is from the inflammatory caspase subfamily, which also includes caspase-4 and caspase-5 in humans and caspase-11 in mice. It is responsible for the direct cleavage and subsequent secretion of the potent inflammatory cytokines IL-1 β and IL-18. Additionally, caspase-1 is an important mediator of the highly inflammatory programmed cell death, pyroptosis (74). Importantly, all inflammasome pathway signalling leads to caspase-1 activation and is therefore a meaningful indicator of various forms of inflammasome-associated inflammation, including NLRP3, NLRP1, NLRP6, NLRC4 and AIM2 (51, 82). All of which can be primed and activated through different stimuli present in the distal oesophagus, resulting in highly pro-inflammatory responses.

The inflammasome pathway has been implicated in various pre-neoplastic conditions, such as liver fibrosis, inflammatory bowel disease (IBD) and chronic obstructive pulmonary disease (COPD) (172, 175, 412, 413). Caspase-1 has been shown to have conflicting roles in tumorigenesis which appear to be dependent on the cancer type. In AOM/DSS-treated mice, caspase-1 deficient mice had enhanced tumour development, suggesting a protective role for caspase-1 early in disease. This is supported by similar studies showing a protective role for IL-18 signalling during colitis-induced mouse models (150, 414). Conversely, using a breast cancer and metastasis mouse model, caspase-1 knockout mice had reduced primary tumour load and significantly less lung metastasis, suggesting a tumour enhancing role (413). These studies highlight the complexity of the inflammasome pathway in cancer.

We hypothesised that inflammasome-mediated inflammation is an important driver of disease progression to Barrett's and OAC. The main objective of this chapter was to characterise the expression patterns of inflammatory caspase-1 during oesophageal disease pathogenesis using several disease model systems. This included resected tissue

from Barrett's-associated OAC patients, a cell line model representing different stages of disease progression and a murine model of Barrett's development. Our findings show for the first time that caspase-1 is highly expressed during Barrett's metaplasia, suggesting a potential role for caspase-1 and inflammasome-mediated inflammation during the early stages of disease.

In Brief:

Hypothesis and aims of Chapter 3

Hypothesis: Inflammatory caspases are expressed at different levels across the sequence of disease progression to Barrett's and OAC.

Specific aims of Chapter 3:

1. Determine the protein expression profiles of inflammatory caspases in areas of normal, metaplasia and OAC from Barrett's-associated OAC patient tissue.
2. Examine the mRNA expression profiles of inflammatory caspases in normal, metaplastic and OAC patient tissue samples.
3. Profile inflammatory caspase expression levels using a cell line model of disease progression.
4. Compare caspase-1 expression levels in different phenotypes of the pL2-IL-1 β Barrett's mouse model.
5. Identify the caspase-1 protein expression profile during stages of disease progression in a transgenic murine model of Barrett's oesophagus.

3.2 Results

3.2.1 Patient oesophageal tissue displays elevated epithelial caspase-1 expression in areas of metaplasia.

Previous work in the lab has identified the expression profiles of inflammatory caspases during inflammatory bowel disease (IBD) and progression to colitis-associated colorectal cancer (CAC). Expression of inflammatory caspases -1, -4 and -5 were shown to be significantly elevated in the stroma of IBD and CAC patients. Stromal expression of caspase-4 and -5 correlated with histological inflammation in IBD patients, and inhibiting caspase-4 activity caused reduced pro-inflammatory cytokine secretion from IBD patient biopsy explant tissue, suggesting that they are involved in the pathogenesis of disease (415). In contrast, epithelial cells from IBD patients expressed caspase-1, but were negative for caspase-4 and -5 expression. However epithelial cells were shown to undergo a dramatic switch in expression during neoplasia, identifying epithelial caspase-4 and -5 as potential biomarkers for CRC (415).

Having identified an involvement of inflammatory caspases in IBD and CRC, we determined whether these observations would also be applicable in other inflammatory-mediated diseases of the gastrointestinal tract. We hypothesised that the inflammatory caspases -1, -4 and -5 are involved in BO pathogenesis and progression to OAC.

To test this hypothesis, histological areas of adjacent normal, Barrett's metaplasia and adenocarcinoma were defined and tissue microarrays (TMAs) were constructed from 32 Barrett's-associated OAC patients who had undergone oesophageal resection surgery in St. James's Hospital, Dublin. These TMAs were then immunohistochemistry (IHC) stained for inflammatory caspases -1, -4 and -5. Stromal and epithelial expression of these proteins were assessed by two blinded reviewers (K. Oficjalska and J. Phelan), using a validated semi-quantitative scoring method where both the intensity and the positivity were scored and multiplied (IxP).

IHC staining revealed that caspase-1 was expressed at low levels in both the epithelium and stroma of adjacent normal tissue (**Figure 3.1**). A significant increase in epithelial

caspase-1 expression was observed in areas of Barrett's metaplasia ($p<0.01$), however significantly elevated expression levels of epithelial caspase-1 were not maintained in OAC tumour tissue (**Figure 3.1A, B**). The stroma represents a score of cells within the connective tissue, beneath the epithelium, and includes all infiltrating immune cells. The graph in **Figure 3.1C** shows that there were no significant differences in levels of stromal caspase-1 between normal and disease areas. These results suggest that epithelial caspase-1 may be involved in Barrett's-associated inflammation and upregulation of this caspase may happen early in progression.

There are many studies that document the negative impact of NAT (neoadjuvant treatment-chemo-radiation therapy) on tissue inflammation, many of which predict a poorer prognosis in patients with more inflammation (416). However, the impact of NAT on caspase-1 expression has not been shown. As 35% of patients had received NAT prior to their oesophageal resection, we separated the cohort into those that had, and had not, received NAT. **Figure 3.2B** shows that treatment naïve patients had significantly increased epithelial caspase-1 expression levels in metaplastic tissue when compared with adjacent normal tissue areas ($p<0.01$). No significant differences were seen in the tumour tissue. Contrary to this, patients that received NAT showed no significant differences in epithelial caspase-1 expression across the different tissue areas (**Figure 3.2C**). Interestingly, there appears to be a trend of higher epithelial caspase-1 expression in normal and tumour tissue in NAT patients compared to treatment naïve patients however this is not significant (**Figure 3.2D**). No significant differences in stromal caspase-1 expression were observed between tissues in treatment naïve (**Figure 3.2E**) and NAT patients (**Figure 3.2F**).

In order to determine whether the observed difference in epithelial caspase-1 expression during metaplasia was due to increased proliferation, oesophageal resected tissue was IHC stained for proliferation marker, Ki-67. Subsequently, correlations between epithelial caspase-1 and Ki-67 scores were carried out (**Figure 3.3**). Ki-67 was scored by two blinded reviewers using a validated semi-quantitative scoring system (G. Barber and H. O'Neill). The representative image for normal oesophageal tissue shows that Ki-67 staining is not concentrated around the basal epithelial layer (dashed line, **Figure 3.3A**), often where regenerative stem cells are located (417). As expected, **Figure**

3.3A, B reveals a significant increase in Ki-67 expression in tumour compared to adjacent normal tissue ($p < 0.05$), confirming that neoplastic epithelial cells had increased rates of proliferation. When Ki-67 scores were separated into NAT untreated and treated, -NAT Ki-67 scores remained elevated in tumour tissue ($p < 0.05$). However, significance was lost between disease groups in +NAT treated patients (**Figure 3.3C**). This may suggest that proliferation is affected by neoadjuvant therapies. However, significance lost may also be due to less patient numbers. Using Spearman's non-parametric correlation analysis, no correlation was observed between epithelial caspase-1 and Ki-67 expression values (Spearman's correlation coefficient, $r_s = 0.1757$, **Figure 3.3D**). This indicates that differences in epithelial caspase-1 expression observed in **Figure 3.1** were not due to increased epithelial proliferation and are likely due to a transcriptional upregulation of caspase-1. In contrast, stromal caspase-1 expression significantly correlated with Ki-67 levels (**Figure 3.3E**). This may suggest that the small trend towards increased stromal caspase-1 expression in OAC tissue may be due to increased cell proliferation.

In contrast to the highly specific pattern of epithelial caspase-4 and -5 expression in malignant colon tissue (415), caspases-4 and -5 are expressed in both stromal and epithelial compartments of normal and metaplastic oesophageal tissue, as well as OAC tissue (**Figure 3.4, 3.5**). Caspase-4 expression was not differentially expressed in healthy and diseased oesophageal tissues, either in the epithelium (**Figure 3.4B**) or the stroma (**Figure 3.4C**). This suggests that caspase-4 is not highly involved in oesophageal inflammation during Barrett's and OAC pathogenesis.

Analysis of caspase-5 revealed that expression was elevated in epithelial metaplastic ($p < 0.01$) and tumour tissue ($p < 0.05$), compared to adjacent normal tissue (**Figure 3.5B**). This suggests a role for epithelial caspase-5 in Barrett's and OAC disease. Despite a similar increasing trend in caspase-5 expression levels in the stroma, differences were not found to be significant (**Figure 3.5C**). **Table 3.1** summarises the characteristics of this OAC patient cohort stating NAT status, tumour differentiation, grade and lymph nodes. Further analysis based on patient characteristics was performed and reported by K. Oficjalska.

Collectively, these findings indicate that epithelial caspase-1 and -5, but not caspase-4, may play a role in Barrett's-associated inflammation and pathogenesis to OAC.

3.2.2 Analysis of public data sets reveal altered inflammatory caspase mRNA levels in Barrett's metaplasia, compared to adjacent normal tissue.

Barrett's metaplasia is defined by a phenotypic switch of cell types in the distal oesophagus. The phenotypic switch involves the replacement of normal stratified squamous epithelium with intestinal-like columnar epithelium (245). Thus, when adjacent normal tissue is compared to Barrett's and OAC tissue, squamous and columnar epithelial cell types are being compared. While this model is currently the best representation available for use with patient samples, it is limited, as the pathological differences are not being compared within one cell type. Cells of the gastric cardia are columnar cells located at the gastroesophageal junction (GOJ) and are often difficult to define. This is also referred to as cardiac mucosa that makes up the 'transitional zone' located distal to the oesophagus, proximal to the simple columnar epithelium of the stomach and are histologically distinct (418). As discussed in the introduction (Section 1.4.4), the migration of immature progenitor cells of the gastric cardia is one of several theories to explain the cellular origin of Barrett's oesophagus (419). Cells of the gastric cardia may represent an alternative 'adjacent normal' control for the comparison of inflammatory caspase expression levels in Barrett's oesophagus. However, some studies provide evidence that this gap between the oesophagus and stomach is not present at birth and is commonly acquired by adulthood, replacing a direct transition of the oesophagus to the stomach. Therefore, rather than being 'normal' tissue, it may be a non-malignant precursor to intestinal metaplasia (219, 220, 420, 421). To compare the relative mRNA expression levels of inflammatory caspases in patient-matched tissue from areas of normal squamous, gastric cardia and Barrett's metaplasia; publicly available gene array data sets were mined and analysed using PubMed Geo Dataset software (see Section 2.4) (422). The graphs in **Figure 3.6A** represent mRNA values for *CASP1*, *CASP4* and *CASP5* within adjacent normal (squamous epithelium), normal gastric cardia (columnar mucosa) and Barrett's metaplasia (intestine-like columnar) tissue. *CASP4* mRNA expression levels were significantly lower in Barrett's tissue compared to normal squamous ($p<0.01$), while *CASP1* ($p<0.01$), *CASP4* ($p<0.05$) and *CASP5* ($p<0.01$) were higher in Barrett's tissue, when compared to gastric cardia. Inflammatory *CASP1* and *CASP4* mRNA levels are significantly lower in normal gastric cardia than in normal

squamous epithelia. This observation highlights a challenge associated with Barrett's patient tissue analysis and use of the appropriate patient matched healthy tissue for comparison.

Gene array expression values taken from a study by Kimchi *et al.* were also analysed for *CASP1*, *CASP4* and *CASP5* mRNA levels in matched oesophageal biopsies of normal squamous, Barrett's and adenocarcinoma (374). We compared a data set with mRNA levels of inflammatory caspases to see if expression levels correlated with our IHC findings (**Figure 3.1, 3.4, 3.5**). **Figure 3.6B** shows increased *CASP5* expression from normal to Barrett's ($p<0.01$) which complements the IHC data shown in **Figure 3.5**. **Figure 3.6B** also shows that no significant differences in *CASP4* mRNA were observed between normal, Barrett's and OAC tissue, which is in agreement with IHC findings observed in **Figure 3.4**. However, in contrast to our immunohistochemistry findings for caspase-1 (**Figure 3.1**), which show increased epithelial caspase-1 expression in metaplasia, no significant differences in *CASP1* mRNA were observed in this data set. Together, IHC findings from this study agree with mRNA expression levels of *CASP4* and *CASP5* from this data set, but not with *CASP1* mRNA levels.

3.2.3 Caspase-1 is strongly expressed in Barrett's metaplastic and dysplastic cell lines and is poorly expressed in OAC cell lines.

Having observed clear differences in caspase-1 and -5 expression levels by IHC staining of patients with oesophageal disease, we next sought to investigate whether the differences in expression levels could be validated in a cell line model representing the disease progression stages to OAC.

The Het-1A cell line was used as a representation of normal squamous epithelium; the QH (CP-A) cell line was derived from Barrett's metaplastic cells; GO (CP-B) cells were derived from high-grade dysplastic oesophageal cells; and the two OAC cell lines, SKGT4 and OE33 were included as representative OAC cell lines (**Figure 3.7A**). The monocytic cell line, THP-1 was used as a positive control, as these cells are known to express high levels of inflammatory caspases. Western blot analysis was performed on cell lysates

and probed for caspase-1, -4 and -5. Protein expression levels of caspase-1, -4 and -5 in each of the cell lines are represented in **Figure 3.7B, C**.

Interestingly, Western blots revealed that caspase-1 is highly expressed in metaplastic ($p < 0.01$) and dysplastic cell lines, while tumour lines have poor expression. This supports data from human oesophageal resected tissue (**Figure 3.1B**), which shows that caspase-1 is upregulated in epithelial cells during Barrett's metaplasia but not in the tumour, suggesting that caspase-1 may play a role in Barrett's-associated inflammation and disease progression. In contrast, significant differences in caspase-4 and -5 expression were not detected across the different cell lines (**Figure 3.7B**). Protein expression for caspase-4 and -5 appeared as double bands which may represent different isoforms or caspase processing and activation. Western blot results were validated in **Figure 3.7C** which represents densitometric analysis from three independent experiments. The results for caspase-4 support our previous observation in human resected tissue, where no expression differences were apparent in diseased oesophageal tissue. However, contrary to IHC results for caspase-5 (**Figure 3.5B**), which show that caspase-5 is upregulated in Barrett's and tumour tissue, no differences were evident between cell lines. It should be noted that these cell lines cannot be directly compared, are not patient matched and are only a representative model of OAC progression.

3.2.4 Caspase-1 is elevated in epithelial cells in the Barrett's region of the squamocolumnar junction in twelve-month-old pL2-IL-1 β mice.

Having determined that epithelial caspase-1 is upregulated in Barrett's resected tissue and in the QH metaplastic cell line, we next sought to determine whether caspase-1 was also upregulated in the transgenic Barrett's murine model, pL2-IL-1 β . All research carried out with the oesophageal mouse model was performed in collaboration with Dr. Michael Quante and his research team, AG Quante, in the Technische Universität München (TUM), Germany. This model is currently the only transgenic murine model available for the study of Barrett's oesophagus. It was originally generated by the Wang research group in Columbia University, New York, USA as described by Quante *et al.*,

2012 (263). The model involves a transgene insert of mature human IL-1 β which is constitutively expressed in oesophageal epithelium, as described in Section 2.7.1 (181).

The unique benefit provided by this model is the opportunity to study the development and progression of disease pathology in Barrett's metaplasia. As these mice age, disease progression occurs. **Figure 3.8** shows the histopathology of 3- and 12-month-old mice at the squamocolumnar junction (SCJ) in the distal oesophagus. At 3 months (**Figure 3.8A**), mice display almost no metaplasia or dysplasia. The normal stratified squamous epithelium meets the simple columnar cells of the stomach, displaying a well-defined SCJ. At 12 months (**Figure 3.8B**) a poorly defined SCJ is observed, which is characterised by the replacement of squamous epithelium with metaplastic columnar cells. These metaplastic cells are phenotypically different to the simple columnar cells of the stomach and are accompanied by an increase in inflammation. This metaplastic region enlarges with disease progression. Barrett's metaplasia is often histologically defined by the presence of goblet cells, as indicated by the arrow (**Figure 3.8B**).

Following results showing increased caspase-1 expression in human metaplastic tissue, we hypothesized that pL2-IL-1 β mice with a developed Barrett's phenotype will have increased epithelial expression of caspase-1, compared with wild-type (WT) mice. Mice of both groups (WT, pL2-IL-1 β) were not litter mates, which may have limited the observation of results. Oesophageal sections from 12-month-old WT and pL2-IL-1 β mice (n=5 per group) were H&E stained and assessed for disease severity by scoring each mouse for inflammation, metaplasia, dysplasia and goblet cell (GC) ratio using the scoring method outlined in Section 2.7.2.3. Scoring was performed by Dr. Michael Quante. Inflammation was assessed by scoring a defined region of tissue around the SCJ, which predominantly consisted of neutrophils. Metaplasia was scored by histological assessment for the presence of mucous cells and metaplastic glands. Dysplasia was assessed by the presence of epithelial atypia and misalignment. GC ratio was assessed by determining the presence or absence of goblet cells within each Barrett's crypt. A decreased GC ratio within the Barrett's region has been associated with an increased tumour score and risk of malignancy (386).

Histological analysis revealed that pL2-IL-1 β mice have a significantly more progressed Barrett's phenotype in comparison to WT controls at 12 months ($p<0.001$, **Figure 3.9**).

No inflammation was observed in WT mice while pL2-IL-1 β mice were found to have high levels of infiltrating immune cells. Predictably, no Barrett's region with columnar epithelium was observed in WT mice and therefore automatically received a score of zero for metaplasia, dysplasia and the GC ratio. Sections were IHC stained for caspase-1 (**Figure 3.10A, B**). Staining was assessed by two independent reviewers using a blinded semi-validated quantitative scoring method (G. Barber, A. Anand). The epithelium and stroma were scored separately for caspase-1 positivity within the Barrett's region. Representative images of caspase-1 expression are shown in **Figure 3.10A** and **B**. Columnar epithelial cells were scored for caspase-1, revealing a statistical difference in caspase-1 expression levels between WT and pL2-IL-1 β mice ($p < 0.001$, **Figure 3.10A, C**). Stromal expression of caspase-1 was assessed by scoring around the Barrett's region of mice. As expected, a significant increase in caspase-1 expression was seen in the inflamed tissue of pL2-IL-1 β mice ($p < 0.05$, **Figure 3.10B, D**).

Collectively, these results confirm that transgenic pL2-IL-1 β mice exhibit significant characteristics of Barrett's pathophysiology at 12 months. These mice have increased caspase-1 expression in both epithelial and stromal cells, when compared to WT mice, further supporting a role for caspase-1 in driving Barrett's disease progression. However, it should be noted that WT controls may not act as the most suitable comparison control for determining caspase-1 expression in pL2-IL-1 β mice, as they lacked a Barrett's region. Although parietal cells of the stomach stained strongly for caspase-1, columnar epithelium expression was zero. A more suitable control would be to compare mice of different ages and therefore difference stages of Barrett's development. This will be addressed in greater detail later in this chapter.

3.2.5 Enhanced epithelial caspase-1 expression levels in the Barrett's region from high fat diet-fed pL2-IL-1 β mice.

Obesity is a leading risk factor for developing Barrett's oesophagus (334). Visceral adipose tissue taken from OAC patients has been shown to secrete many pro-angiogenic and inflammatory factors which are thought to promote disease and tumorigenesis (423). Research by Quante and colleagues has shown that metaplastic disease

progression is accelerated in high fat diet (HFD)-fed pL2-IL-1 β mice (364). Taking these factors into consideration, we hypothesized that caspase-1 expression levels would be elevated in HFD-fed pL2-IL-1 β mice, compared to those fed on a standard fat diet (SFD).

Groups of mice (n=5) were fed a SFD or a HFD for 12 months, as described in Section 2.6.1. Oesophageal sections were H&E stained and histologically scored for inflammation, metaplasia, dysplasia and GC ratio, using the scoring systems outlined for **Figure 3.9** (detailed in Section 2.7.2.3). Histological scoring was performed by Dr. Michael Quante. Graphs in **Figure 3.11** show histological scores comparing 12-month-old pL2-IL-1 β mice fed a SFD and HFD. Scores for inflammation and metaplasia were identical for both phenotypes (**Figure 3.11A, B**). There was a trend towards increased dysplasia scores in HFD-fed mice, compared to SFD-fed mice (**Figure 3.11C**). Although dysplasia was not shown to be significantly different between these groups, research by Quante's group have shown that HFD-fed mice overall have increased dysplasia and advanced disease progression at 12 months (364). HFD pL2-IL-1 β mice had a decreased GC ratio compared to the SFD control mice ($p<0.05$), suggesting accelerated disease progression and increased cancer risk (**Figure 3.11D**).

Murine tissue sections were also IHC stained for caspase-1 expression. The epithelium and stroma were scored for caspase-1 positivity within the Barrett's region by two independent reviewers. Representative images are shown in **Figure 3.12A** and **B**. Notably, epithelial caspase-1 is increased in HFD mice in comparison to SFD mice ($p<0.05$, **Figure 3.12C**), while there was no significant difference seen in expression in the stroma (**Figure 3.12D**).

Taken together, these observations indicate that caspase-1 expression is further upregulated by a HFD and this may be contributing to Barrett's-associated inflammation during disease progression.

3.2.6 pL2-IL-1 β FXR^{-/-} mice have upregulated epithelial caspase-1 and reduced levels of stromal caspase-1 in metaplastic tissue, compared to pL2-IL-1 β mice.

Several clinical studies have shown that bile acid (BA) reflux into the oesophagus contributes to injury and disease pathogenesis in Barrett's oesophagus and OAC (424-426). Bile acids are a natural ligand for farnesoid X receptor (FXR). This is a nuclear receptor that positively regulates genes involved in BA synthesis and homeostasis. Research has also shown that FXR activation inhibits pro-inflammatory cytokine production in mice (227, 228). Interestingly, FXR^{-/-} mice backcrossed onto the pL2-IL-1 β mice have been reported to more rapidly develop Barrett's metaplasia (Quante, unpublished). In collaboration with the Quante lab, we sought to investigate whether caspase-1 was involved in inflammation observed in this model and whether caspase-1 expression was upregulated in the pL2-IL-1 β FXR^{-/-} phenotype compared to pL2-IL-1 β mice.

Groups (n=5) of pL2-IL-1 β and pL2-IL-1 β FXR^{-/-} mice were sacrificed at 12 months. Histological analysis was carried out on H&E staining, formalin-fixed, paraffin-embedded oesophageal tissue. Each mouse was scored for inflammation, metaplasia, dysplasia and GC ratio (**Figure 3.13**). Scores for inflammation and metaplasia in pL2-IL-1 β FXR^{-/-} mice were very similar to pL2-IL-1 β mice (**Figure 3.13A, B**). However, pL2-IL-1 β FXR^{-/-} mice were significantly more dysplastic, indicating an advanced pathological phenotype (**Figure 3.13C**). Supporting this, the GC ratio was lower in pL2-IL-1 β FXR^{-/-} mice (**Figure 3.13D**). This data reveals that the pL2-IL-1 β FXR^{-/-} mice have a more progressed Barrett's phenotype to their pL2-IL-1 β counterparts at 12 months, suggesting that knocking out the FXR gene has accelerated disease.

Sections were taken and IHC stained for caspase-1. The epithelium and stroma were scored for caspase-1 positivity within the Barrett's region using a validated semi-quantitative scoring method. Representative images are shown in **Figure 3.14A** and **B**. Results show caspase-1 was elevated in the epithelium of pL2-IL-1 β FXR^{-/-} mice compared to the pL2-IL-1 β mice (**Figure 3.14C**). However, pL2-IL-1 β FXR^{-/-} mice display reduced caspase-1 expression in the stroma (**Figure 3.14D**). These results indicate that caspase-1 may play a larger role within the epithelium.

3.2.7 Caspase-1 expression levels are reduced in pL2-LKR mice, compared to pL2-IL-1 β mice, at 12 months.

Disease progression in pL2-IL-1 β mice to Barrett's metaplasia occurs as the mice age. However, this progression is slow, and mice do not present with OAC-like characteristics until they are approximately 20 months (263). To develop a more rapidly dysplastic mouse model, pL2-IL-1 β mice were crossed with Kras^{G12D} and RB1 mice using the Cre-recombinase system to generate L2-LKR (L2-IL-1b/Lgr5CreTM/Kras^{G12D}/Rb1^{fl/fl}) mice described in Section 2.7.1, KRAS (Kirsten-RAS) is a proto-oncogene belonging to the RAS superfamily. KRAS mutations are prevalent in cancers such as pancreatic (>80%) and colon cancer (40-50%) but are less common in Barrett's and OAC (10.3%) (427-429). RB is a tumour suppressor gene that is involved in regulating cell division and has been shown to be inactivated in some OAC patients (~36%) (430, 431). Unpublished work in Quante's lab has shown these pL2-LKR mice progress to OAC in a shorter time period while severity of disease increases more rapidly (Quante, unpublished).

Our observations to date suggest that metaplastic oesophageal tissue and cell lines express elevated levels of caspase-1, however tumour cell lines appear to express little or no caspase-1. We therefore hypothesized that caspase-1 would be decreased in the more dysplastic pL2-LKR mouse model when compared to the metaplastic, but less dysplastic pL2-IL-1 β mice.

Groups (n=5) of pL2-LKR and pL2-IL-1 β mice were sacrificed at 12 months. Sections were histologically scored for inflammation, metaplasia, dysplasia and GC ratio (**Figure 3.15**). Histological analysis showed that inflammation and metaplasia were very similar between both mice groups (**Figure 3.15A, B**). Scores for dysplasia show that the pL2-LKR mice had a significantly more dysplastic phenotype to their pL2-IL-1 β mice counterparts (**Figure 3.15C**). A more progressed phenotype was also supported by a lower GC ratio (**Figure 3.15D**). Tissue sections were IHC stained for caspase-1 (**Figure 3.16A, B**) and scored for epithelial and stromal caspase-1 expression levels. Results revealed that both epithelial and stromal caspase-1 expression was reduced in pL2-LKR mice compared to pL2-IL-1 β mice (**Figure 3.16C, D**). This supports our hypothesis that a more dysplastic phenotype, which was incurred by mutating oncogenes KRAS and RB, resulted in less

caspase-1 expression. This suggests that caspase-1 may be actively downregulated during tumorigenesis but may have a role in the earlier stages of metaplastic disease.

3.2.8 Time course of caspase-1 expression in pL2-IL-1 β mice at three, six, nine and twelve months.

Having observed that caspase-1 expression is upregulated in patient metaplastic oesophageal tissue and in the metaplastic cell line, QH, we sought to determine the expression levels of caspase-1 during the establishment and development of metaplasia in the murine pL2-IL-1 β model. Although we have previously shown that caspase-1 is significantly upregulated in pL2-IL-1 β mice at 12 months compared to WT controls (**Figure 3.10**), it was noted that WT mice were not a satisfactory comparison because there was no Barrett's region established in these mice. To this end, it was resolved that pL2-IL-1 β mice sacrificed at different ages would represent better comparative controls.

pL2-IL-1 β mice were sacrificed at 3 months (n=4), 6 months (n=5), 9 months (n=5) and 12 months (n=5). **Figure 3.17** shows representative H&E images of tissue taken from each age group at the SCJ. Histological analysis of mice at 3 months revealed that inflammation and early evidence of metaplasia had developed. However, almost no dysplasia and goblet cells were evident (**Figure 3.17A**, **Figure 3.18**). Six-month-old representative histology images clearly show that morphological changes occurred at the SCJ. Arrow indicates metaplasia, defined by the presence of columnar epithelium (**Figure 3.17A**). Histological analysis of 6-month-old mice revealed established Barrett's metaplasia but little dysplasia. At this time point, goblet cells were present indicating a specialised intestinal metaplasia phenotype had developed (**Figure 3.17A**, **Figure 3.18**). Representative images of 9-month-old mice show typical Barrett's histology. The arrow indicates goblet cells characterised by reduced nucleus size, polarised to the base of the cell (**Figure 3.17B**). At 9 months, histological scores of disease severity reveal well established metaplasia ($p<0.001$) and significant dysplasia ($p<0.01$, **Figure 3.18**). Representative images and histological scores for 12-month-old mice reveal a very inflamed ($p<0.05$) and metaplastic histology ($p<0.001$), with a significant expansion of the Barrett's region accompanied by disorganised crypt structure (**Figure 3.17B**, **Figure**

3.18). Arrows indicate dysplastic columnar epithelium, characterised by misalignment of cellular nuclei, and goblet cells (**Figure 3.17B**). Mice had more established dysplasia ($p<0.01$) and a significantly reduced goblet cell ratio ($p<0.05$). This indicates that 12-month-old mice had well established Barrett's metaplasia and were at higher risk of neoplasia. A reduction in goblet cell ratio ($p<0.05$) indicates mice were more susceptible to the occurrence of neoplasia (386). Collectively, histological scoring shows that pL2-IL-1 β mice develop metaplasia and advance to dysplasia with age.

Sections from the same mice were IHC stained and scored for caspase-1 expression in the epithelium and stroma by two independent reviewers (G. Barber, J. Phelan), using a validated semi-quantitative scoring system. Strikingly, scoring of epithelial caspase-1 revealed that expression was substantially upregulated in 9-month-old mice compared to 3-month-old mice ($p<0.001$). However, this increase was not seen in 12-month-old mice (**Figure 3.19**). This discrepancy may be explained by examining the histological scores of these mice. Mice at 9 months had well established metaplasia, but less dysplasia. While at 12 months, the mice presented with more dysplasia. This result supports our previous data which shows an increase in epithelial caspase-1 in metaplastic human tissue, but not in tumour tissue (**Figure 3.1**). In support of this, metaplastic cell lines had significantly elevated levels of caspase-1, while tumour cell lines poorly expressed caspase-1 (**Figure 3.7**). Correlation analysis was performed using the Spearman's correlation coefficient to compare epithelial caspase-1 scores to histological values (**Figure 3.20**). Results show that caspase-1 expression significantly correlates with dysplasia ($p<0.01$), further suggesting a role for caspase-1 in disease progression.

Results for stromal caspase-1 showed a stepwise increase in expression with increasing age groups, with the highest values overall in 12-month-old mice (**Figure 3.21**). Correlation of histological values with caspase-1 in these mice (**Figure 3.22**) showed significant correlations with inflammation and dysplasia ($p<0.05$), suggesting that caspase-1 is expressed by infiltrating immune cells associated with Barrett's inflammation and further disease progression. Representative images of negative controls for caspase-1 IHC staining are shown in **Figure 3.24A-C**.

To assess for proliferation, murine tissue was also stained and scored for Ki-67 expression (**Figure 3.23**). Representative image of Ki-67 IHC negative control is shown in **Figure 3.24D**. As predicted, Ki-67 expression increased with age ($p<0.001$), indicating that disease progression induces cellular proliferation (**Figure 3.23B**). No significant correlation was found between Ki-67 and epithelial caspase-1 expression (**Figure 3.23C**), suggesting that disease-associated increased caspase-1 expression is not due to increasing proliferation, but is likely induced transcriptionally.

Collectively, the results of this time course analysis of pL2-IL-1 β mice reveal some very interesting results. Firstly, epithelial caspase-1 is highly upregulated in 9-month-old mice, but not 12-month-old mice. Taking into consideration previous results which showed upregulation of caspase-1 in epithelial oesophageal resected tissue and metaplastic cell lines, we hypothesise that caspase-1 may be playing a key role in driving metaplastic inflammation, but epithelial caspase-1 is not as involved in neoplasia. Secondly, stromal expression of caspase-1 correlates with histological inflammation and dysplasia scores, suggesting that stromal caspase-1 is contributing to disease progression.

3.2.9 Summary of main findings of Chapter 3

- In human resected OAC tissue, epithelial caspase-1 expression is significantly increased in metaplastic tissue when compared to normal adjacent tissue. Additionally, epithelial caspase-5 expression is significantly increased in both metaplastic and tumour tissue when compared to normal adjacent tissue.
- *In vitro*, caspase-1 is highly expressed in metaplastic cells, but has little expression in tumour cell lines.
- In the Barrett's mouse model (pL2-IL-1 β), epithelial caspase-1 increases in expression from 3 to 9 months old, however in more dysplastic tissue (12-month-old) caspase-1 is not significantly elevated. Additionally, stromal caspase-1 correlates with histological scores for inflammation during disease progression.
- A high fat diet in pL2-IL-1 β can accelerate disease progression and induces higher expression levels of epithelial caspase-1 compared to mice fed on a standard fat diet.
- pL2-IL-1 β FXR^{-/-} mice have increased epithelial caspase-1 and decreased stromal caspase-1.
- pL2-LKR mice express decreased levels of caspase-1 in the epithelium and stroma.
- In the Barrett's mouse model, epithelial caspase-1 increases in expression from 3 to 9 months old, however in more dysplastic tissue (12-month-old) caspase-1 is not significantly elevated. Additionally, stromal caspase-1 correlates with histological scores for inflammation during disease progression.

3.3 Discussion

Barrett's and associated OAC are inflammatory-driven diseases. Studies indicate risk factors such as obesity and chronic exposure to gastric reflux drive inflammation and subsequent disease progression. The inflammatory mechanisms which promote disease progression are still not understood, and this study was designed and performed to investigate the expression profiles of inflammatory caspases during Barrett's and OAC. In Barrett's-associated OAC patient tissue, epithelial caspase-1 expression was significantly upregulated in Barrett's tissue compared to matched adjacent normal mucosa. This observation is supported by results from a cell line model of disease progression and a transgenic murine Barrett's model. In contrast to Barrett's, epithelial expression of caspase-1 did not appear to be elevated in tumour resected OAC patient tissue. Decreased epithelial caspase-1 expression was also evident in tumour cell lines and in 12-month-old pL2-IL-1 β mice which display a dysplastic, rather than metaplastic, phenotype. A similar trend for epithelial caspase-1 expression has been observed by our laboratory in inflamed and tumorigenic colon tissue, with elevated caspase-1 expression observed in inflamed IBD patient biopsy tissue, but decreasing levels were observed in CRC patient tissue (415). Furthermore, unpublished data from our lab has shown an inverse correlation between caspase-1 expression and tumour stage during CRC. In the context of non-GI adenocarcinomas, caspase-1 expression is also significantly lower in tumour compared to adjacent normal tissue in both breast and prostate (432, 433). These observations allow us to speculate that caspase-1 activity may play a more redundant role in later disease or perhaps that tumour-induced suppression of caspase-1 expression may be occurring.

Observations regarding elevated caspase-1 during metaplasia suggest that epithelial caspase-1 may be playing a role during inflammation-induced pathogenesis in Barrett's metaplasia and earlier stages of disease. In support of our findings, other studies have shown that IL-1 β is increased in Barrett's tissue (95, 226). As IL-1 β is directly activated by caspase-1 as part of the inflammasome pathway (88, 434), this study agrees with this observation and indicates that significant inflammasome activity occurs during Barrett's metaplasia.

Interestingly, when OAC patients were divided into cohorts of those who had and had not received NAT, we observed a trend of increasing caspase-1 expression in normal and tumour tissue of NAT patients, when compared to naïve patients. This finding may prove to be significant if a larger cohort of patients were examined, or experimental power was increased. Indeed, several studies have implicated inflammasome activation in response to chemotherapeutics. These findings have resulted in various outcomes involving anti- and pro-tumorigenic immunity depending on the nature of the cancer (435-437). One such study showed that 5-FU activates caspase-1 and the NLRP3 inflammasome pathway in myeloid-derived suppressor cells (MDSCs) and subsequently limits the anti-tumour efficacy of these chemotherapeutics in an EL4 (lymphoma) tumour murine model (435). While paclitaxel was capable of inducing NLRP3 inflammasome activation in macrophages in mice (438). Moreover, cisplatin efficacy was shown to be reduced by NLRP3 priming and activation, however the addition of an IL-1R antagonist (Anakinra) partially restored drug resistance in a malignant mesothelioma xenograph mouse model (439). These studies have investigated chemotherapeutics that are routinely used to treat OAC during the CROSS and MAGIC regimens (204, 209), so are important indicators of inflammasome-associated responses during treatments. Indeed, elevated inflammatory mediators have been identified as predictive markers of poor treatment response during OAC (270, 440, 441). Increased complement system proteins, C3a and C4a serum levels correlated with poor pathological response to neoadjuvant chemoradiation therapy (441). While elevated levels of the IL-6 type cytokine, leukaemia inhibitory factor (LIF) was also associated with poor patient response rates to neoadjuvant treatment (440). Of note, our results show that caspase-1 remains significantly elevated in metaplastic tissue of treatment naïve patients, but not in NAT patients. We propose this may be due to increased caspase-1 expression being induced following NAT treatment in normal tissue, resulting in a loss of significance between groups.

This study has shown that in a cell line model representing stages of disease progression to OAC, that inflammatory caspase-1, but not caspase-4 and -5, is highly expressed in metaplastic cell lines. To support this, an insightful study by Nadatani *et al.* showed that the NLRP3 inflammasome pathway is primed in a Barrett's cell line (BAR-T) and activated

following LPS stimulation (48), resulting in IL-1 β -mediated inflammation. A study by Souza *et al.* observed that when squamous oesophageal cells were exposed to bile acids there was significant induction of IL-1 β and IL-8 expression (297). Similarly, Fitzgerald and colleagues showed that pulsatile bile acid exposure induces IL-1 β in Barrett's metaplasia (226). These studies provide evidence that oesophageal tissue responds to external stimuli in an inflammasome-associated manner.

Traditionally, bile acid exposure was assumed to act as a chemical burn, incurring direct tissue damage and injury to the squamous mucosa (442). However, in a review by Rhonda F. Souza (409), it was proposed that gastric and bile acids induce immune-mediated injury, rather than caustic injury. This is initiated by the release of pro-inflammatory cytokines such as IL-1 β , which in turn mediates tissue injury and disease progression. This proposed mechanism for Barrett's development supports the argument that inflammasome activation contributes to chronic inflammation induced by risk factors such as obesity and gastric reflux, resulting in immune-mediated injury (263, 297, 364, 409). To support this, our study shows that knockout of the bile acid receptor FXR in pL2-IL-1 β mice resulted in elevated epithelial caspase-1 levels. The FXR receptor is important for maintaining bile acid and lipid homeostasis and reducing inflammation (228, 384). The receptor has been identified as a potential target for reducing colitis in the intestine, and therefore may also be a suitable target during Barrett's (227).

Although our results and work by others have reported significant increases in caspase-1 and IL-1 β expression (226), this chapter does not provide functional evidence to support inflammatory mediation of disease progression. This will be further addressed in Chapter 4. However, current evidence to support this theory that caspase-1 mediated inflammation is involved in Barrett's disease initiation and progression is shown by Quante and colleagues. They have developed a transgenic Barrett's mouse model where IL-1 β -mediated disease progression is characterised by chronic inflammation. This transgenic model overexpresses mature IL-1 β specifically targeting the squamous epithelium of mice, leading to the development of Barrett's-like features at the SCJ over time. Eventually, as the mice age to approximately 20 months, they spontaneously develop OAC-like features (181, 263). The premise of this model supports the argument

that inflammation mediated by IL-1 β signalling alone is capable of inducing Barrett's pathogenesis, without the presence of additional risk factors such as bile acid exposure and obesity. However, bile acids and a HFD have also been shown to accelerate disease progression (263, 364). Findings from this study show that epithelial caspase-1 is upregulated during metaplasia in pL2-IL-1 β mice and epithelial expression correlates with dysplasia scores. This aligns with results generated from OAC patient tissue and oesophageal cell lines. Stromal caspase-1 expression levels were shown to correlate with histological inflammation during disease progression, suggesting that inflammasome activity also contributes to inflammation mediated by infiltrating immune cell infiltration

Further evidence for the role of the inflammasome during disease is found in genetic studies. Two studies have linked IL-18 and IL-1 β with susceptibility to Barrett's. Holla *et al.* reported that certain haplotypes of the IL-1 β gene cluster are associated with development of GORD, oesophagitis and Barrett's (443). Moreover, Babar *et al.* strongly implicated polymorphisms of IL-18 and IL-18RAP with susceptibility to Barrett's and OAC (444). Considering that IL-1 β and IL-18 activity are completely dependent upon caspase-1, these studies provide further convincing evidence that the inflammasome pathway is associated with Barrett's pathogenesis.

Currently, there is little evidence in the literature which identifies the possible inflammasome pathway that may be involved in Barrett's-associated IL-1 β inflammation, or the molecular mechanisms involved in triggering inflammasome activation. Our study reveals that there is elevated expression of caspase-1 in the Barrett's cell line, QH. In support of our finding, a study by Nadatani *et al.* also showed that the NLRP3 inflammasome pathway was upregulated in the Barrett's cell line, BAR-T. They showed that, following LPS stimulation, ROS-mediated activation of the NLRP3 inflammasome occurred in these cells, resulting in caspase-1 activation and subsequent IL-1 β secretion (48). Several other studies have reported increased intracellular reactive oxygen species (ROS) in Barrett's and OAC (445). Wetscher *et al.* studied oxidative stress in oesophagitis patients pre- and post-anti-reflux surgery and found that the highest levels of oxidative stress were in Barrett's patients, concluding that Barrett's is a severe form of oxidative damage (446). This finding was supported by O'Farrell *et al.*, who

reported elevated oxidative stress markers in Barrett's tissue, compared to OAC patient tissue. Interestingly, they associated reduced oxidative stress expression with disease progression (388). Taking into consideration ROS is thought to trigger NLRP3 activation (447-449), it is reasonable to speculate that IL-1 β secretion during Barrett's may be arising due to ROS mediated NLRP3 inflammasome activation. As cell lines are strictly only representative of Barrett's pathology, more investigation is required before conclusions can be made.

The canonical inflammasome pathway has been implicated in many other inflammation-induced cancers. For example, IL-1 β has been implicated in breast cancer and has been proposed as a biomarker of progression to associated bone metastasis (450, 451). Moreover, Coleman *et al.* showed that expression of IL-1 β in breast cancer patients strongly correlated with re-occurrence and relapse in bone (452). During a study using an orthotopic mammary gland model, inflammasome-deficient and IL-1R knockout mice were both found to have less primary tumour growth and lung metastasis, suggesting IL-1 and inflammasome involvement in breast tumour growth and metastasis (413).

Inflammasomes have also been implicated in gastric carcinogenesis, which arise due to persistent *H. pylori* infections. This bacterium is associated with chronic, unresolved inflammation and elevated levels of IL-1 β and IL-18. IL-1 β can reduce acidity in the stomach, creating a hypo-acidic environment which has the potential to eventually develop into gastric carcinoma (179, 183). Moreover, the NLRP3 inflammasome and IL-1 β have been associated with both lung fibrosis and COPD. Activation may be triggered by a variety of substrates, such as inhalation of asbestos, silica and cigarette smoke (412). One study showed that healthy smokers had significantly elevated levels of IL-1 β compared to healthy non-smokers, this was seen in a cigarette-dose dependent manner (453). There are many more examples of the canonical inflammasome pathway driving inflammation-associated carcinogenesis, such as fibrosarcoma, lung metastasis, liver fibrosis and liver cancer (169, 413). When these studies are reviewed together, the caspase-1 canonical inflammasome is exposed as a common instigator and driver of disease and the potential therapeutic value of targeting IL-1 β or its upstream signalling pathway becomes clear.

In support of this theory, Tu *et al.* developed a transgenic mouse model of gastric cancer which overexpresses IL-1 β in the stomach. Similar to the Barrett's mouse model, it is characterised by chronic gastritis, metaplasia and eventual HGD/carcinoma. Using this model, they showed that IL-1 β -mediated inflammation alone was sufficient to initiate cancer. Unsurprisingly, addition of IL-1Ra, the natural antagonist to the IL-1R, significantly reduced gastric inflammation and inhibited carcinoma. Interestingly, this inhibitory effect by IL-1Ra was also witnessed in *H. felis*-infected WT mice, suggesting that inhibition is not limited to the transgenic model (181). *H. felis* is a bacterium of the *Helicobacter* species and is used in small animals to mimic active chronic gastritis known to occur in humans during *H. pylori* infection (454). As this gastric cancer model by Tu *et al.* is very similar to the Barrett's model, it suggests that administration of IL-1Ra in the Barrett's model would likely reduce disease progression. Further work investigating the therapeutic benefit of an upstream inhibitor of the canonical pathway, such as the NLRP3 inhibitor MCC950, may provide interesting insights into the inflammatory mechanisms promoting carcinogenesis and will be further investigated in later chapters.

Biomarkers of inflammation can be used to identify Barrett's and associated-OAC and therefore can be used for early detection and risk stratification of patients. One common tissue biomarker in use in the clinic today are goblet cells. These are fully-differentiated cells which represent the presence of intestinal metaplasia, however their usefulness as a biomarker is under constant debate (455). Trefoil factor 3 (TFF3) is currently in clinical trials as a tissue biomarker. Samples are retrieved using a sampling device called a cytosponge, which is swallowed by the patient and pulled back up to retrieve cells. Studies so far report 89.7% sensitivity in those who swallowed the sponge twice (456).

Non-invasive biomarker tests are also available for use. For example, CancerSEEK have recently released a commercial, multi-analyte blood test for early clinical detection and localisation of eight different cancers: ovary, liver, stomach, pancreas, colorectal, lung, breast and oesophagus. The test involves high-sensitivity detection of plasma DNA and protein biomarkers common to the cancers. Trials with the test reported accurate detection of five types of the cancers (ovary, liver, stomach, pancreas and oesophagus)

in 69-98% of cases (457). These are promising results for a preliminary non-invasive test, however its use in clinic will continue to be limited until higher levels of accuracy and specificity are obtained. For full incorporation into the clinic these multi-analyte tests need to give a reliable indication of cancer in all patients. Failing to do so means that standard current methods of diagnosis will still need to be carried out in order to reasonably confirm a patient status. Among protein biomarkers used in this test were several inflammatory proteins such as IL-6 and IL-8, confirming that biomarkers of inflammation can be successfully used for early detection and identification of cancers such as OAC (457).

Several other studies have attempted to identify circulating, pre-diagnostic markers that could potentially be used in the clinic, such as TNFR2, IL-6 and CRP (411, 458). Certainly, it has long been established that systemic inflammation is observed in the majority of solid epithelial malignancies and at the time of diagnosis up to 50% of patients have an elevated acute phase protein response (APPR) (459, 460). Oesophageal cancer patients that have elevated serum CRP and decreased albumin at time of diagnosis are predicted to have reduced survival (461, 462). One study by Deans *et al.* showed that in gastro-oesophageal cancer, IL-1 β expression was increased 10-100-fold in tumour tissue compared to healthy controls and protein levels correlated significantly with systemic CRP. The study revealed that IL-1 β overexpression was an independent risk factor associated with reduced survival. The authors suggest that IL-1 β could potentially be used alongside CRP as a marker of reduced survival in gastro-oesophageal cancer (459). In contrast to this study, our data has shown that caspase-1 expression was not significantly increased from adjacent normal to tumour in human resected tissue. However, it was noted that our study compared matched resected tissue, while Deans *et al.* compared tumour tissue from cancer patients with healthy controls, therefore the two studies cannot be directly compared (459). Nonetheless, our data shows there is significant elevation of caspase-1 expression in Barrett's metaplasia compared to adjacent normal resected tissue. Additionally, we have shown that caspase-1 is consistently overexpressed in other models such as in the Barrett's cell line, QH and the pL2-IL-1 β mouse model. Similar to studies described above, we suggest that caspase-1 may represent an inflammatory biomarker of Barrett's. Further work is necessary to

elucidate the role of caspase-1 in Barrett's-associated inflammation and whether elevated caspase-1 expression correlates with increased risk of progression. If so, then caspase-1 could become a valued biomarker used to predict and stratify Barrett's patients into progressors and non-progressors.

In this research study we also hypothesised that obesity was enhancing inflammation in disease progression to Barrett's and OAC *via* a caspase-1 pathway. As previously mentioned, obesity is a major risk factor for Barrett's. It has been reported that central obesity confers just under a two-fold risk of developing the disease (344). One study undertaken by Vandanmagasar *et al.* showed that the NLRP3 inflammasome was activated by obesity-associated danger signals in mice, therefore contributing to obesity-instigated inflammation (463). In support of this hypothesis, our results reveal that epithelial caspase-1 is significantly elevated in HFD-fed pL2-IL-1 β mice compared to those fed on normal chow at 12 months. Quante's group have observed an accelerated disease phenotype in HFD-fed mice (364). Taken together, we can speculate that a HFD further induces chronic inflammation in pL2-IL-1 β mice by activating the canonical inflammasome pathway and subsequently advance pathogenesis. Notably, from a young age pL2-IL-1 β mice fed on a HFD were significantly lower in weight than their normal chow counterparts (364), suggesting that: (i) the IL-1 β signalling pathway is somehow involved in regulating lipid storage; and (ii) activators of the canonical inflammasome pathway may be derived directly from a HFD rather than from stressed VAT depots. Indeed, Stienstra *et al.* observed that both caspase-1 and NLRP3 knockout mice were resistant to HFD-induced obesity. Further studies by the same author reported that inflammasome-mediated caspase-1 activation regulates adipocyte differentiation and function through IL-1 β signalling (464, 465). These studies highlight the strong crosstalk between metabolism and inflammasome-associated inflammation and how adverse conditions such as a HFD may contribute to disease.

To conclude, the findings of this chapter have identified a potential role for the canonical inflammasome pathway in inflammation-mediated progression to Barrett's and associated OAC. Results show that epithelial caspase-1 levels are elevated in Barrett's metaplasia, but not in tumour. Stromal caspase-1 correlates with inflammation during

disease progression in the Barrett's mouse model. Additionally, we show upregulated epithelial caspase-1 at the SCJ of pL2-IL-1 β mice fed a HFD.

Results from this study have unveiled further questions, such as identifying the mechanistic role that caspase-1 plays in Barrett's development and pathogenesis. The proceeding experiments of this thesis will look at whether caspase-1 mediates inflammation during Barrett's. Active IL-1 β and IL-18 secretion will be assessed using a caspase-1 inhibitor with Barrett's patient explants and mouse organoid models. Although these models will provide details of how caspase-1 functionally effects inflammation during Barrett's disease, they are limited as they do not provide information as to whether this inflammation is disease progressing.

Additionally, the inflammasome pathway that is upregulated alongside caspase-1 during metaplasia will also be assessed. This information will allow us to determine whether specific inflammasome inhibitors such as MCC950 (NLRP3) could be useful as a future preventative therapy (466). However, many inflammasomes do not currently have any specific inhibitors currently available which may limit possible future studies (467).

This chapter's results showed that caspase-1 was elevated in HFD-fed Barrett's mice. How obesity is promoting disease will be further investigated by further examining HFD-fed Barrett's mice and using a cell line model stimulated with patient adipose conditioned media (ACM). These experiments will give us further insights into the confirmed relationship between Barrett's and obesity (334, 468). However, it is likely that obesity and a HFD are not the sole drivers of disease but are part of a larger combination of risk factors causing pathogenesis (341). These questions will be further addressed in the following chapters of this study.

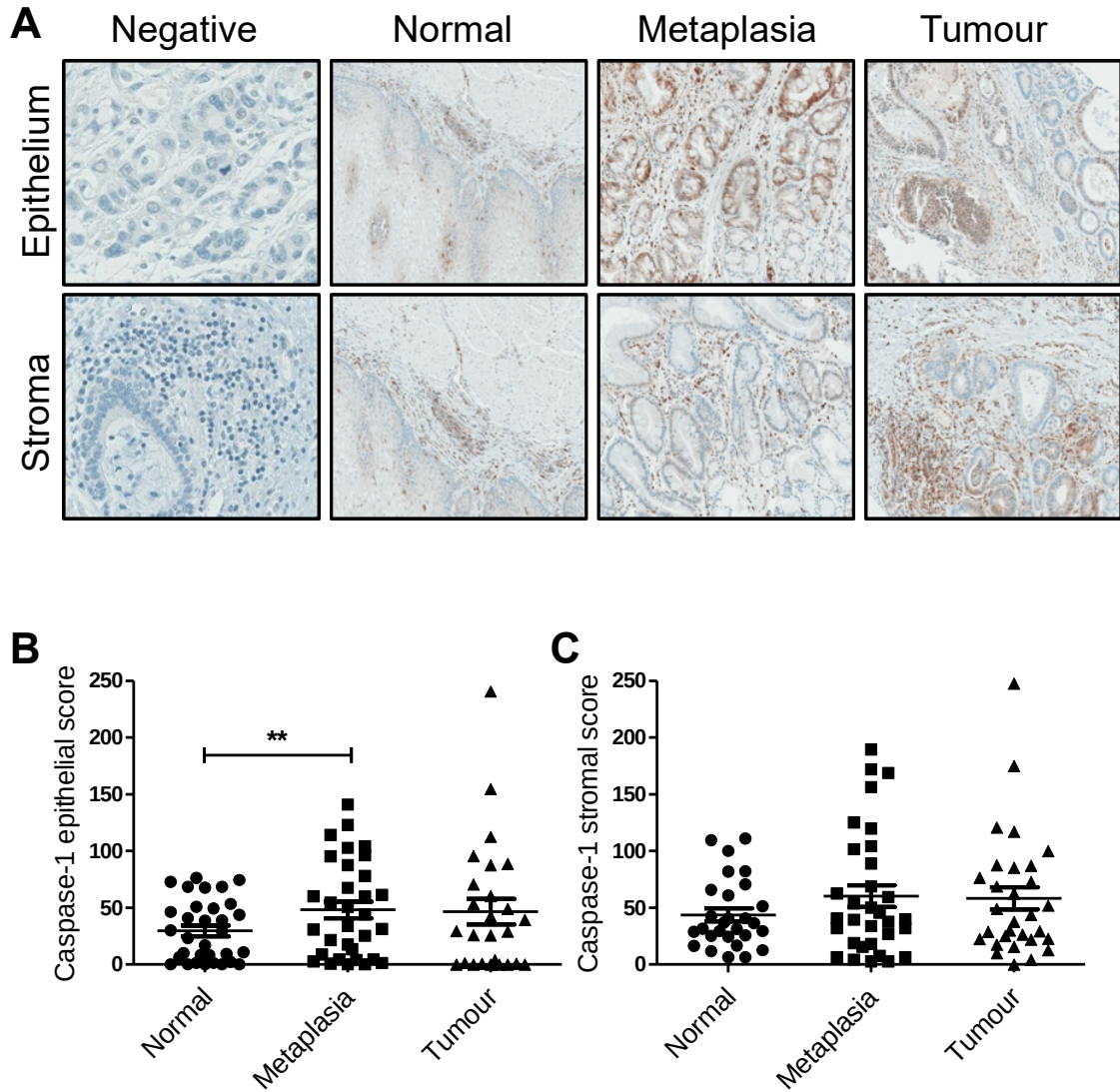


Figure 3.1: Patient oesophageal tissue displays elevated epithelial caspase-1 expression in areas of metaplasia.

IHC stained for caspase-1 expression was performed on formalin-fixed, paraffin-embedded (FFPE) oesophagectomy tissue from 32 Barrett's-associated OAC patients. Histologically defined areas of adjacent normal, metaplasia and tumour tissue were identified for each patient. Caspase-1 expression was assessed by two blinded reviewers (J. Phelan, K. Oficjalska) using a validated semi-quantitative scoring method, where both intensity and positivity were scored and multiplied (IxP). **A)** Representative images of negative control and caspase-1 expression in epithelial cells (top panel) and stromal cells (bottom panel) in defined areas of normal, metaplasia and tumour tissues. Magnification 20X. **B)** and **C)** IHC scores for caspase-1 expression in epithelium (normal, metaplasia and tumour (n = 32, 32, 26, respectively) and stroma (normal, metaplasia and tumour (n = 28, 32, 30, respectively)). Data represents mean $\pm$ SEM. Wilcoxon signed-rank test: ** $p < 0.01$.

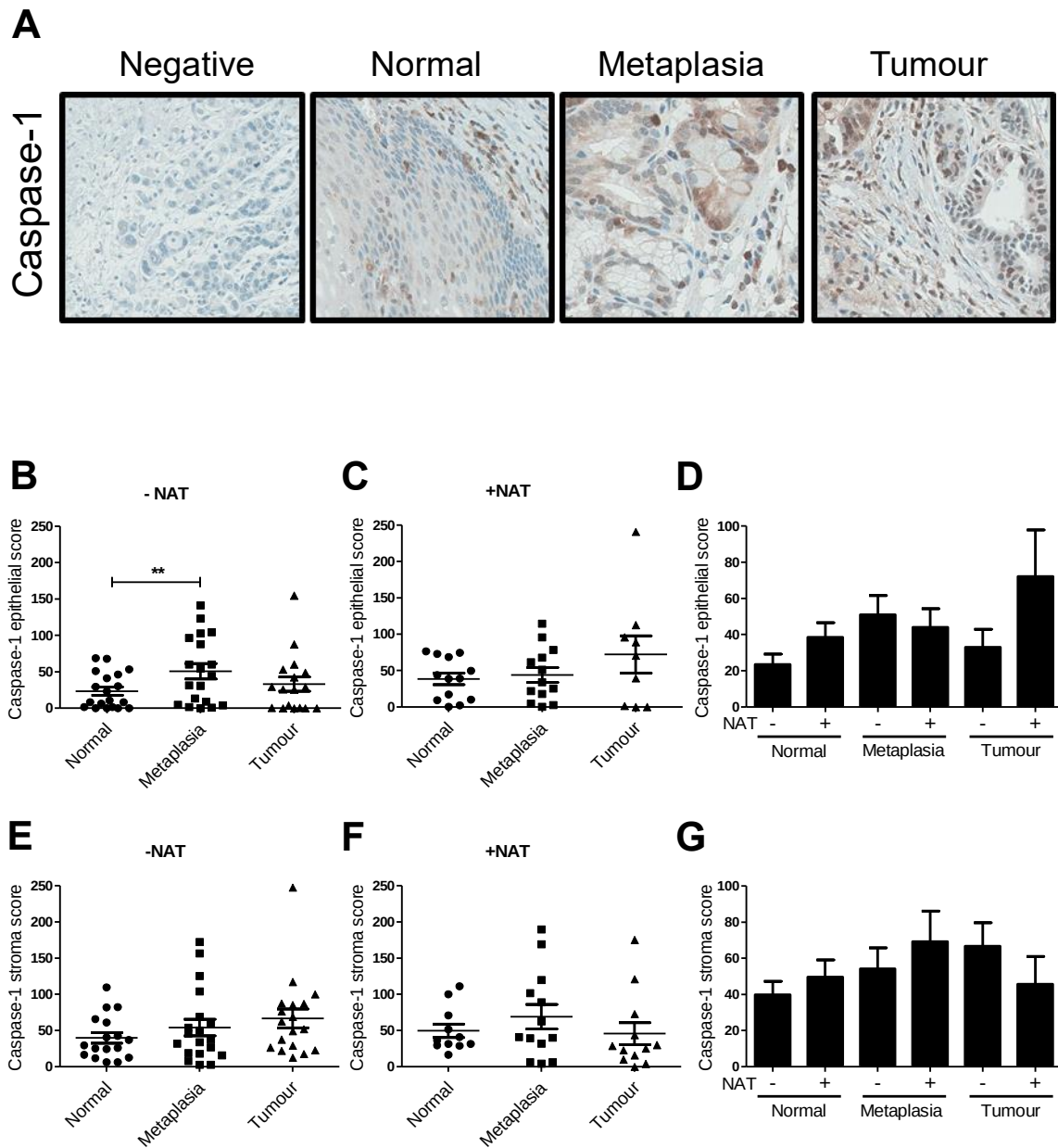


Figure 3.2: Epithelial caspase-1 expression is upregulated in metaplastic tissue from treatment naïve patients.

A) Representative images of IHC caspase-1 expression patterns in the negative control and in defined areas of normal, metaplasia and tumour oesophageal tissues in OAC patients. Expression of caspase-1 in the epithelium of patients who B) have not received neoadjuvant treatment (-NAT, n=19) and C) who have received NAT (n=13) prior to resection surgery. D) and G) Bar chart comparing mean expression values of epithelial and stromal caspase-1 in treatment naïve and NAT patients, respectively. Stromal caspase-1 expression is shown in E) NAT naïve patients and F) NAT patients. Data represents mean $\pm$ SEM. Wilcoxon signed-rank test: ** $p < 0.01$.

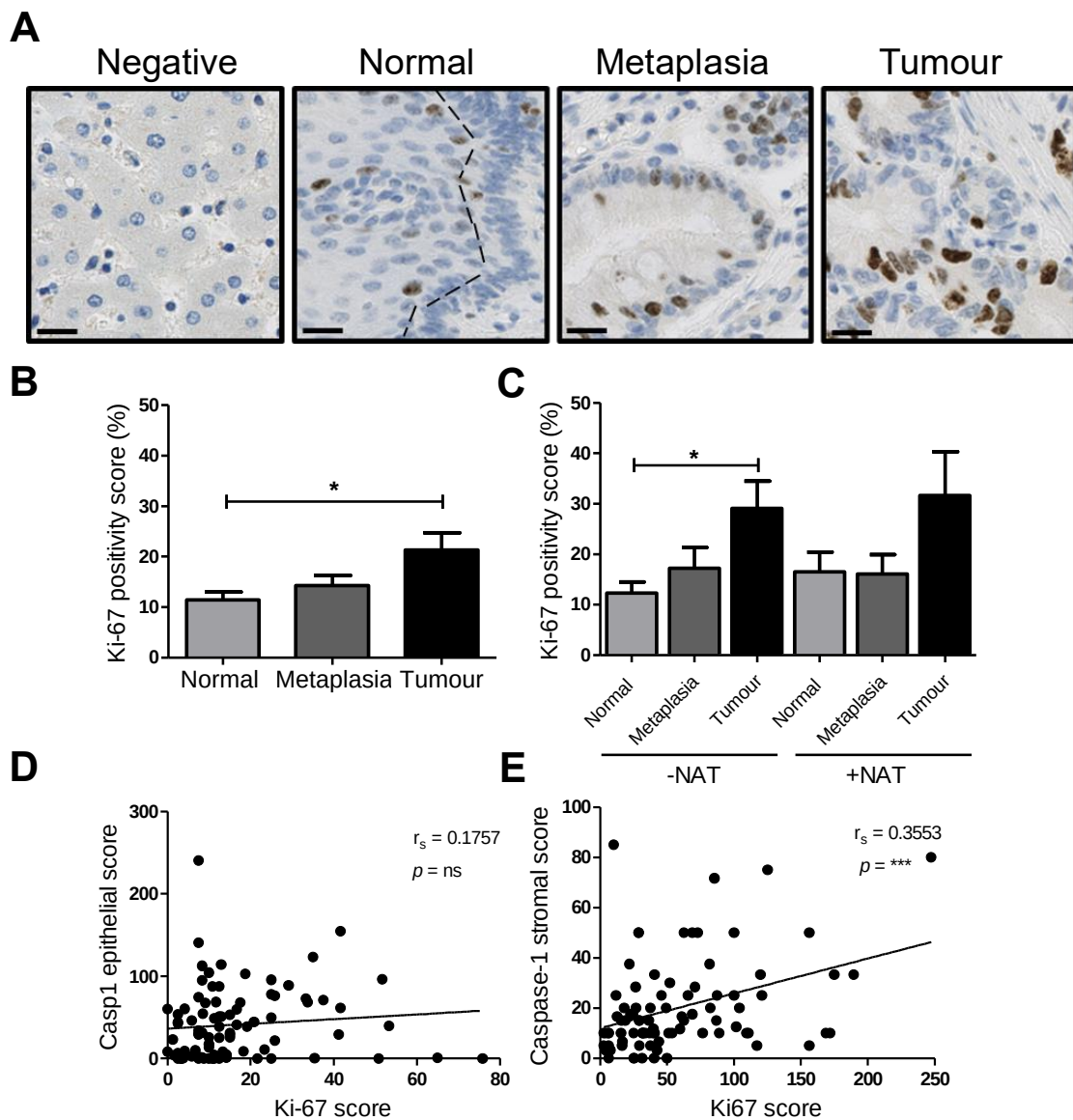


Figure 3.3: No correlation between epithelial caspase-1 expression and Ki-67 in human oesophageal tissue.

A) Representative images of IHC Ki-67 expression patterns in the negative control and in defined areas of normal, metaplasia and tumour oesophageal tissues in OAC patients. Dashed line shows basal epithelial. Ki-67 expression was assessed by two blinded reviewers (G. Barber, H. O'Neill) using a validated semi-quantitative scoring method. Images: 50X magnification, scale bars = 20 μ m. **B)** IHC scores for Ki-67 expression (normal, metaplasia and tumour (n = 32, 32, 26, respectively). **C)** Ki-67 scores separated into patient groups of +/- NAT. Correlation of Ki-67 positivity (%) verses caspase-1 **D)** epithelial and **E)** stroma expression levels in patient OAC tissue. Data represents mean $\pm$ SEM. One-way ANOVA followed by Tukey's post hoc test, r_s = Spearman's correlation coefficient: p = non-significant (ns), * p <0.05, *** p <0.001.

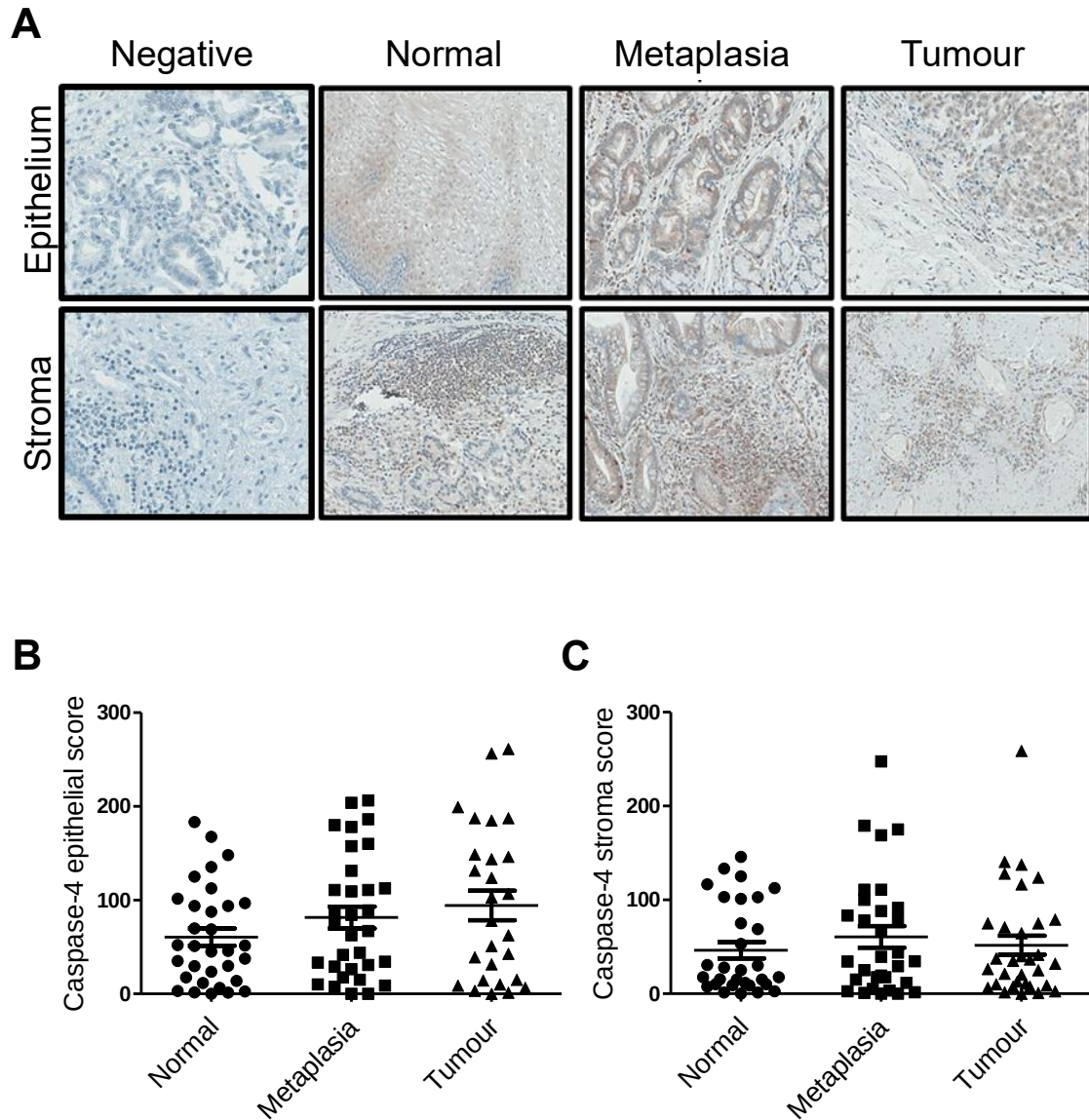


Figure 3.4: No significant differences in caspase-4 expression levels in areas of normal, metaplasia and OAC from oesophageal resected tissue.

A) Representative images of negative control and caspase-4 expression in epithelial cells (top panel) and stromal cells (bottom panel) in defined areas of normal, metaplasia and tumour resected oesophageal tissues. Magnification 20X. **B)** and **C)** IHC scores for caspase-4 expression in epithelium (normal, metaplasia and tumour (n = 32, 32, 27, respectively)) and stroma (normal, metaplasia and tumour (n = 30, 31, 32, respectively)). Data represents mean $\pm$ SEM. Wilcoxon signed-rank test: non-significant.

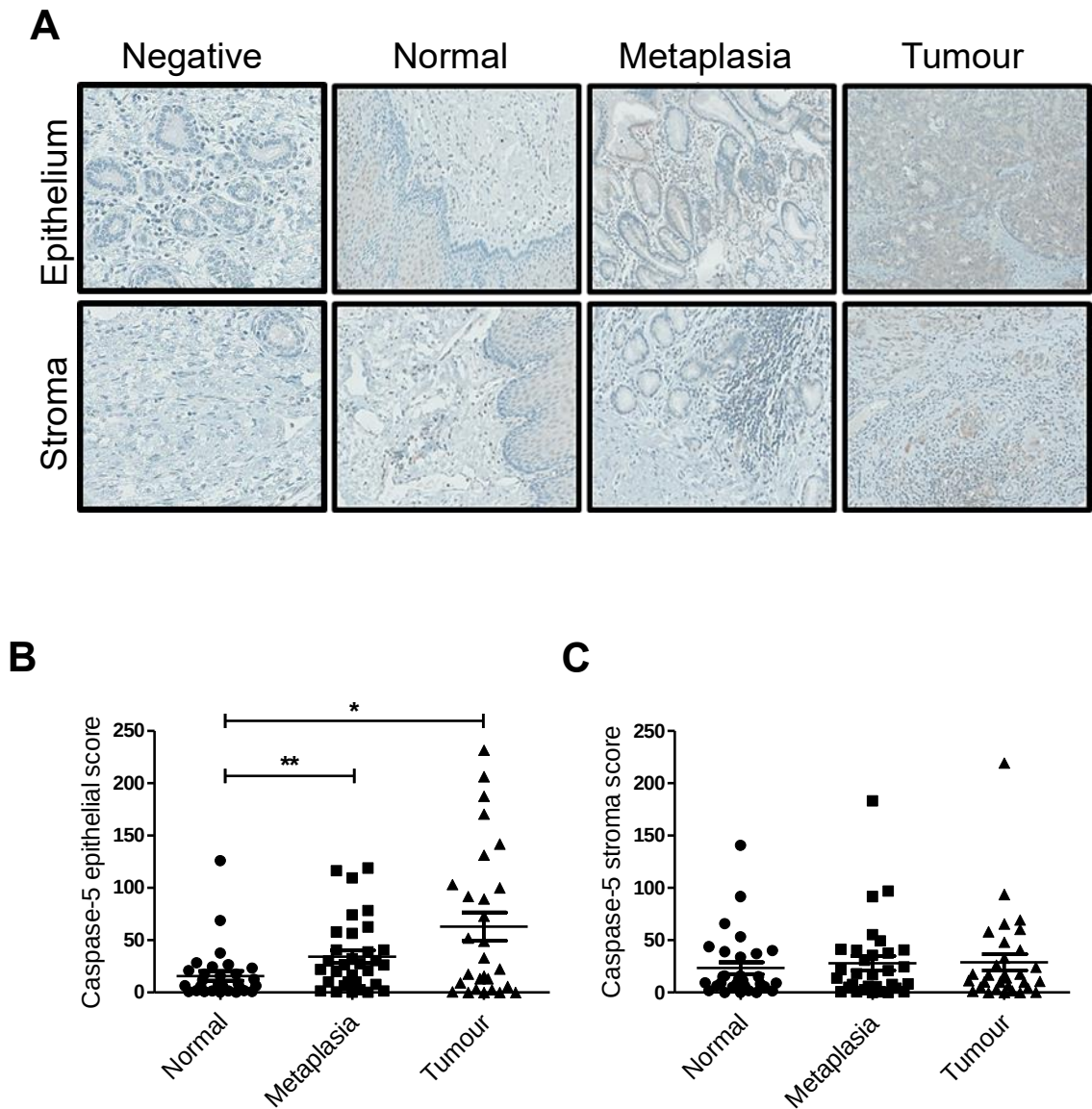


Figure 3.5: Epithelial expression of caspase-5 is significantly elevated in metaplastic and tumour, compared to adjacent normal tissue.

A) Representative images of negative control and caspase-5 expression in epithelial cells (top panel) and stromal cells (bottom panel) in defined areas of normal, metaplasia and tumour resection oesophageal tissues. Magnification 20X. **B)** and **C)** IHC scores for caspase-5 expression in epithelium (normal, metaplasia and tumour (n = 31, 32, 29, respectively)) and stroma (normal, metaplasia and tumour (n = 30, 31, 31, respectively)). Data represents mean $\pm$ SEM. Wilcoxon signed-rank test: * $p < 0.05$, ** $p < 0.01$.

Table 3.1: Characteristics of OAC patients that were IHC stained for caspase-1, 4 and -5 expression.

Patient no.	NAT	Differentiation	Grade	Lymph node
1	No	Poor	Not stated	2/8 positive
2	Yes	Moderate	Not stated	Negative
3	No	Moderate	Not stated	19 negative
4	No	Poor	Not stated	11 negative
5	Yes	Moderate	ypT2N0Mx	Negative
7	No	Moderate	Not stated	5/17 positive
9	Yes	Poor	yPT3N1Mx	1 positive
10	No	Well	pT1N0Mx	Negative
11	Yes	Poor	ypT3, N1	3/10 positive
12	No	Moderate	pT3N1Mx	4/6 positive
14	No	Moderate	Not stated	7 negative
15	No	Moderate to poor	T1cN0MX	25 negative
16	No	Moderate	pT3N1Mx	4/14 positive
17	No	Poor	pT3N1Mx	4/10 positive
18	No	-	pT1N0Mx	22 negative
19	Yes	Poor	ypT3N1Mx	1/10 positive
20	Yes	Moderate	yPT2N0Mx	40 negative
21	No	Poor	pT3N0(ITC)Mx	19 negative
22	Yes	-	ypT2N0Mx	5 negative
23	No	Well	pT3N0Mx	10 positive
24	No	Moderate - poor	pT3pN1Mx	5/12 positive
25	No	Poor	pT2N0Mx	14 negative
27	Yes	Moderate	yPT3N1	1/8 negative
28	No	Moderate	ypT3N0Mx	12 negative
29	Yes	Moderate	ypT1N1Mx	2/7 positive
30	Yes	Poor	pT3N1Mx	2/17 positive
31	Yes	Moderate	ypT3N1Mx	3/18 positive
32	Yes	Moderate - poor	pT3N2Mx	6/17 positive
34	Yes	Poor	ypT3N1Mx	1/9 positive
35	No	Moderate - well	pT1	Negative
38	No	Moderate	pT1bN0Mx	Negative

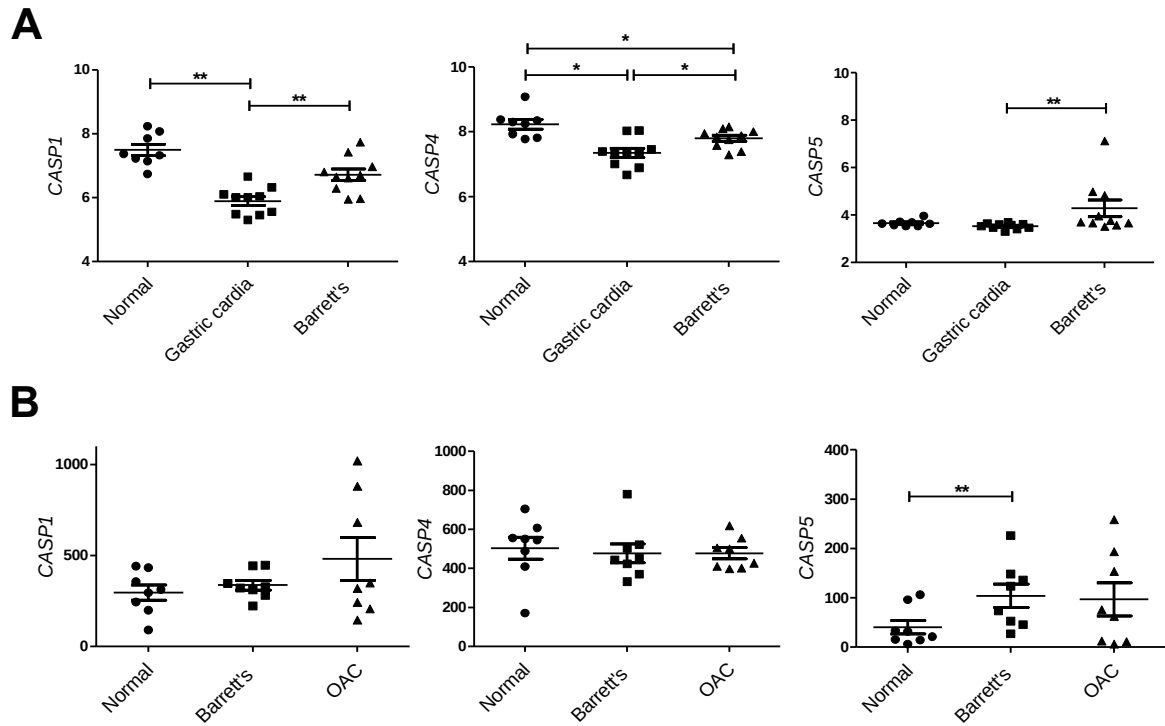


Figure 3.6: Analysis of public data sets reveal inflammatory caspase mRNA levels are higher in Barrett's metaplasia compared to adjacent normal tissues.

Graphs represent relative mRNA inflammatory *CASP1*, *CASP4* and *CASP5* expression values analysed using publicly available data sets. **A)** mRNA expression microarray profile of inflammatory caspases in matched patient biopsies from normal squamous (n=8), normal adjacent gastric cardia (n=10) and Barrett's oesophagus (n=10) tissue (di Pietro M. *et al.* PNAS, 2012 [GDS4350], (373)). **B)** mRNA expression microarray profile of inflammatory caspases in matched biopsies (n=8) of normal squamous, Barrett's oesophagus and oesophageal adenocarcinoma (Kimchi ET *et al.* 2005 [GDS1321], (374)). Data represents mean $\pm$ SEM. Wilcoxon signed-rank test was used to evaluate significant differences between two groups. * $p < 0.05$, ** $p < 0.01$.

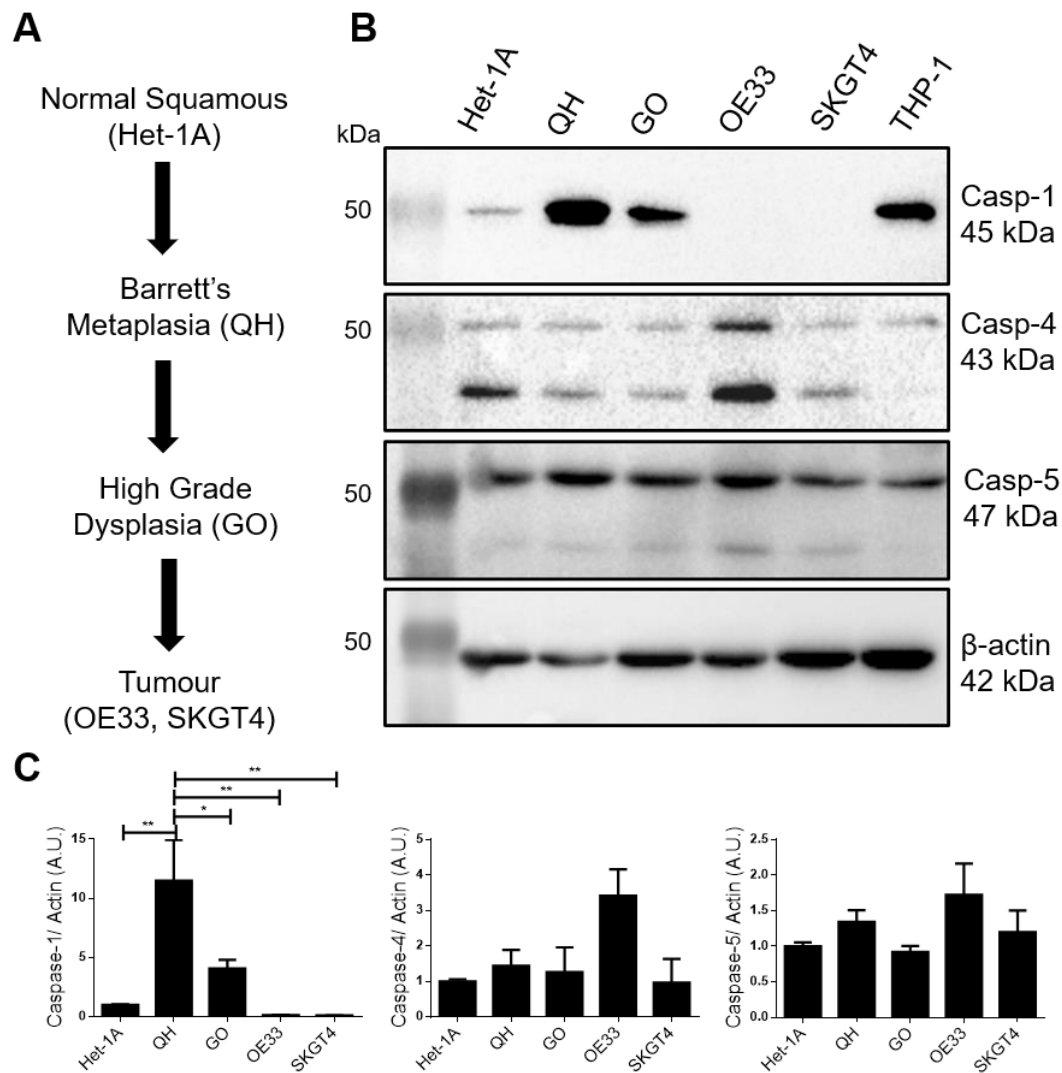


Figure 3.7: Caspase-1 is strongly expressed in Barrett's metaplastic and dysplastic cell lines and is poorly expressed in OAC cell lines.

A) Oesophageal cell lines representing progression; Het-1A (normal squamous), QH (Barrett's metaplasia), GO (high-grade dysplasia), OE33 and SKGT4 (OAC) and THP-1 (human monocyte - positive control). Western blot analysis **B)** and subsequent densitometric analysis of **C)** inflammatory caspase-1 (45 kDa), caspase-4 (43, 36 kDa), and caspase-5 (49.7, 33 kDa) in oesophageal cell lines. 20 µg lysate was loaded into each well of a 12% SDS-PAGE gel. β-actin was probed as a loading control. Immunoblots are representative of three independent experiments. Data represents mean ± SEM. One-way ANOVA followed by Tukey's post hoc test: * $p < 0.05$, ** $p < 0.01$.

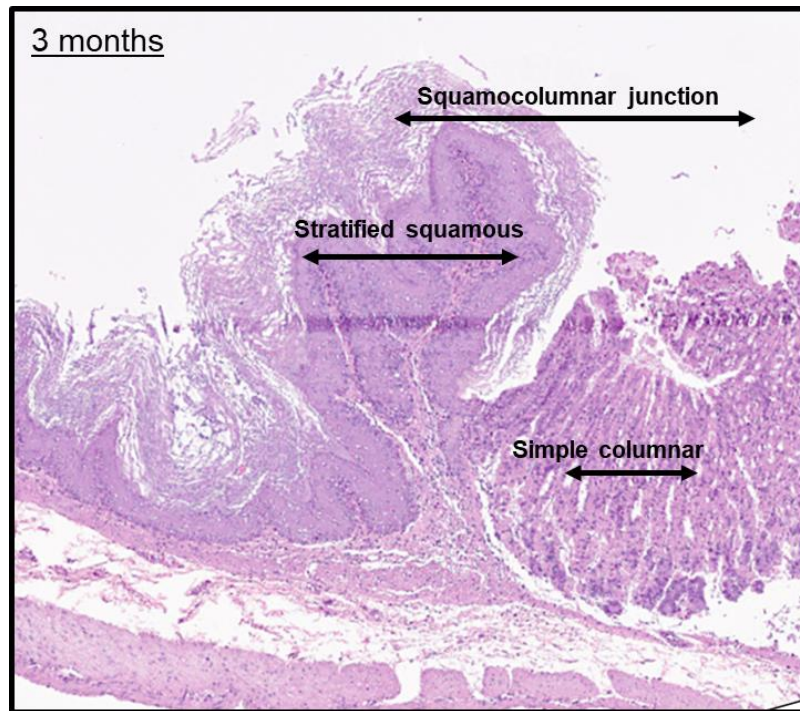
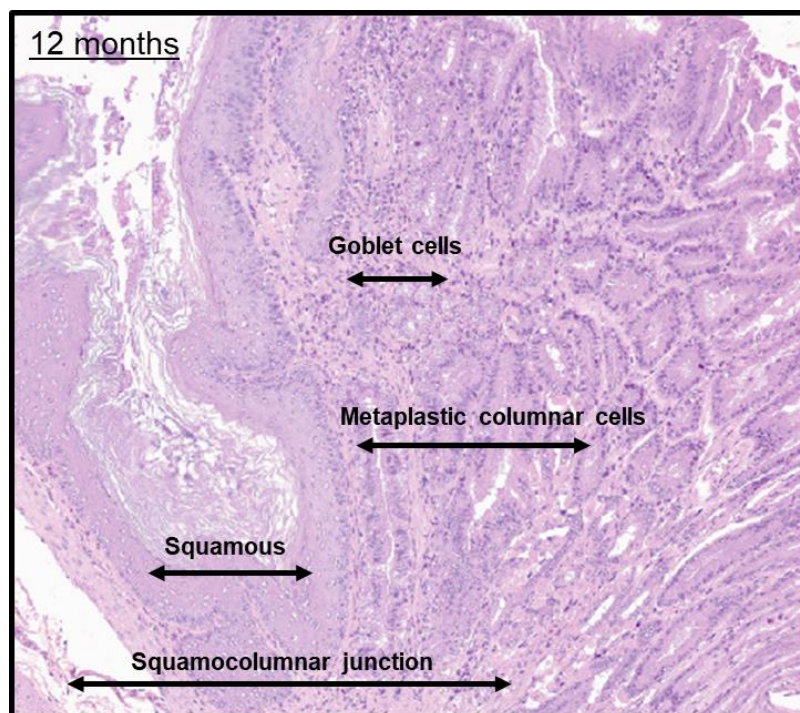
A**B**

Figure 3.8: Histology of pL2-IL-1 β mice at age three months and twelve months.

A) Histopathology of a 3-month-old pL2-IL-1 β mouse. Arrows indicate normal stratified squamous epithelium of the oesophagus and simple columnar epithelium of the stomach at the SCJ. **B)** Histopathology of a 12-month-old pL2-IL-1 β mouse. Arrows indicate: Squamous epithelium, goblet cells and metaplastic columnar cells and simple columnar cells of the stomach.

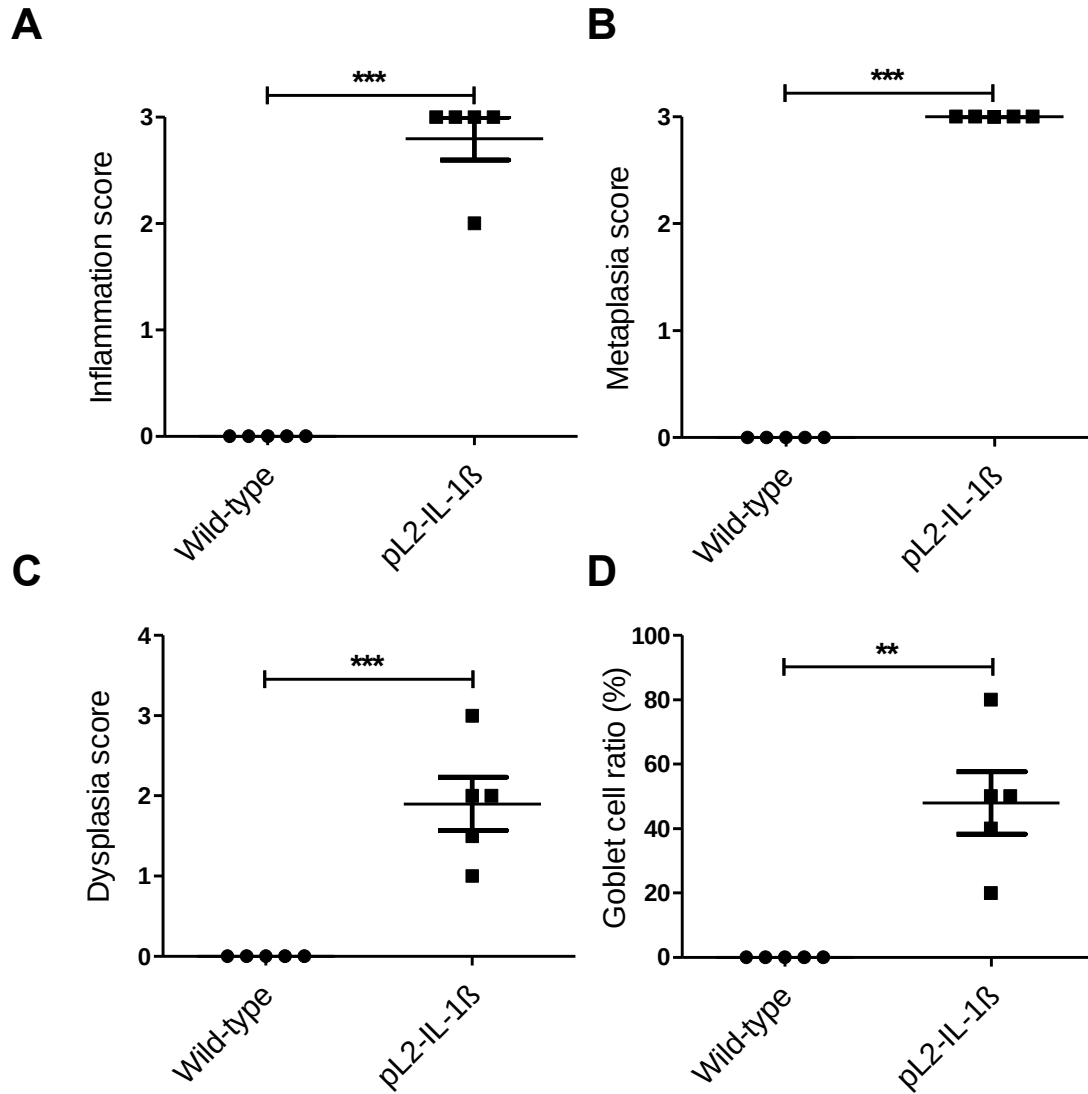


Figure 3.9: Histological scoring of H&E stained tissue confirms a Barrett's phenotype in pL2-IL-1 β mice at 12 months of age.

Disease severity scoring of wild-type (n=5) and pL2-IL-1 β mice at 12 months (n=5) using H&E staining. Graphs represents histological scores for: **A)** Inflammation (scale: 0 = no inflammation, 1 = mild, 2 = moderate, 3 = severe); **B)** Metaplasia (scale: 0 = no metaplasia, 1 = rare mucous cells, 2 = single metaplastic glands, 3 = multiple metaplastic glands); **C)** Dysplasia (scale: 0 = no dysplasia, 1 = superficial epithelial atypia, 2 = atypia with granular complexity, 3 = low-grade dysplasia, 4 = high-grade dysplasia); **D)** Goblet cell ratio (%) calculated as a percentage of goblet cell positivity within each crypt of the Barrett's region. Error bars represent mean $\pm$ SEM. Unpaired Student's *t* test: ** $p < 0.01$, *** $p < 0.001$.

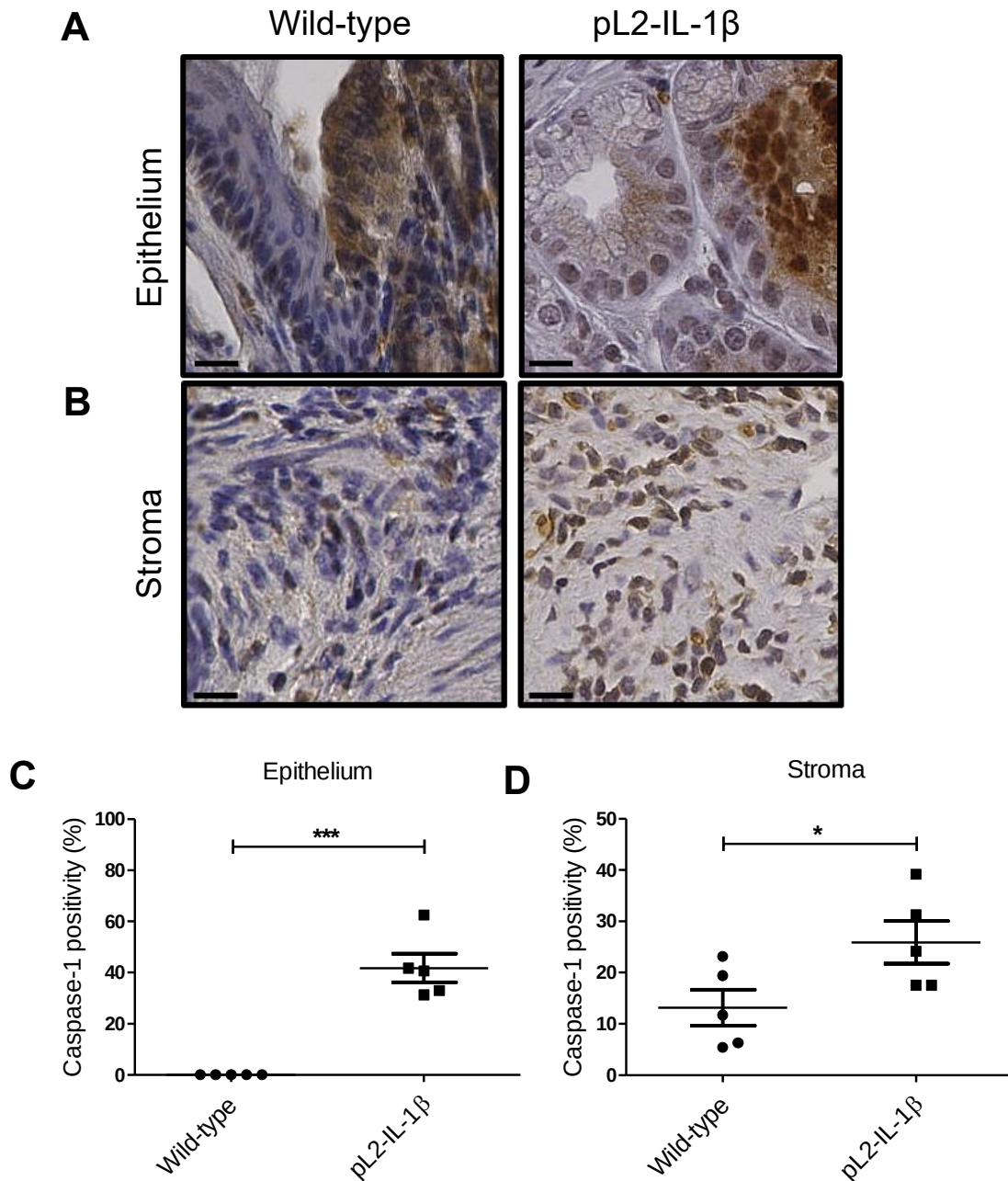


Figure 3.10: Caspase-1 is elevated in both epithelial and stromal cells of the Barrett's region in 12-month-old pL2-IL-1 β mice.

pL2-IL-1 β mice (n=5) and wild-type mice (n=5) were sacrificed at 12 months ($\pm$ two weeks). Subsequently, the oesophagus and stomach were resected and tissue was formaldehyde fixed for 24 hours and paraffin embedded. IHC staining for caspase-1 was performed and expression was assessed by two reviewers using a validated semi-quantitative scoring method (G. Barber, J. Phelan). **A**) Epithelium and **B**) stroma representative images of IHC caspase-1 expression patterns in wild-type and pL2-IL-1 β mice. Images taken at 20X magnification, scale bars = 10 μ m. Graphs represent caspase-1 positivity (%) in the **C**) columnar epithelium of the Barrett's region and **D**) the stroma at the SCJ. Data represents mean $\pm$ SEM. Unpaired Student's *t* test: **p*<0.05, ****p*<0.001.

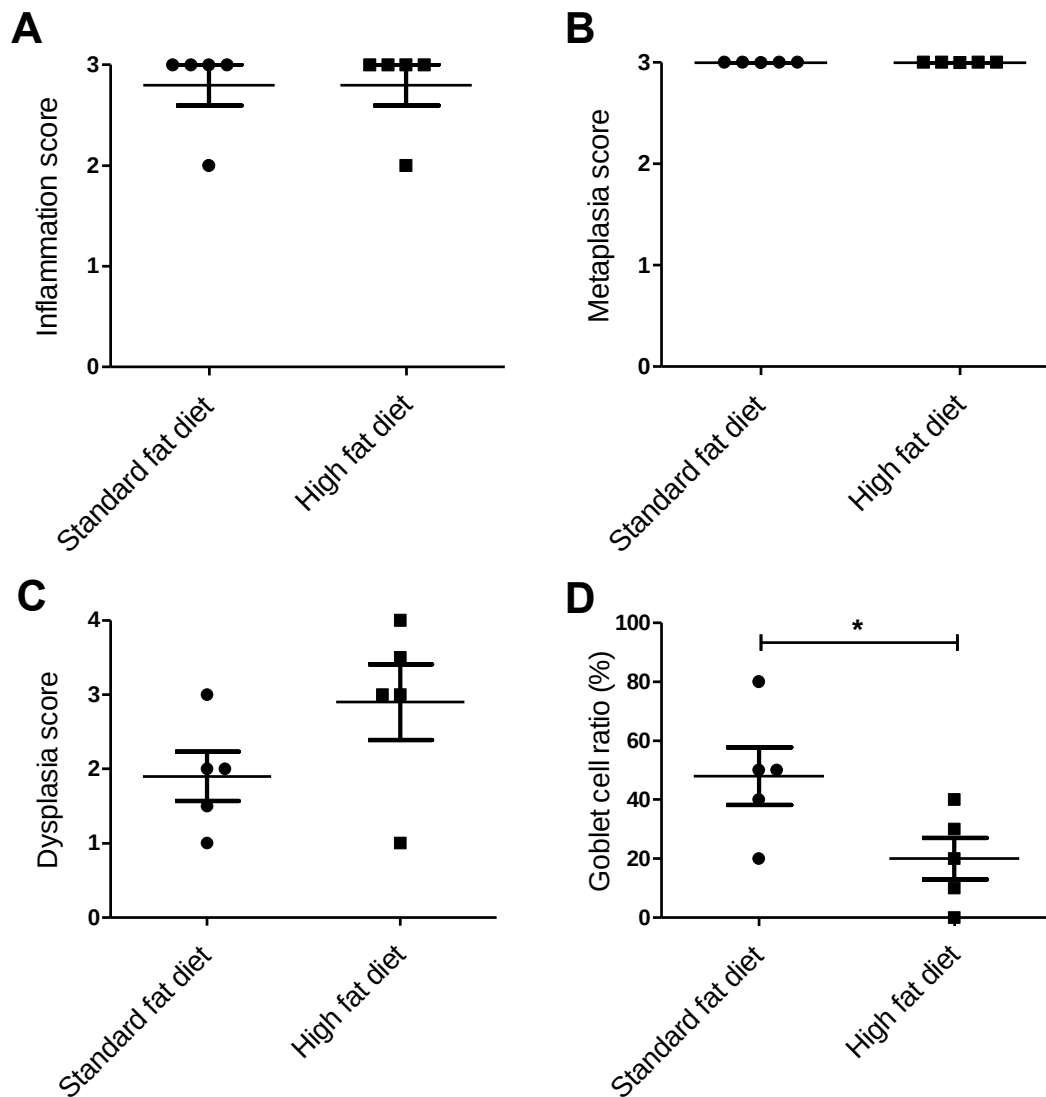


Figure 3.11: Histological comparison of oesophageal tissue in 12-month-old pL2-IL-1 β mice fed on a standard fat or high fat diet.

Graphs represent histology scores of pL2-IL-1 β mice fed on standard fat and high fat diets (n=5, 12 months) using H&E staining. **A)** Inflammation scores. **B)** Metaplasia scores. **C)** Dysplasia scores. **D)** Goblet cell ratio (%). Error bars represent mean $\pm$ SEM. Unpaired Student's *t* test: **p*<0.05.

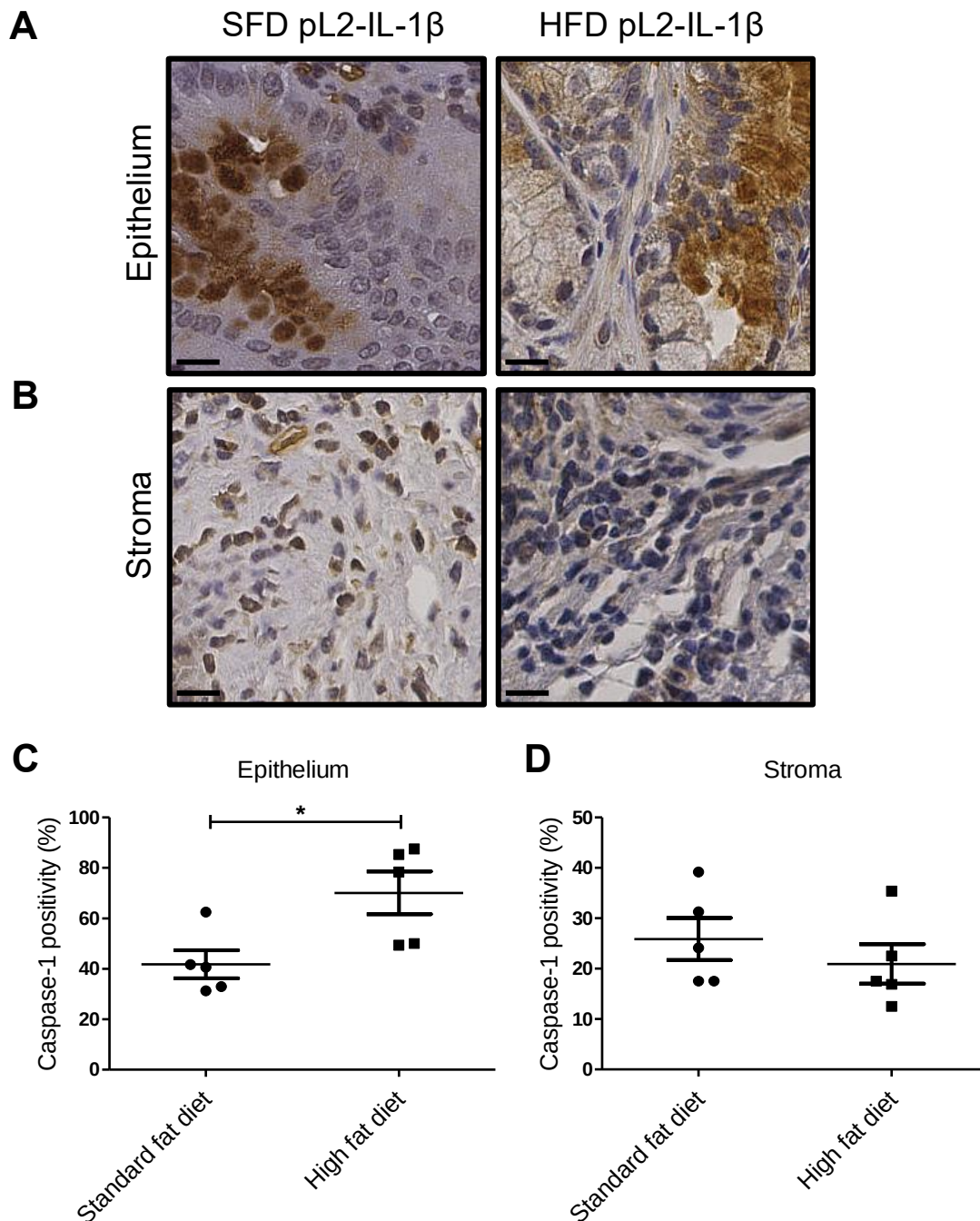


Figure 3.12: Enhanced expression of epithelial caspase-1 in the Barrett's region of high fat diet-fed, pL2-IL-1 β mice.

Tissue sections from pL2-IL-1 β mice fed a standard fat or high fat diet (n=5, 12 months) were IHC stained for caspase-1 and scored by two blinded reviewers. Representative images of caspase-1 expression patterns in **A**) epithelium and **B**) stroma of pL2-IL-1 β SFD and HFD at the SCJ. Images taken at 20X magnification, scale bars = 10 μ m. Graphs represent caspase-1 positivity (%) in the **C**) columnar epithelium of the Barrett's region and **D**) the stroma at the SCJ. Data represents mean $\pm$ SEM. Unpaired Student's *t* test: **p*<0.05.

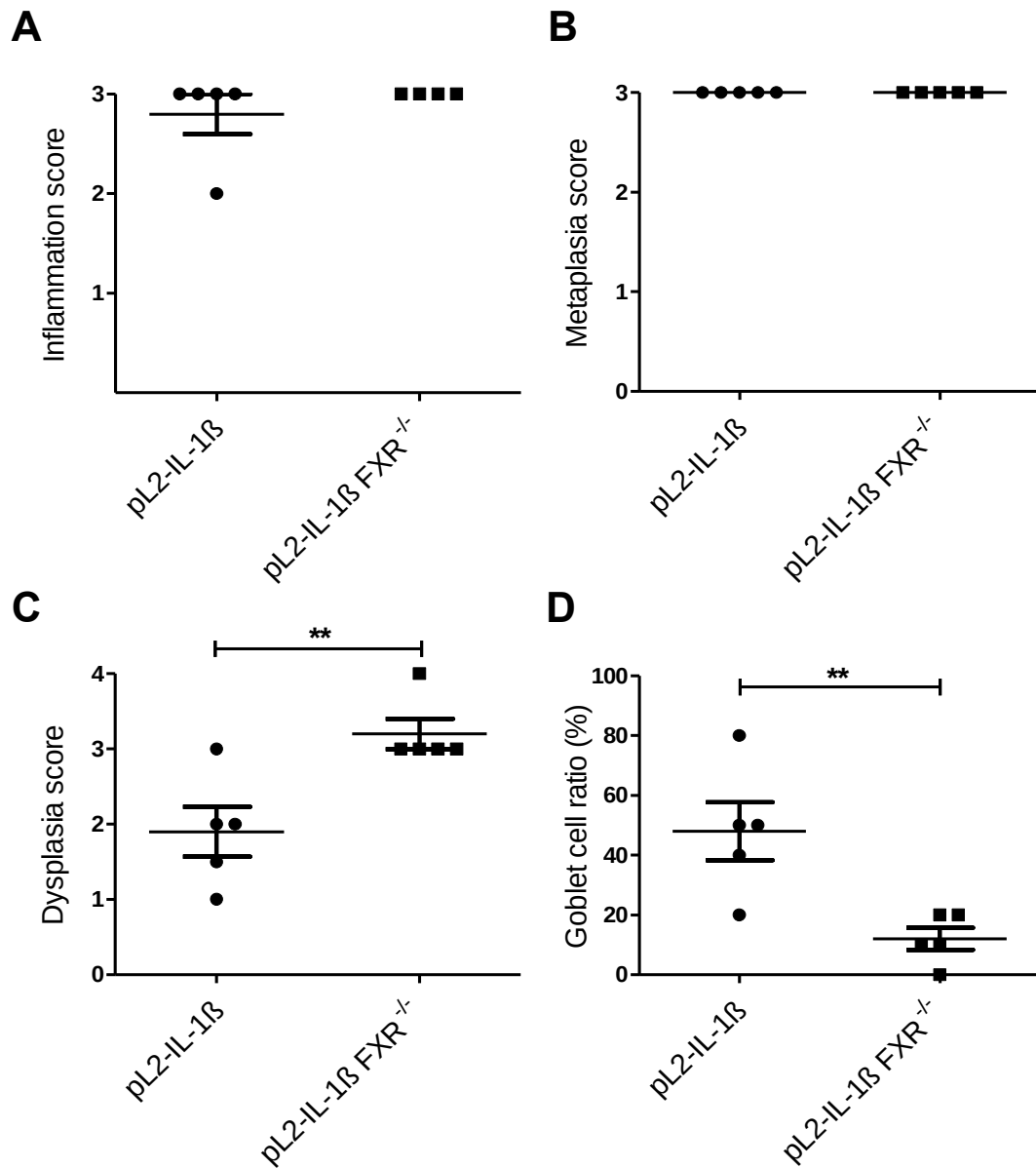


Figure 3.13: Barrett's tissue from pL2-IL-1 β FXR^{-/-} mice display higher levels of dysplasia than pL2-IL-1 β mice at 12 months.

Graphs represent histology scores of pL2-IL-1 β mice and pL2-IL-1 β FXR^{-/-} mice (n=5, 12 months) using H&E staining. **A)** Inflammation scores. **B)** Metaplasia scores. **C)** Dysplasia scores. **D)** Goblet cell ratio (%). Error bars represent mean $\pm$ SEM. Unpaired Student's *t* test: ***p*<0.05.

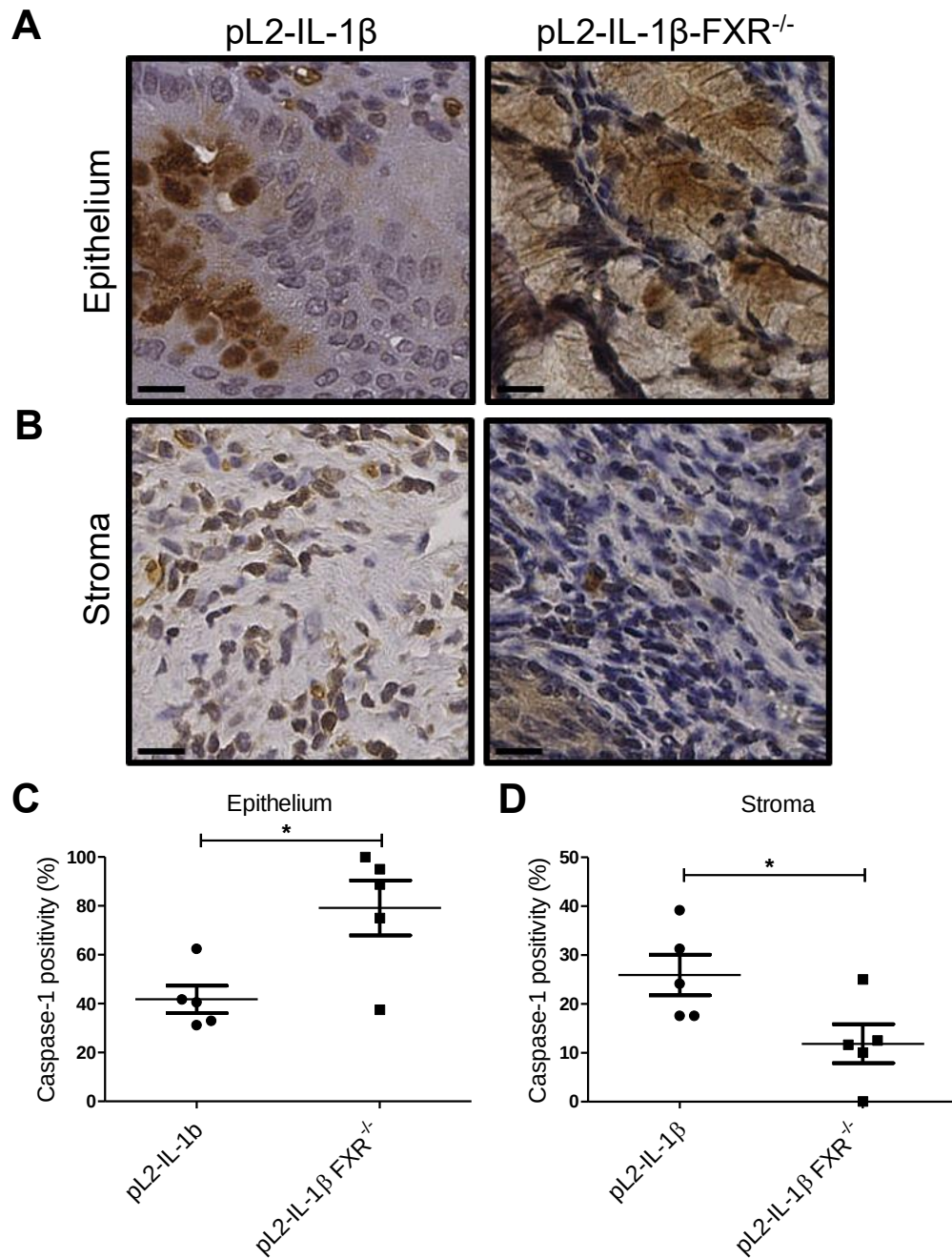


Figure 3.14: pL2-IL-1 β FXR $^{-/-}$ mice have upregulated caspase-1 in the metaplastic epithelium, when compared to pL2-IL-1 β mice, but have reduced levels of caspase-1 in stromal tissue.

pL2-IL-1 β mice (n=5) and pL2-IL-1 β FXR $^{-/-}$ mice (n=5) were sacrificed at 12 months. pL2-IL-1 β FXR $^{-/-}$ tissue was fixed in zinc formalin fixative for one week and paraffin embedded. IHC staining for caspase-1 was performed. **A)** Epithelium and **B)** stroma representative images of IHC caspase-1 expression patterns in pL2-IL-1 β mice and pL2-IL-1 β FXR $^{-/-}$ mice. Images taken at 20X magnification, scale bars = 10 μ m. Graphs represent caspase-1 positivity (%) in the **C)** columnar epithelium of the Barrett's region and **D)** the stroma at the SCJ. Data represents mean $\pm$ SEM. Unpaired Student's t test: * p < 0.05.

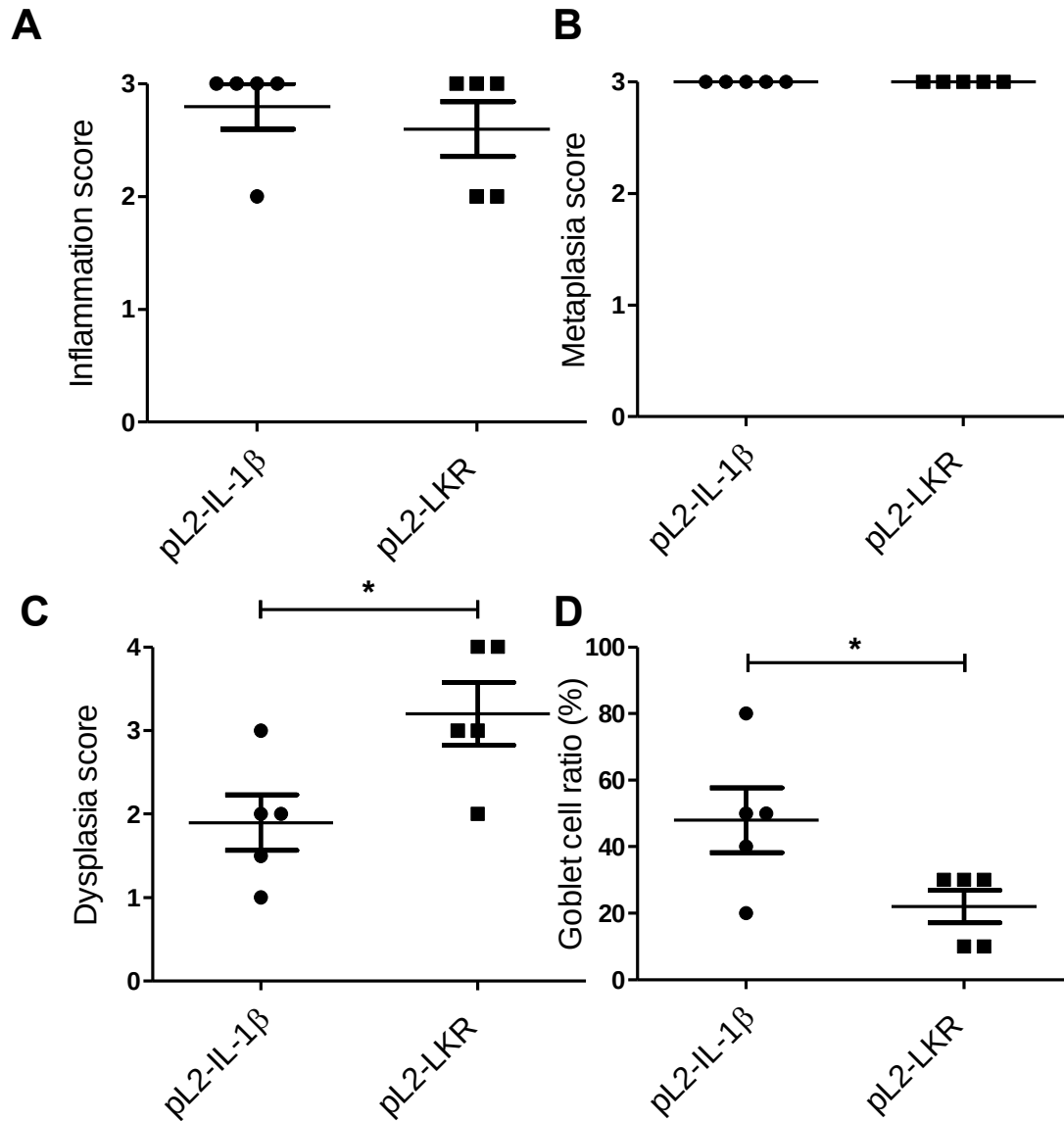


Figure 3.15: Barrett's tissue from pL2-LKR mice display higher levels of dysplasia than pL2-IL-1 β mice at 12 months.

Graphs represent histology scores of pL2-IL-1 β mice and pL2-LKR mice (n=5, 12 months) using H&E staining. **A)** Inflammation scores. **B)** Metaplasia scores. **C)** Dysplasia scores. **D)** Goblet cell ratio (%). Error bars represent mean $\pm$ SEM. Unpaired Student's *t* test: **p*<0.05.

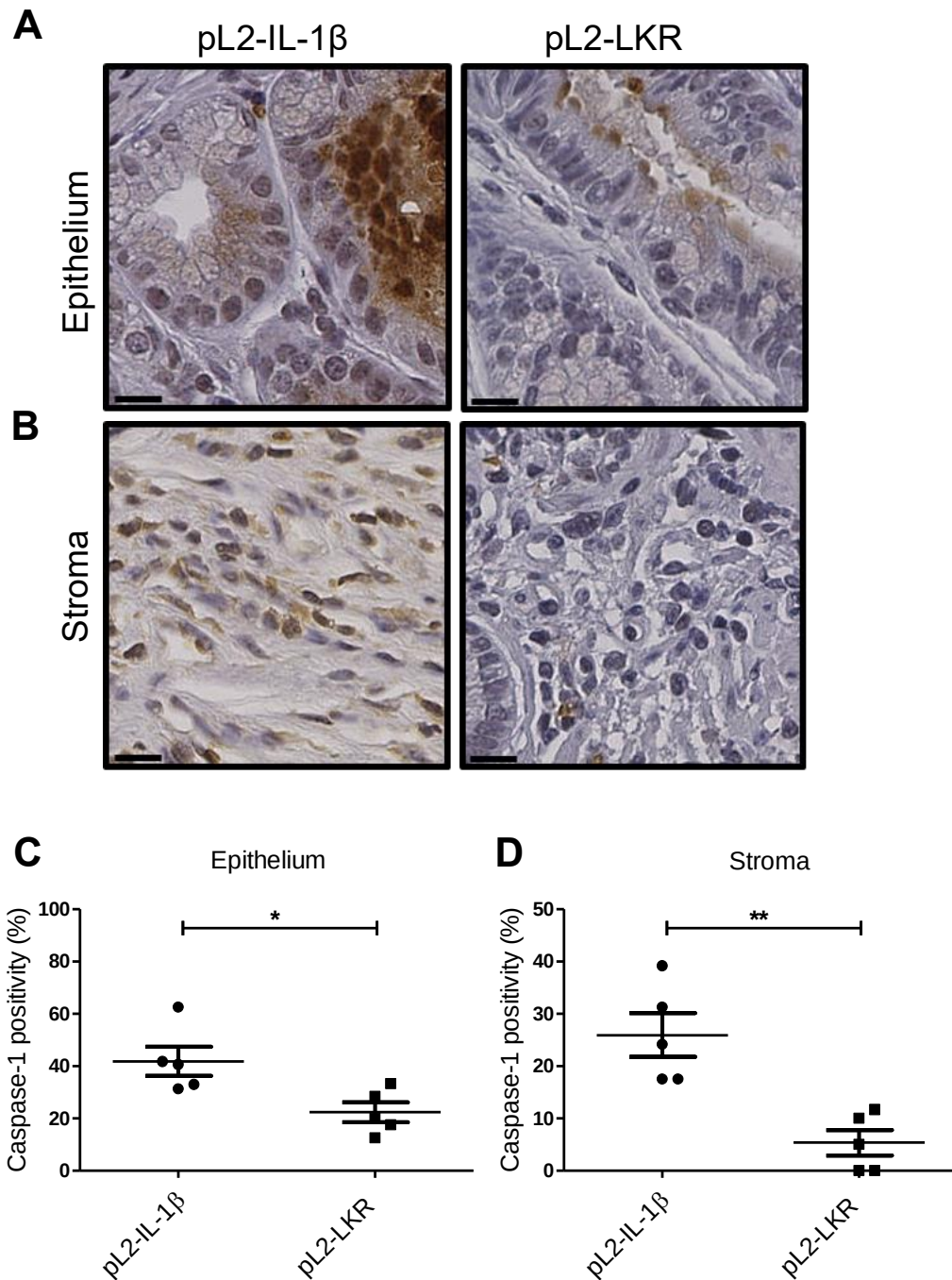


Figure 3.16: Caspase-1 expression levels are reduced in pL2-LKR mice, compared to pL2-IL-1 β mice, at 12 months.

pL2-IL-1 β mice (n=5) and pL2-LKR mice (n=5) were sacrificed at 12 months ($\pm$ two weeks). Resected tissue was formaldehyde fixed for 24 hours and paraffin embedded. IHC staining for caspase-1 was performed. **A)** Epithelium and **B)** stroma representative images of IHC caspase-1 expression patterns in pL2-IL-1 β mice and pL2-LKR mice. Images taken at 20X magnification, scale bars = 10 μ m. Graphs represent caspase-1 positivity (%) in the **C)** columnar epithelium of the Barrett's region and **D)** the stroma at the SCJ. Data represents mean $\pm$ SEM. Unpaired Student's *t* test: **p*<0.05, ***p*<0.001.

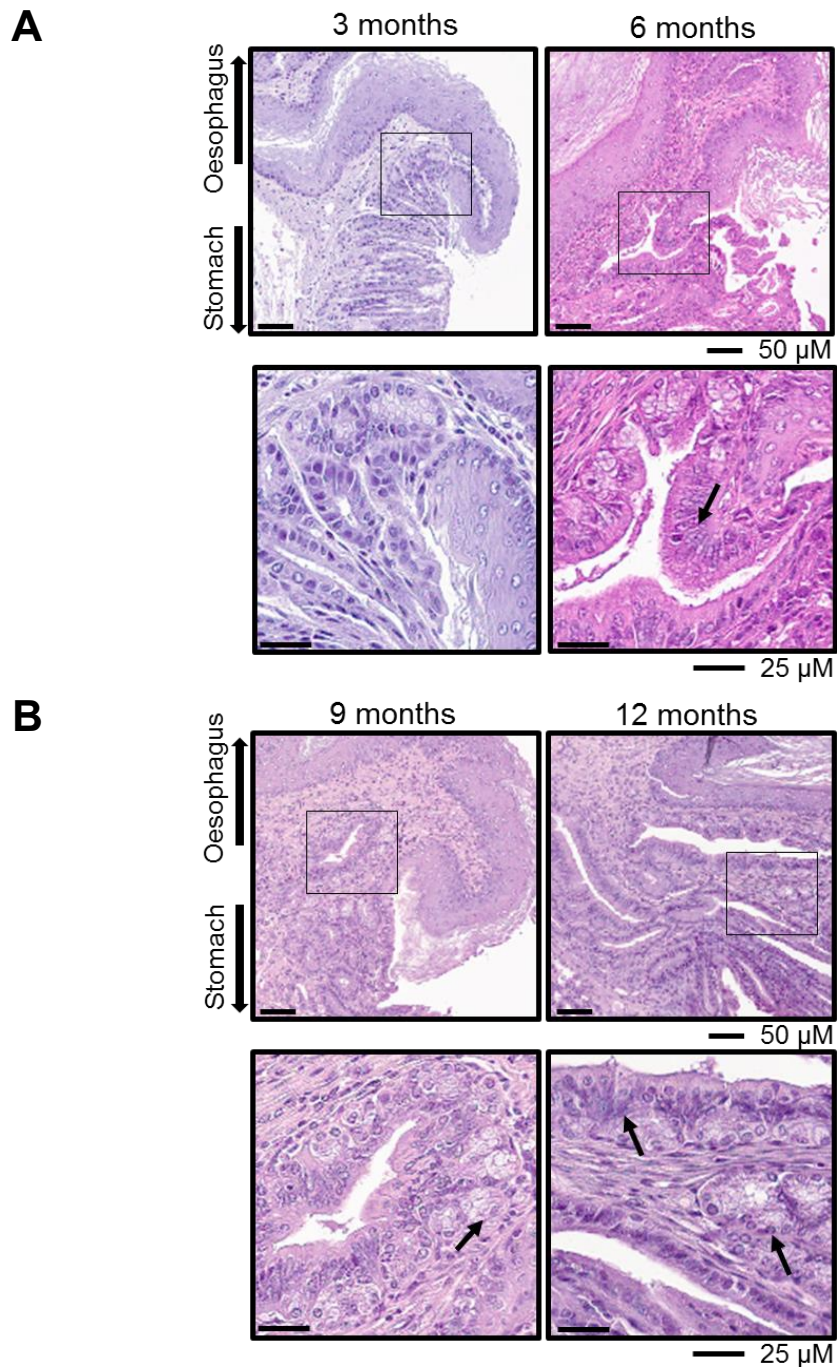


Figure 3.17: Histological characterisation of disease progression in pL2-IL-1 β mice over 12 months.

Resected oesophageal and stomach tissue from pL2-IL-1 β mice were sacrificed at 3, 6, 9 and 12 months ($\pm$ 2 weeks, n=5 per age group) was formaldehyde fixed and paraffin embedded. Representative H&E stained images of: **A)** the Barrett's region at the SCJ at 3 and 6 months. Arrow at 6 months indicates metaplasia in columnar epithelium; **B)** the Barrett's region at the SCJ at 9 and 12 months. Starting from the left, arrow at 9 months indicates goblet cells. First arrow at 12 months indicates dysplastic columnar epithelium. Second arrow at 12 months indicates more goblet cells. Scale bars = 25 μ m and 50 μ m.

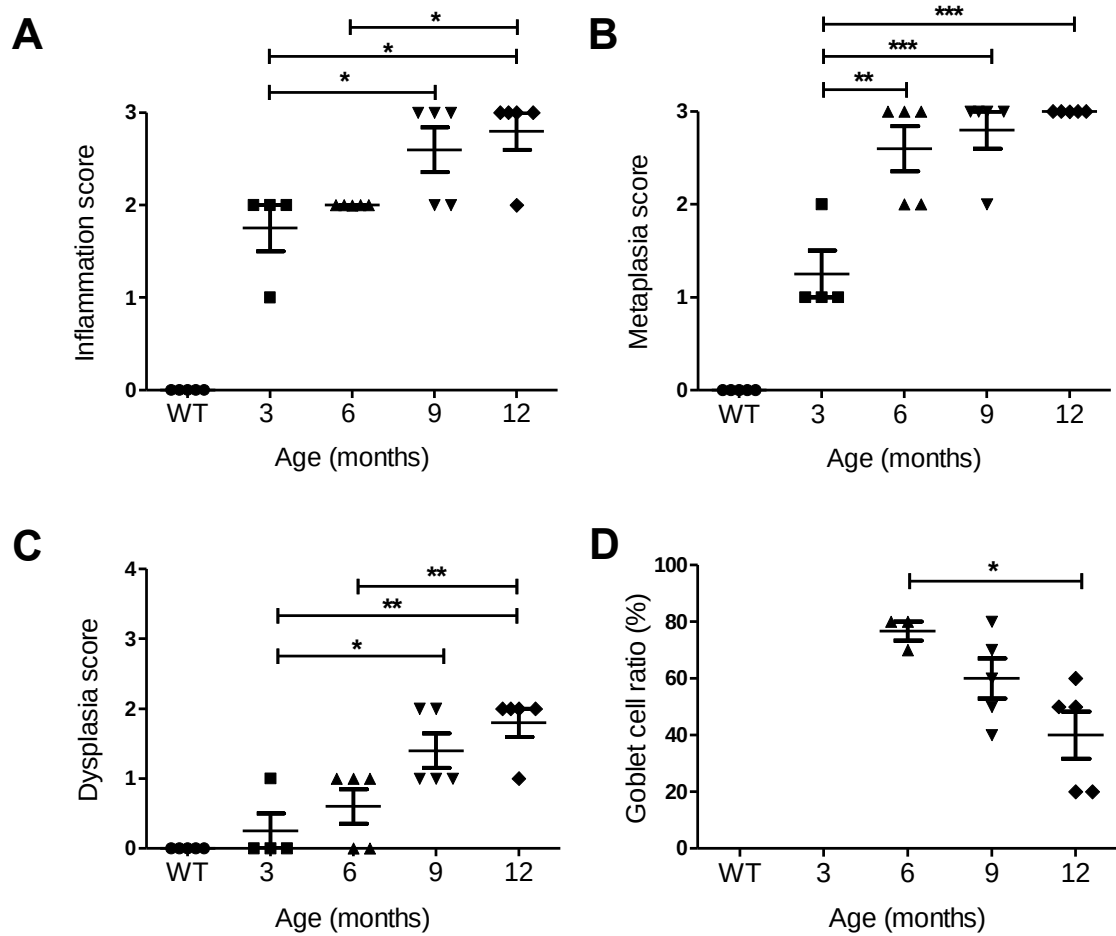


Figure 3.18: Stepwise progression of disease severity with age in pL2-IL-1 β mice.

Disease severity scoring of pL2-IL-1 β mice of different age groups (n=5). Graph represents histological scores for **A)** inflammation using the following scale: 0 = no inflammation, 1 = mild, 2 = moderate, 3 = severe. **B)** Metaplasia using the following scale: 0 = no metaplasia, 1 = rare mucous cells, 2 = single metaplastic glands, 3 = multiple metaplastic glands. **C)** Dysplasia using the following scale: 0 = no dysplasia, 1 = superficial epithelial atypia, 2 = atypia with granular complexity, 3 = low-grade dysplasia, 4 = high-grade dysplasia. **D)** Goblet cell ratio (%) calculated as a percentage of goblet cell positivity within each crypt of the Barrett's region. Data represents mean $\pm$ SEM. One-way ANOVA followed by Tukey's post hoc test: * p <0.05, ** p <0.01, *** p <0.001.

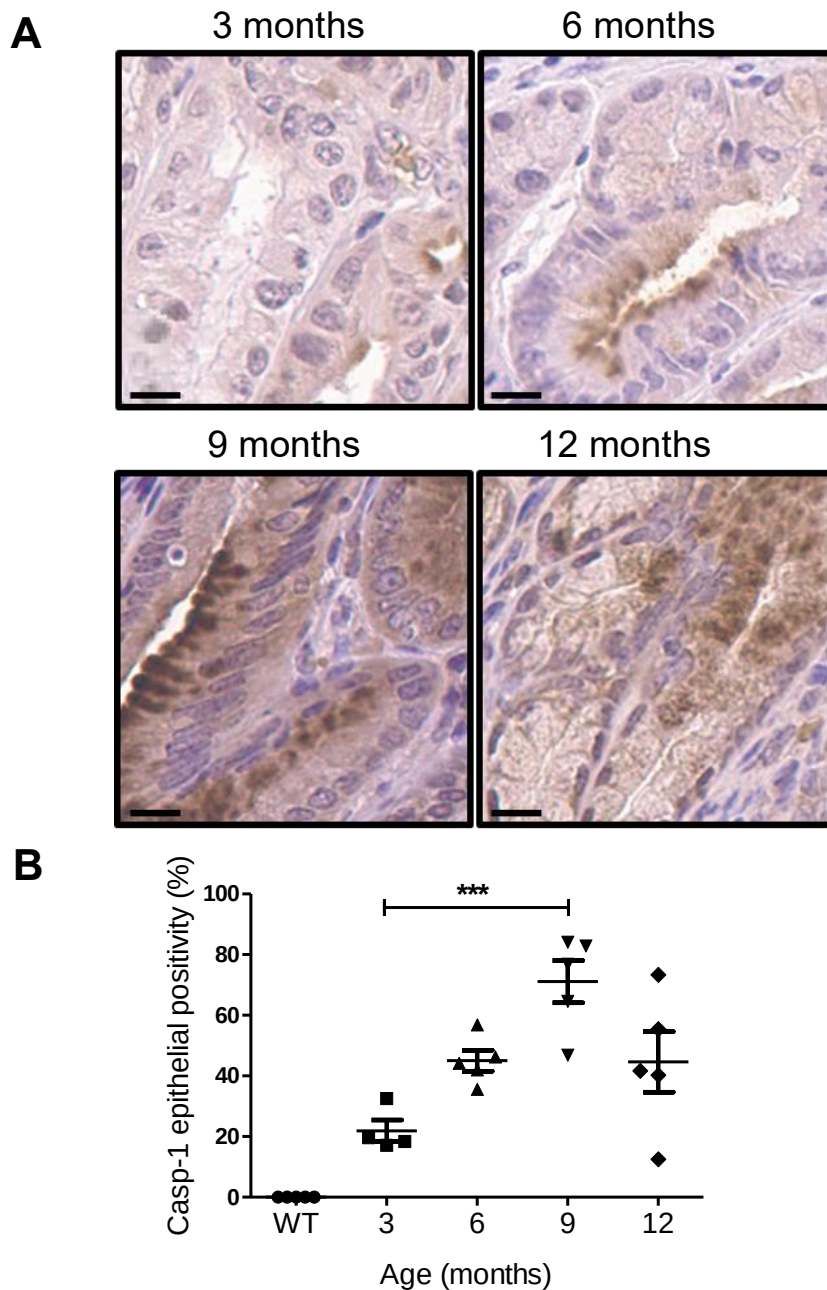


Figure 3.19: IHC analysis shows upregulated epithelial expression of caspase-1 in nine-month-old pL2-IL-1 β mice.

pL2-IL-1 β mice were sacrificed at 3, 6, 9 and 12 months ($\pm$ 2 weeks) and wild-type mice (n=5) were sacrificed at 12 months. Subsequently, the oesophagus and stomach were resected and the tissue was formalin-fixed and paraffin-embedded. IHC staining for caspase-1 was performed and expression was assessed by two independent reviewers using a validated semi-quantitative scoring method (G. Barber, A. Anand). **A)** Representative images of IHC caspase-1 expression patterns of different age groups. Images taken at 20X magnification, scale bars = 10 μ m. **B)** Graph represents cytoplasmic caspase-1 % positivity of columnar epithelium at the SCJ. Data represents mean $\pm$ SEM. One-way ANOVA followed by Tukey's post hoc test: ** p <0.01, *** p <0.001.

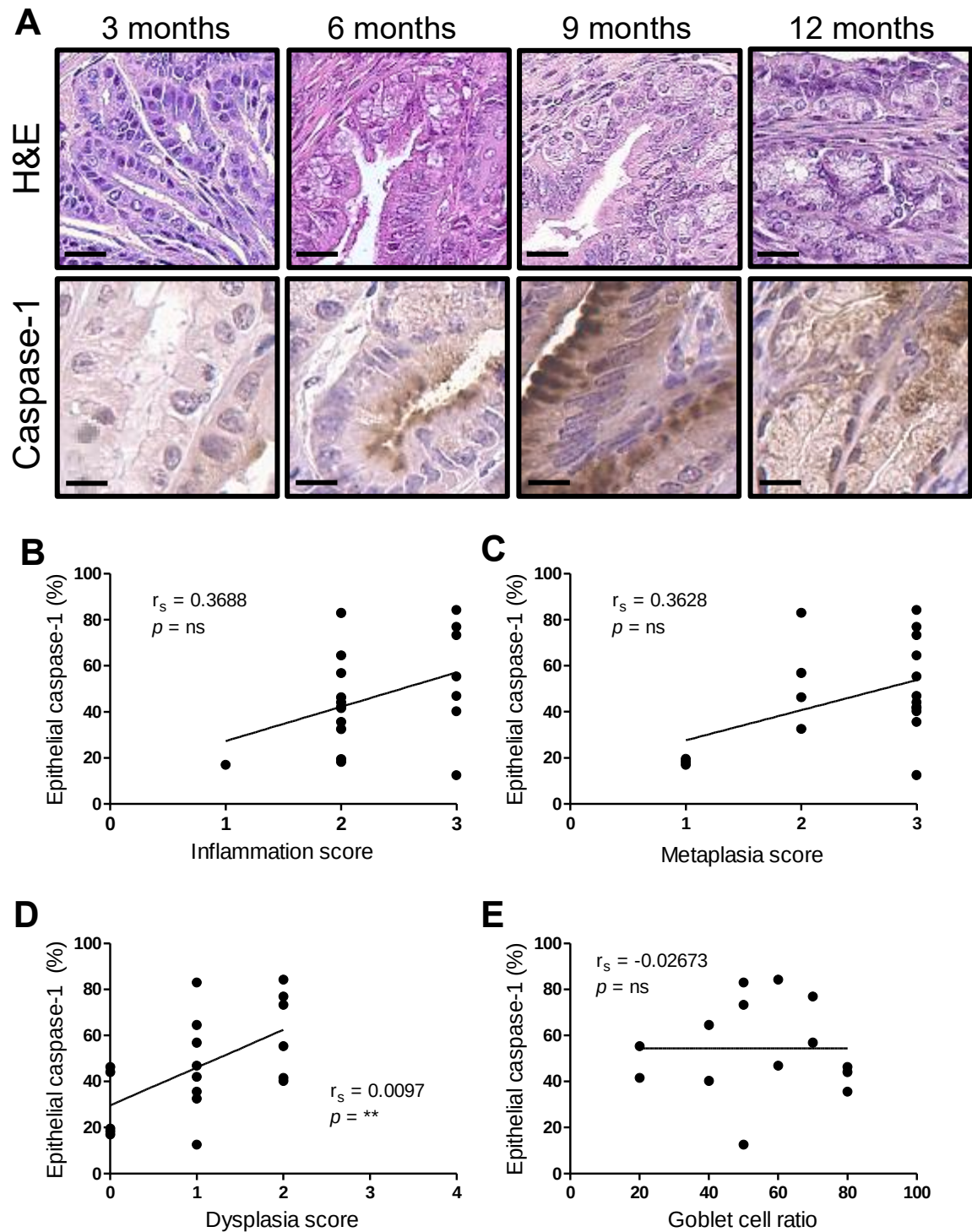


Figure 3.20: Positive correlation of epithelial caspase-1 expression with histological scores for dysplasia in pL2-IL-1 β mice.

A) Representative images for H&E and epithelial caspase-1 IHC staining. Images taken at 20X magnification, scale bars = 10 μ m. **B-E)** Correlation of epithelial caspase-1 expression (%) verses scores for **B)** inflammation, **C)** metaplasia **D)** dysplasia and **E)** goblet cell ratio in pL2-IL-1 β mice. r_s = Spearman's correlation coefficient: p = non-significant (ns), $**p < 0.01$.

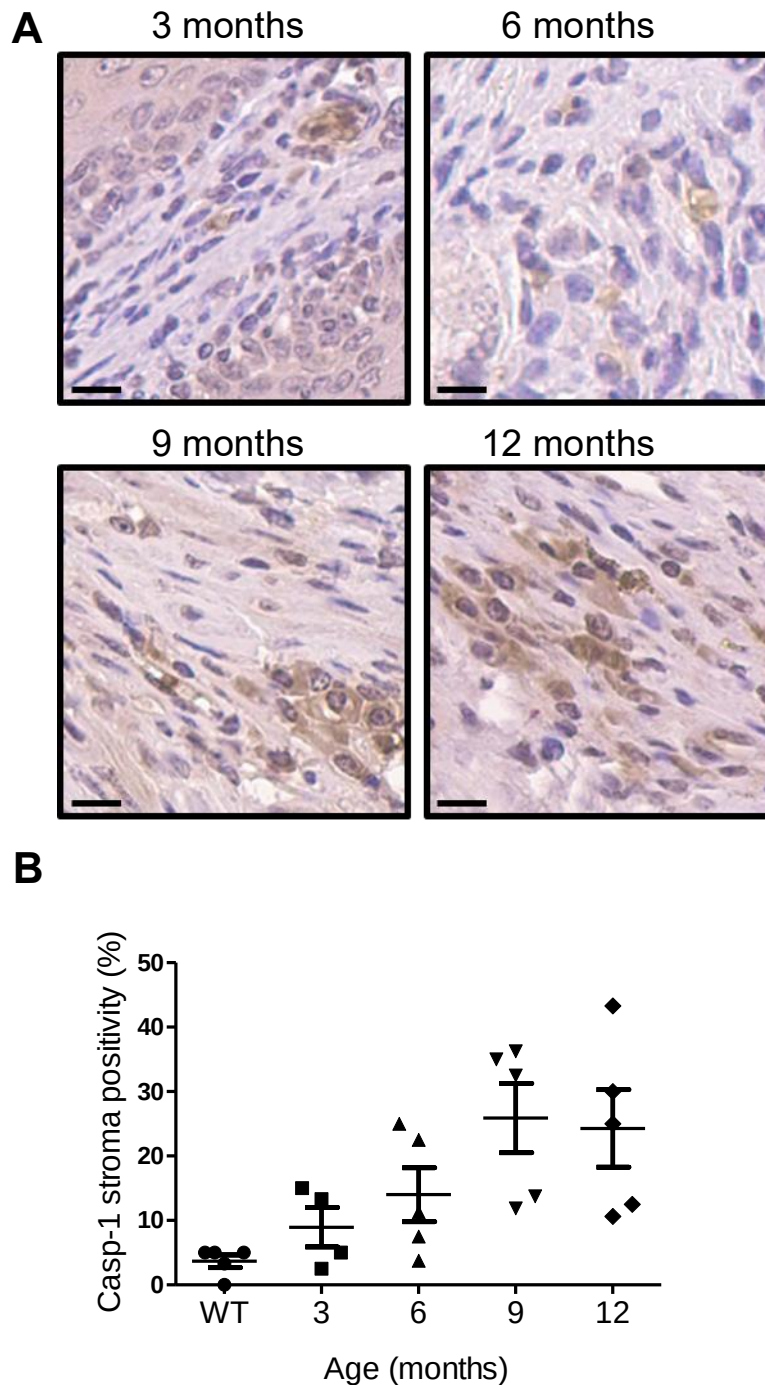


Figure 3.21: IHC analysis identifies age-dependent increase in stromal caspase-1 expression in pL2-IL-1 β mice.

IHC staining for caspase-1 was performed and each group was scored for stromal caspase-1 % positivity at the SCJ. **A)** Representative images of IHC caspase-1 expression patterns of different age groups. Images taken at 20X magnification, scale bars = 10 μ m. **B)** Graphs represents caspase-1 % positivity in the stroma at the SCJ. Data represents mean $\pm$ SEM. One-way ANOVA followed by Tukey's post hoc test: non-significant.

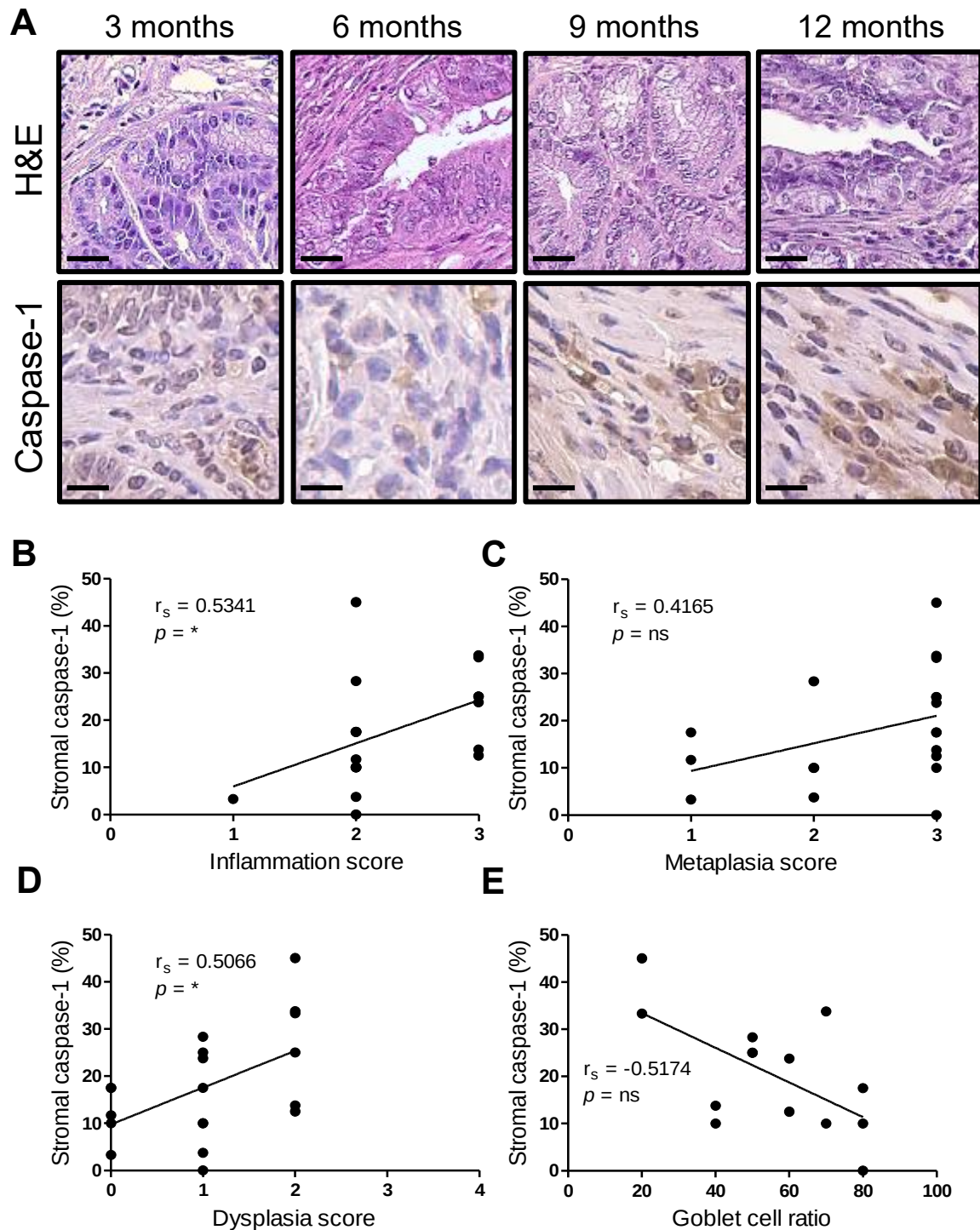


Figure 3.22: Correlation analysis of pL2-IL-1 β mice shows that scores for inflammation and dysplasia correlate with caspase-1 expression.

A) Representative images for H&E and stromal caspase-1 IHC staining. Images taken at 20X magnification, scale bars = 10 μ m. **B-E)** Correlation of stromal caspase-1 expression (%) versus scores for **B)** inflammation, **C)** metaplasia **D)** dysplasia and **E)** goblet cell ratio in pL2-IL-1 β mice. r_s = Spearman's correlation coefficient: p = non-significant (ns), $*p < 0.05$.

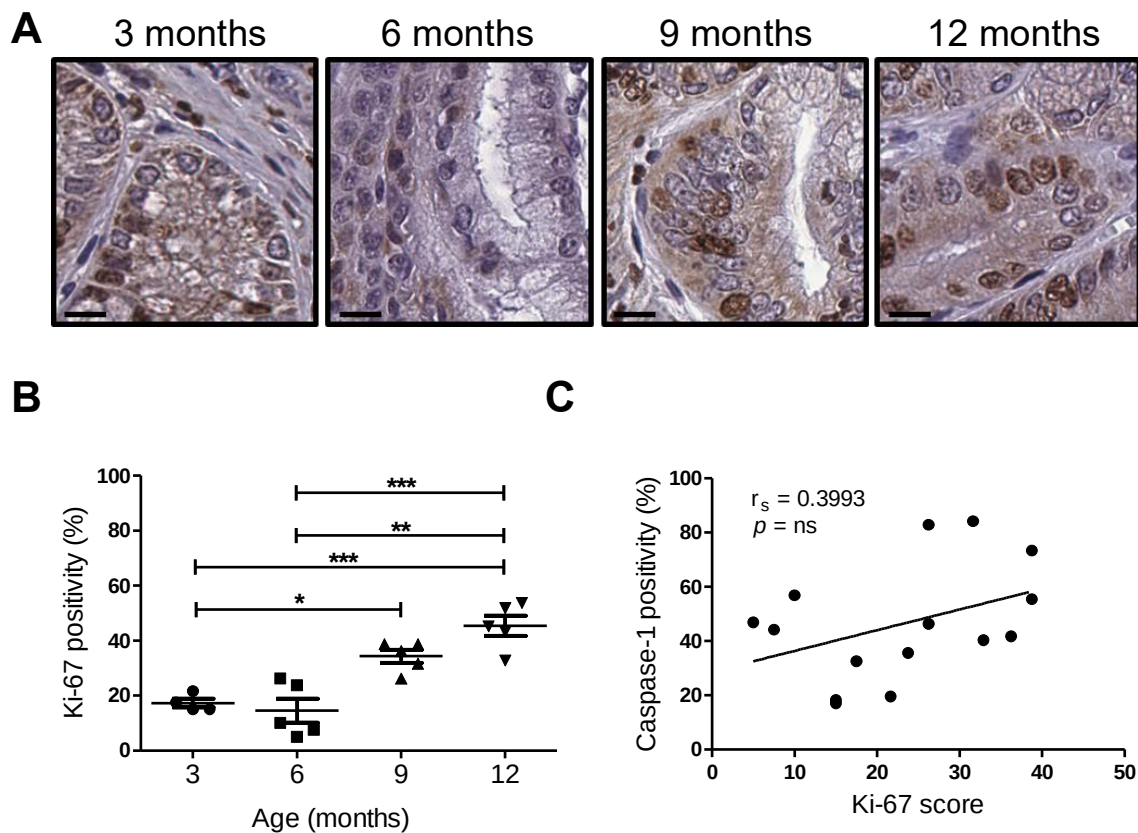


Figure 3.23: No correlation between epithelial caspase-1 expression and Ki-67 in Barrett's tissue from pL2-IL-1 β mice.

A) Representative images of IHC Ki-67 expression at the SCJ of pL2-IL-1 β mice at ages 3, 6, 9 and 12 months. Images taken at 20X magnification, scale bars = 10 μ m. **B)** Ki-67 nuclear positivity (%) of the columnar epithelium within the Barrett's region of pL2-IL-1 β mice (n=19). **C)** Correlation of caspase-1 epithelial expression (%) verses Ki-67 positivity (%) in pL2-IL-1 β mice. Data represents mean $\pm$ SEM. One-way ANOVA followed by Tukey's post hoc test, r_s = Spearman's correlation coefficient, p = non-significant (ns), * p <0.05, ** p <0.01, *** p <0.001.

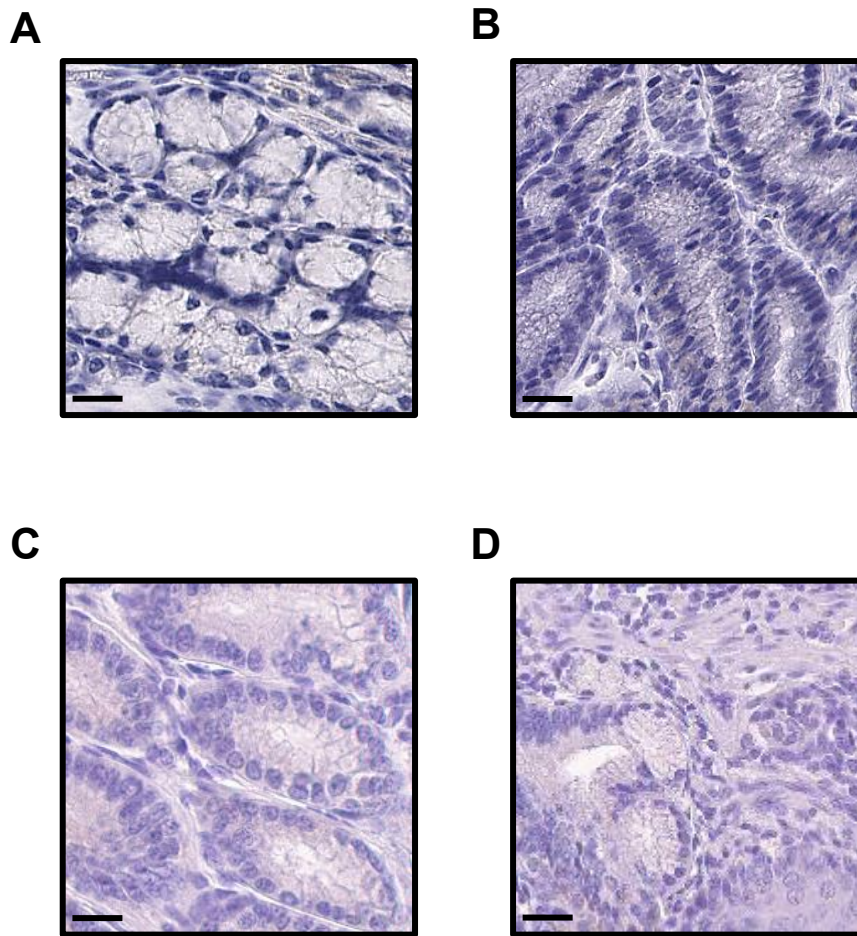


Figure 3.24: Negative controls for pL2-IL-1 β mice IHC.

Representative images of IHC negative control expression (exclusion of primary) at the SCJ of pL2-IL-1 β mice. Negative representative images for caspase-1 staining (anti-rabbit alone) in **A)** Figures 3.10 and 3.12, **B)** Figures 3.14 and 3.16, **C)** Figures 3.19 and 3.21. Negative Ki-67 staining (anti-rabbit alone) for **D)** Figure 23. Images taken at 20X magnification, scale bars = 10 μ m.

Chapter 4

4.1 Introduction

Inflammasomes are important multimeric complexes that mediate acute and chronic inflammation during infection and disease. First described by Martinon *et al.* (2002) as a caspase activating and IL-1 β processing pathway (85), inflammasomes have since become implicated in tumorigenesis. The pathway has since been identified as a potential target for immunotherapy (467). Inflammasome signalling requires two signals. Signal one, 'priming' occurs when PAMPs and DAMPs are recognised by PRRs such as TLRs and NLRs (87, 469). These molecules initiate a signalling cascade that results in activation of transcription factor pathways such as NF- κ B, leading to transcriptional upregulation of inflammasome components, such as NLRP3 and pro-IL-1 β (470). Signal two, 'activation' is triggered by specific stimuli which can trigger inflammasome sensor protein activation, including ATP and DNA (80, 124, 471). This results in the oligomerisation of inflammasome components into a complex, in most cases incorporating ASC. This promotes autocatalytic cleavage of caspase-1 and subsequent activation of IL-1 β and IL-18, leading to their secretion from the cell (74). Alternatively, caspase-1 cleaves gasdermin D inducing its pore forming activity, resulting in pyroptosis (60).

Conflicting roles have been attributed to the inflammasome during tumorigenesis and are specific to the cancer type. Inflammasome activity during disease development of OAC is not well understood (48, 93, 95), however we can form hypotheses based on studies of other cancers. For example, in melanoma, NLRC4 has been reported to suppress tumour growth in a subcutaneous murine model, suggesting a protective role (472). Similarly, increasing AIM2 upregulation by an exogenous AIM2 promoter was shown to inhibit proliferation and invasion potential in renal carcinoma cell lines (473). Inhibition of NLRP1 also showed protective effects in chronic myelogenous leukaemia (CML), where proliferation was suppressed and apoptosis increased in primary cells (474). Furthermore, a range of studies have provided evidence for a protective effect of the inflammasome pathway during colorectal carcinogenesis (150, 175, 475, 476).

In contrast to this, tumour development and progression may also be facilitated by inflammasome activity. In pancreatic ductal cancer, inhibiting NLRP3, ASC and caspase-1 suppressed tumour growth and metastasis in mice, indicating a promoting affect (477).

Indeed, AIM2, an inflammasome originally believed to be protective due to its DNA sensing functions, has been reported to have tumour promoting effects in non-small cell lung carcinoma (478). These studies among others show the contrasting roles inflammasome signalling plays both in cancer development and progression. This highlights the need to investigate each cancer type independently, and at all stages of development.

Many instigators of inflammasome activity are derived from obesity and diet, and together they are thought to enhance carcinogenesis. Obesity is a risk factor for Barrett's and OAC and has been attributed as a leading cause of disease (334, 479). Obesity is simply defined as excess body weight and is highly preventable. Despite this, obesity-related mortality is prevalent in developed countries worldwide. Excess body weight is caused by increased intake of energy dense foods and elevated adiposity, which often results in metabolic and cardiovascular complications (480). In Ireland, obesity is considered a major public health concern, where 43% of men and 31% of women are overweight. Adding to this, 25% of men and 22% of women are obese (481). Therefore, more than half the population is above what is considered a normal body weight. Measurement of waist circumference (WC) has become important in clinical settings as it provides valuable additional patient information about abdominal obesity (324, 482). This can also be measured using visceral fat area (VFA), often used in the clinic after cancer diagnosis (326). In the context of obesity, increases in abdominal circumference are predominantly caused by increased production of visceral adipose tissue (VAT). This fat is located around the internal organs of the abdomen and is important for insulation and protection. Recent studies have revealed that VAT is also highly metabolically active and performs important roles in energy homeostasis throughout the body (354, 483). During obesity, VAT becomes stressed and produces elevated levels of pro-inflammatory and pro-tumorigenic factors such as IL-6 and VEGF (368). These studies identify the need for further investigation into the relationship between inflammation and obesity during oesophageal disease.

In Brief:

Hypothesis and aims of Chapter 4

Hypothesis: The inflammasome pathway, mediated through caspase-1: (i) contributes to inflammation and disease progression to Barrett's and OAC; (ii) is instigated by obesity.

Specific aims of Chapter 4:

1. Confirm and characterise the inflammatory phenotype associated with Barrett's patient tissue.
2. Determine the extent of caspase-1-mediated inflammation during Barrett's oesophagus, by co-culturing patient biopsies with a caspase-1 inhibitor.
3. Assess caspase-1 mediated inflammation in cardia organoids taken from the squamocolumnar junction of pL2-IL-1 β mice using a caspase-1 inhibitor.
4. Demonstrate TLR2 mediated inflammation in cardia organoids taken from the squamocolumnar junction of pL2-IL-1 β mice using a TLR2 blocking antibody.
5. Identify potential inflammasome pathways which may initiate caspase-1-related inflammation during oesophageal disease.
6. Investigate the effects of adipose conditioned media from obese OAC patients on inflammasome component expression and inflammatory signalling, using an oesophageal cell line model representing stages of disease progression.

4.2 Results

4.2.1 Biopsies from Barrett's regions secrete higher levels of inflammatory mediators than adjacent normal biopsies.

Published literature has shown that Barrett's tissue has an increased inflammatory phenotype. Fitzgerald *et al.* observed that IL-1 β was elevated in Barrett's (93), this work was supported by Kavanagh *et al.* who characterised the inflammatory secretion profile in oesophageal disease tissues, which included evidence that Barrett's tissue produced increased levels of IL-1 β , IL-6 and IL-10 (95).

For the purpose of this project, we sought to further define the inflammatory phenotype of Barrett's tissue and to confirm inflammasome involvement during Barrett's. To do this, biopsies were taken from histologically confirmed Barrett's patients (n=10) undergoing routine surveillance endoscopies in St. James's Hospital as part of the National Barrett's oesophagus registry and biobank. Biopsies (~2 mm) were taken from identified regions of Barrett's (x4) and adjacent normal squamous tissue (x4) from each patient, and were subsequently cultured for 16 hours *ex vivo* in the presence or absence of a caspase-1 inhibitor (described in detail below).

Table 4.1 shows Barrett's patient characteristics for this study. Median age among patients was 63 years, 70% were male and 80% were overweight or obese, as per BMI (kg/m²). Biopsy tissue (two normal and two Barrett's biopsies/patient) was cultured *ex vivo* for 16 h in M199 media (10% FBS, 1% Pen/Strep, 1 mg/mL insulin). Culture supernatants were analysed using either Multiplex analysis (MSD; IL-1 β , IL-6, TNF α) or standard singleplex ELISA (BioLegend; IL-8, IFN- γ , IL-1 α , R&D Systems; IL-18). Cytokine levels for each biopsy were normalised to the biopsy protein concentration (μ g/mL). Cytokine secretion levels from areas of normal and Barrett's are shown for each patient (**Figure 4.1**). Average patient cytokine levels for areas of normal (n=2 biopsies) and Barrett's (n=2 biopsies) are shown. Analysis of culture supernatants confirmed that Barrett's explant tissues secrete higher levels of IL-1 β , IL-6, IL-8 and IFN- γ ($p < 0.01$), when compared to adjacent normal tissue (**Figure 4.1A, C-E**). In contrast, levels of IL-18

secretion were reduced ($p<0.01$) in Barrett's explants compared to adjacent normal tissue (**Figure 4.1B**). In the literature, levels of IL-18 secretion are unreported in Barrett's tissue. This result initially contradicts expectations as IL-1 β secretion levels are elevated, while both IL-18 and IL-1 β maturation are directly dependent on caspase-1 (82).

Analysis of pro-*IL1B* and pro-*IL18* mRNA expression in two publicly available data sets may provide an explanation for the decreased levels of IL-18 secretion observed in Barrett's biopsies (373, 374). Although pro-*IL1B* mRNA was significantly increased during Barrett's, pro-*IL18* mRNA levels were significantly lower in Barrett's tissue, compared to adjacent normal (**Figure 4.2A-D**). Thus, it appears that metaplastic tissue has lower constitutive IL-18 expression compared to adjacent normal squamous tissue. Additionally, graphs show levels of both pro-*IL1B* and pro-*IL18* mRNA expression were decreased during OAC, consistent with findings reported in Chapter 3.

Collectively, these results confirm that Barrett's tissue has an inflammatory phenotype, with increased levels of IL-1 β , IL-6, IL-8 and IFN- γ secretion, compared to adjacent normal tissue. Results also show a decrease in IL-18 mRNA and protein expression levels. Findings suggest that IL-1 β -mediated inflammation may play a role in development and progression of Barrett's.

4.2.2 Caspase-1 inhibitor, WEHD.CHO optimally inhibits IL-1 β secretion from THP-1 cells at 10 μ M.

Results generated thus far have shown that caspase-1 is upregulated in a Barrett's metaplastic cell line (QH), in the Barrett's mouse model, and in areas of Barrett's from human resected tissue. These results provide evidence for inflammasome priming during Barrett's. Results also have shown that Barrett's tissue is inflammatory and secretes increased levels of pro-inflammatory cytokines, which may be driving disease. These observations suggest that caspase-1 has a functional role during Barrett's-mediated inflammation. To explore this hypothesis, we examined whether levels of pro-inflammatory cytokine secretion from Barrett's biopsy tissue are altered in the presence of a caspase-1 inhibitor. Barrett's patient explant tissues were cultured in the presence

or absence of a caspase-1 inhibitor for 16 hours, and levels of inflammatory cytokine secretion were determined by ELISA.

First, the efficacy of two caspase-1 inhibitors were compared and a dose response was carried out in a THP-1 cell line. Inhibitor optimisation was not performed using Barrett's tissue due to limited availability of patient samples. Oesophageal cell lines were not used for optimisations as they did not secrete quantifiable amounts of IL-1 β when stimulations were attempted. In **Figure 4.3A**, caspase-1 inhibitors YVAD.fmk (Z-Tyr-Val-Ala-Asp (OMe)-fluoromethylketone) and WEHD.CHO (Trp-Glu-His-Asp-aldehyde) were compared for their ability to inhibit caspase-1-mediated cleavage of IL-1 β and secretion from PMA-differentiated THP-1 macrophages. The cells were pre-treated (1 hour) with 1 μ M inhibitor, primed with LPS (1 μ g/mL) overnight and the NLRP3 inflammasome was subsequently activated with ATP (5 mM) or Nigericin (5 μ M) for 30 minutes. Supernatants were removed and analysed for IL-1 β secretion by ELISA. A control LPS/DMSO sample was missing which may have limited the observations of this experiment. Caspase-1 inhibition was achieved using both YVAD.fmk and WEHD.CHO, as both inhibitors reduced IL-1 β secretion to similar levels (**Figure 4.3A**).

YVAD and WEHD are selective tetrapeptide inhibitors whose sequences are designed to be specifically recognised by caspase-1 active site residues where proteolytic cleavage normally occurs. The inhibitors contain aspartic acid in the P1 position that mimics the cleavage site of pro-IL-1 β and causes hydrogen bonding to occur (484). As all caspases are similarly structured it is challenging to design an inhibitory compound which is specific to caspase-1 and does not inhibit other caspases, such as caspase-4 and -5. A study published by Rano *et al.* showed that the WEHD sequence is the most favoured tetrapeptide recognition motif for caspase-1. The K_{cat}/K_m value for WEHD cleavage by caspase-1 was found to be approximately 50-fold higher than for YVAD, and WEHD binding affinity was 14-fold higher due to an increase in the lifetime of the enzyme-inhibitor complex ($t_{1/2}$ YVAD = 0.66h, $t_{1/2}$ WEHD = 12.8h) (485). A similar study involving HPLC analysis, shows WEHD is the most favourable substrate for caspase-1 under competitive reaction conditions (486). Although YVAD.fmk was initially thought to be a very potent inhibitor of caspase-1, its widespread use as a specific caspase-1 inhibitor has been questioned following research published by Rozman-Pungercar *et al.* reporting that

YVAD.fmk can also efficiently inhibit cysteine cathepsins, including cathepsin B (487). Taking these studies into account, it was decided that WEHD.CHO would be used as a caspase-1 inhibitor in future Barrett's patient explant experiments.

Next, a dose response (1, 5 and 10 μ M) of WEHD.CHO dissolved in H₂O was carried out in activated THP-1 macrophages revealed that caspase-1 activity was optimally inhibited at 10 μ M ($p < 0.001$), as indicated by reduced levels of IL-1 β secretion (**Figure 4.3B**).

4.2.3 Caspase-1 inhibition results in reduced inflammatory cytokine secretion in biopsies from obese Barrett's patients.

Oesophageal biopsies from each patient (n=10) were cultured *ex vivo* in the presence or absence of the caspase-1 inhibitor, WEHD.CHO for 16 hours. Attempts were made to analyse protein lysates from the biopsies for biomarkers of Barrett's by immunoblotting. Villin and CDX2 have previously been identified as protein biomarkers for metaplasia (488, 489). They were therefore chosen to confirm the presence of Barrett's metaplasia in patient biopsies. However, technical difficulties arose using this method, with multiple non-specific bands present due to immunoglobulin cross-reactivity. This made visualisation of villin and CDX2 expression very difficult and was further limited by the lack of available protein lysates due to the very small size of patient biopsies (data not shown).

Protein lysates were further analysed for inflammatory caspase-1 and pro-IL-1 β expression. **Figure 4.4A** shows representative immunoblot images for caspase-1 and pro-IL-1 β expression in the presence or absence of WEHD.CHO. Some variability between loading control samples for each patient was observed, which limited visualising differences between treatment groups. Subsequent densitometric analysis was carried out assessing expression profiles from 6 patients (**Figure 4.4B, C**). Protein was not available from the other four remaining patients due to small biopsy size. As expected, treatment with the caspase-1 inhibitor had no significant effect on the expression levels of caspase-1 or pro-IL-1 β . A trend towards elevated caspase-1 expression in adjacent normal biopsy lysates was observed, compared to Barrett's

biopsies. This did not agree with results reported in Chapter 3, which showed increased caspase-1 levels in the epithelium. Differences may be due to biopsies consisting of many cell types, including fibroblasts, immune cells and epithelium. In contrast, expression of pro-IL-1 β was consistently upregulated in Barrett's biopsies, suggesting inflammasome priming consistent with the literature (48, 93). This result also indicated that the Barrett's biopsies were derived from Barrett's tissue.

Cytokine secretion levels for each biopsy were normalised to the biopsy protein concentration ($\mu\text{g}/\text{mL}$). Cytokine secretion levels from areas of normal and Barrett's, in the presence or absence of WEHD.CHO, are shown for each patient (**Figure 4.5**). Average patient cytokine levels are shown, based on the results from two biopsies/patient/group (i.e. adjacent normal +/- inhibitor; Barrett's +/- inhibitor). Analysis of inflammatory cytokines ($\text{pg}/\mu\text{g}$) from the supernatants revealed varying levels of caspase-1 inhibitor efficacy (**Figure 4.5A-G**). 50% of patients showed reduced IL-1 β secretion in response to inhibitor treatment, highlighting patient-to-patient variability and biopsy heterogeneity. Analysis of supernatants from the Barrett's biopsy cultures revealed no significant differences in LDH secretion between WEHD.CHO-treated and -untreated groups, indicating that WEHD.CHO was not having any overt cytotoxic effect (**Figure 4.5H**).

To further analyse results, cytokine secretion profiles were separated based on different patient characteristics, including cytokine secretion profile, age and obesity status per patient. First, the inflammatory profile of each independent patient was visualised by graphing overall cytokine secretion levels per patient (**Figure 4.6A-J**) and secretion levels for each cytokine (**Figure 4.6K**). Graphs reveal high levels of patient-to-patient variability and significant differences in levels of inflammatory cytokines between patients. Following this, cytokine secretion data was separated based on patient age (≥ 60 years old). Barrett's diagnosis is more commonly made in older individuals and is considered a risk factor (267, 490). Additionally, the term 'inflammaging' has recently been coined to describe the low-grade chronic inflammation present in accumulative tissue damage of aging individuals (491). Inflammatory cytokine secretion of IL-1 β and IL-6 were decreased in 3/4 patients under 60 years old, suggesting that caspase-1 inhibition may be more effective at limiting inflammation in younger patients (**Figure 4.7A, C**). Cytokine

secretion profiles were also separated based on the length of the Barrett's segment, defined as the long-segment (≥ 3 cm) or short segment (< 3 cm) (**Figure 4.8**). A longer segment has been associated with increased risk of disease progression (241). However, only 2/10 patients were classified as having a short segment. No associations were observed between caspase-1 inhibition and Barrett's length.

Obesity has been associated with Barrett's development and is thought to be a major driver of disease (340, 342). Graphs in **Figure 4.9** represent cytokine secretion separated by patient obesity status (BMI, obese ≥ 30 kg/m²). Red symbols indicate inhibition. Results revealed that 4/6 obese Barrett's patients had reduced IL-1 β secretion in both normal and Barrett's biopsies in response to caspase-1 inhibition. No obvious differences were observed for IL-18 expression levels (**Figure 4.9A, B**).

Overall, separation of results based on patient characteristics revealed that inhibition of caspase-1 resulted in moderate, although not significant, reduction in IL-1 β secretion in obese patients compared to non-obese, suggesting that caspase-1 inhibition may be more effective in patients with obesity status. IL-1 β and IL-18 levels from untreated biopsies were separated by obesity status to determine the effect of obesity on inflammasome-regulated cytokine secretion levels within this patient cohort (**Figure 4.10A, B**). Results show that there is a trend towards increased IL-1 β and IL-18 secretion levels in biopsies from obese patients. Cytokine secretion ratios in the presence/absence of inhibitor were also plotted for each biopsy (WEHD.CHO-treated:untreated) (**Figure 4.10C, D**). Secretion values less than 1 indicate inhibition. Results suggest that IL-1 β secretion is reduced in the presence of the caspase-1 inhibitor in obese patients, although not significantly.

To summarise, *ex vivo* culture of Barrett's biopsy tissue in the presence of a caspase-1 inhibitor appears to be effective at limiting IL-1 β secretion in obese patients. This suggests that targeting inflammasome or caspase-1 activity in obese Barrett's patients may represent a potential therapy to limit inflammation and disease progression.

4.2.4 TLR2 and caspase-1 mediate inflammation in Barrett's organoids from pL2-IL-1 β mice.

To further assess a role for caspase-1 mediated inflammation during Barrett's we employed a Barrett's organoid model using pL2-IL-1 β mice. This was done in collaboration with the Quante lab in Munich. Barrett's organoid cultures are self-organising epithelial 3D tissue models that are derived from Lgr5⁺ stem cells which grow continuously *in vitro* (392). TLR2 activity can lead to upregulation of inflammasome components mediated through NF- κ B (492). Expression of TLR2 was shown to increase with oesophageal disease progression in patient tissue and has been associated with IL-8 secretion from a Barrett's cell line (BAR-T) (318). Ongoing research in our lab has also shown TLR2 mediates inflammatory crosstalk between OAC cell lines and macrophages (E. Flis, unpublished).

Barrett's organoids were grown from crypts taken from the cardia region at the SCJ of 12-month old pL2-IL-1 β mice, which typically have well-established metaplasia and initial dysplasia (**Figure 3.18C**, (263)). Before carrying out a full organoid experiment, caspase-1 and TLR2 inhibitor optimisation experiments were carried out (**Figure 4.11**). A 12-month old pL2-IL-1 β (n=1) was sacrificed and the cardia region of the stomach resected. Crypts were seeded and cultured continuously for 3 weeks (500 μ L L-WRN media, 50 μ L Matrigel in a 24 well plate), with passaging once a week (as described in Section 2.9.2). During week 4, the organoids were pre-treated (1 hour) with either caspase-1 inhibitor, WEHD.CHO (50 μ M or 150 μ M) or TLR2 blocking antibody, OPN-301 (1 μ g/mL or 10 μ g/mL). As no significant differences were observed previously between WEHD.CHO treatment groups in the Barrett's biopsies, higher inhibitor concentrations were used over concerns of inhibitor penetration issues. Organoids were then stimulated with LPS (1 μ g/mL), Pam2 CSK4 (500 ng/mL) or IFN- γ (40 ng/mL) for 24 hours. The next day, media was replaced and re-treated and stimulated for a further 24 hours. After 48 hours of treatment, WEHD.CHO-treated organoids were pre-treated again (1 hour), before activating the NLRP3 inflammasome with Nigericin (5 μ M, 1 hour). Organoids were harvested and supernatants were assessed for inflammatory cytokine secretion levels.

Figure 4.11A, B shows secretion levels of IL-8 orthologs, MIP-2 and CXCL1. Pam2 CSK4 stimulation resulted in increased cytokine levels, which appeared to be inhibited by TLR2 blocking antibody at both concentrations (1 and 10 µg/mL). **Figure 4.11C, D** shows CXCL1 and MIP-2 secretion levels following LPS and LPS + Nigericin stimulations. Secretion levels appeared to be inhibited following WEHD.CHO treatment at 50 and 150 µM, following stimulation with LPS. IL-6, IL-1β and IL-18 levels were also assessed using standard commercial ELISA kits. However, levels were too low to detect.

The optimisation results suggested that 10 µg/mL TLR2 blocking antibody in combination with Pam2 CSK4 stimulation, and 50 µM WEHD.CHO caspase-1 inhibitor in combination with LPS and LPS + Nigericin stimulation, should be used in future organoid experiments.

Next, pL2-IL-1β mice (n=3) at 12 months old were sacrificed and cultured in the same manner as described previously (Section 2.9.2). Organoid treatments and stimulations were carried out in exactly the same way as described for the optimisation experiment using caspase-1 (50 µM) and TLR2 (10 µg/mL) inhibitors. Therefore, the optimisation mouse was included in analysis as the fourth mouse to increase statistical power. Samples from this mouse were re-analysed and included on the same ELISA plates as the other 3 mice for consistency.

Figure 4.12A-D shows the secretion levels of inflammatory cytokines IL-1β, IL-6, MIP-2 and CXCL1. As IL-1β and IL-6 levels were expected to be very low, samples were analysed using a highly sensitive MSD V-PLEX assay (as described in Section 2.5.5). IL-8 orthologs, CXCL1 and MIP-2 levels were assessed using standard R&D Systems ELISA kits.

Unfortunately, analysis of IL-1β secretion using the highly sensitive MSD V-PLEX resulted in values that were extremely low, where most were below the stated lower limit of detection (LLOD). The specific LLOD for this plate was 0.0419 pg/mL, therefore values below this cannot be accurately quantified and differentiated from potential background observed at the lower end of the analysis curve. MSD defines the LLOD as 'a calculated concentration corresponding to the signal 2.5 standard deviations above the background (zero calibrator)', (MSD V-PLEX protocol). For this reason, IL-1β values cannot be viewed as definitive values but provide an indication of caspase-1 inhibition.

A significant decrease in IL-1 β levels was observed ($p<0.01$) in the presence of WEHD.CHO in LPS-treated organoids (**Figure 4.12A**). However, results also show a trend towards increased basal IL-1 β levels with caspase-1 inhibition in unprimed organoids (**Figure 4.12A**), suggesting that these are likely to be background values. All values for IL-6 were within the certified range, however no significant differences were observed between treatment groups (**Figure 4.12B**).

Following LPS stimulation and inflammasome priming, CXCL1 and MIP-2 secretion were elevated ($p<0.01$) indicating TLR4 activity (**Figure 4.12C, D**). Pre-treatment of LPS-treated organoids resulted in reduced CXCL1 secretion ($p<0.05$), and a trend towards reduced MIP-2 secretion (**Figure 4.12C, D**). NLRP3 inflammasome activation with Nigericin did not lead to any further increase in cytokine secretion. LDH secretion levels were also analysed and no significant differences between groups were detected, suggesting no significant cytotoxicity occurred in the organoids during these treatments (**Figure 4.12E**). Growth curve analysis was performed out to assess the effect of treatments on organoid proliferation and growth. Organoid diameters (μm) were assessed before treatments started (0 hours), during treatments (24 hours) and before organoids were harvested (48 hours), and are represented by images shown in **Figure 4.13A**. Results show organoid sizes from 3 mice in **Figure 4.13B**. Statistical analysis of the growth curves was not carried out as 3 mice were considered to be of insufficient statistical power. However, data was included for comparison with other organoid results and transparency.

Figure 4.14 shows results following organoid treatment for 48 hours with TLR2 blocking antibody, OPN-301 (10 $\mu\text{g}/\text{mL}$), with or without Pam2 CSK4 stimulation (500 ng/mL). No significant differences in IL-1 β and IL-6 secretion levels were observed (**Figure 4.14A, B**). Similarly, no differences in LDH secretion were detected, suggesting that the treatments have little cytotoxic effect (**Figure 4.14E**). In contrast, organoids significantly respond to the TLR2 agonist, Pam2 CSK4 to produce robust secretion of the IL-8 orthologs, MIP-2 ($p<0.001$) and CXCL1 ($p<0.01$). Inhibition of TLR2 resulted in complete reduction of MIP-2 to basal levels ($p<0.001$), suggesting direct regulation of MIP-2 *via* the TLR2 signalling pathway. CXCL1 was also inhibited following TLR2 inhibition ($p<0.05$), although secretion did not return to basal levels (**Figure 4.14C, D**). Interestingly, in the absence

of stimulation, basal CXCL1 secretion was upregulated during TLR2 inhibition, which may suggest that TLR2 is a regulator of CXCL1 activity during Barrett's disease, or that the TLR2 neutralising antibody may have low levels of endotoxin that are stimulating the organoids. **Figure 4.15A, B** shows organoid representative images and growth curve analysis at 0 hours and following treatments at 24 hours and 48 hours from 3 mice.

Together, findings show that murine Barrett's organoids are responsive to LPS and Pam2 CSK4 stimulation. Additionally, CXCL1 was reduced in the presence of a caspase-1 inhibitor, suggesting that inflammasome activation may be occurring in LPS-treated organoids. A TLR2 neutralising antibody was also shown to reduce secretion levels of MIP-2 and CXCL1 in Pam2 CSK4-stimulated organoids. Findings confirm that TLR2 and caspase-1 mediated IL-8 signalling is functional in murine Barrett-tissue organoids.

4.2.5 Inflammasome proteins NLRP3 and AIM2 are strongly expressed in oesophageal tissue and cell lines.

Following pathogen or danger signal recognition, the inflammasome assembles into a multimeric complex capable of activating caspase-1. Subsequently facilitating the processing and secretion of IL-1 β and IL-18 (493). In Chapter 3, we showed that caspase-1 is robustly upregulated in metaplastic tissue. Taking into consideration caspase-1 activation is completely dependent upon the inflammasome pathway, we hypothesized that other components of inflammasome complexes would also be upregulated during Barrett's metaplasia.

To initially screen for inflammasome sensor protein upregulation, the Protein Atlas Repository was analysed for protein expression levels of inflammasome components in normal oesophageal tissue in humans (494)(www.proteinatlas.org). Results reveal that NLRP1, NLRP3 and AIM2 are expressed in normal oesophageal tissue, while NLRP6 and NLRC4 expression levels were undetectable (**Figure 4.16A**).

Inflammasome components AIM2 and NLRP3 were chosen to further investigate as potential inflammasome-associated mediators of inflammation. NLRP3 is the most studied of the inflammasomes and it has been reported to have both protective and tumorigenic roles in other cancers (82). Activation of NLRP3 has also previously been

demonstrated in a Barrett's cell line, warranting further investigation (48). AIM2 involvement has been reported during the early stages of carcinogenesis. Studies have observed crucial roles for AIM2 in pancreatitis and prostate hyperplasia (495, 496). Additionally, following findings observed in Figure 4.13 showing TLR2 mediating inflammation in Barrett's organoid culture, we wanted to further assess the role of TLR2 during oesophageal pathogenesis.

The oesophageal cell line model representing stages of disease progression was used to determine the basal protein expression levels of NLRP3, TLR2, AIM2 and IL-1 β by Western blot (**Figure 4.16B**) and densitometric analysis (n=3, **Figure 4.16C-F**). Results show that all four proteins were robustly expressed in the metaplastic cell line, QH. This correlates with caspase-1 expression levels in this model (**Figure 3.7**) and provides further evidence for an inflammasome-related pro-inflammatory phenotype during metaplasia. Moreover, poor expression was observed in HGD and OAC cell lines, with exception of TLR2 levels. Results show differential expression of TLR2 in the cell line model. Well-differentiated OE33 cells displayed elevated levels of TLR2, suggesting a role during later stages of cancer.

Together these results provide further evidence for a role of the inflammasome and TLR2 signalling during Barrett's-associated inflammation but suggest that inflammasomes may not be as involved during later disease stages.

4.2.6 Inflammasome-related genes are upregulated in OE33 tumour cells when co-cultured with adipose tissue explants from obese cancer patients.

Previous results suggest a potential role for caspase-1-mediated inflammation in obese Barrett's patients (**Figure 4.10**), therefore the role of obesity in oesophageal disease pathogenesis was investigated further. As detailed in the introductory section, obesity is a major risk factor for developing Barrett's and OAC, and it is thought to drive a pro-inflammatory phenotype in the distal oesophagus (411, 423). A meta-analysis study by Singh *et al.* reported that central (visceral) obesity conferred just under a two-fold risk

of Barrett's (497). Taking this research into consideration, we hypothesized that obesity may be causing inflammasome-mediated inflammation during metaplasia and OAC.

To determine whether inflammatory mRNA expression levels were affected by visceral adipose tissue in OAC, publicly available gene array data sets were mined using Pubmed Geo Dataset software (see Section 2.4) (498). OAC cell line, OE33 was co-cultured with VAT explants taken from viscerally obese OAC patients (n=6). Graphs in **Figure 4.17** show differences in inflammasome mRNA expression in OE33 cells when co-cultured with adipose tissue, compared to M199 media alone controls. Adipose tissue explants were taken from obese OAC patients, chopped up and conditioned in M199 media for 72 h. Conditioned supernatants with adipose tissue were cultured with OE33 cells, using a non-contact transwell system. Inflammatory *CASP1*, *IL1B* and *AIM2* mRNA ($p<0.05$) were upregulated following co-culture with adipose tissue (**Figure 4.17**). This suggests that OE33 cells become primed for AIM2 inflammasome activity in response to VAT.

A second data set (**Figure 4.18**) from the same study was analysed which involved co-culturing OE33 cells with adipocytes that were isolated from VAT explants from obese OAC patients (n=6) (498). When compared to media alone control, OE33 cells co-cultured with adipocytes did not express altered inflammatory mRNA. Interestingly, this suggests that the ligands which stimulated upregulation of inflammasome mRNA in OE33 cells shown in **Figure 4.17** did not originate from adipocytes. The result suggests that inflammasome priming ligands are present in the stroma of the explants and may be derived from infiltrating immune cells.

Collectively, these results show that co-culture of OAC cell line, OE33 with visceral adipose explants, but not with adipocytes, results in the upregulation of inflammasome-associated mRNAs. This indicates a potential association between visceral obesity and inflammasome-mediated inflammation during OAC.

4.2.7 OE33 cell line culture with adipose conditioned media (ACM) results in upregulation of caspase-1 and IL-1 β .

To investigate the ability of VAT to contribute to inflammation-associated disease progression from Barrett's to OAC, we used a cell line model representing disease progression (shown in **Figures 3.7 and 4.16**). Cells representative of each disease stage were incubated with ACM taken from obese, OAC patients. Expression levels of inflammatory caspases and other inflammasome components were examined for ACM-mediated alterations.

To carry out these experiments, optimisations were first required. OE33 cells were first incubated for 24 hours with varying concentrations of ACM taken from viscerally obese OAC patients (**Figure 4.19**). As ACM is derived directly from patient samples, and treatment naïve samples are of highest demand, optimisation experiments were carried out with ACM from patients that had received neo-adjuvant treatment (NAT). Cells were cultured with undiluted ACM, or dilutions of 1:2 and 1:4, to determine the minimum amount of sample required to detect any effects on inflammatory protein expression. Where indicated OE33 cells were co-stimulated with LPS (1 μ g/mL) to prime cells for possible inflammasome activation via ACM. Expression of caspases-1, -4, -5 and IL-1 β were assessed by immunoblotting, as shown in **Figure 4.19**. Results show that caspase-1 is upregulated when incubated with ACM. Undiluted ACM was required for maximum caspase-1 upregulation, while priming with LPS did not result in differences in caspase-1 upregulation. Expression of caspases-4 and -5, which appear to be constitutively expressed in OE33 cells, were not significantly affected by the presence of ACM. Pro-IL-1 β expression was increased when cultured with all dilutions of ACM and at 2 μ g/mL LPS, suggesting that factors within ACM are capable of priming OE33 cells for inflammasome activation.

As results in **Figure 4.19** show that caspase-1 and IL-1 β protein expression are induced in OAC cells in the presence of ACM. It was decided that undiluted ACM, without LPS would be used for future experiments.

Supernatants from the ACM-stimulated OE33 experiment described in **Figure 4.19** were also assessed for inflammatory cytokine secretion. As the supernatants contained ACM, a control that consisted of ACM alone without OE33 cells was used for comparison. **Figure 4.20** shows the secreted levels of IL-1 β and IL-1 α as measured by ELISA. Results revealed that minimal levels of IL-1 β and IL-1 α were secreted in response to ACM (**Figure 4.20**). Results suggest that ACM does not induce secretion of inflammatory cytokines IL-1 α and only weakly induces IL-1 β secretion. As pro-IL-1 β protein expression was clearly elevated in OE33 cells cultured in ACM (**Figure 4.17**), these results may indicate that ACM is priming, but not activating an inflammasome pathway. Alternatively, it may be the case that these epithelial tumour cells are not programmed to secrete significant amounts of inflammatory cytokines.

4.2.8 Cross-reactivity of secondary anti-rabbit antibody with heavy chain immunoglobulins present in ACM produces non-specific bands in caspase-1 Western blots.

To confirm that caspase-1 upregulation occurs in OE33 cells cultured with ACM, OE33 cells were cultured for 24 hours with ACM. IFN- α was used as a positive control for caspase-1 upregulation. As OE33 cells do not appear to upregulate caspase-1 in response to LPS (1 μ g/mL), we used LPS as a control to show that endotoxin contamination of ACM was not responsible for any caspase-1 upregulation observed (**Figure 4.21**). In this experiment, it was noted that double bands similar to the molecular weight of caspase-1 (at ~50 kDa) appeared in lanes where ACM alone were run as controls on the immunoblot probed for caspase-1, indicated by arrows (**Figure 4.21A**). As these protein bands were not observed in the immunoblots for caspase-5, it was suggested that significant levels of caspase-1 may be already present in the ACM. Another possibility was that high levels of heavy chain immunoglobulin (Ig, 50 kDa) were present in the ACM, and were being detected by the anti-rabbit IgG secondary antibody, but not by the anti-mouse IgG secondary antibody (used for caspase-4 and -5 immunoblots). We therefore sought to determine what the protein bands in the ACM

only lanes represented, and to establish whether caspase-1 was being truly upregulated in OE33 cells cultured with ACM.

To investigate whether antibody cross-reactivity may be responsible for the additional bands observed in the ACM only lanes (**Figure 4.21A**), immunoblots were probed directly with secondary antibodies, in the absence of any primary antibody. **Figure 4.21B** reveals that the secondary anti-rabbit IgG antibody appears to be strongly binding at approximately 50 kDa. While anti-mouse secondary antibody was weakly binding at the same molecular weight. Furthermore, in lanes containing lysates from ACM-treated OE33 cells, the same size protein band was present in the immunoblot probed with secondary anti-rabbit IgG antibody only. This result suggests that heavy chain IgG's present in patient ACM are detected by the anti-rabbit IgG antibody. When this immunoblot was probed for caspase-1 (**Figure 4.21A**), we observed that the 50 kDa band appears just above that of caspase-1, which may impede our ability to accurately assess caspase-1 expression in this context.

We therefore sought to source an alternative caspase-1 antibody, to replace the anti-caspase-1 polyclonal rabbit antibody (SC-622) used to date. Despite an exhaustive search for a more specific monoclonal anti-mouse alternative, no acceptable substitute could be found. Subsequently, direct HRP conjugation to caspase-1 SC-622 primary antibody was carried out, as described in Section 2.5.2. **Figure 4.22** shows immunoblot results for caspase-1 probed with unconjugated primary and anti-rabbit secondary (**Figure 4.22A**) and caspase-1 probed using the directly conjugated HRP antibody (**Figure 4.22B**). **Figure 4.22A** shows typical double bands in the lane with ACM alone lysate, the higher band corresponding to IgG in the ACM. Contrary to this, in **Figure 4.22B** only one band appears, representing caspase-1 expression in the ACM. This experiment confirms that direct HRP-conjugation of the primary caspase-1 antibody eliminates the previous issues of IgG cross-reactivity in the ACM. It also reveals that significant levels of caspase-1 may be present in ACM samples from certain patients. Therefore, HRP-conjugated caspase-1 antibody was used for all future Western blots involving the analysis of ACM cultured cells.

4.2.9 Upregulation of inflammasome proteins when oesophageal cell lines are stimulated with ACM from obese OAC patients.

Next, we investigated the relationship between obesity and the inflammasome by stimulating a cell line model representing stages of disease progression with ACM from obese OAC patients. Patient characteristics are shown in **Table 4.2**. ACM was derived from VAT taken from six NAT naïve OAC patients, all of whom were obese males. Patients were selected using VFA as an indicator of abdominal obesity, using cut-off values outlined by Doyle *et al.* (326). The cut-off value, 163.8 cm², was selected based on gender and the presence of gastrointestinal cancer and is representative of the Irish OAC patient cohort used in this study.

Immunoblot controls are represented in **Figure 4.23**, which show the stimulation of oesophageal cell lines with LPS (1 µg/mL) and IFN-γ (40 ng/mL), confirming the ability of these cell lines to respond to stimulation by upregulating the proteins of interest. Results also show that responses to stimulations vary substantially between cell lines. Overall, the metaplastic cell line, QH expressed inflammatory proteins most significantly, and also responded to LPS and IFN-γ most robustly with upregulated NLRP3, TLR2, caspase-1 and IL-1β. Interestingly, IFN-γ specifically upregulated significant expression of AIM2 in the columnar-derived cell lines QH, GO and OE33, but not in the squamous epithelial cells, Het-1A. This upregulation corresponded with elevated caspase-1, but not pro-IL-1β levels. Processing of caspase-5 was also increased in cell lines following stimulations suggesting a role for the non-canonical inflammasome pathway.

Following this, ACM samples were cultured for 24 hours with the oesophageal cell lines; Het-1A (normal squamous), QH (Metaplasia), GO (HGD) and OE33 (OAC). Western blot analysis was initiated by probing all six sets of ACM cultured cells for a range of inflammatory proteins, including NLRP3, TLR2, caspase-1, -4 -5, AIM2, pro-IL-1β and cleaved IL-1β. B-actin was probed as a loading control, while LPS-treated THP-1 monocyte cells were used as a positive control (**Figure 4.24A-F**). Densitometric analysis was subsequently carried out using ImageLab software (Bio-Rad) (**Figure 4.24G**). Analysis of immunoblots revealed that substantial upregulation of NLRP3 occurred in

the normal squamous cell line, Het-1A, when cultured with ACM, and this was seen consistently with all six patient ACM samples ($p < 0.01$). NLRP3 was already primed and upregulated in the metaplastic cell line, QH, and was not upregulated further in response to ACM stimulation. ACM cultured with GO (HGD) cells, showed increased NLRP3 with all six ACM samples ($p < 0.05$), particularly in patient samples 2, 5 and 6. These results provided some interesting observations, in particular the appearance of two bands for NLRP3 running at approximately 110 kDa and slightly lower, suggesting that ACM is inducing upregulation of two NLRP3 isoforms. Expression of each isoform appears to be specific to the cell line and the patient ACM. This argues against suggestions that either bands are unspecific or derived from the ACM which strongly expresses NLRP3. There are six reported isoforms of NLRP3 which can be identified by molecular weight: 83.5 kDa, 106 kDa, 111.8 kDa, 112.2 kDa, 116 kDa, 118 kDa (www.uniprot.org). Although it is difficult to identify precise molecular weights of bands by Western blot, it is unlikely that either of the bands are 83.5 kDa. We speculate that the two bands represent isoforms 118 kDa and 106 kDa. Isoform 118 kDa is known as the canonical sequence and is the most commonly expressed isoform, while the 106 kDa isoform, known as the intermediate isoform, has been reported on rare occasions (499, 500). It is also possible the appearance of two NLRP3 bands are due to post-translational modifications occurring causing slightly different molecular weights. These bands may indicate priming, activation or regulation of NLRP3 (501).

Following the ACM stimulation with oesophageal cell lines, we observed that TLR2 (89.8 kDa) was elevated in the normal squamous cell line, Het-1A ($p < 0.05$). Although stimulation of metaplastic cell line, QH, did not result in any differences in expression, it was observed that TLR2 was robustly expressed in this cell line. Results may suggest a potential role for TLR2-mediated inflammasome priming during the early stages of disease.

Western blot analysis of cell lines cultured with ACM revealed that no significant differences in pro-caspase-1 (45 kDa) expression occurred, when compared to the basal expression levels of each specific cell line (**Figure 4.24**). Caspase-1 is present in the cytosol as an inactive zymogen and Lin *et al.* has indicated that it is constitutively expressed in monocytes, where protein and mRNA levels were not affected by LPS

stimulation (154). Although ACM does not result in increased expression of caspase-1, expression is already upregulated in unstimulated metaplastic (QH) and dysplastic (GO) cell lines. Interestingly, caspase-4 expression (43.2 kDa) was upregulated in all cell lines in response to ACM stimulation, particularly the Het-1A cell line ($p<0.001$). Furthermore, caspase-5 (49.7 kDa) was also significantly upregulated in the normal cell line, Het-1A ($p<0.01$). As inflammatory caspases-4 and -5 are part of the non-canonical inflammasome pathway (74), these findings suggest this pathway is involved during the early stages of disease development in obese patients.

Supporting previous results in this chapter, AIM2 expression (39 kDa) was significantly upregulated with ACM stimulation in all cell lines (Het-1A: $p<0.05$, GO: $p<0.001$, OE33: $p<0.05$) (**Figure 4.24**), with exception of the QH cell line, where it was significantly expressed prior to stimulation (**Figure 4.24**). This result shows that ACM is capable of priming the AIM2 inflammasome and supports our hypothesis that the AIM2 inflammasome is involved in mediating obesity-related inflammation.

Finally, pro-IL-1 β (30.75 kDa) and cleaved IL-1 β (17.5 kDa) expression levels were determined. Two densitometric graphs represent differences in pro-IL-1 β expression (**Figure 4.24G**). Due to the range of pro-IL-1 β expression levels across the different cell lines, analysis was performed on each immunoblot twice. The graph labelled 'IL-1 β long exposure' represents a longer chemiluminescent exposure time, where the metaplastic cell line expression is overexposed. The second 'IL-1 β short exposure' graph represents all values within the acceptable exposure range. This combination approach allowed for full analysis of all cell lines in response to ACM stimulation, revealing that pro-IL-1 β levels were upregulated in all cell lines (Het-1A: $p<0.05$, QH: $p<0.05$, GO: non-significant, OE33: $p<0.05$). Immunoblots were further assessed for cleaved (mature) IL-1 β expression levels. The presence of cleaved IL-1 β indicates the activation of the inflammasome pathway (502). Interestingly, only the metaplastic cell line, QH, displays weak expression of cleaved IL-1 β , which is significantly increased when stimulated with ACM ($p<0.05$). This suggests two important findings for this experiment. Firstly, ACM is capable of priming the inflammasome pathway in oesophageal cell lines but does not cause activation. Secondly, an exception to this observation is seen when cells are

already 'primed'. In this scenario, ACM is capable of inflammasome activation when the QH cell line is already 'primed' prior to stimulation.

Figure 4.25 represents the cytokine secretion profiles from oesophageal cell lines following ACM stimulation. No significant differences were observed, however a trend towards increased levels of IL-1 β and IL-18 secretion in Het-1A cells was noted (**Figure 4.25A, B**). IL-1 α was strongly expressed in metaplastic QH cells, but no significant differences were observed following stimulation (**Figure 4.25E**). IL-1 α is an alarmin and a potent pro-inflammatory cytokine. Secretion of the mature form is thought to be dependent on caspase-1 activation (503). Notably, this observation is not supported by previous data shown in **Figure 4.2G**, where IL-1 α secretion was undetectable from Barrett's patient biopsies. **Figure 4.25F** represents the cytokine levels present in ACM alone, where IL-18 has the highest expression levels of this secretion profile.

Overall, a comprehensive assessment of the results shown in **Figure 4.24** and **Figure 4.25** reveals that ACM causes upregulation of inflammasome components in oesophageal cell lines representing stages of progression. ACM stimulation had the most significant effects during the earlier stages of disease, specifically in the normal squamous cell line, Het-1A, where differences observed were the most substantial. In contrast, inflammatory cytokine secretion was not increased by ACM stimulation. These results indicate that obesity is priming the oesophagus for inflammation, which may be subsequently activated by a second stimulus, such as gastric reflux.

4.2.10 Summary of the main findings of Chapter 4.

- IL-18 expression levels are reduced in Barrett's patient tissue, compared to adjacent normal tissue.
- Caspase-1 inhibition in Barrett's patient explants results in a trend towards reduced IL-1 β secretion in obese patients.
- Inhibition of caspase-1 in LPS-stimulated murine oesophageal Barrett's organoids leads to reduced inflammatory cytokine secretion.
- MIP-2 and CXCL1 secretion is inhibited by a TLR2-blocking antibody in Pam2-stimulated murine Barrett's organoids.
- The Barrett's cell line (QH) expresses significant levels of inflammatory proteins, NLRP3, TLR2, AIM2 and IL-1 β .
- LPS and IFN- γ stimulation of oesophageal cell lines causes upregulation of inflammasome proteins.
- Obese OAC adipose conditioned media promotes upregulation of inflammasome proteins in oesophageal cell lines, particularly in normal cells (Het-1A).

4.3 Discussion

The inflammasome pathway is a crucial mediator of inflammation during infection and disease. However, chronic upregulation and activation of this pathway can lead to disease development and tumorigenesis (82). Many inflammasome pathways exist, but all pathways lead to caspase-1 activation and subsequent IL-1 β and IL-18 processing, or pyroptosis (103). Therefore, caspase-1 expression and activity are a valuable indicator of inflammasome-mediated inflammation. It is known that obesity is an instigator of inflammation during oesophageal disease (359). The relationship between obesity and inflammasome involvement is previously unreported during Barrett's and OAC and is characterised for the first time in this study.

Ex vivo culture of Barrett's and adjacent normal patient biopsies confirmed the inflammatory status of Barrett's tissue, with elevated secretion levels of IL-1 β , IL-6, IL-8 and IFN- γ , as has been reported in earlier studies (48, 93, 95). Elevated levels of IL-1 β supports our hypothesis that inflammation is mediated by the inflammasome pathway in Barrett's. Interestingly, our study shows that IL-18 secretion is reduced in Barrett's explants, when compared to adjacent normal tissue. This result is previously unreported and initially seems counterintuitive as both IL-1 β and caspase-1 are elevated in Barrett's. In order to explain this finding, basal mRNA levels of pro-IL-1 β and pro-IL-18 in normal and Barrett's tissue were determined using publicly available data sets (373, 374). Constitutive pro-*IL18* mRNA expression was significantly lower and pro-*IL1B* mRNA was significantly higher in metaplastic tissue, when compared to normal tissue, corresponding with secreted levels. Importantly, pro-IL-1 β upregulation is induced following priming and activation of the transcription factor NF- κ B, in contrast pro-IL-18 is constitutively expressed in cells. These results provide an explanation for the observed lower levels of IL-18 secretion from Barrett's tissue. Additionally, levels of both pro-*IL1B* and pro-*IL18* mRNA expression are decreased when OAC develops, consistent with expression levels of caspase-1 reported in Chapter 3.

Like IL-1 β , it is probable differences in IL-18 secretion are contributing to disease pathogenesis as this cytokine has many important and varied physiological roles. IL-18 is a direct product of the inflammasome pathway and is important for stimulating IFN- γ production and a Th1 cell response (82, 504). It has a key role in the promotion of

epithelial barrier function, stimulating epithelial regeneration and repair which may be relevant in oesophageal mucosa (99, 100, 173). We further speculate about the role of IL-18 during Barrett's in the general discussion (Section 6.1).

Culture of Barrett's explants with a caspase-1 inhibitor led to a reduction in IL-1 β secretion in 50% of explant tissue. These results reflect the heterogeneous and variable nature of patient studies. To further analyse this, data was stratified based on patient characteristics to identify any trends within this cohort. We observed a trend towards greater IL-1 β inhibition in obese patient explants, compared to those from non-obese patients. This was an interesting observation, as both Barrett's and OAC risk are strongly related to obesity and weight gain (344-346). This result suggests that obesity causes an increased localised inflammatory response in oesophageal tissue which may promote disease. Quante's group have reported increased expression of inflammatory factors in oesophageal tissue from HFD-fed pL2-IL-1 β mice (32). These findings provide further reasons for investigating the role of obesity-associated inflammation in Barrett's development. Positive correlations between IL-1 β and metabolic markers have been shown in Barrett's (389). Furthermore, Western and high-fat diets are reported to instigate systemic inflammation and inflammasome activity (364, 505).

Interestingly, obese patients responded better to caspase-1 inhibition. The observed increased efficacy of the inhibitor may be due to differences in the immune environment within Barrett's tissue during obesity. Quante's research group observed that there were increased levels of neutrophils and myeloid cells, and decreased NK cells in lower oesophageal tissue taken from pL2-IL-1 β mice during a high fat diet (364). Alternatively, the enhanced inhibitor efficacy in obese patient explants may be accounted for by increased epithelial barrier permeability in obese individuals that would aid inhibitor penetration (506). A more detailed analysis of caspase-1 activity in obese and non-obese Barrett's patients will need to be performed before any firm conclusions can be made. Overall, this finding highlights the importance of patient stratification into groups based on likelihood to respond in a clinical setting.

In an attempt to further investigate and fully confirm caspase-1-mediated inflammation during Barrett's, we treated Barrett's organoids taken from 12-month old pL2-IL-1 β mice with a caspase-1 inhibitor for 48 hours. Organoid culture is a relatively new

technology first developed by the Clevers lab (392). The technique allows for long term culture of a 3D tissue model containing phenotypically different self-organising epithelial cells derived from Lgr5⁺ stem cells (392). Use of this model allows us to confirm epithelial-mediated signalling to support our findings showing epithelial upregulation of inflammasome components in Barrett's tissue. Additionally, the cardia organoids contain more than one epithelial cell-type (including columnar and mucous-producing cells) and are self-organised in a manner reflecting physiological conditions (formation of a lumen) (391). Therefore, results provide more complex insights into ongoing inflammatory mechanisms occurring during metaplasia.

Analyses of results from our caspase-1 inhibitor organoid experiment reveals several things. Firstly, Barrett's metaplasia organoids from pL2-IL-1 β mice secrete high levels of the IL-8 orthologs, CXCL1 (KC) and MIP-2 (CXCL2), suggesting their role in disease. Secondly, organoids are responsive to LPS stimulation, confirming TLR4 is expressed and active in metaplastic tissue. Thirdly, CXCL1 and potentially IL-1 β secretion were inhibited in LPS-treated organoids following caspase-1 inhibitor treatment, suggesting that caspase-1 mediates inflammation during Barrett's metaplasia in mice. IL-1 β secretion is notoriously difficult to detect *ex vivo* and the levels detected from this experiment were extremely low (507-509). We therefore cannot conclude that caspase-1 inhibition resulted in significantly lower levels of IL-1 β secretion. However, CXCL1 inhibition was also observed in the same context, supporting the evidence for caspase-1-mediated inflammation.

The reduced CXCL1 levels observed following caspase-1 inhibition suggests that either IL-18 or IL-1 β signalling effected CXCL1 secretion. Studies have documented IL-1 β as a modulator of IL-8 activity in epithelial cells. For example, Eskin *et al.* reported that IL-1 β modulated IL-6 and IL-8 inflammatory activity following TLR4 signalling in response to *P. gingivalis* infection in human primary epithelial gingival cells (510). While human gastric epithelial cells stimulated with IL-1 β resulted in increased IL-8 level secretion (511). Additionally, intestinal crypts isolated from ulcerative colitis patients produced significant transcription of CXCL1 and CXCL2 in response to IL-1 β (512). Although alveolar epithelial cells stimulated with IL-1 β induced CXCL1 and MIP-2, only moderate induction occurred when stimulated with IL-18 (513). Unfortunately, we were unable to

quantify levels of IL-18 due to undetectable levels using a standard ELISA. However, the following studies identify a relationship between IL-1 β and IL-8 during inflammation.

Due to the minimum IL-1 β levels observed it must also be considered that although the inflammasome pathway is significantly primed during Barrett's metaplasia, activity may not be as important within the Barrett's columnar epithelium. A second activating signal such as gastric reflux may lead to further activation. We and other studies have shown that IL-1 β secretion is elevated in human Barrett's tissue, however the source of IL-1 β has not been elucidated and may be fully derived from the stroma (95, 226). Additionally, Quante's group has shown induced IL-1 β production in the normal squamous epithelium is required for spontaneous metaplastic development in pL2-IL-1 β mice, however IL-1 β production in the metaplastic columnar epithelium may not be necessary for disease pathogenesis (263).

Interestingly, LPS-stimulated TLR4 activity in Barrett's organoids resulted in robust upregulation of murine IL-8 orthologs CXCL1 and MIP-2, but not IL-6. This was surprising as Quante *et al.* reported the pL2-IL-1 β model was dependent on an IL-1 β –IL-6 signalling cascade at the SCJ of the oesophagus, where IL-6 deficiency abrogated IL-1 β -induced metaplastic development (263). IL-6 signalling has been highly associated with carcinogenesis in other cancers as well (514), however based on results from this chapter IL-6 is not derived from the Barrett's epithelium.

Notably, these results also implicate TLR4 signalling during Barrett's. LPS is derived from gram negative bacteria and is a well-known activator of TLR4 (515). Organoid response to this ligand suggests that metaplastic tissue is sensitive to the microbial environment in the oesophagus which may influence inflammatory responses. In this chapter, we also show that the Barrett's cell line (QH) is responsive to LPS stimulation, leading to upregulation of IL-1 β , caspase-1 and AIM2 expression. To further support this, Nadatani *et al.* showed that LPS stimulation of a Barrett's cell line (BAR-T) lead to increased NLRP3 inflammasome activation and IL-1 β secretion. While Huhta *et al.* revealed that TLR4 expression increased with oesophageal disease progression in patient tissue, which positively correlated with poor prognosis (316). Findings by Verbeek *et al.* agreed with this, also showing increased TLR4 expression with disease in patient tissue. Notably, they demonstrated that LPS stimulation of biopsies and Barrett's BAT-T cells led to NF-

κB activation and IL-8 secretion in a dose dependent manner (516). This is in agreement with our organoid stimulation. Findings from our study and others suggest that Barrett's inflammation may be influenced by TLR4-mediated microbiome activity to promote carcinogenesis.

It was not surprising IL-8 orthologs, CXCL1 and MIP-2 were strongly induced by TLR2 and TLR4 stimulation in Barrett's organoids, based on other research. CXCL1 has already been implicated in Barrett's disease progression by Quante's research group. They proposed that elevated CXCL1 at the SCJ of pL2-IL-1β mice was induced by a HFD and promotes the accelerated disease phenotype in pL2-IL-1β mice through neutrophil recruitment. Additionally, they show that IL-8 secretion increases with disease in oesophageal cell lines representing progression and in oesophageal patient tissue (364). Additionally, several studies have shown that bile acid stimulation leads to IL-8 secretion in oesophageal cell lines (297, 517, 518).

CXCL1 and IL-8 have been associated with proliferation and immune cell recruitment (neutrophil and myeloid cells) (519, 520). In ulcerative colitis, CXCL1 is highly expressed and has been proposed as a marker of disease activity (521). Similarly, human IL-8 signalling was implicated in gastric and colorectal tumour development in mice, through local and systemic immune cell recruitment and inflammation (519). In the oesophagus, Fitzgerald *et al.* showed that IL-8 and IL-1β secretion were elevated in Barrett's patient tissue, compared to adjacent normal tissue (226). IL-8 and IL-1β mRNA levels were also shown to be elevated in oesophagitis patient tissue (306). Moreover, IL-8 mRNA levels correlated with oesophagitis patient endoscopic grades and neutrophil infiltration scores (522). Systemically, circulating IL-8 levels were significantly higher in Barrett's patients, compared to controls (411). Thrift *et al.* associated elevated IL-8 levels with Barrett's risk in patients (523). These studies agree with our findings and identifies IL-8/CXCL1 as a mediator of inflammation at local and systemic levels during oesophageal disease.

Building on preliminary research by Quante's research group, findings in this chapter further implicate TLR2-mediated IL-8 signalling during Barrett's. Quante and colleagues proposed that TLR2-IL-8 signalling is responsible for the accelerated phenotype observed in HFD-fed pL2-IL-1β mice (364). We show that there is significant basal

expression of TLR in a Barrett's cell line (QH). Additionally, we demonstrate Pam2-instigated TLR2 activity results in highly elevated secretion levels of murine IL-8 orthologs, CXCL1 and MIP-2 in Barrett's organoids.

Indeed, other publications have already implicated TLR2 involvement during Barrett's and OAC. Huhta *et al.* revealed that TLR2 is increasingly upregulated with oesophageal disease progression (316). While Verbeek *et al.* identified TLR2 as an important mediator of chronic inflammation in a Barrett's cell line (BAR-T) (318). Like TLR4, TLR2 activity in Barrett's organoids implies Barrett's epithelial cells are sensitive to the microbial environment. TLR signalling may be important for maintaining immune homeostasis and response during infection (30, 524). We show that during TLR2 inhibition, basal CXCL1 levels are increased, however following stimulation organoid secretion of CXCL1 is reduced. This suggests TLR2 is a regulator of CXCL1 during disease. Aside from inflammation, CXCL1 has other functions such as mediating epithelial growth, proliferation and migration (525, 526). Although we see no significant differences in organoid growth. Bolitho *et al.* showed that recombinant CXCL1 induced transactivation of the epidermal growth factor receptor (EGFR) leading to increased proliferation in human ovarian cancer cell lines (526). A similar effect of CXCL1 was shown in prostate cancer cell lines, where increased proliferation, migration and invasion was observed (525). Additionally, CXCL1 has been associated with angiogenesis in a xenograph mouse model (527). These studies support a tumour promoting role of CXCL1 which may be mediated through both TLR2 and caspase-1 activity.

Similarly, in this chapter we also show that MIP-2 (CXCL2) secretion is highly induced following Pam2 stimulation. Surprisingly, this response was completely suppressed following TLR2 inhibition, which implies TLR2 signalling can directly regulate MIP-2 induction. Other studies have reported a TLR2-MIP-2 signalling cascade during inflammation, specifically in regards regulation of neutrophil recruitment (528). Mole *et al.* showed that TLR2/MIP2 signalling was crucial for neutrophil recruitment during acute and chronic murine liver injury (529). Similarly, TLR2-MIP-2 signalling was found to be crucial for mediating neutrophil recruitment during specific cornea and lung infections in mice (530, 531). Neutrophils are important during early acute immune responses to infections and found in large numbers in oesophageal tissue taken from

the SCJ of pL2-IL-1 β mice (364, 532). In our study, we show that Barrett's metaplasia epithelial cells are a major source of neutrophil chemoattractants, CXCL1 and MIP-2 following stimulation of Barrett's organoids.

Findings related to TLR2 inhibition in Barrett's organoids allow us to consider the therapeutic potential of targeting TLR2 to suppress inflammation during carcinogenesis. Indeed, pre-clinical mouse studies have evaluated the efficacy of TLR2 inhibition during gastric cancer (533). The study revealed STAT3 drives significant upregulation of epithelial TLR2 in gastric tumours. Treatment with the TLR2-neutralising antibody OPN-301 resulted in dramatic tumour growth reduction, independent of inflammation (533). Interestingly, Quante *et al.* have previously proposed an IL-1 β -IL-6-STAT3 signalling cascade promoted tumorigenesis in the pL2-IL-1 β model (263). In contrast to gastric cancer, Quante's study and our data support a role for TLR2 in inflammation during oesophageal disease progression. Additionally, no changes in organoid growth were observed following 48 hours of inhibitor treatment. However, a longer OPN-301 incubation period or inclusion of more mice to increase statistical power may result in proliferation differences.

Similar to TLR2 activity, our results demonstrate an association between caspase-1 signalling and Barrett's-mediated inflammation, calling into question the therapeutic potential of targeting the inflammasome pathway in Barrett's patients. Blocking IL-1 signalling, via Anakinra, Rilonacept or Canakinumab, has been FDA-approved to treat a broad spectrum of diseases, including rheumatoid arthritis, Crohn's disease and type II diabetes (534). Recently, findings of the CANTOS trial (Canakinumab Anti-inflammatory Thrombosis Outcome Study) have been published (535). This was the largest, placebo-controlled trial ever designed in anti-cytokine therapeutics, recruiting 10,061 participants. It sought to determine whether Canakinumab could reduce secondary incidence of heart attack, stroke or cardiovascular death in patients with prior myocardial infarction combined with high-sensitivity CRP ≥ 2 mg/L (a biomarker of inflammation and cardiovascular risk).

The CANTOS trial reported a 15% reduction in major adverse cardiovascular events in the 150-mg treatment cohort. Interestingly, it was the 300-mg cohort that revealed the most impressive result of the trial. The authors noted a dramatic 66% reduction in total

lung cancer incidences and a 77% reduction in fatal lung cancers. Additionally, total cancer mortality was 50% lower in the Canakinumab 300-mg cohort in comparison to the placebo group. Patients with increased levels of CRP and IL-6 were at the highest risk of incidence, while those who saw greatest reductions gained the most benefit. Although the rate of fatal infection and sepsis was significantly higher in treatment groups, there was no significant differences in all-cause mortality between the two groups due to the striking reduction in cancer incidence (535). Lung cancer is an epithelial cancer highly associated with chronic inflammation (412). Similarly, Barrett's and associated OAC are epithelial derived and associated with chronic inflammation (270).

Taking into consideration our data shows caspase-1 and inflammasome proteins are robustly upregulated in Barrett's and previously mentioned research implicates IL-1 β in disease (95, 226, 263), we can speculate that Canakinumab may have potential efficacy in Barrett's oesophagus patients most at risk of progressing. In a similar manner to the CANTOS trial, Barrett's patients would need to be stratified into groups according to risk of progressing, and biomarkers of inflammation need to be considered to help identify those most likely to respond. To consider a trial of this nature, only dysplastic Barrett's patients would likely be considered. Patients with HGD have a 30-40% chance of progressing to OAC within five years (256), identifying them as potential candidates. However, the current standard care of HGD is radiofrequency ablation, which has recently been shown to have effective long-term outcomes, with only 3% of HGD and early cancer reoccurring after seven years (536).

Other inhibitors of caspase-1 exist, some of which have been incorporated into clinical trials for various diseases but have failed to reach phase III (537). Pralnacasan (VX-740) was assessed for efficacy in rheumatoid arthritis patients. However, it was terminated during Phase IIb due to liver toxicity observed after long term use in animals, despite these adverse effects not being seen in patients (537, 538). Belnacasan (VX-765) was assessed in clinical trials for epilepsy and psoriasis (537). Tolerance and safety in patients were proven acceptable, however the drug was ineffective for epilepsy treatment and has not been brought forward for psoriasis treatments (537, 539, 540).

Caution must be taken when considering inhibition of caspase-1 and the inflammasome pathway for therapeutic potential in cancer and prevention. Due to reduced levels of caspase-1 during later disease, our data allows us to speculate that the inflammasome pathway is actively suppressed within established OAC tissue. Therefore, there is a risk that anti-IL-1 or inhibitor administration could accelerate disease progression in certain Barrett's patients that have already begun progression to OAC. Fitzgerald *et al.* reported reduced inflammation in the distal segment of Barrett's, characterised by increased anti-inflammatory IL-10 expression and a Th2 response (226). IL-10 and Th2-type responses have been proposed to facilitate tumorigenesis by dampening anti-tumour responses (541). Indeed, Fitzgerald and colleagues remarked that HGD patients in this study had elevated IL-10 levels. Furthermore, of Barrett's patients that developed cancer, 86% of cases arose in the distal portion of the segment, where inflammation and IL-1 β expression were lower (226). This may partly explain why tumour occurrence at the GOJ are twice as common as OACs (>2cm from the GOJ) (542, 543). Additionally, results shown in Chapter 3 in pL2-LKR mice with progressed disease had reduced epithelial and stromal caspase-1 expression, suggesting a suppressing mechanism later in disease. These studies may suggest that reduced inflammasome signalling is part of an immunomodulatory mechanism contributing to carcinogenesis and further blocking of this signalling could possibly have tumour promoting effects.

In further support of this theory, one study found that the tumour suppressor p63 protein actively binds the caspase-1 promoter. P63 is an important transcription factor during epithelial development, but also displays anti-tumour functions. Cancer survival estimation analysis revealed that functional interaction of p63 and caspase-1 is indicative of a positive survival outcome in patient prostate, bladder and breast cancer (544). Once cancers have developed, inflammatory responses can be both anti- and pro-tumorigenic. Haabeth *et al.* proposed that Th1 responses associated with pro-inflammatory cytokines; IL-1 α , IL-1 β , IL-6 and TNF α repress tumour growth (545). IP injection of IL-1 β enabled complete regression of subcutaneous SA1 sarcoma and L5178Y lymphoma in mice (546). Moreover, caspase-1 deficient mice developed early incidence of papilloma in a model of skin SCC (547). Together, these studies reveal the potential anti-tumour actions of the inflammasome pathway that may be relevant

during OAC carcinogenesis and highlight the complex ongoing interplay between inflammation and cancer.

In contrast, chronic inflammation and inflammasome activation are often associated with promoting carcinogenesis (82). Several studies indicate that inflammasome involvement has further tumour promoting effects when cancer has developed. For example, using a model of breast cancer metastasis one study showed that mice administered IL-1 receptor antagonist, Anakinra following paclitaxel treatment had reduced primary tumour burden, but enhanced spontaneous metastasis (548). Guo *et al.* observed similar findings using a model of lung metastasis in breast cancer (413). Supporting this, chemotherapies gemcitabine and 5-FU were reported to have higher anti-tumour activity in NLRP3 and caspase-1 knockout mice bearing EL4 (lymphoma) tumour burdens (435). These conflicting studies highlight the complexities of targeting the inflammasome pathways once cancer has developed, particularly when used in combination with different chemotherapies.

Perhaps a less costly and less efficacious therapeutic alternative for Barrett's is aspirin (acetylsalicylic acid). Aspirin's mechanism of action directly inhibits COX enzymes (COX-1 and COX-2). COX-2 is involved in pro-inflammatory functions such as inducing prostaglandin E2 and has been implicated in aiding development and progression of many types of cancers, including Barrett's and associated-OAC (549). Several studies have observed elevated levels of COX-2 in Barrett's and dysplasia and have proposed it as an inflammatory marker indicting malignant risk in Barrett's (449, 550, 551). A study by Hua *et al.* has shown that COX-2 regulates NLRP3 inflammasome-derived IL-1 β production. Inhibition of COX-2 resulted in reduced levels of IL-1 β and caspase-1 activity, suggesting a close relationship between these two highly inflammatory pathways (552). Interestingly, a recently published study by Jovani *et al.* examining the association of aspirin and Barrett's onset found that regular aspirin use reduced the risk of developing Barrett's in women. This benefit appeared to positively correlate with higher dosage and longer duration of use (275). In support of this, results recently released from the AspECT trial found that high dose PPI (Esomeprazole) and Aspirin use were most beneficial as treatment for Barrett's patients (276). We can therefore speculate that regular aspirin usage could have therapeutic benefit for treatment of

Barrett's onset by decreasing general and NLRP3-inflammasome-mediated inflammation. Indeed, these studies are indicative of a disease enhancing role of the inflammasome pathway during pathogenesis.

In this chapter, we identified NLRP3 and AIM2 as potential mediators of inflammation and were chosen for further investigation in this research project. This decision was made based on evidence showing expression was upregulated in normal oesophageal tissue, and in our cell line model (www.proteinatlas.org)(494). As mentioned, preliminary work has implicated NLRP3 in Barrett's metaplasia (48). However, little else has been published in Barrett's and OAC involving this molecular pathway. In a Barrett's cell line, QH, our results show that NLRP3, AIM2 and IL-1 β are robustly upregulated, while relative expression in OAC cell lines is low or undetectable. This supports our data implicating caspase-1 activity during Barrett's. Similar to Barrett's and OAC, expression levels of AIM2 in prostate tissue are increased in hyperplasia, while both AIM2 and caspase-1 are reduced in patient tumour tissue (433, 495). The role of NLRP3 and AIM2 in carcinogenesis is conflicting and tissue specific. NLRP3 and AIM2 activity have reported protective functions in colitis and colon cancer in mice (173, 475). Both are also known to have important functions in gut homeostasis in mice (553-555). In contrast, studies of pancreatitis show NLRP3 and AIM2 activity exacerbate disease in mice (556, 557). These differences highlight the need to assess inflammasome activity independently in specific tissue types, but also in the context of disease, such as during obesity or microenvironment conditions.

Obesity involves the excessive production of insulin, glucose and fatty acids, creating a nutrient rich environment pre-conditioning for cancer development. This is combined with a systemic state of chronic low-grade inflammation, constituting tumour promoting adipokine secretion and immune cell activation (2, 3). Visceral obesity is a significant risk factor for Barrett's and OAC development, caused by increased levels of pro-inflammatory and pro-tumorigenic factors (129, 366). In Chapter 3 we showed that 12-month-old pL2-IL-1 β HFD-fed mice exhibited increased histological inflammation and dysplasia, alongside higher levels of epithelial caspase-1. Following this, we generated results to suggest that caspase-1 is mediating inflammation during Barrett's, particularly

in obese patients. Next, we hypothesized that obesity is inducing inflammasome-mediated inflammation during Barrett's and OAC pathogenesis.

In this chapter, we demonstrated that culturing oesophageal cell lines representative of disease progression in the presence of ACM induced a pro-inflammatory phenotype. ACM-induced inflammation was most prominent in the cell lines representing the early stages of disease, particularly in the normal squamous cell line, Het-1A. Although there were no differences in caspase-1 expression in ACM-stimulated cell lines, a robust upregulation of NLRP3, AIM2 and IL-1 β expression was observed. This suggests that obesity-associated factors are capable of mediating inflammasome priming. However, as the ACM was derived from adipose tissue taken from OAC patients, we also consider that stimulatory factors specific to cancer may also be driving inflammation. Caspase-4 and -5 were also upregulated by ACM in normal cells (Het-1A), indicating involvement of the non-canonical inflammasome pathway in early disease. Inflammasome 'priming' may be mediated in part by TLR2, which was also shown to be upregulated in response to ACM in normal cells.

Work conducted by Lysaght *et al.* has sought to characterise the OAC patient ACM used in this study and provides clues as to what ligands may be inducing inflammasome upregulation, such as elevated levels of IL-6, VEGF and IFN- γ (368, 558). IL-6 is a downstream target of IL-1 β , and their functional relationship is synergistic (559). For example, indolent multiple myeloma patients treated with anti-IL-1 therapy, Anakinra, showed reduced IL-6 with greater efficacy than neutralizing IL-6 (560). IFN- γ can upregulate AIM2 expression in several diseases and has also been associated with NLRP3 signalling in obesity (115, 495). NLRP3^{-/-} mice showed reduced IFN- γ secretion, associated with improved metabolic outcomes and insulin signalling in obesity (463). VEGF has been identified as a critical regulator of angiogenesis and vascular permeability and has been highly associated with aiding tumour growth and survival (561, 562). Interestingly, many studies are published regarding a positive or negative regulatory relationship between IL-18 and VEGF, depending on the disease tissue and circumstances (563-568). For example, increased IL-18 suppresses VEGF signalling activity in the eyes of mice and patients, and VEGF also negatively regulated IL-18 signalling (564). In contrast, IL-18 signalling was required for VEGF-mediated migration

of gastric cell lines and metastasis in a gastric cancer mouse model (567, 568). These studies provide clues as the immune-stimulatory potential of ligands present in the OAC ACM used in this study.

Studies are emerging that identify inflammasome activators in obesity, many of which are cell-stress associated metabolites. As mentioned in the introduction, it is speculated that NLRP3 is activated indirectly by signalling pathways related to cellular stress, such as K^+ efflux, phagosomal and lysosomal destabilisation, calcium signalling and mitochondrial ROS production (105). Vandanmagsar *et al.* reported that NLRP3 could induce caspase-1 activation through sensing obesity-associated danger signals in mice, such as lipotoxic ceramides (463). Other NLRP3 activating ligands include oxidised low-density lipoprotein (LDL) and cholesterol crystals (569). Further, Karasawa *et al.* demonstrated that excess saturated fatty acids, such as palmitic and stearic acid, undergo intracellular crystallisation, leading to lysosomal dysfunction and NLRP3 activation (570). In contrast, AIM2 is a sensor of dsDNA. During type 2 diabetes, a disease highly associated with obesity, significantly increased levels of cell free mitochondrial DNA (mtDNA) are present in patient circulation, which lead to AIM2 upregulation and activation (571). In general, fibrosis or tissue damage is common under obese conditions, leading to DNA leaking and inflammation (572, 573).

Toll-like receptors have long been known to mediate inflammation during obesity. Unlike NLRs, TLRs are capable of mediating inflammation both inter- and intracellularly. Interestingly, TLRs have been suggested to be able to 'sense' nutrient levels and control metabolic pathways accordingly (574). A study by Miura *et al.* implicated a direct association of palmitic acid with TLR2 which instigates signalling through the inflammasome pathway and was essential for NASH development in mice (575). Palmitic acid is a saturated long chain FA found in palm oil, dairy and meat, and is common in high calorie diets. Davis *et al.* showed TLR2 deficiency protects against HFD associated inflammation but may also be a regulator of glucose uptake in adipocytes (576). To support this, Nguyen *et al.* showed that TLR2 and -4 can sense levels of free FAs and activate macrophages, promoting inflammation and insulin resistance (577). These studies provide preliminary evidence for TLR2 involvement during obesity-

associated inflammation during oesophageal disease, however further experimentation is required and will be further discussed in Chapter 5.

In contrast to normal squamous cells, OAC cells appeared to poorly express NLRP3 and caspase-1 when cultured with ACM. However, AIM2 and IL-1 β expression levels were increased in a patient specific manner, suggesting that AIM2-mediated rather than NLRP3-mediated inflammation may be involved in established OAC. Alternatively, the tumour may be actively suppressing caspase-1 and NLRP3 activity, as was discussed previously. Indeed, in mouse models of chemotherapy, NLRP3-dependent IL-1 β activation was essential for induction of dendritic cell anti-tumour immunity (436). While in liver cancer, NLRP3-mediated IL-18 secretion was shown to suppress colorectal metastasis (578). Interestingly, new research is emerging that obesity can have protective effects once cancer has developed, as it can enhance response to therapy. In OAC, obesity correlated with improved radiosensitivity and response in cell lines and in patients receiving chemoradiation therapy (579). This is further supported by work by Wang's research group, who reported enhanced anti-tumour efficacy and survival during obesity following checkpoint blockade immunotherapy in patients and mouse models of cancer, including melanoma and lung carcinoma (580). Increased numbers of T regulatory (Treg) cells are present in OAC (314), which promote 'immune privilege' sites for tumour cells (185). These paradoxical studies of obesity suggest that boosting pro-inflammatory responses, while blocking suppressive immunity in obesity-primed immune systems could improve OAC survival.

Although the NLRP3 inflammasome is the best characterised of the inflammasomes, functional studies are lacking describing the less commonly expressed NLRP3 isoforms. This study observed that ACM induced the upregulation of two NLRP3 bands by immunoblot, suggesting the upregulation of two different NLRP3 isoforms at approximately 110 kDa and slightly lower. These bands are likely to correspond to two of the six known isoforms of NLRP3. The most commonly reported NLRP3 isoforms in the literature are the 118 kDa and the 83.5 kDa isoforms, while the 106 kDa isoform has been observed on rare occasions (499, 500). We have yet to confirm which isoforms are being upregulated by ACM in oesophageal cells lines. Work by the Latz research group has shown that NLRP3 function is partly controlled through posttranscriptional

regulation involving abundant alternative splicing variations of LRRs. Some of these NLRP3 isoforms (eg. exon 5) do not associate with NEK7 and subsequently were thought to be dysfunctional (581). Later unpublished work from the group observed that priming of different isoforms required different lengths of time to occur. NLRP3 exon 5 was re-discovered as functional but activity was time delayed due to alternative splicing (Latz oral presentation, ISI, 2019). Different isoforms of NLRP3 may prove to be important during inflammasome signalling or may have other unknown functions, some of which may be relevant in obesity-instigated pathogenesis in Barrett's. However, more work is required before further speculation.

Overall this chapter provides evidence for inflammasome-mediated inflammation during Barrett's, particularly during obesity. Additionally, we show TLR2 activity mediates IL-8 ortholog signalling during Barrett's using a murine organoid model. These findings suggest a therapeutic benefit for targeting caspase-1 or TLR2 during oesophageal disease development and progression. We also show preliminary results demonstrating the stimulatory effect of obesity on inflammasome activity using an oesophageal cell line model representing disease progression. Findings suggest obesity may be priming cells for inflammation associated with oesophageal disease, especially during the early stages of pathogenesis.

Patient characteristics						
Age (median)		63	(51-86)			
Male gender (n) (%)		7	70%			
BMI (median) (kg/m ²)		28.69	(24.43-37.78)			
Weight (median) (kg)		89.6	(56.3-111.6)			

Patient	Age	Gender	Weight (kg)	BMI (kg/m ²)	Prague	Vienna
					classification	grade
1	51	M	107.8	35.61	C0M3	1
2	77	F	89.2	40.72	C1M3	1
3	69	M	71.5	23.62	C12M12	3
4	64	F	101.2	43.8	C5M9	1
5	62	M	92	31.1	C0M5	1
6	77	F	56.6	24.66	C2M3	1
7	57	M	97.9	29.9	C1M2	1
8	86	M	81.9	28.01	C1M5	1
9	59	M	99.7	34.5	C1M2	4
10	49	M	108.9	37.6	C0M6	1

Table 4.1: Characteristics of Barrett's patients.

Histologically confirmed Barrett's patients were prospectively recruited from St. James's Hospital as part of the National Barrett's oesophagus registry and biobank. Biopsy tissues were subsequently analyzed for inflammatory markers in the presence/absence of a caspase-1 inhibitor.

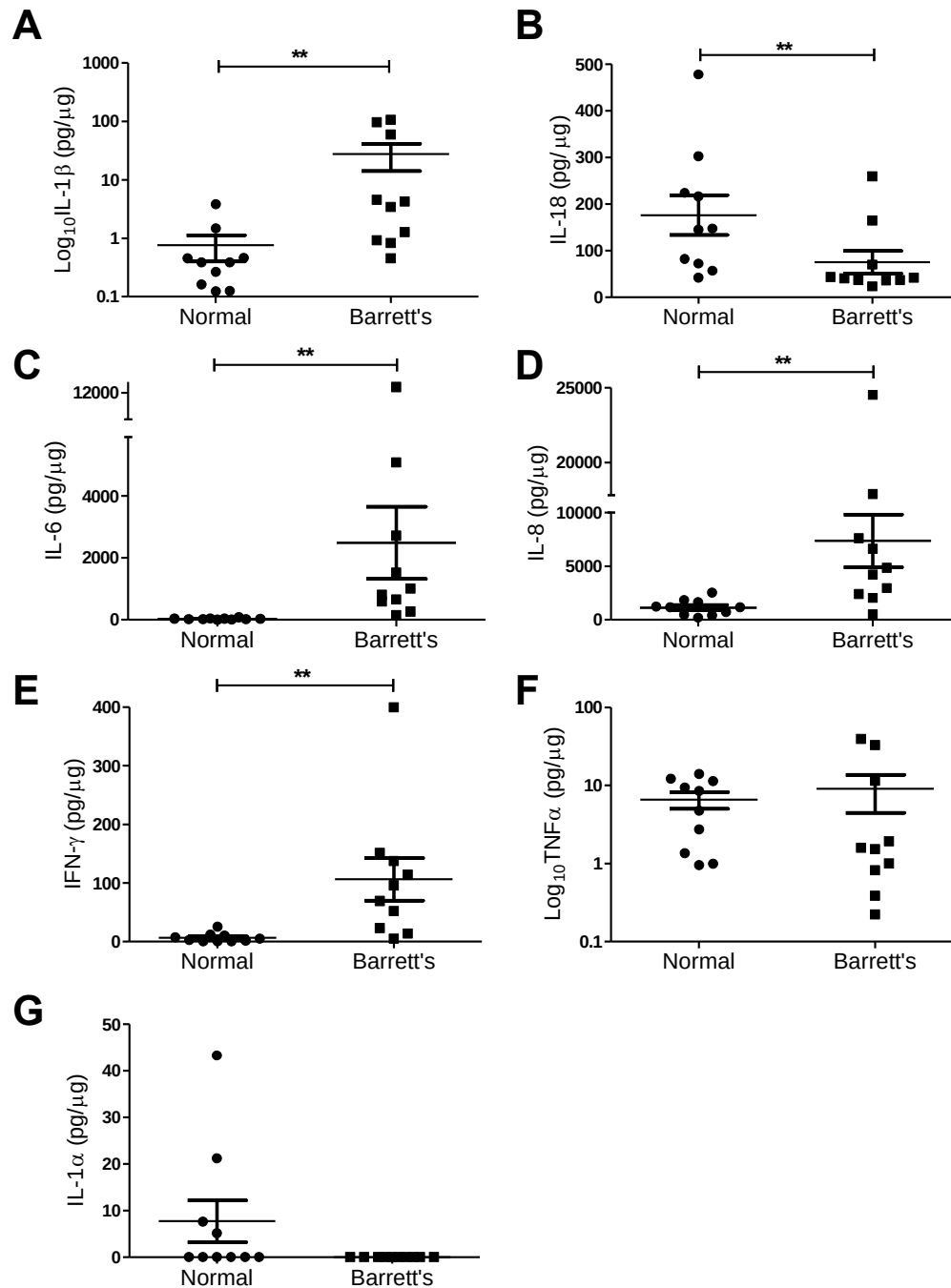


Figure 4.1: Biopsies from Barrett's regions secrete higher levels of inflammatory mediators than adjacent normal tissue.

Matched oesophageal biopsies were taken from diagnosed Barrett's patients (n=10) from regions of Barrett's and adjacent normal tissue. Explant biopsies were cultured for 16 hours (500 μ L M199) and culture supernatants were subsequently measured for levels of inflammatory mediators and normalised to total protein as determined using the BCA method (pg/ μ g): **A)** IL-1 β , **B)** IL-18, **C)** IL-6, **D)** IL-8, **E)** IFN- γ , **F)** TNF α and **G)** IL-1 α . Cytokines were measured using MSD multiplex analysis (A, C, F), R&D Systems (B) and BioLegend ELISA (D, E, G). Data represents mean $\pm$ SEM. Wilcoxon signed-rank test: ** p <0.01.

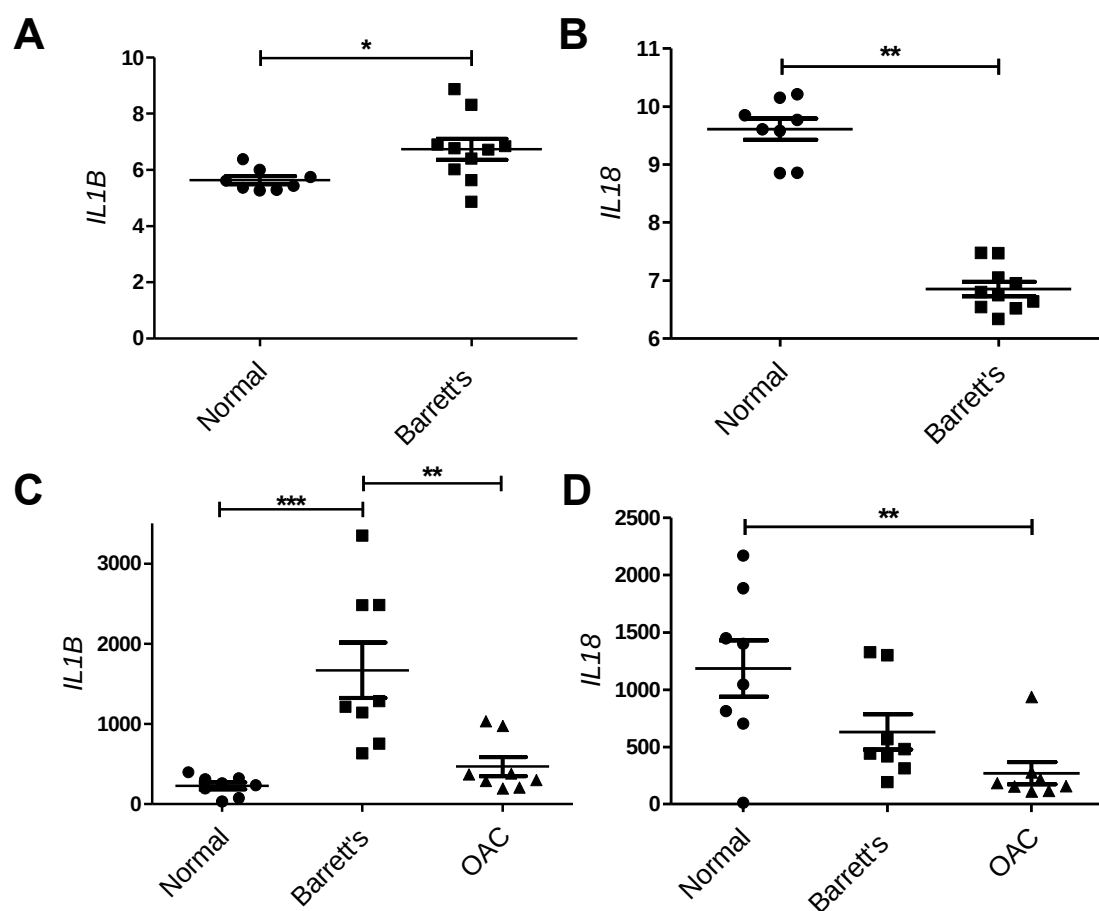


Figure 4.2: Analysis of public data sets reveal *IL1B* mRNA levels are upregulated in Barrett's, while *IL18* mRNA levels are decreased in Barrett's and OAC.

Graphs represent relative mRNA expression values for IL-1 β and IL-18 analysed using publicly available data sets. mRNA expression microarray profile of **A)** *IL1B* and **B)** *IL18* in matched patient biopsies from normal squamous (n=8) and Barrett's oesophagus (n=10) tissue. Wilcoxon signed-rank test (Di Pietro *et al.* 2012, [GSE34619], (373)). mRNA expression microarray profile of **C)** *IL1B* and **D)** *IL18* in matched biopsies (n=8) of normal squamous, Barrett's and OAC (Kimchi ET *et al.* 2005 [GSE1420], (374)). Data represents mean $\pm$ SEM. One-way ANOVA followed by Tukey's post hoc test: * p <0.05, ** p <0.01, *** p <0.001.

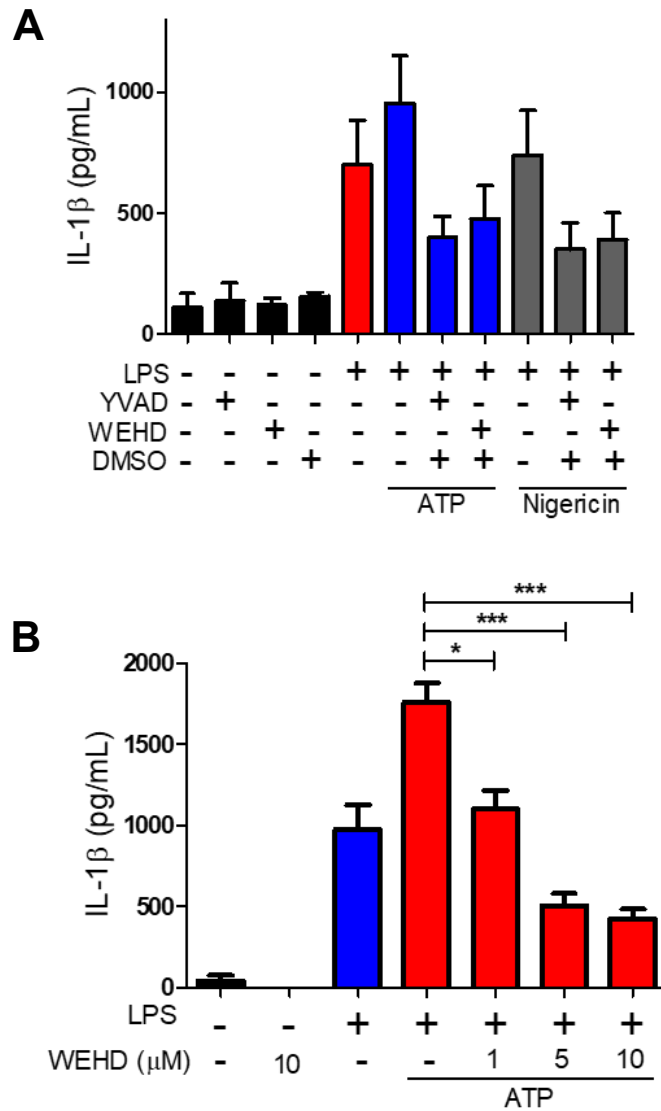


Figure 4.3: Caspase-1 inhibitor, WEHD.CHO optimally inhibits IL-1 β secretion from THP-1 cells at 10 μ M.

THP-1 cells were PMA-stimulated (10 ng/mL, 48 h) prior to LPS stimulation (1 μ g/mL, 18 h), followed by ATP (5 mM) or Nigericin (5 μ M) stimulations where indicated (30 min). **A**) Comparison of IL-1 β secretion inhibition using 1 μ M of tetrapeptide caspase-1 inhibitors; YVAD.fmk (Z-Tyr-Val-Ala-Asp (OMe)-fluoromethylketone) and WEHD.CHO (Trp-Glu-His-Asp-aldehyde) in THP-1 cells. DMSO (1 μ M) was used as vehicle control. **B**) Dose response of caspase-1 inhibitor, WEHD.CHO (Trp-Glu-His-Asp-aldehyde) (1 μ M, 5 μ M, 10 μ M) in the THP-1 cell line. H₂O was used as vehicle control. Graphs A and B are representative of three independent experiments. Paired Student's *t* test: **p*<0.05, ****p*<0.001. Data represents mean $\pm$ SEM.

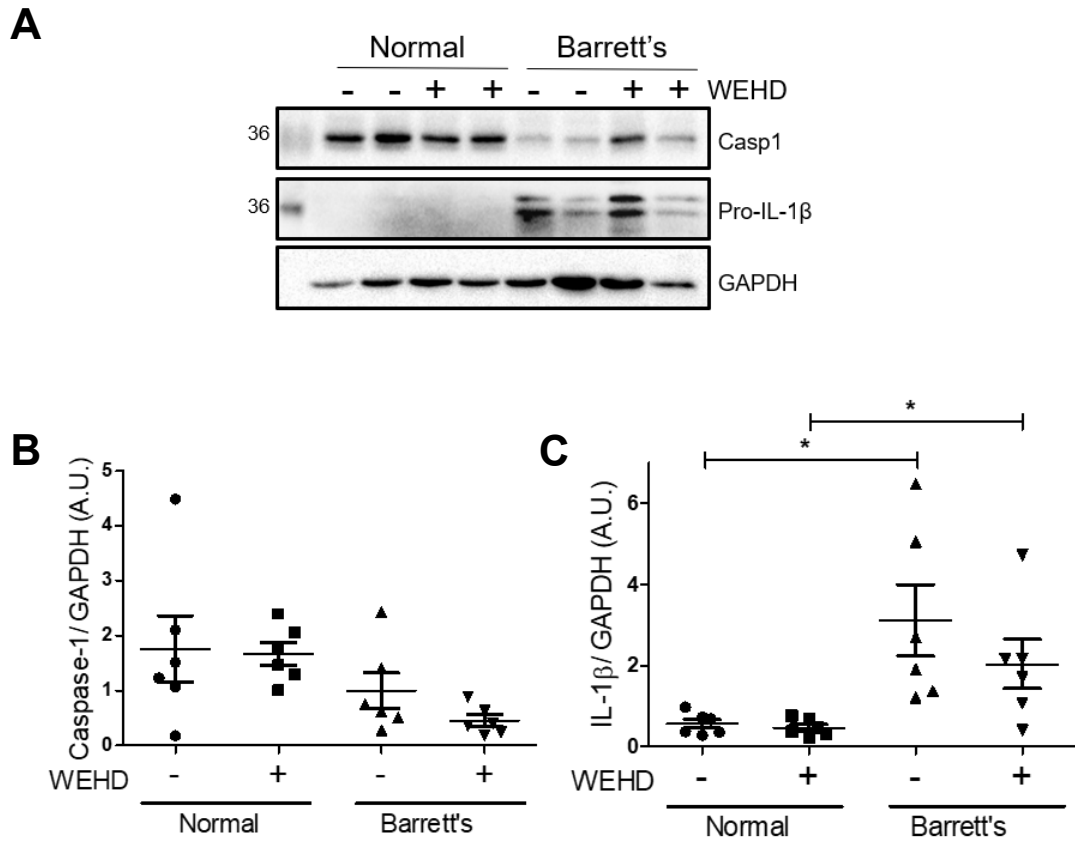


Figure 4.4: Specific upregulation of pro-IL-1 β in Barrett's patient tissue, but not in normal tissue.

Matched oesophageal biopsy explants were taken from areas of Barrett's and adjacent normal tissue from previously diagnosed Barrett's patients (n=10). Oesophageal explants were cultured with or without caspase-1 inhibitor (WEHD.CHO, 10 μ M, 500 μ L M199) for 16 hours. Protein lysates from 6 patients were resolved on a SDS-PAGE gel and wet transferred. Immunoblots were probed for caspase-1 (45 kDa), pro-IL-1 β (30.75 kDa) and loading control GAPDH (36 kDa). **A**) Representative immunoblots of caspase-1 and pro-IL-1 β expression, followed by densitometric analysis (arbitrary units) of **B**) caspase-1 and **C**) pro-IL-1 β levels normalised to loading control, GAPDH. Wilcoxon signed-rank test: * p <0.05.

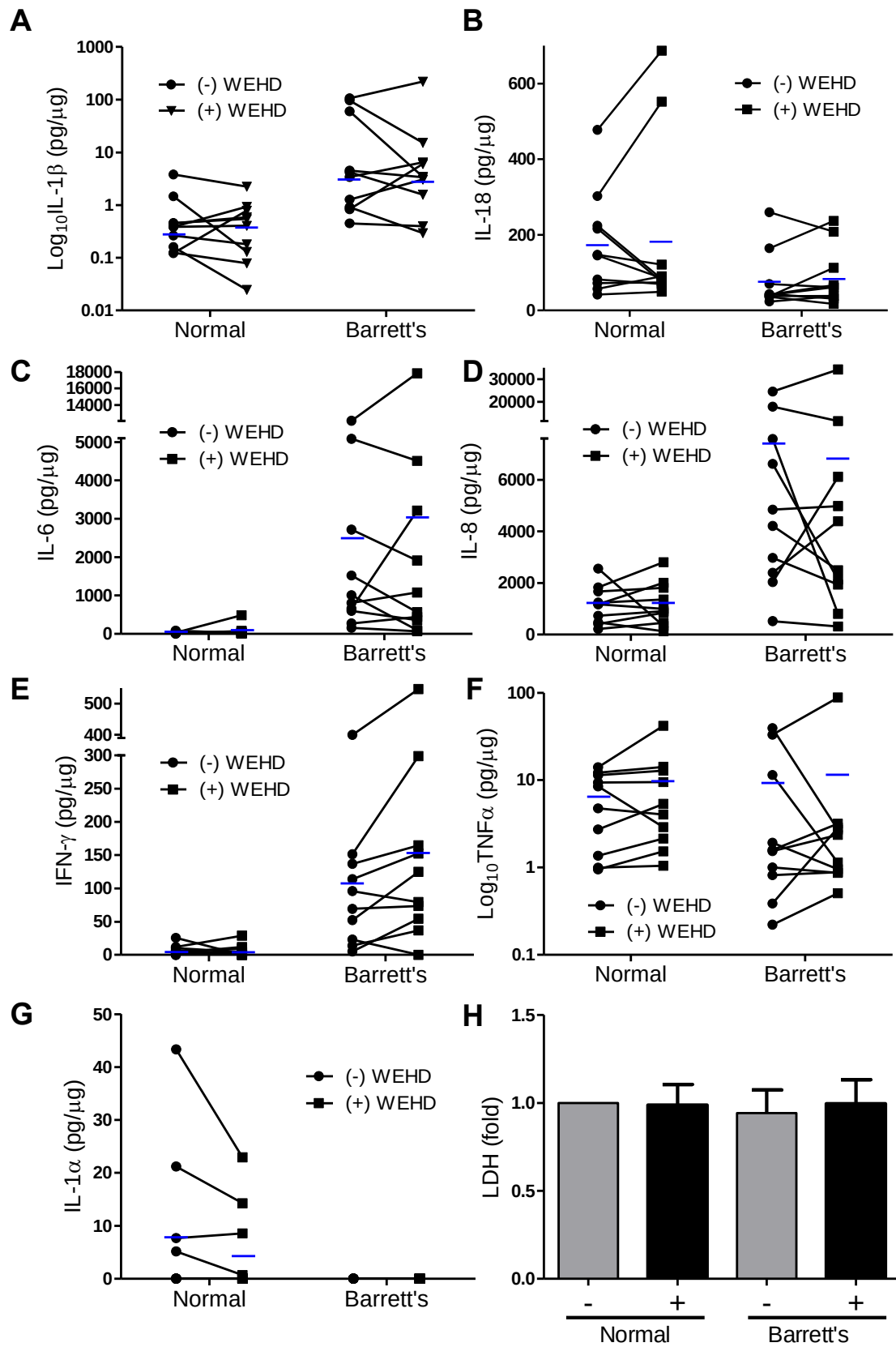
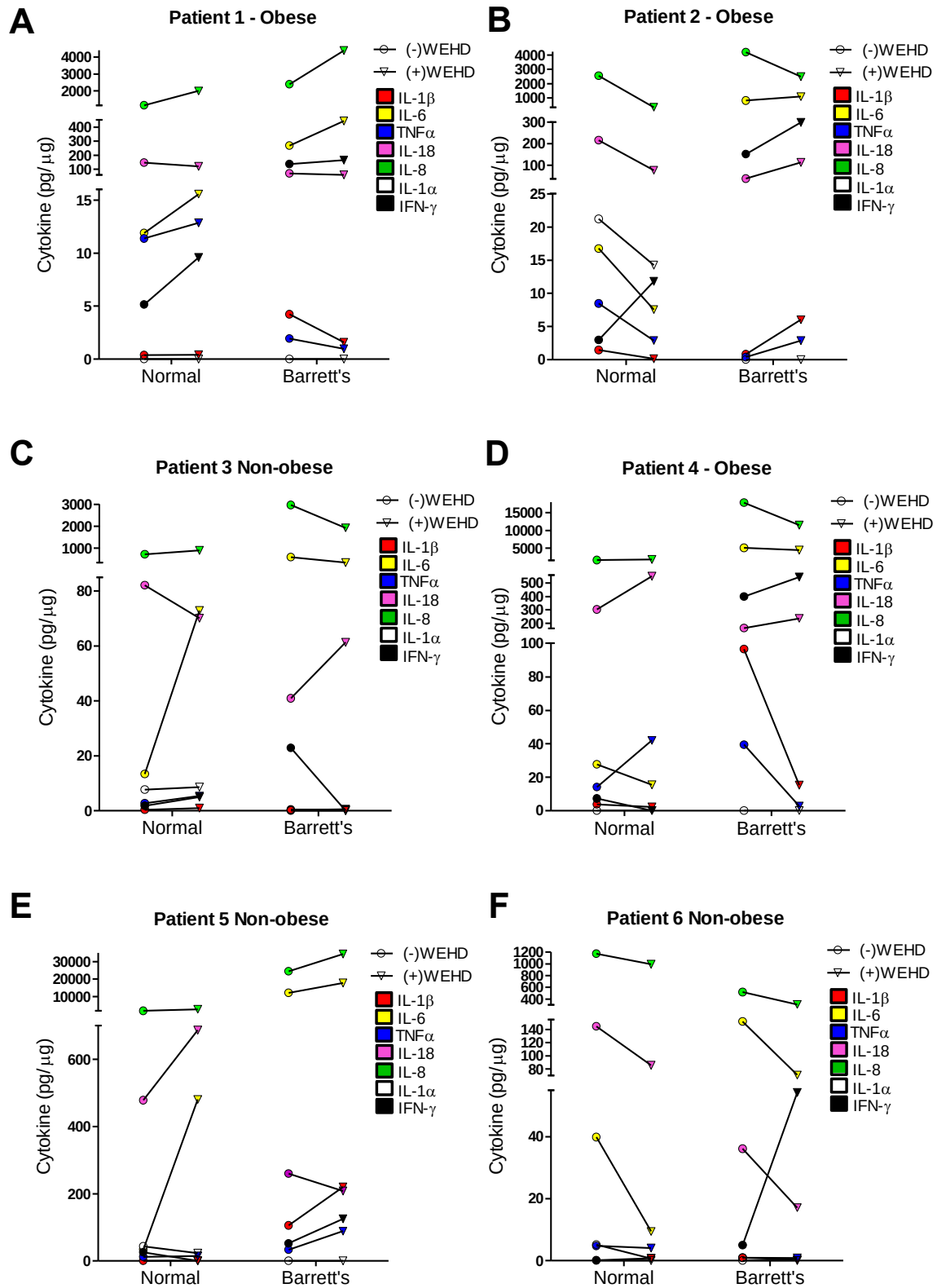


Figure 4.5: Caspase-1 inhibition has varied levels of efficacy in reducing inflammatory cytokine secretion from Barrett's biopsies.

Matched oesophageal biopsy explants were taken from areas of Barrett's and adjacent normal tissue from previously diagnosed Barrett's patients (n=10). Oesophageal explants were cultured with or without caspase-1 inhibitor (WEHD.CHO, 10 μ M) for 16 hours (500 μ L M199). Secretion of inflammatory mediators **A)** IL-1 β , **B)** IL-18, **C)** IL-6, **D)** IL-8, **E)** IFN- γ , **F)** TNF α and **G)** IL-1 α (pg/ μ g) were measured using MSD multiplex analysis (A,C,F), R&D Systems (B) and BioLegend ELISA (D,E,G). **H)** Cytotoxicity assay measuring LDH levels in supernatants from adjacent normal and Barrett's explants. Blue lines represent mean. Wilcoxon signed-rank test comparing inhibitor treated to untreated: non-significant. Data represents mean $\pm$ SEM.

Cytokine secretion by patient



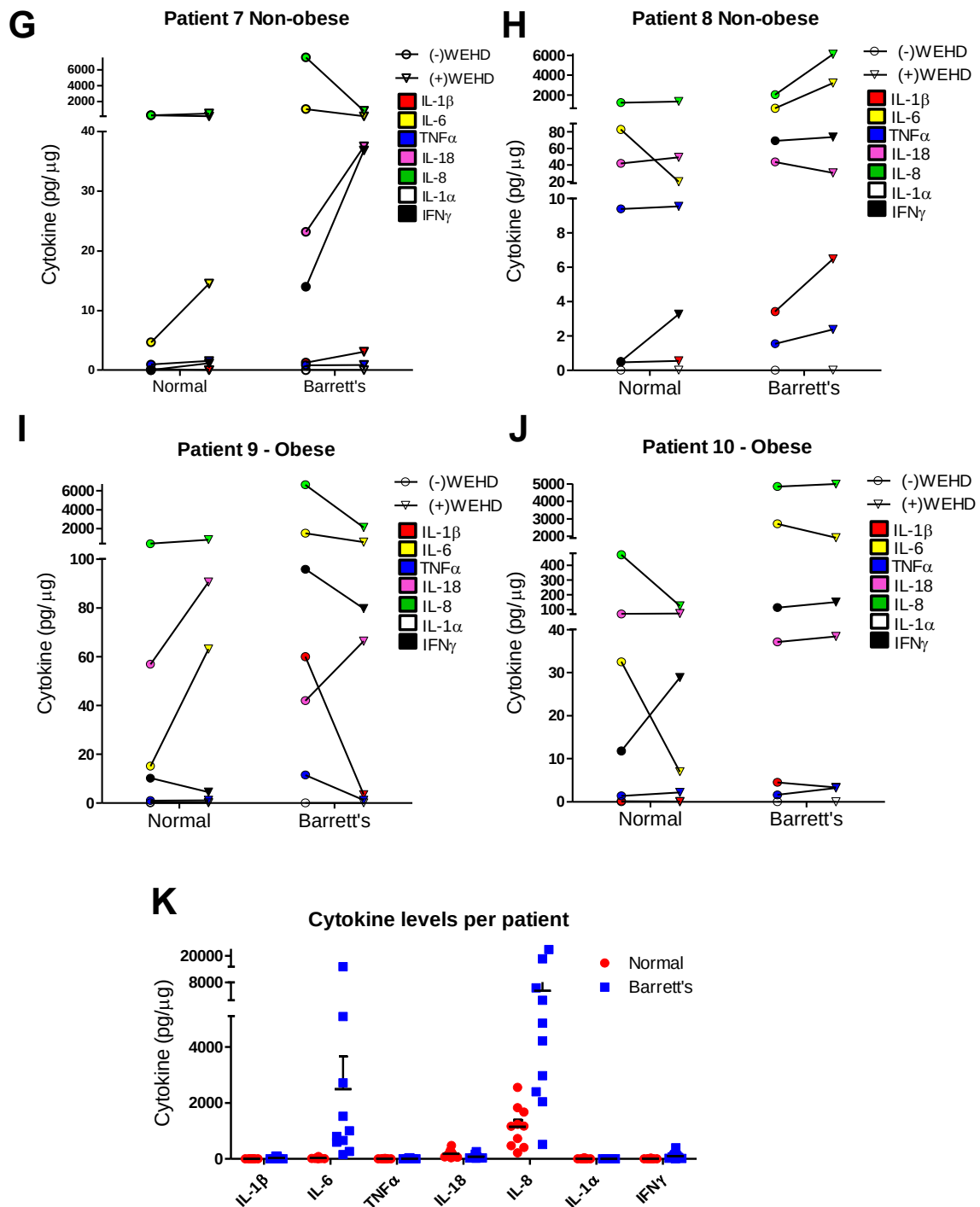


Figure 4.6: Graphical representation of cytokine secretion profiles of each individual patient highlights patient-to-patient variation.

Matched oesophageal biopsy explants were taken from areas of Barrett's and adjacent normal tissue from previously diagnosed Barrett's patients (n=10). Oesophageal explants were cultured with or without caspase-1 inhibitor (10 μ M) for 16 hours. Graphs represent cytokine secretion (pg/ μ g) from each patient. **A)** patient 1, **B)** patient 2, **C)** patient 3, **D)** patient 4, **E)** patient 5, **F)** patient 6, **G)** patient 7, **H)** patient 8, **I)** patient 9 and **J)** patient 10, **K)** Cytokine levels per patient.

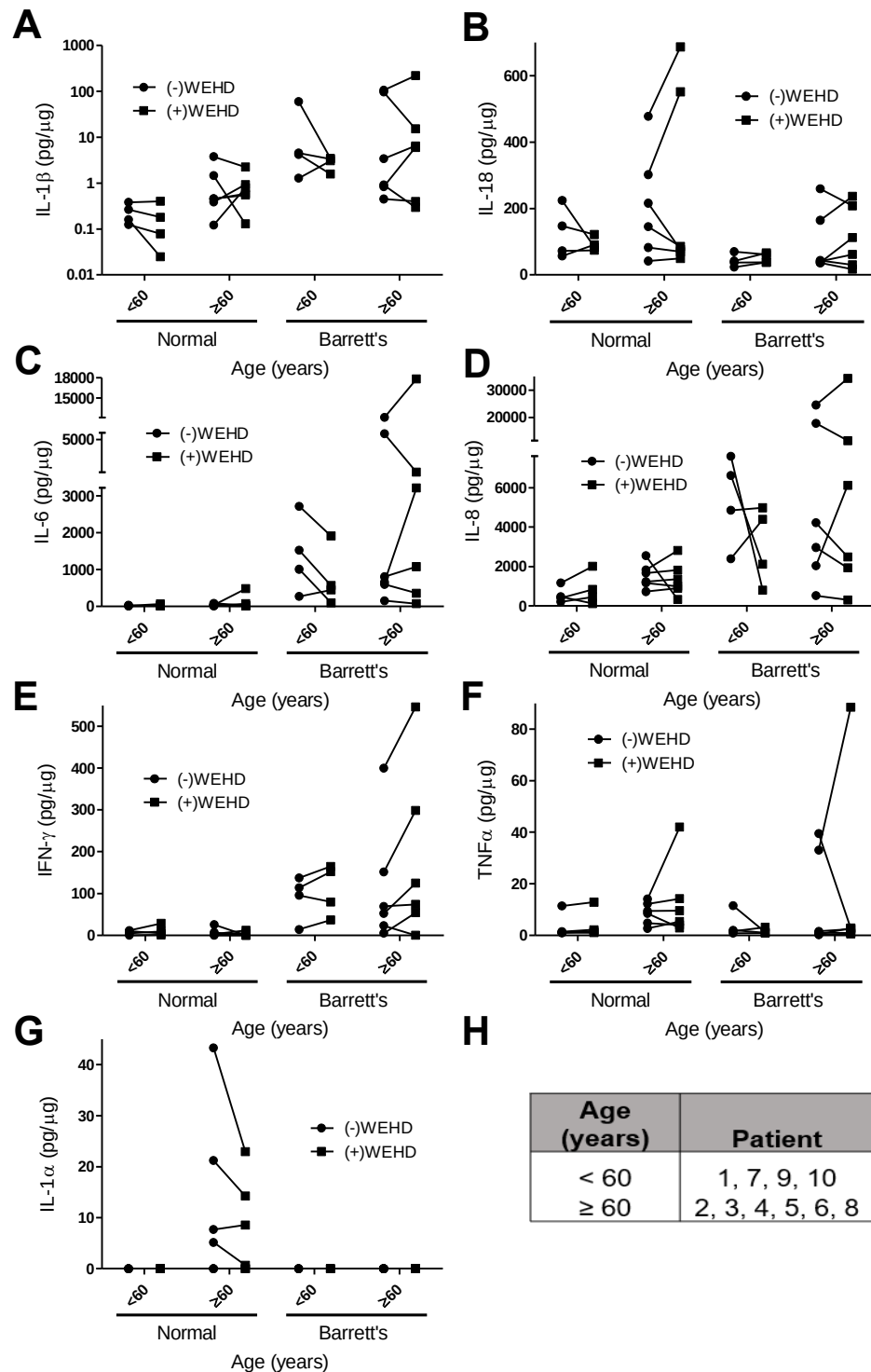


Figure 4.7: Inflammatory cytokine secretion of IL-1 β and IL-6 are decreased in patients under 60 years old in the presence of caspase-1 inhibition. Matched Barrett's patient biopsies (n=10) were taken from areas of Barrett's and adjacent normal tissue and cultured *ex vivo* with or without caspase-1 inhibitor (WEHD.CHO, 10 μ M, 500 μ L M199) for 16 hours. Data is separated based on patient age (years) and is represented by graphs **A**) IL-1 β , **B**) IL-18, **C**) IL-6, **D**) IL-8, **E**) IFN- γ , **F**) TNF α and **G**) IL-1 α (pg/ μ g). **H**) Table showing the age of individual patients. Wilcoxon signed-rank test comparing treated to untreated: non-significant.

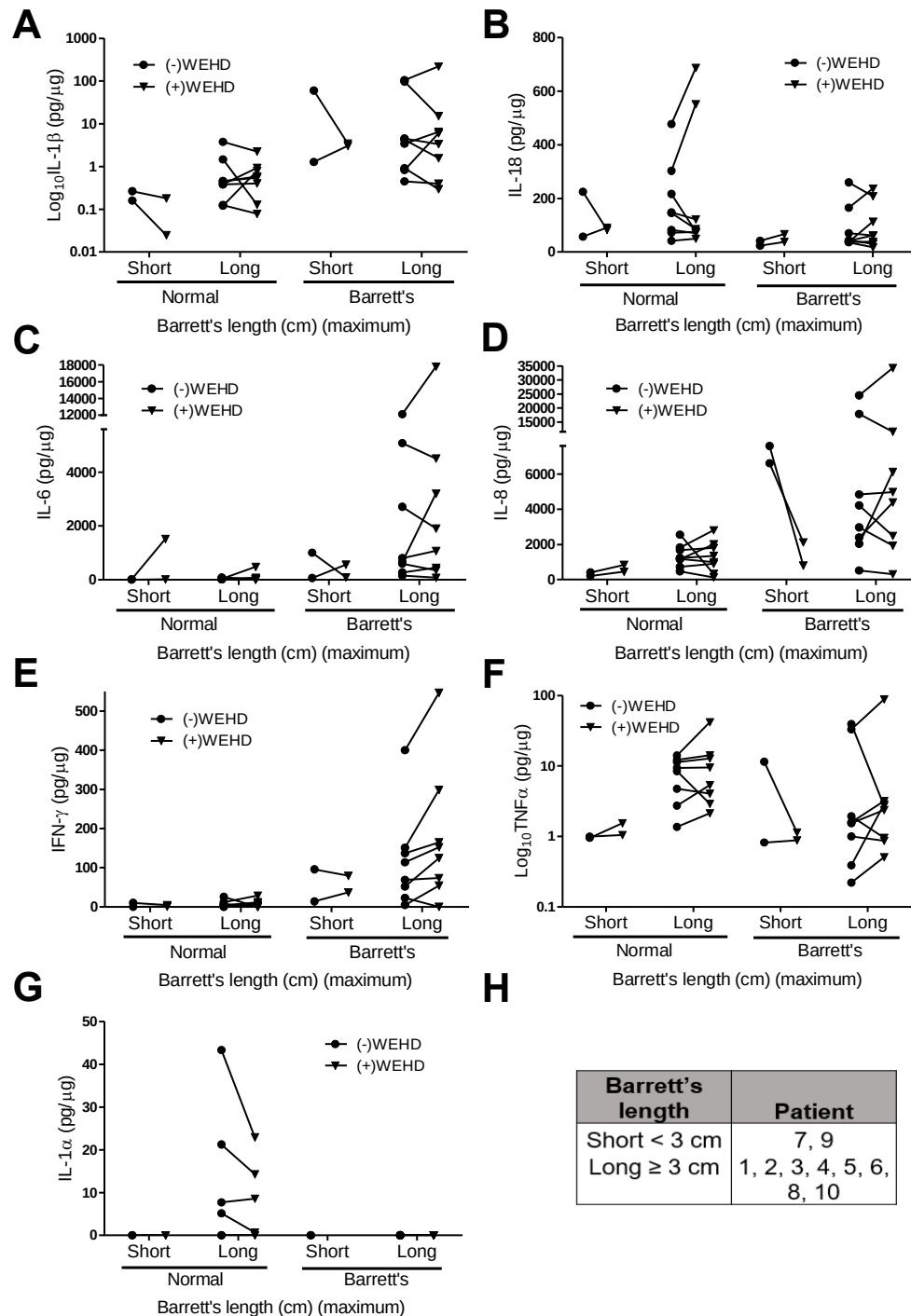


Figure 4.8: No significant differences in cytokine secretion in the presence of caspase-1 inhibition when patients are separated based on Barrett's length. Matched Barrett's patient biopsies (n=10) were taken from areas of Barrett's and adjacent normal tissue. Biopsy explants were cultured *ex vivo* with or without caspase-1 inhibitor (WEHD.CHO, 10 μM, 500 μL M199) for 16 hours. Data is separated based on defined Barrett's length (long ≥ 3cm) and is represented by graphs **A**) IL-1β, **B**) IL-18, **C**) IL-6, **D**) IL-8, **E**) IFN-γ, **F**) TNFα and **G**) IL-1α (pg/μg). **H**) Table showing Barrett's length of individual patients. Wilcoxon signed-rank test comparing inhibitor treated to untreated: non-significant.

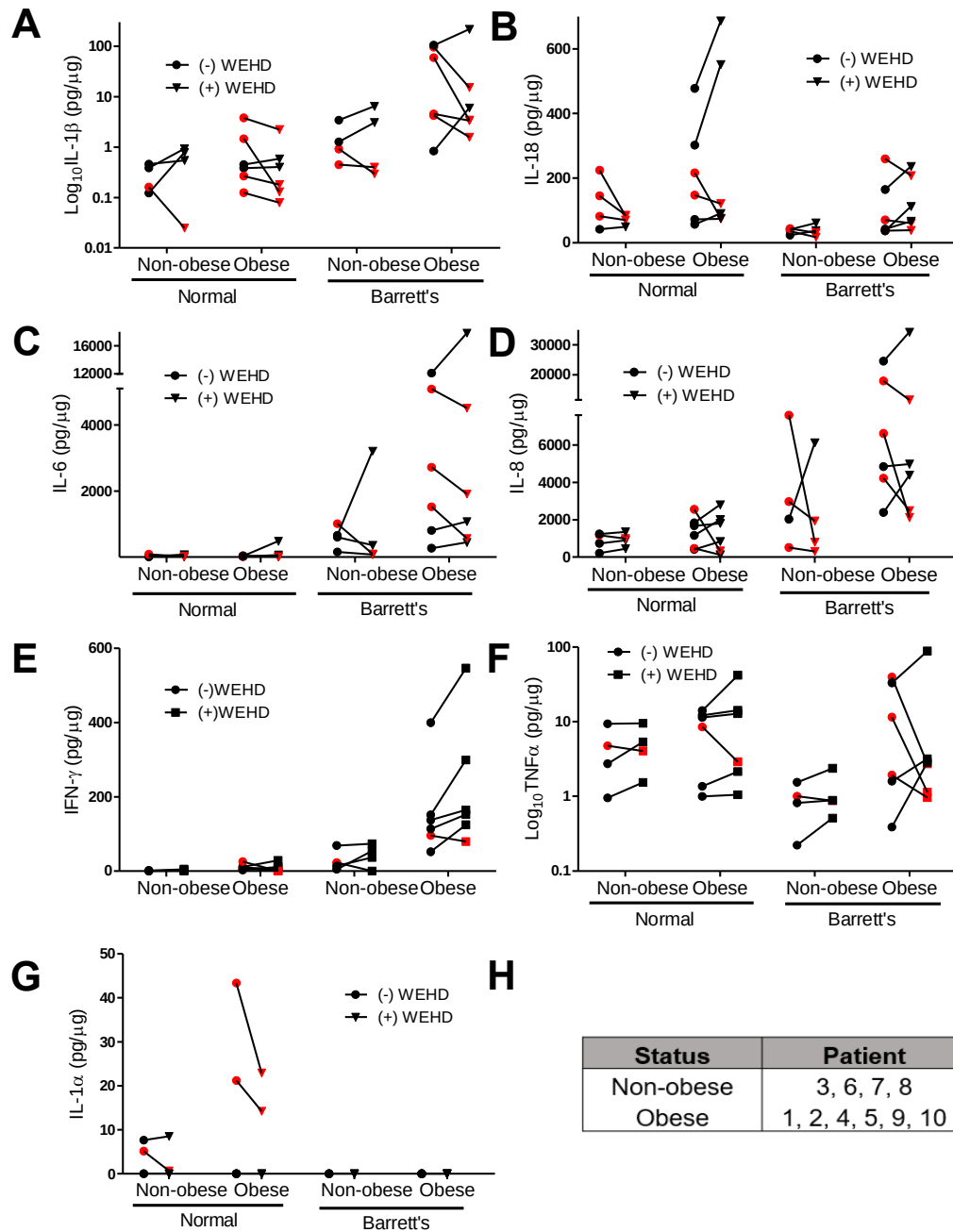


Figure 4.9: Caspase-1 inhibition results in reduced inflammatory cytokine secretion in Barrett's biopsies from obese patients.

Matched oesophageal biopsy explants were taken from areas of Barrett's and adjacent normal tissue from previously diagnosed Barrett's patients (n=10). Oesophageal explants were cultured with or without caspase-1 inhibitor (WEHD.CHO, 10 μM , 500 μL M199) for 16 hours. Data is separated based on patient BMI (obese $\geq 30 \text{ kg/m}^2$) and is represented by graphs. Red symbols indicate inhibition. **A)** IL-1 β , **B)** IL-18, **C)** IL-6, **D)** IL-8, **E)** IFN- γ , **F)** TNF α and **G)** IL-1 α (pg/ μg). **H)** Table of obesity status per patient. Wilcoxon signed-rank test comparing inhibitor treated to untreated: non-significant.

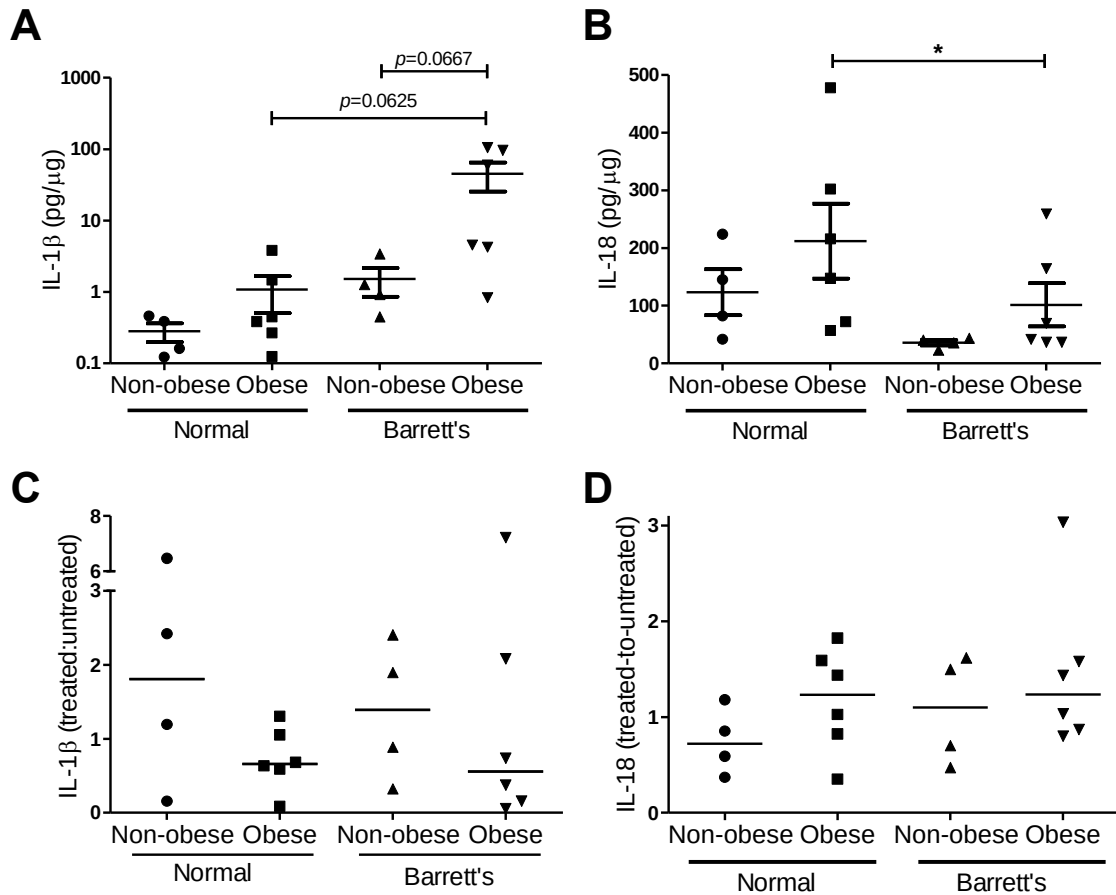


Figure 4.10: Obese Barrett's patients trend towards elevated levels of IL-1 β , which are reduced following caspase-1 inhibition.

Cytokine secretion values from untreated matched Barrett's patient biopsies (n=10) cultured *ex vivo* were separated based on patient BMI (obese ≥ 30 kg/m²) and are represented by graphs **A**) IL-1 β and **B**) IL-18 (pg/ μ g). Data represents mean $\pm$ SEM. Patient biopsies (n=10) cultured *ex vivo* with or without WEHD.CHO (10 μ M, 500 μ L M199) were separated based on patient BMI and are represented by graphs using a WEHD.CHO treat:untreated ratio for **C**) IL-1 β and **D**) IL-18. Data represents the median. Wilcoxon signed-rank test comparing normal to Barrett's. Mann Whitney U test comparing non-obese to obese: * $p < 0.05$.

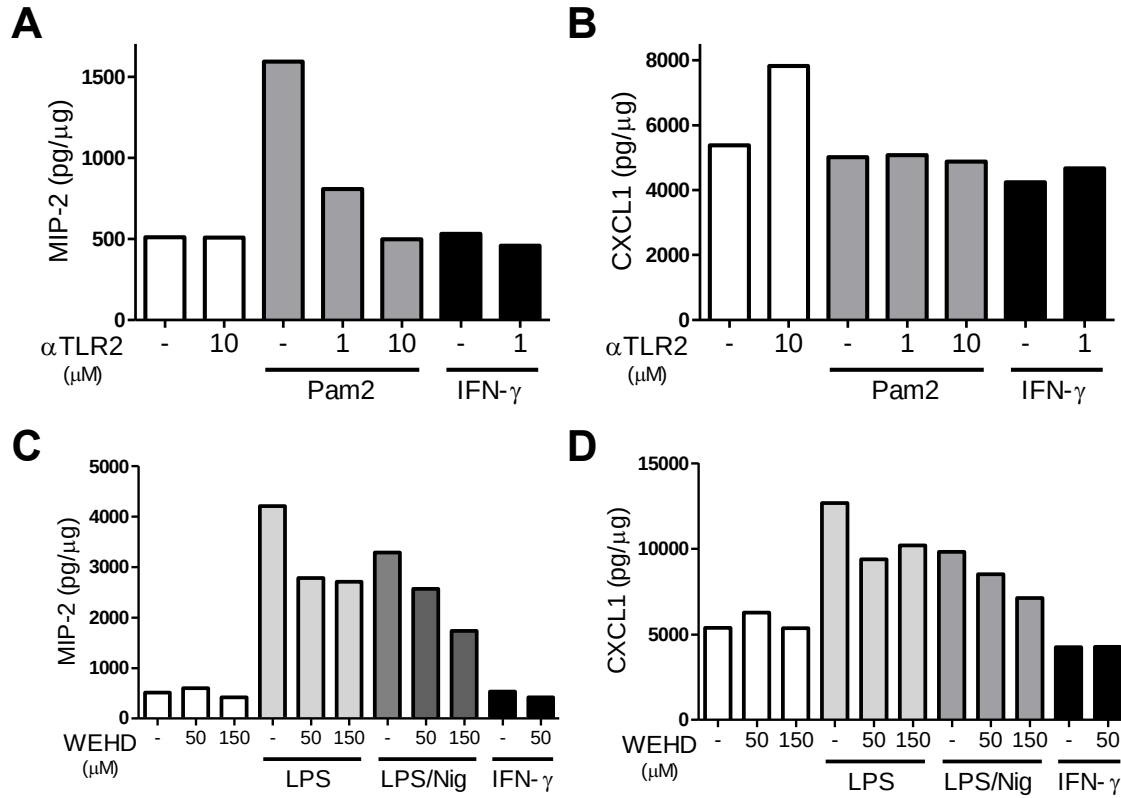


Figure 4.11: Optimisation of caspase-1 and TLR2 inhibitor concentrations for use with organoids taken from the SCJ of pL2-IL-1 β mice.

The oesophagus and stomach of a pL2-IL-1 β mouse (n=1) was resected at 12 months and cells were taken from the cardia region at the SCJ. Following 3 weeks of growth, the organoids were pre-treated with caspase-1 inhibitor, WEHD.CHO (50 and 150 μ M) or TLR2 blocking antibody, OPN301 (1 and 10 μ g/mL, 1 hour), before stimulation with LPS (1 μ g/mL), Pam2 (500 ng/mL) or IFN- γ (40 ng/mL) for 24 hours (400 μ L L-WRN media). Media was replaced and re-treated for a further 24 hours. On day 3, WEHD.CHO organoids were re-treated (1 hour), and stimulated with Nigericin (5 μ M, 1 hour). Supernatants were analysed for secretion levels of MIP-2 following treatment with **A**. OPN301 and **C**. WEHD.CHO and for secretion of CXCL1 following treatment with **B**. OPN301 and **D**. WEHD.CHO.

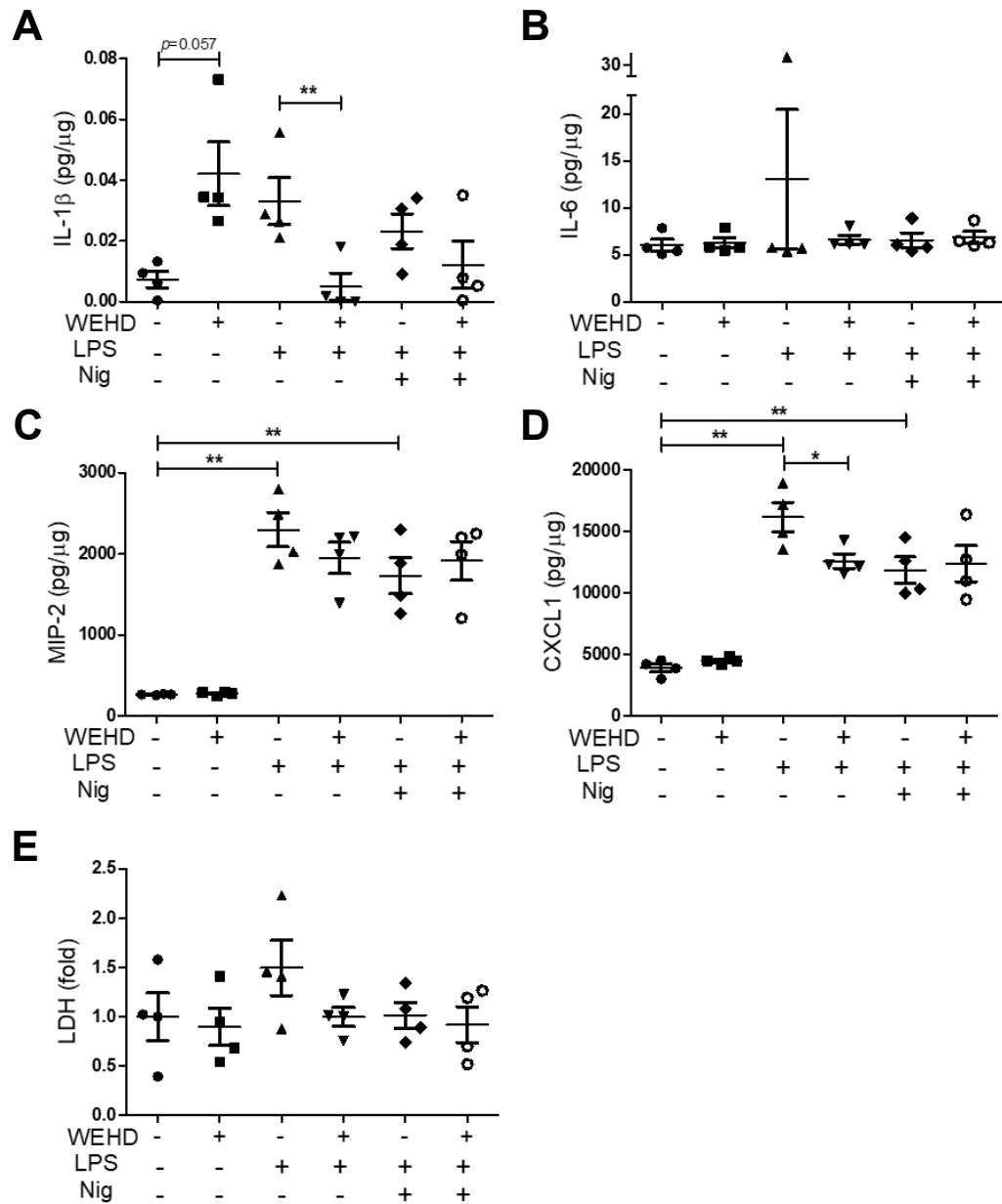


Figure 4.12: Caspase-1 inhibition in pL2-IL-1 β cardia organoids results in reduced levels of CXCL1 secretion.

Cells were taken from the cardia region at the SCJ of 12-month old pL2-IL-1 β mice (n=4) and cultured as 3D organoids for 3 weeks. 3 days prior to harvesting, organoids were pre-treated (1 hour) with caspase-1 inhibitor, WEHD.CHO (50 μ M) before 24-hour stimulation with LPS (1 μ g/mL). The following day, media was replaced and re-treated with inhibitor and LPS for a further 24 hours. On day 3, organoids were re-treated with WEHD.CHO (1 hour), before treating with inflammasome activating Nigericin (1 hour, 5 μ M) where indicated. Organoids were harvested and supernatants were analysed for secretion levels of **A**) IL-1 β , **B**) IL-6, **C**) MIP-2, **D**) CXCL1 and **E**) LDH. Data was normalised to total organoid protein (pg/ μ g) as determined using the BCA method and is represented as $\pm$ SEM. Paired Student's *t* test comparing inhibitor treated to untreated, and unstimulated to stimulated: **p*<0.05, ***p*<0.01

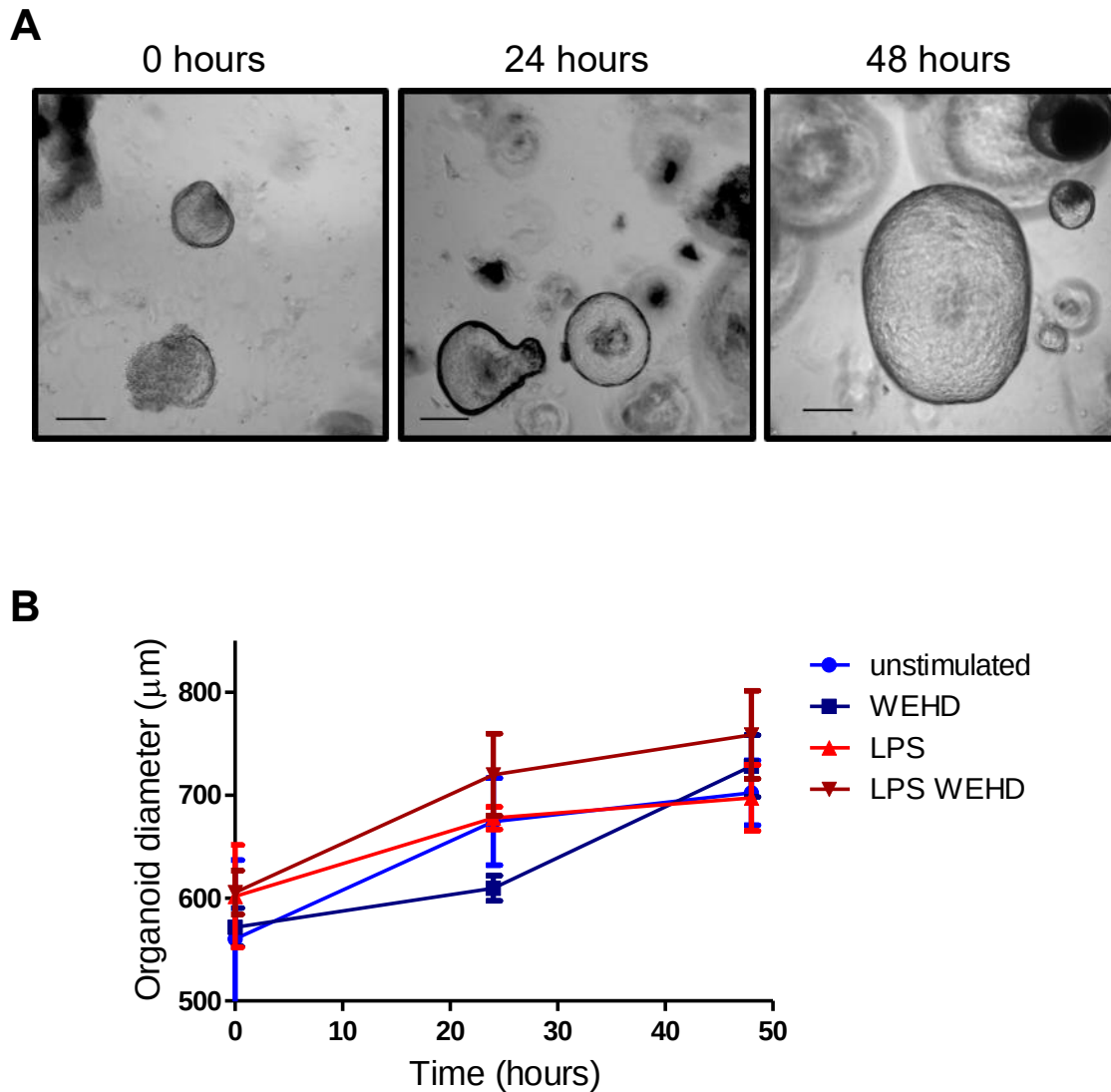


Figure 4.13: No differences in growth were observed following caspase-1 inhibition in pL2-IL-1 β Barrett's organoids.

Cells were taken from the cardia region at the SCJ of 12-month-old pL2-IL-1 β mice (n=3) and cultured as 3D organoids for 3 weeks. 3 days prior to harvesting, the organoids were pre-treated (1 hour) with caspase-1 inhibitor, WEHD.CHO (10 μ M) before stimulation with LPS (1 μ g/mL) for 24 hours. The following day, media was replaced and re-treated for a further 24 hours. Organoid diameters (μ m) were assessed under a light microscope (Zeiss, Axiovert 200M) and analysed using ImageJ software (Fiji) at 0 h (before treatment), 24 h (during treatment) and 48 h (before harvest). **A**) Representative images of organoid morphologies. Magnification: 5X. Scale bars: 200 μ m. **B**) Growth curve analysis of organoids following treatments at 0h, 24h and 48h. Data is represented as $\pm$ SEM.

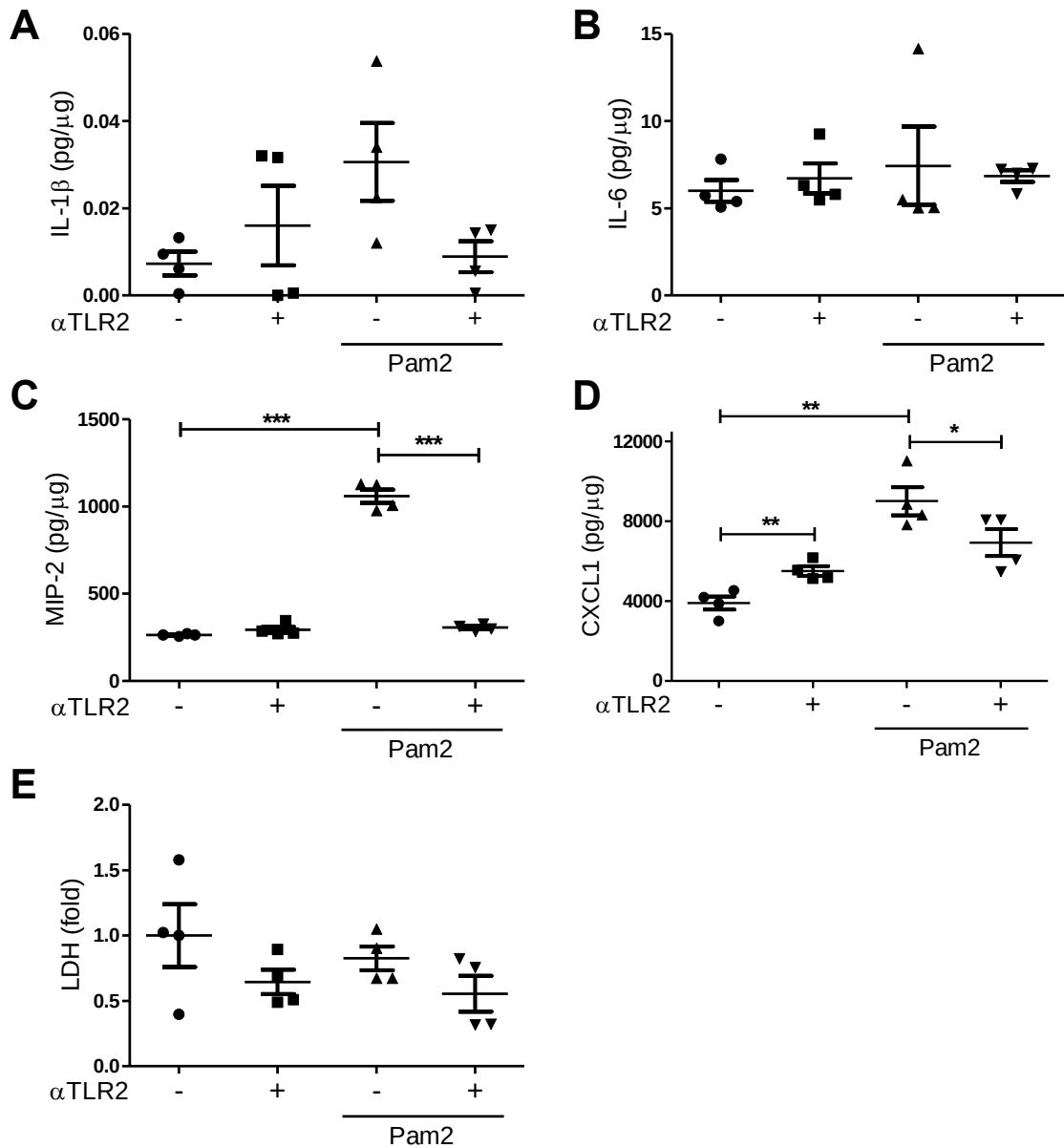


Figure 4.14: TLR2 inhibition in Pam2-stimulated Barrett's organoids reduces secretion of the IL-8 orthologs, CXCL1 and MIP-2.

Cells were taken from the cardia region at the SCJ of 12-month-old pL2-IL-1 β mice (n=4) and cultured as 3D organoids for 3 weeks. 3 days prior to harvesting, organoids were pre-treated (1 hour) with TLR2 blocking antibody, OPN-301 (10 μ g/mL) before stimulation with Pam2 CSK4 (40 ng/mL) for 24 hours. On treatment day 2, media was replaced and re-treated for a further 24 hours. Organoids were harvested and supernatants were analysed for secretion levels of **A**) IL-1 β , **B**) IL-6, **C**) MIP-2, **D**) CXCL1 and **E**) LDH. Data was normalised to total organoid protein (pg/ μ g) as determined using the BCA method and is represented as $\pm$ SEM. Paired Student's *t* test comparing inhibitor treated to untreated, and unstimulated to stimulated: **p*<0.05, ***p*<0.01, ****p*<0.001.

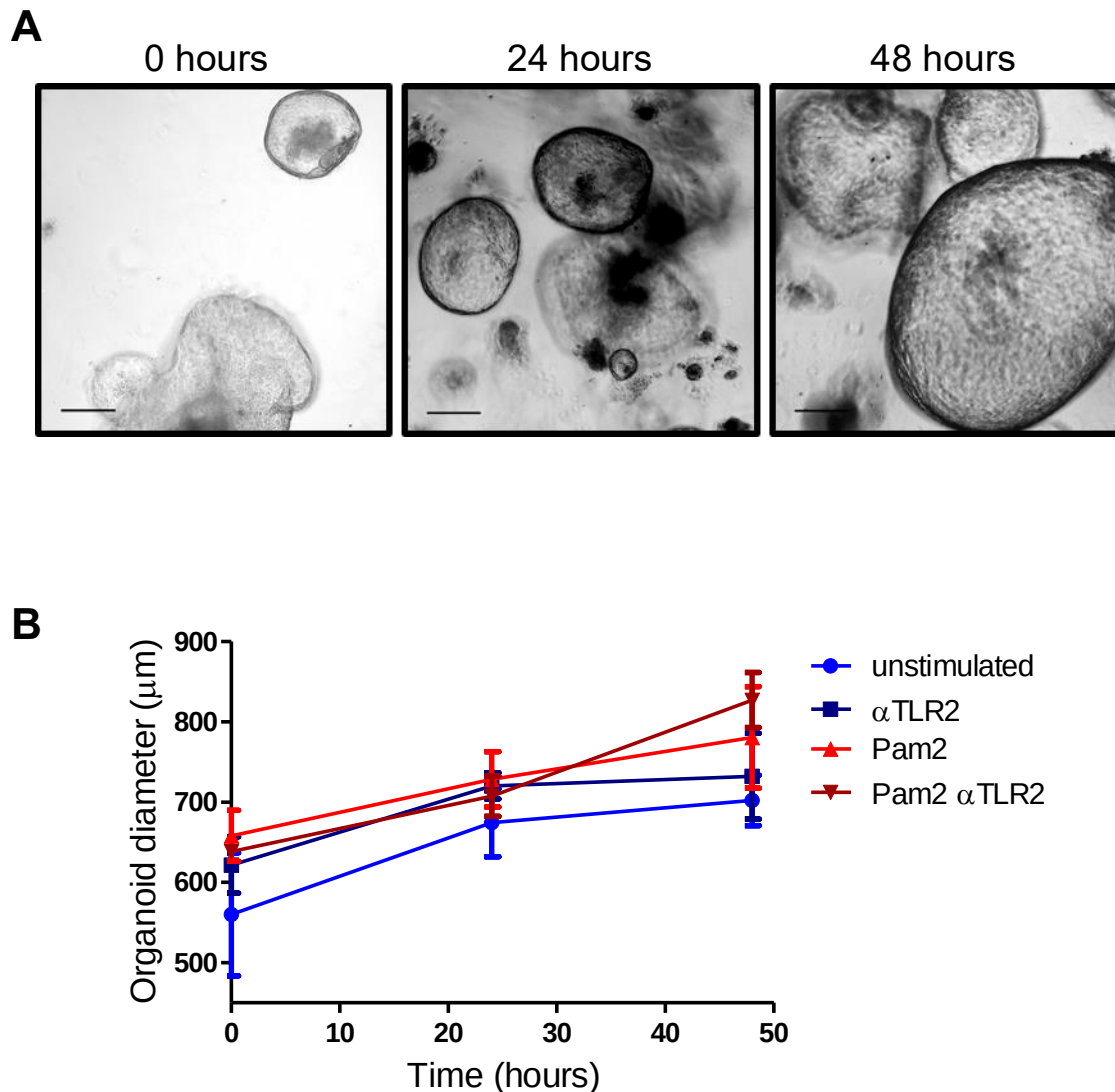


Figure 4.15: TLR2 inhibition does not significantly alter growth of pL2-IL-1 β Barrett's organoids after 48 hours.

Cells were taken from the cardia region at the SCJ of 12-month old pL2-IL-1 β mice (n=3) and cultured as 3D organoids for 3 weeks. 3 days prior to harvesting, the organoids were pretreated (1 hour) with caspase-1 inhibitor, WEHD.CHO (10 μ M) before stimulation with LPS (1 μ g/mL) for 24 hours. The following day, media was replaced and re-treated for a further 24 hours. Organoid diameters (μ m) were assessed under a light microscope (Zeiss, Axiovert 200M) and analysed using ImageJ software (Fiji) at 0 h (before treatment), 24 h (during treatment) and 48 h (before harvest). **A**) Representative images of organoid morphologies. Magnification: 5X. Scale bars: 200 μ m. **B**) Growth curve analysis of organoids following treatments at 0h, 24h and 48h. Data is represented as $\pm$ SEM.

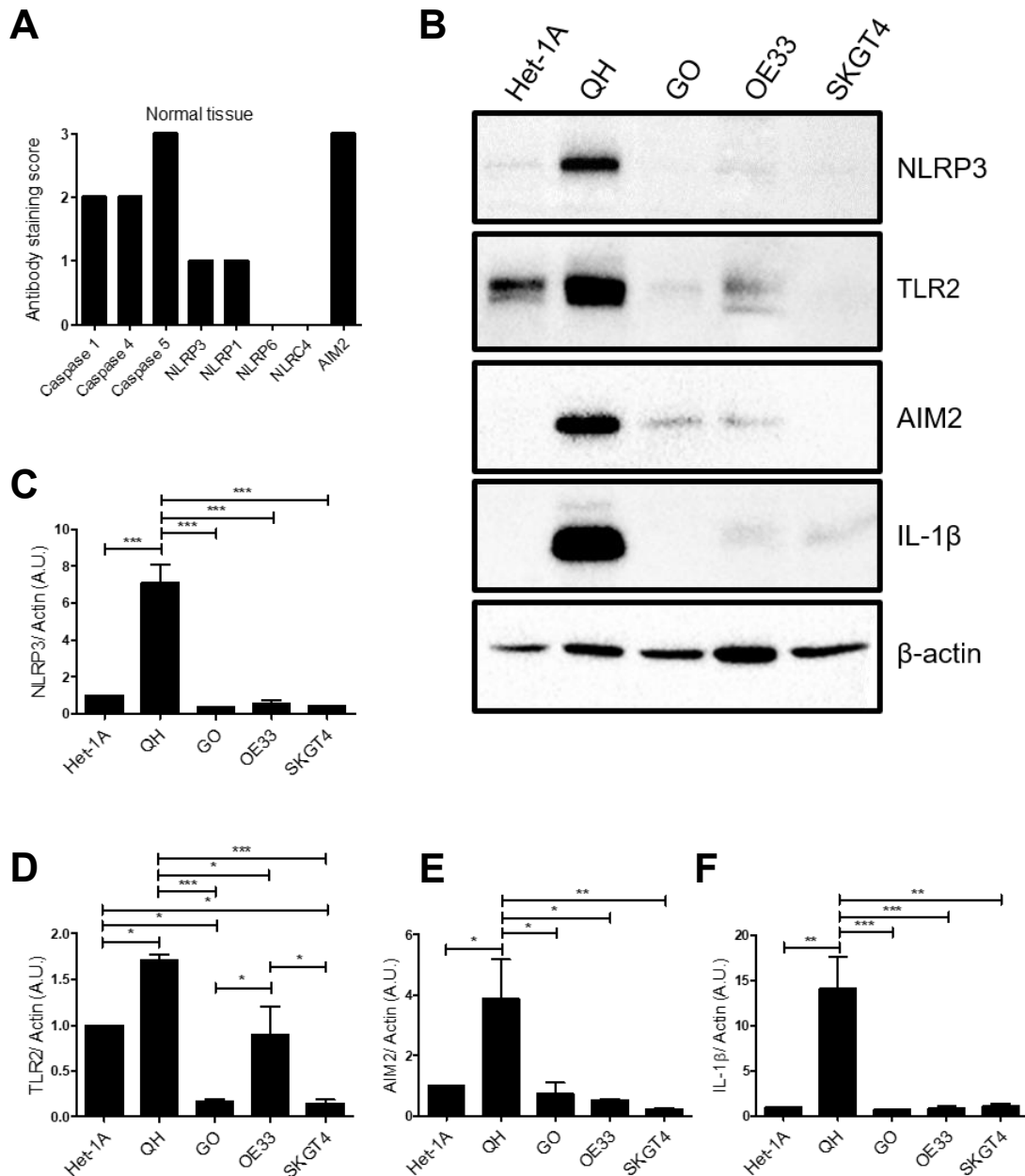


Figure 4.16: Inflammatory proteins are strongly expressed in the Barrett's metaplasia cell line, QH.

A) The Protein Atlas repository was analysed for protein expression values of inflammasome-associated components in human normal oesophageal tissue (www.proteinatlas.org). **B)** Immunoblot analysis of NLRP3 (110, 106, 83.5 kDa), TLR2 (89.8 kDa), AIM2 (38.9 kDa) and IL-1β (30.7 kDa) expression in oesophageal cell lines representing disease progression. Densitometric analysis (n=3) of **C)** NLRP3, **D)** TLR2, **E)** AIM2 and **F)** IL-1β was performed by normalising values to loading control, β-actin (42 kDa), relative to Het-1A cell line expression. Lysates (20 μg/well) were resolved on a 12% SDS-PAGE gel. Data represents mean ± SEM. One-way ANOVA followed by Tukey's post hoc test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

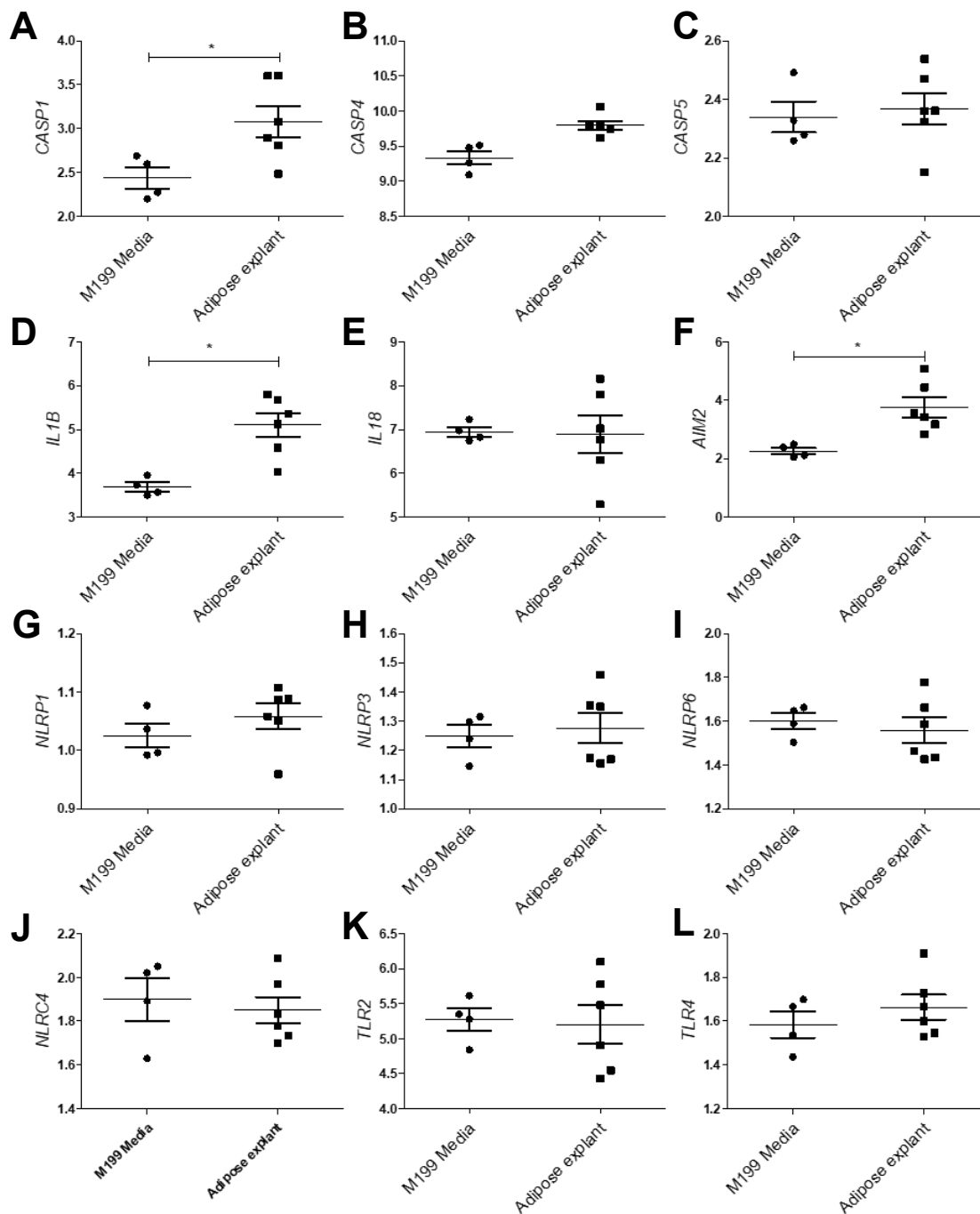


Figure 4.17: Specific inflammasome-related genes are upregulated in OE33 tumour cells when co-cultured with adipose tissue explants from obese OAC patients.

Graphs represent relative inflammasome mRNA expression values analysed using a publicly available data set (Allott EH. *et al.*, 2012 [GSE36841], (498)). An affymetrix microarray was used to profile mRNA expression in OE33 OAC cells following co-culturing with visceral adipose tissue explants taken from visceraally obese OAC patients (n=6) or media control (n=4) for 24 hours. **A) – L)** Gene values for *CASP1*, *CASP4*, *CASP5*, *IL1B*, *IL18*, *AIM2*, *NLRP1*, *NLRP3*, *NLRP6*, *NLRC4*, *TLR2* and *TLR4*, respectively. Data represents mean $\pm$ SEM. Paired Student's *t* test: **p* < 0.05.

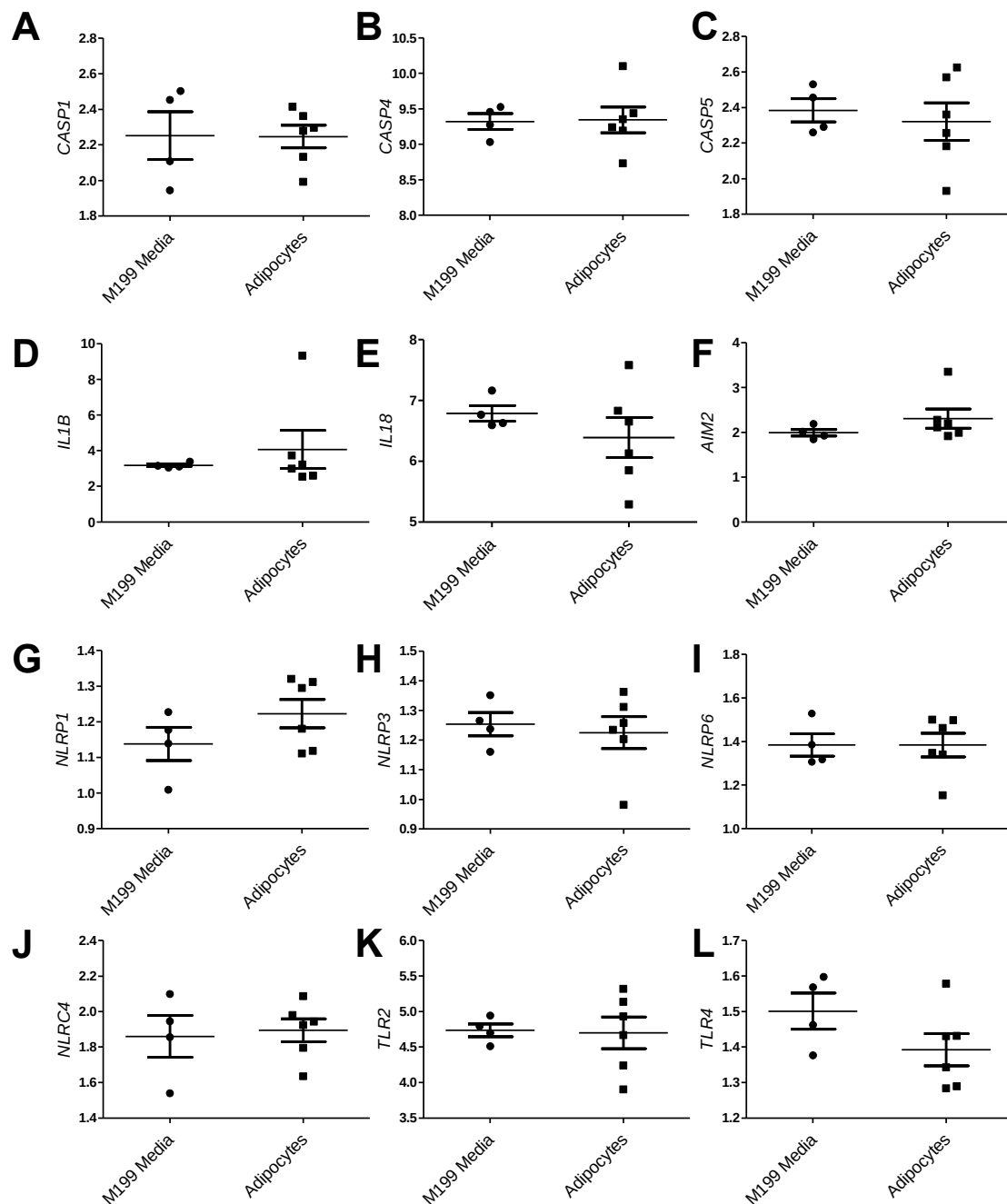


Figure 4.18: No significant alterations in inflammasome-related gene expression in OE33 cells when co-cultured with adipocytes taken from obese OAC patients.

Graphs represent relative inflammasome mRNA expression values analysed using a publicly available data set (Allott EH. *et al.* 2012 [GSE36841], (498)). An affymetrix microarray was used to profile gene expression in OE33 OAC cells following co-culture with adipocytes isolated from visceraally obese OAC patients (n=6) or media control (n=4) for 24 hours. **A) – L)** Gene values for *CASP1*, *CASP4*, *CASP5*, *IL1B*, *IL18*, *AIM2*, *NLRP1*, *NLRP3*, *NLRP6*, *NLRC4*, *TLR2* and *TLR4*, respectively. Data represents mean $\pm$ SEM. Paired Student's *t* test: no significance.

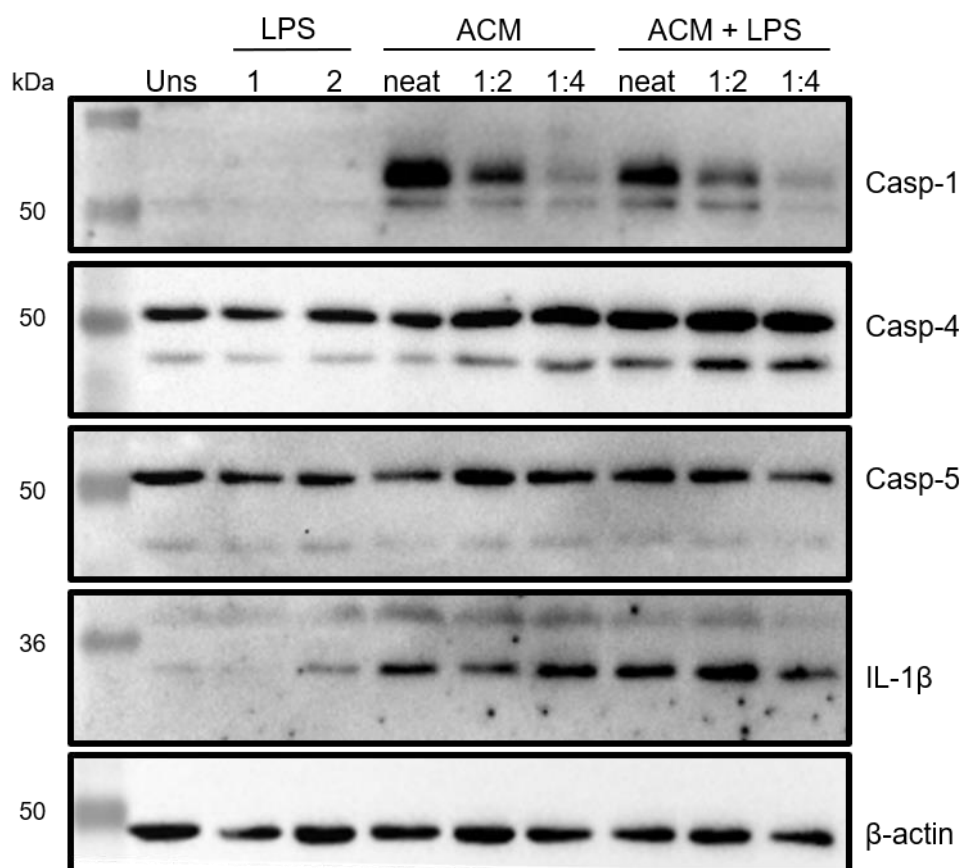


Figure 4.19: Culturing OE33 cells with adipose conditioned media (ACM) results in the upregulation of caspase-1 and IL-1 β .

OE33 cells were seeded at 7×10^4 cells/mL 24 hours prior to stimulation. Cells were stimulated for 24 hours with LPS (1 μ g/mL and 2 μ g/mL) or with the following dilutions of ACM: neat, 1:2 and 1:4. Patients were viscerally obese, OAC males (BMI > 30 kg/m², WC > 94 cm) and have both received neoadjuvant treatment. Protein lysate (25 μ g) was resolved by 12% SDS-PAGE, wet transferred to nitrocellulose and probed for caspase-1 (45 kDa), caspase-4 (43 kDa), caspase-5 (47 kDa), IL-1 β (30.7 kDa) and loading control, β -actin (42 kDa). Immunoblot is representative of two independent experiments from two different patient ACMs.

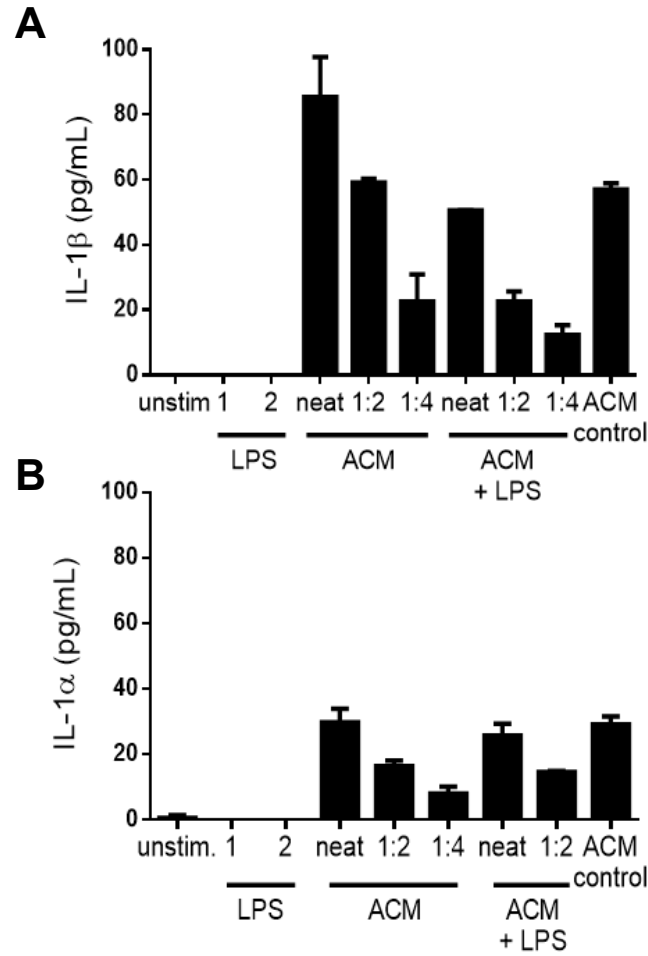


Figure 4.20: OE33 cells secrete minimum pro-inflammatory cytokines following incubation with ACM.

OE33 cells, p19 were seeded at 7×10^4 cells/mL 24 hours prior to stimulation. Cells were stimulated for 24 hours with LPS (1 μ g/mL and 2 μ g/mL) or with the following dilutions of ACM: neat, 1:2 and 1:4. Cytokine quantification of **A**) IL-1 β and **B**) IL-1 α were determined by ELISA. Patients were viscerally obese, OAC males (BMI > 30kg/m², WC > 94 cm, n=2) and have both received neoadjuvant treatment. Graphs represent data from one patient and is representative of two independent experiments. Data represents mean $\pm$ SD.

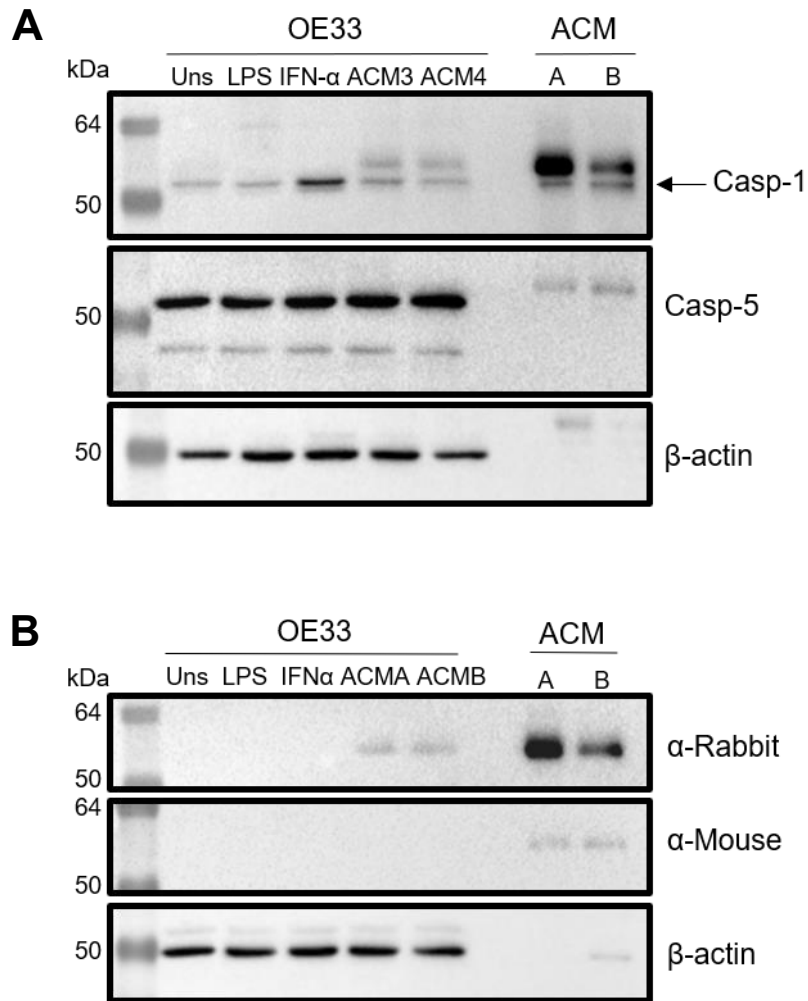


Figure 4.21: ACM-derived proteins appear to cross-react with anti-rabbit IgG generating unspecific bands at 50 kDa.

Cells were stimulated for 24 hours with 1 µg/mL LPS, 40 ng/mL IFN-α or ACM (A and B, n=2 patients). Patients were viscerally obese, OAC males (BMI > 30 kg/m²) and were both treatment naive. **A)** Protein lysate (25 µg) was resolved by 12% SDS-PAGE, wet transferred to nitrocellulose (50 mins, 200 mA) and probed for caspase-1 (45 kDa), caspase-5 (47 kDa) and loading control, β-actin (42 kDa). **B)** Immunoblot probed alone with anti-rabbit and anti-mouse secondary antibody conjugated to HRP for one hour and developed using a BioRad Gel DocTMXR+System.

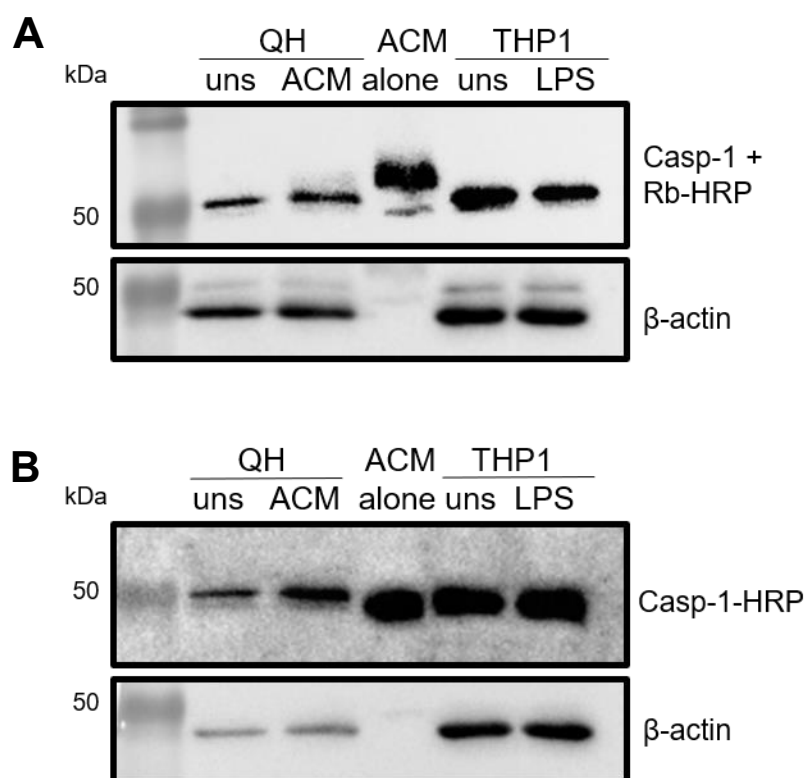


Figure 4.22: Direct HRP conjugation to primary caspase-1 antibody eliminates issues with IgG cross-reactivity in ACM.

A) Immunoblot probing for caspase-1 (45 kDa) using unconjugated caspase-1 SC-622 primary antibody with secondary antibody anti-rabbit conjugated to HRP. **B)** Immunoblot probing for caspase-1 using SC-622 primary antibody directly conjugated to HRP using the Lightning-Link HRP conjugation kit (Expedeon).

Patient	Sex	Neoadjuvant treatment	Waist circumference (cm)	Weight (kg)	Height (cm)	BMI (kg/m ²)	VFA (cm ²)	TNM Staging
1	M	No	94	NA	NA	26.3	217.1	T1, N0, M0
2	M	No	88	NA	NA	22.86	227	NA
3	M	No	115	98.9	167.64	35.2	291.6	T2, N0, M0
4	M	No	97	65	161	25.1	200.38	NA
5	M	No	NA	NA	NA	NA	227.01	T1a
6	M	No	NA	NA	NA	NA	201.6	T1b, N0, M0

Table 4.2: Characterisation of the OAC patient cohort used to generate ACM.

Adipose tissue was taken from viscerally obese (VFA>160 cm²), OAC patients undergoing oesophageal resection surgery. All patients were male and treatment naïve (n=6).

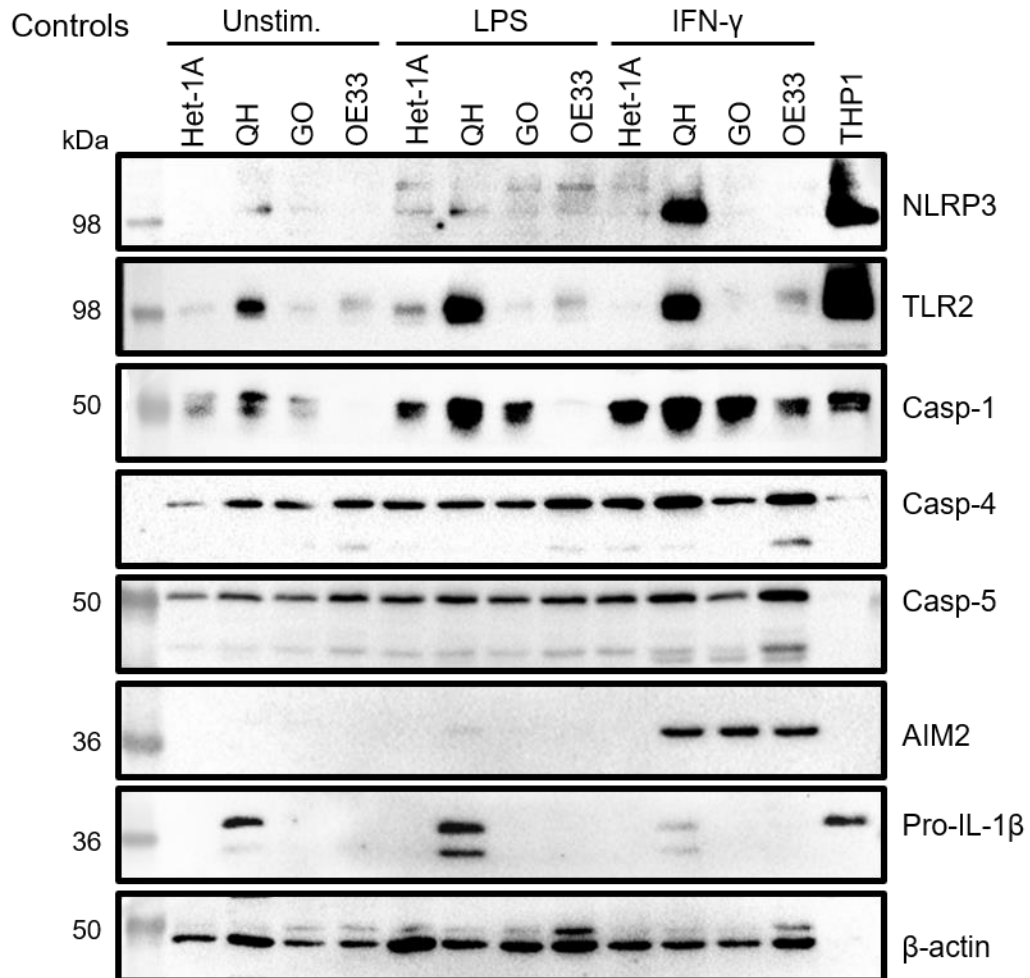
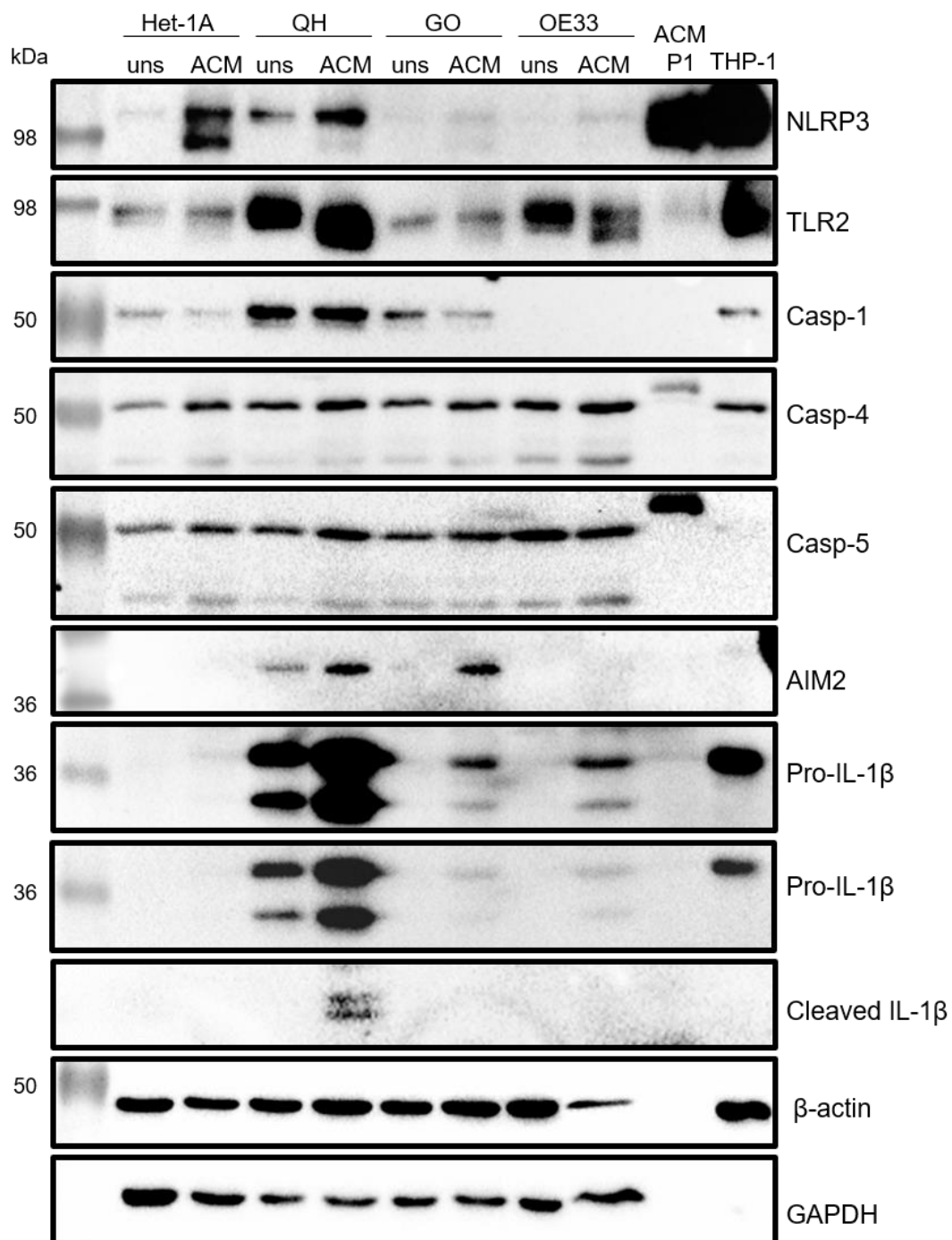


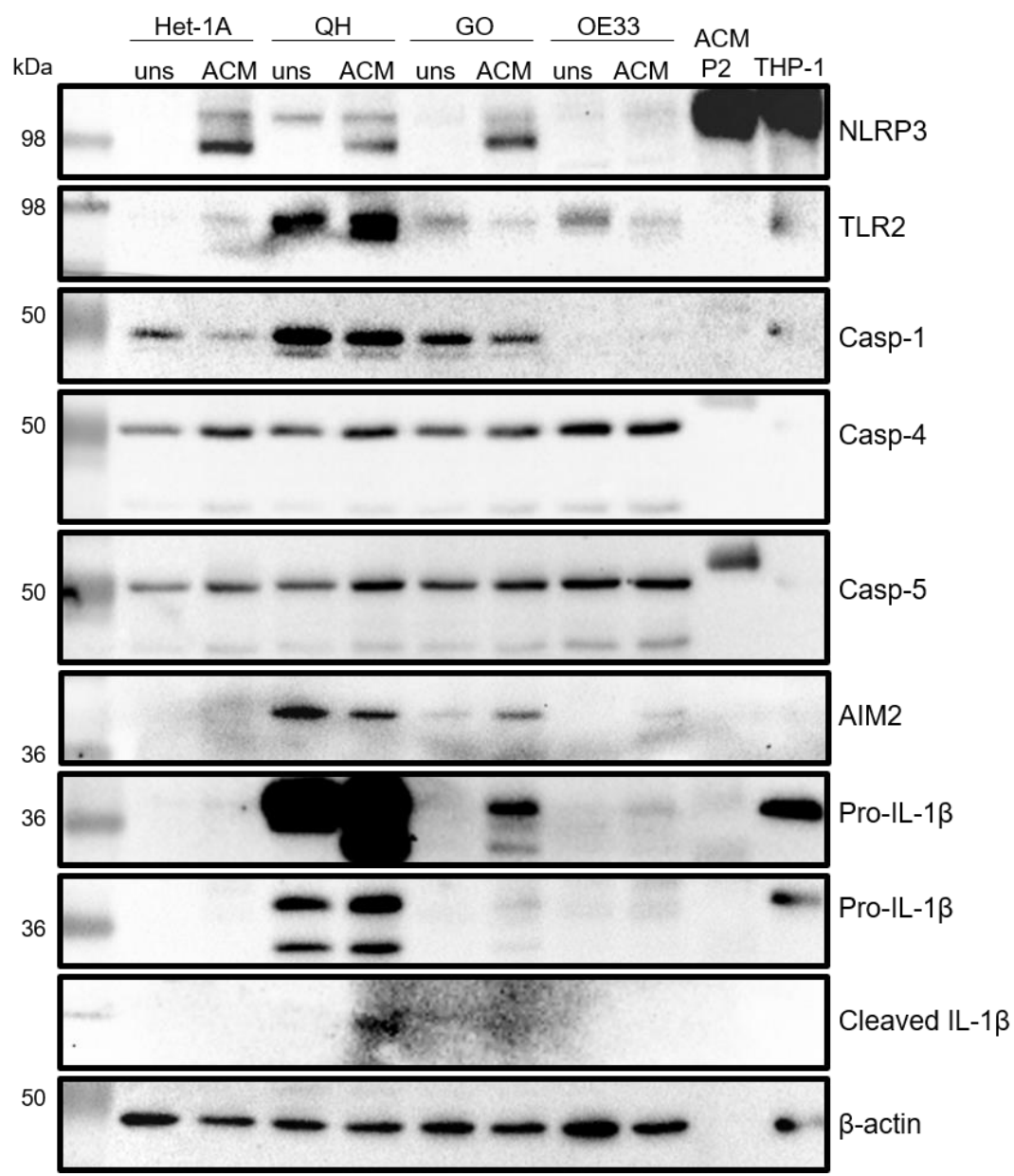
Figure 4.23: Stimulation of oesophageal cell lines with LPS and IFN-γ causes upregulation of inflammatory proteins.

A) Oesophageal cell lines; Het-1A, QH, GO and OE33 were stimulated with LPS (1 µg/mL) or IFN-γ (40 ng/mL) for 24 hours. Experimental control immunoblots were probed for NLRP3 (110, 106, 83.5 kDa), TLR2 (89.8 kDa), caspase-1 (45 kDa), caspase-4 (43.2 kDa), caspase-5 (49.7 kDa), AIM2 (39 kDa), pro-IL-1β (30.75 kDa) and loading control, β-actin (42 kDa). 20 µg of protein was resolved on a SDS-PAGE gel, (6 µg THP-1 sample) and wet transferred onto a nitrocellulose membrane.

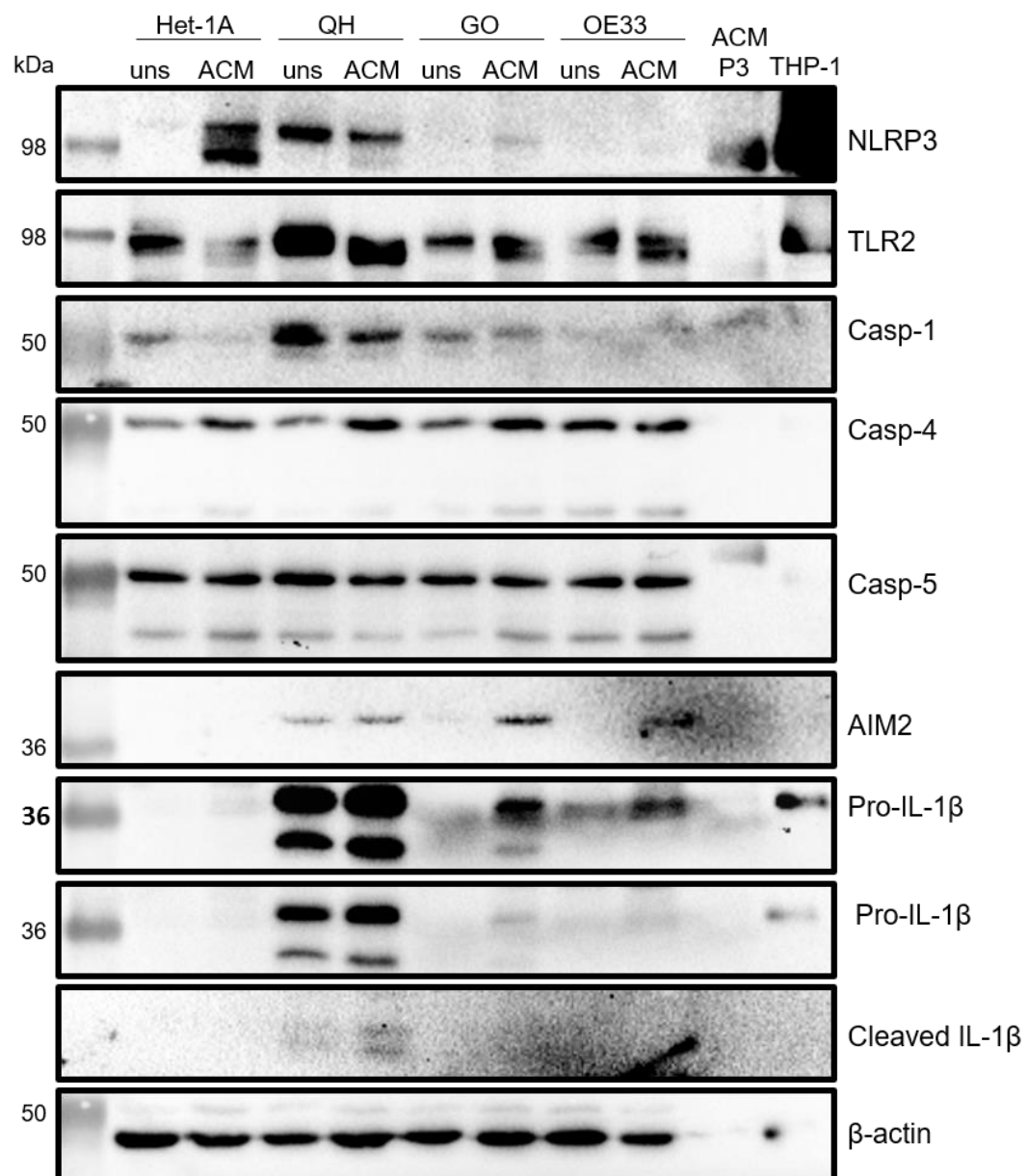
A Patient 1



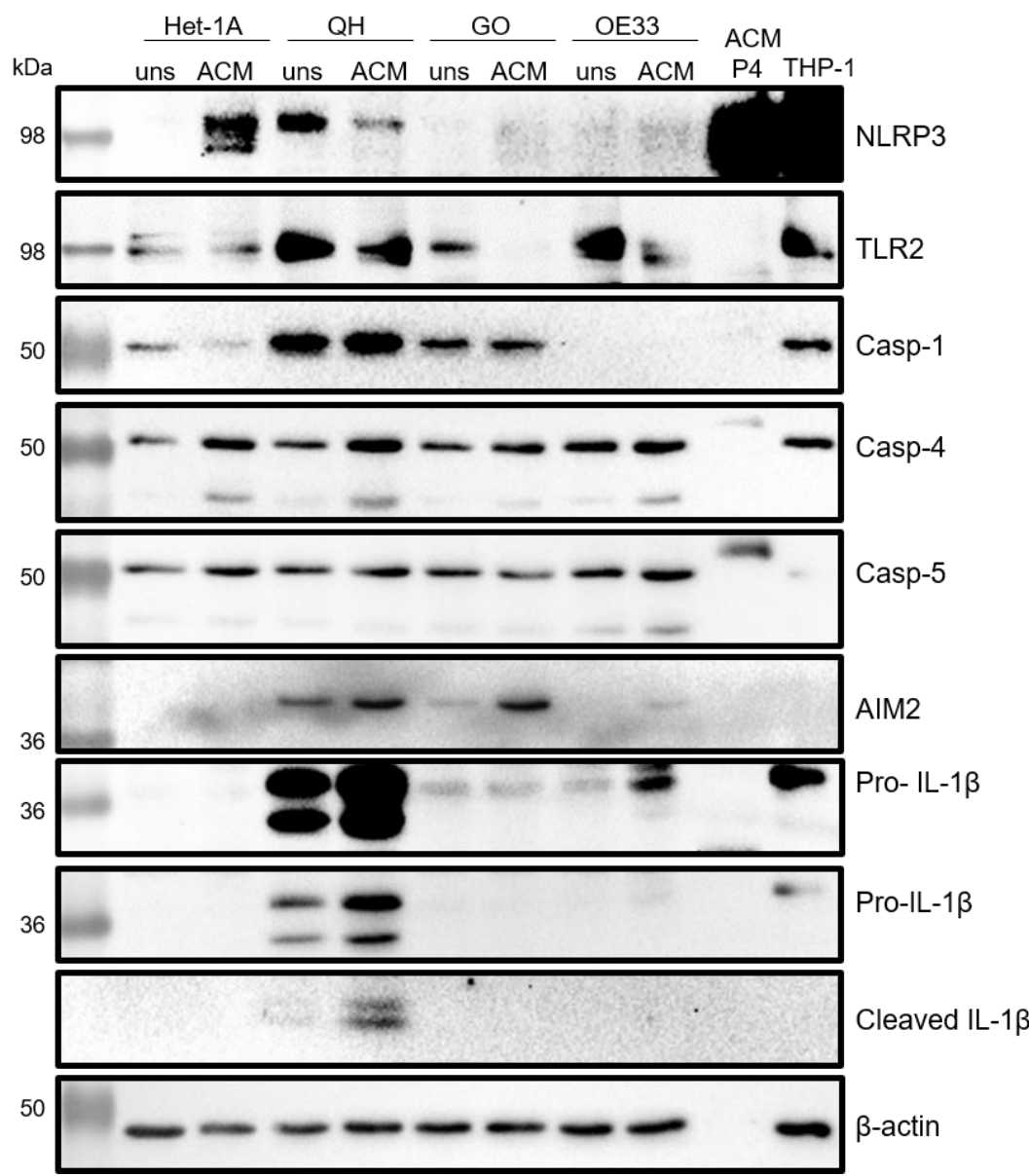
B Patient 2



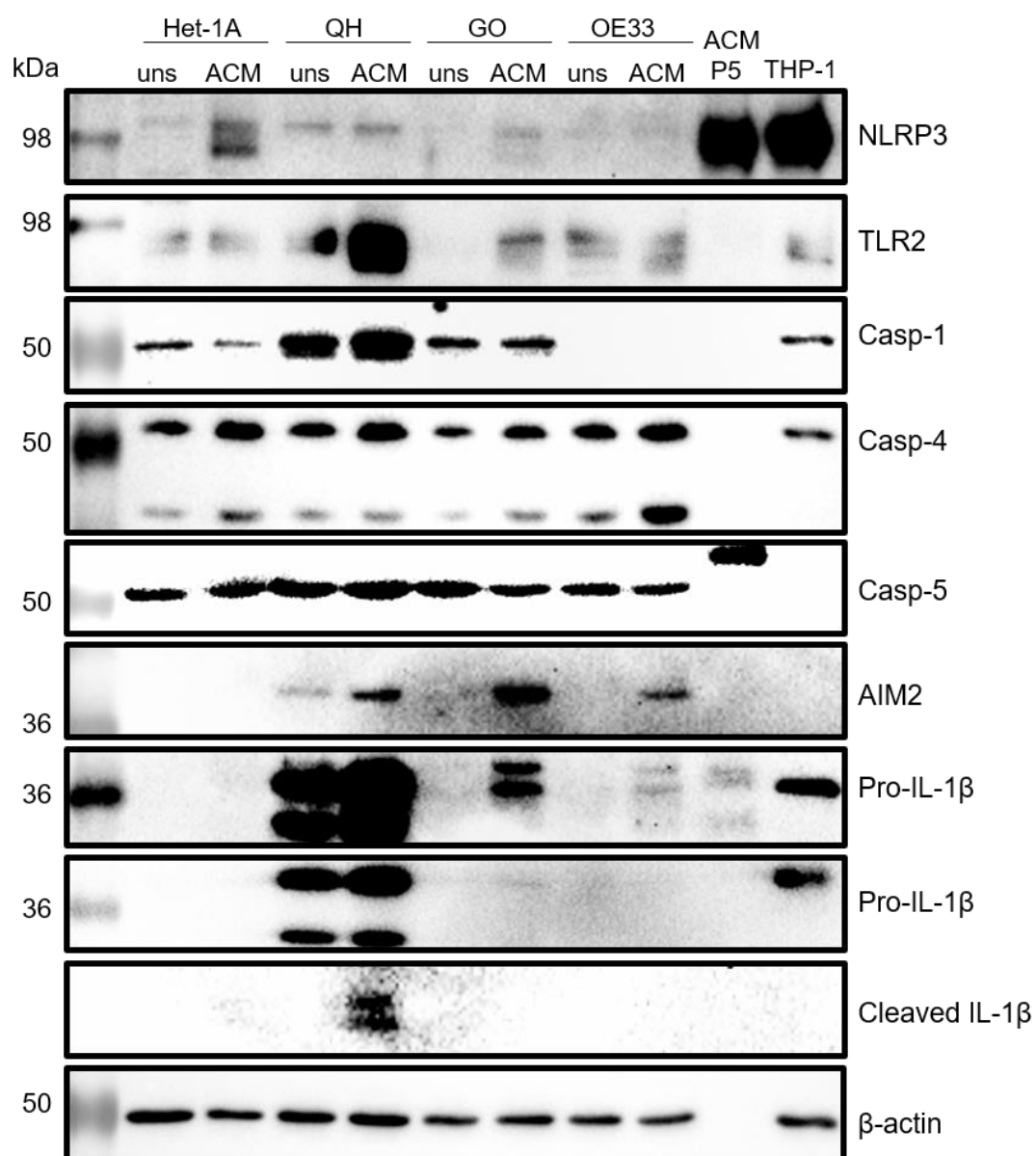
C Patient 3



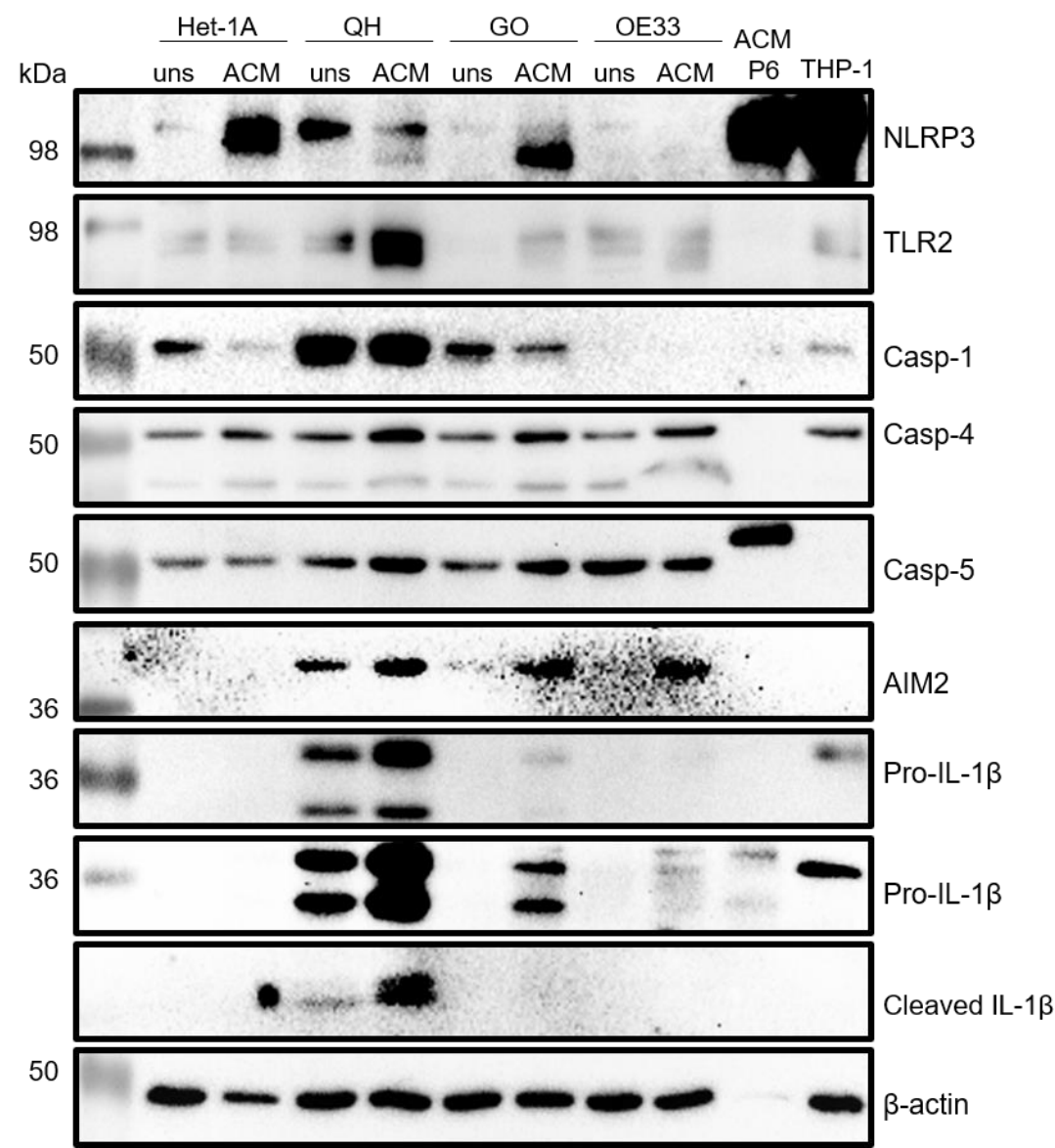
D Patient 4



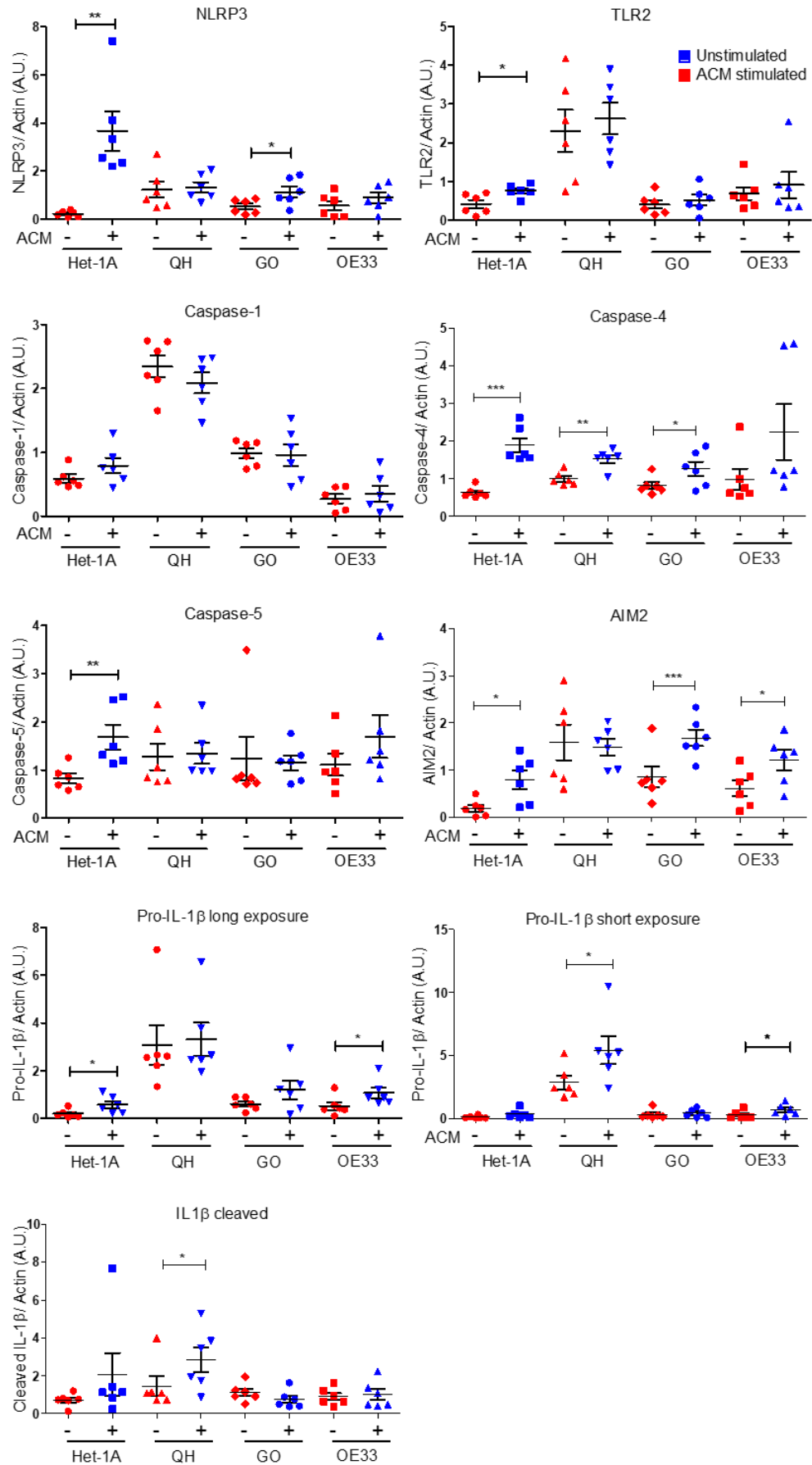
E Patient 5



F Patient 6



G



F

	Cell line			
	Het-1A	QH	GO	OE33
NLRP3	**	-	*	-
TLR2	*	-	-	-
Casp1	-	-	-	-
Casp4	***	**	*	-
Casp5	**	-	-	-
AIM2	*	-	***	*
Pro-IL-1β	*	*	-	*
Cleaved IL-1β	-	*	-	-

Figure 4.24: Upregulation of inflammasome proteins when oesophageal cell lines are stimulated with ACM from obese OAC patients.

Cell lines were seeded at the following concentrations: Het-1A (4.3×10^5 cells/mL), QH (4.25×10^5 cells/mL), GO (4×10^5 cells/mL) and OE33 (3.5×10^5 cells/mL). At 70% confluency, cells were incubated for 24 hours with visceral ACM taken from viscerally obese OAC patients (VFA > 160 cm², n=6 patients). Protein lysate (20 μ g) was resolved on a SDS-PAGE gel, (6 μ g THP-1 sample) and wet transferred onto a nitrocellulose membrane. **A)-F)** Immunoblots represent unstimulated (blue) and ACM stimulated (red) expression of NLRP3 (110, 106, 83.5 kDa), TLR2 (89.8 kDa), caspase-1 (45 kDa), caspase-4 (43.2 kDa), caspase-5 (49.7 kDa), AIM2 (39 kDa), pro-IL-1 β (30.75 kDa), cleaved IL-1 β (17.5 kDa) and loading controls, β -actin (42 kDa), GAPDH (36 kDa). **G)** Densitometric analysis of immunoblots relative to loading control (arbitrary units). **F)** Table displaying significant differences in protein expression when stimulated with ACM. Data represents mean $\pm$ SEM. Paired Student's *t* test of unstimulated to ACM stimulated: **p*<0.05, ***p*<0.01, ****p*<0.001.

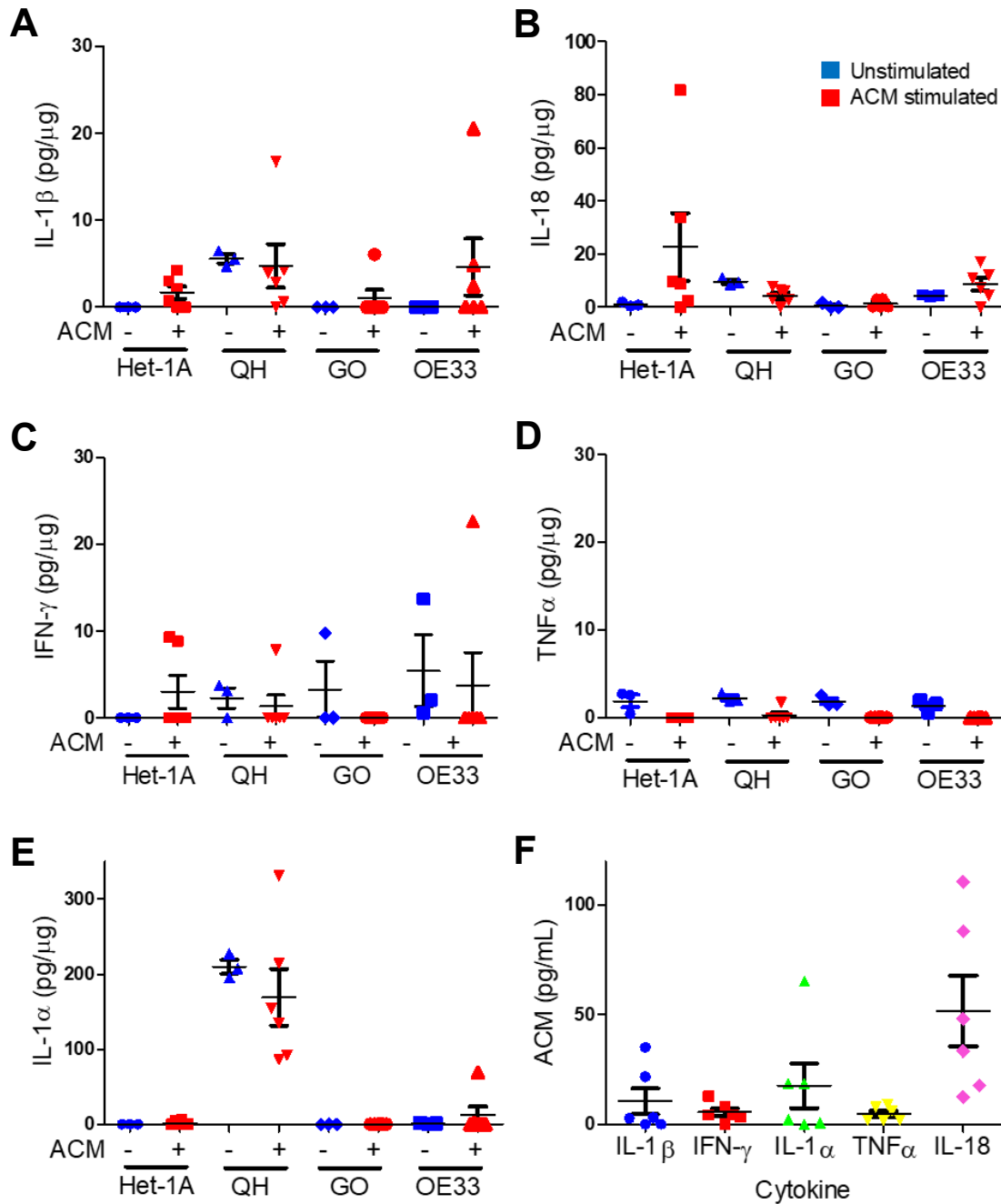


Figure 4.25: ACM stimulation does not induce differences in inflammatory cytokine secretion from oesophageal cell lines.

Oesophageal cell lines (Het-1A, QH, GO and OE33) were incubated for 24 hours with ACM taken from visceroally obese, OAC patients (VFA>160 cm², n=6 patients). Inflammatory cytokine secretion from unstimulated (blue) and ACM stimulated (red) cells was measured using ELISA and normalised to total protein. Graphs represent **A)** IL-1β, **B)** IL-18, **C)** IFN-γ, **D)** TNFα, **E)** IL-1α (pg/μg) and **F)** cytokine levels in ACM alone (pg/mL). Data represents mean ± SEM. Paired Student's *t* test comparing unstimulated to ACM stimulated: non-significant.

Chapter 5

5.1 Introduction

Results from Chapter 4 suggest that caspase-1 is mediating inflammation during Barrett's, particularly in obese patients. As previously discussed, obesity is a risk factor for Barrett's and OAC, although the mechanisms through which it accelerates disease are still not completely understood (497). Our initial observation, linking obesity with caspase-1 mediated inflammation during disease, led to further investigation into disease involvement of inflammasome pathways. Using a cell line model of disease progression, we observed that normal and early disease stage cell lines are highly responsive to adipose-conditioned media (ACM), which caused the upregulation of several inflammasome pathway components.

Gastro-oesophageal reflux disease (GORD) represents the most prevalent risk factor leading to Barrett's development, affecting approximately 20% of the Western population (582, 583). During GORD, reflux oesophagitis may arise involving inflammation and irritation of the mucosa. Prolonged exposure leads to erosive reflux disease in approximately 30% of patients (583). In most cases symptoms will regress, however Barrett's can arise as a complication in approximately 5-15% of cases. This number is markedly higher in men over 60 years old (584).

A number of contributing risk factors are associated with causing oesophagitis, including alcohol consumption, hiatus hernia, obesity, a high fat diet (HFD), smoking and medications such as NSAIDs (582). Historically, 'acid burn' was thought to drive erosive oesophagitis, however recent proposals theorise that inflammatory processes are the main drivers of disease, which may be promoted by a number of risk factors, including obesity-associated inflammation (213, 297).

Notably, obesity and a HFD are differentiated as two independent risk factors for oesophagitis (582). Symptoms of gastric reflux and heartburn are most severe during the postprandial period, indicating a relationship with consumption and diet (585, 586). Fried and spicy foods, alongside alcohol were the largest precipitating factors, indicative of a role for food composition (586). Causative mechanisms of reflux occurred through transient lower oesophageal sphincter pressure and increased abdominal pressure, which may be caused by increased waist circumference and over-eating during meals

(324, 585). Indeed, many studies have correlated visceral fat and abdominal obesity with oesophagitis and Barrett's (497, 587). Some studies suggest that obesity can cause disruption of gastro-oesophageal junction (GOJ) integrity, increasing the risk of hiatal hernia and reflux in patients (588). Aside from abdominal pressure and the physical effects of obesity, the effects of 'meta-inflammation' derived from obesity may be a leading driver of oesophagitis and Barrett's (213). Visceral fat and leptin have been shown to increase the risk of oesophagitis through increased IL-6, IL-1 β and IL-8 signalling (305). Other studies observed increased MCP-1, IL-8 and RANTES expression with NF- κ B activation in the oesophageal mucosa during oesophagitis (297, 589, 590). Interestingly, IL-8 was reduced after treatment with the proton pump inhibitor (PPI), Lansoprazole (590).

PPIs are used to treat gastric reflux and are highly effective in healing oesophagitis but may not be as effective at relieving symptoms of heartburn (271, 591). Further, the long-term benefits of PPI use remain unclear with regard to Barrett's and OAC prevention (246). Potential side effects of long term PPI therapy include kidney disease, enteric infection and micronutrient deficiency (Iron, B12, Calcium), however these findings are subject to poor quality studies (592, 593). Despite this, PPIs are currently the first-line drugs available to patients, highlighting the need for more effective preventative treatments (582). Many emerging studies are suggesting alternative, mainly diet-related methods for treating oesophagitis. One study showed that high dietary fat and cholesterol were associated with GORD symptoms in patients with a body mass index (BMI) >25 kg/m². In contrast, increased fibre intake inversely correlated with symptoms. This study was unable to determine if the effects of food composition were independent of obesity (594). Nonetheless, this research provides evidence for an interventive role for diet during oesophageal disease development. Similarly, risk of OAC development was associated with higher fat intake (595, 596). Although there was no association between Barrett's and dietary fat in these studies, another trial revealed that Barrett's risk was increased by 79% and 71% respectively, when compared to sucrose and sugar intake (595, 597). These studies emphasize highly attainable methods of disease prevention therapy, if provided with the correct patient support and encouragement to maintain medical adherence.

In this chapter we sought to further understand the role of inflammasome-mediated inflammation during the earlier stages of disease under HFD conditions. We investigate the inflammatory profile of the oesophagus in HFD-fed mice during normal conditions and during Barrett's development. Furthermore, we considered possible mechanisms of inflammatory signalling by using specific inhibitors of TLR2 and NLRP3 as therapies.

In brief:

Hypothesis and aims of Chapter 5

Hypothesis: A high fat diet can 'prime' or instigate inflammasome-mediated inflammation during the early stages of Barrett's disease development.

Specific aims of Chapter 5:

Aim I: To compare the inflammatory status of oesophageal tissue from wild-type mice fed a standard fat and a high fat diet. Experiments were set up to:

1. Determine whether oesophageal explants from HFD-fed mice have higher levels of inflammatory cytokine secretion or inflammasome component upregulation than SFD-fed mice.
2. Examine whether inflammatory responses of oesophageal explants to TLR2 ligands are altered in HFD-fed mice, compared to SFD-fed mice.
3. Determine whether inflammatory cytokine secretion from oesophageal explants can be altered by the presence of NLRP3 or TLR2 inhibitors.

Aim II: To characterise the expression of inflammatory proteins, AIM2 and TLR2, in a murine model of Barrett's disease progression, under both standard and high fat diet conditions.

Experiments were set up to compare:

1. AIM2 expression levels between 6- and 12-month-old pL2-IL-1 β mice fed on a SFD or HFD.
2. TLR2 expression levels between 6- and 12-month-old pL2-IL-1 β mice fed on a SFD or HFD.

5.2 Results

5.2.1 Priming of inflammatory components in normal oesophageal tissue during a high fat diet.

In Chapter 4 we observed that ACM-stimulated oesophageal cell lines increased expression of inflammatory components, particularly in the cell line representing normal squamous epithelial cells, Het-1A (598). This suggested that obesity or a high fat diet may lead to inflammasome priming early in disease, which could subsequently be driving disease development. To further investigate this hypothesis, we set up a collaboration with the Lynch research lab (TCD), to study the effect of a high fat diet on inflammatory protein expression in WT mice. We hypothesized that oesophageal explants from HFD-fed mice would have enhanced inflammatory protein expression, compared to SFD mice. Additionally, we speculated that inflammatory cytokine secretion could be inhibited using inhibitors of NLRP3 (MCC950) and TLR2 (OPN-301).

An optimisation experiment was initially carried out on SFD WT mice to establish the experimental conditions for oesophageal explant culture and inhibitor concentrations (**Figure 5.1**). Mice were treated with the dsRNA analogue, poly I:C (100 µg), *via* IP injection for four hours prior to their sacrifice, to mimic experimental trial conditions, which will include this immunostimulatory step. Oesophageal explants were harvested from mice and transferred to 200 µL DMEM in a 96 well plate. Explants were left untreated or pre-treated with TLR2 blocking antibody OPN-301 (1 and 10 µg/mL), or the NLRP3 inhibitor, MCC950 (10 µM), for one hour prior to stimulation with LPS or Pam2 CSK4 for 18 hours to 'prime' explants for inflammasome activation. Nigericin (5 µM, 30 mins) was used where indicated to activate the inflammasome. Secretion of IL-1β and IL-6 levels were assessed and normalised to explant total protein (pg/µg). Results did not consistently reveal inflammatory cytokine secretion in response to TLR stimulation, and therefore did not show inhibition when treated with inhibitors. Based on these results, it was determined to treat unstimulated explants with MCC950 (10 µM) and OPN-301 (1 µg/mL) during the trials.

Data from two trials (n=5 and n=4 per group, respectively) were combined and are represented in **Figures 5.2-7**. These trials were performed as part of a larger experimental trial being performed by the Lynch group. Mice used for this study made up the control groups of the larger trial. Mice were set up in two groups of 4 or 5 mice, with one group fed on a standard fat, chow diet (SFD) and the second group fed on a 60% lard HFD for 9 weeks. Mice were administered daily vehicle (PBS/DMSO) IP injections for the final seven days prior to sacrificing the mice, and a single IP injection of poly I:C (100 µg) four hours before harvesting the oesophagi. Therefore, results from these trials are considered preliminary observations. **Figure 5.2A** shows that mice fed a HFD were significantly heavier than those fed a SFD, pre-IP injections. Mice were slightly less heavy following 7 daily IP PBS/DMSO injections, likely due to ongoing cage fighting occurring between the HFD-fed mice during week 8 of trial 1. Cage fighting, due to the presence of a male bully, is a common occurrence in male-only cages of HFD-fed mice (599, 600). However, overall IP-injected HFD-fed mice were significantly heavier when a direct statistical comparison was made (**Figure 5.2B**).

To determine the protein expression of inflammasome related proteins, immunoblot analysis of oesophageal explant homogenates from SFD and HFD mice was carried out. Representative blots taken from trial two are shown in **Figure 5.3A**. Densitometric analysis from combined trial results show significant upregulation of AIM2, TLR2 and caspase-1 expression in HFD-fed mice, compared to SFD-fed mice (**Figure 5.3C-E**, $p<0.01$, $p<0.05$). Additionally, caspase-11 expression was elevated ($p=0.0596$), but not significantly. This supports our hypothesis that a HFD induces inflammasome priming in oesophageal tissue.

Analysis of *ex vivo* cultured oesophageal explant supernatants was carried out to characterise the inflammatory cytokine secretion profile of HFD- and SFD-fed mice. Results show a trend towards increased inflammatory cytokine secretions for TNF α and IL-6 in HFD-fed mice (**Figure 5.4C, H**). However, this was not significant due to variations in cytokine secretion levels from HFD-fed oesophageal explants. Interestingly, HFD-fed mice had significantly decreased IL-18 secretion levels ($p<0.05$, **Figure 5.4B**). This observation was consistent between both trials. Mature IL-18, like IL-1 β , is a direct

product of the inflammasome pathway and is important for stimulating IFN- γ production, which was also decreased in six out of nine oesophageal explants (82).

To determine whether HFD-mediated inflammatory priming could boost inflammatory secretion, oesophageal explants were stimulated with the TLR2 agonist, Pam2 CSK4. **Figure 5.5** shows the inflammatory secretion profile of oesophageal explants from mice fed a SFD and HFD, following stimulation with Pam2 CSK4. Results reveal that *ex vivo* biopsies from both SFD- and HFD-fed mice did not respond significantly to TLR2 stimulation. The poor response suggests that there is low TLR2 expression on the normal oesophageal mucosa, which may be due to microbial tolerance mechanisms (318).

In Chapter 4, we observed that TLR2 was upregulated in the normal squamous cell line, Het-1A, in response to ACM stimulation. TLR2 is a PRR that when activated can prime the inflammasome pathway through NF- κ B-mediated transcription (82). To investigate whether TLR2 is mediating oesophageal inflammation following HFD-feeding, pre-treatment of explants with a TLR2 neutralising antibody (OPN-301, 1 μ g/mL) was employed to assess for an effect on inflammatory cytokine secretion. Explants were cultured for 18 hours with OPN-301, and subsequent ELISA analysis of supernatants revealed no significant differences between untreated and TLR2-inhibited samples (**Figure 5.6**).

A study by Nadatani *et al.* has shown that stimulation of a Barrett's cell line, BAR-T, with LPS causes priming and activation of the NLRP3 inflammasome (48). Findings in Chapter 4 showed that oesophageal cell lines representing normal squamous (Het-1A) and Barrett's (QH) epithelial cells upregulate NLRP3 and IL-1 β when cultured with ACM from obese OAC patients. We therefore hypothesized that NLRP3 inflammasome signalling is mediating inflammation of the oesophagus during obesity.

Murine oesophageal explants were cultured for 18 hours in the presence or absence of the small molecule NLRP3 inhibitor, MCC950 (10 μ M) (466). Culture supernatants were assessed for inflammatory cytokine secretions (**Figure 5.7**). Results show that preincubation with the NLRP3 inhibitor had no significant effect on inflammatory cytokine secretion levels. The read-out for NLRP3 activity is measured by the secretion of inflammasome activation products, IL-1 β and IL-18. A trend towards decreased IL-1 β

levels in SFD- and HFD-fed mice was observed. Cytokine secretion levels were very low in this system, therefore suggesting that robust NLRP3 inflammasome activation was not occurring. However, a trend towards reduced IL-18 levels in MCC950-treated oesophageal tissue from SFD-fed mice was observed, but paradoxically are elevated in some HFD-fed mice (two mice per trial) (**Figure 5.7B**), suggesting that the NLRP3 inflammasome may regulate basal IL-18 secretion in oesophageal tissue.

Collectively, results presented in Chapter 5 to date have shown that a HFD in mice leads to upregulated expression of the inflammatory proteins AIM2, TLR2 and caspase-1 in oesophageal tissue, suggesting inflammasome priming. We also show that IL-18 secretion was reduced in oesophageal biopsies from HFD-fed mice, which may promote disease progression by affecting epithelial integrity (173, 553). However, there was little basal secretion of inflammasome-regulated cytokines, particularly IL-1 β , suggesting that the inflammasome is primed but not significantly activated under these conditions.

5.2.2 Histological comparison of disease development in pL2-IL-1 β mice fed a SFD and HFD.

Evidence shows that obesity is a risk factor for the development of Barrett's (497). Our research demonstrated that inflammasome components were upregulated in oesophageal cell lines stimulated with ACM, particularly in cell lines which are representative of normal and early disease stages, Het-1A and QH. We have also shown that a HFD in mice can lead to the upregulation of inflammatory proteins, AIM2, TLR2 and caspase-1, in normal oesophageal tissue. We next sought to determine whether inflammatory proteins TLR2 and AIM2 were also upregulated by a HFD in a murine disease model of Barrett's oesophagus.

This aspect of the study was performed in collaboration with the Quante research lab (Munich), which gave us access to murine Barrett's tissue sections for IHC analysis. We received tissue sections from pL2-IL-1 β mice aged 6 and 12 months fed a SFD diet, and mice aged 6, 9 and 12 months fed a HFD. No SFD-fed mice aged 3 or 9 months, and no HFD-fed mice aged 3 months were available and therefore could not be included in this analysis. All histological scoring was performed by Dr. Michael Quante, Munich. **Figure**

5.8 shows the histological scores for 6- and 12-month-old SFD-fed mice. Mice that were 6 months old represented early establishment of Barrett's disease. While at 12 months Barrett's was well-established and further developed, with a significantly lower ($p<0.05$) GC ratio indicating significant disease progression. There was also a trend towards increased dysplasia, although not significant in this cohort. However, this was determined to be significant in the cohort shown in Chapter 3 and in larger groups previously characterised by Quante (263). Overall, using these mice we can compare early and later stages of Barrett's disease.

In **Figure 5.9**, the histological scores of HFD-fed pL2-IL-1 β mice aged 6, 9 and 12 months are shown. Well-established inflammation and metaplasia were observed in all groups (**Figure 5.9B, C**). Mice at 12 months had a decreased GC ratio compared to mice at 6 months ($p<0.05$), indicating significant disease progression (**Figure 5.9E**). Similar to SFD-fed mice, there were no significant differences between groups when comparing the dysplasia scores. However, there was a clear trend towards step-wise increased dysplasia from 6 to 12 months (**Figure 5.9D**). **Figure 5.10** compares the histological scores for mice fed a SFD and HFD diet at 6 and 12 months. Scores for inflammation and metaplasia are similarly high in both SFD and HFD pL2-IL-1 β mice. However, at 12 months, scores for dysplasia and GC ratio suggest enhanced disease progression in HFD-fed mice. Despite these differences not being significant, the recently published paper by Quante's research group (*Gastroenterology*, 2019) showed that these differences are significant with increased statistical power (364). These findings confirm that a HFD accelerates Barrett's disease progression, compared to mice fed a SFD.

5.2.3 Optimisation of AIM2 and TLR2 antibodies for IHC analysis in murine Barrett's tissue sections.

Before staining oesophageal tissue for inflammasome protein expression, antibodies were tested and optimised. In Chapter 4, we observed significantly elevated NLRP3 expression in a Barrett's cell line, QH. Additionally, Nadatani *et al.* showed that a Barrett's cell line (BAR-T) was primed and activated in response to LPS. They hypothesized that NLRP3 may mediate inflammation during Barrett's disease (48). We

wanted to investigate NLRP3 expression further in the pL2-IL-1 β model and if expression was affected by a HFD. However, no commercially available antibody was identified as being specific for IHC staining of NLRP3. The following six antibodies were assessed and found to be non-specific: Abcam (ab214185), Adipogen (AG-20B-0014-C100), Cell signaling (D4D8T), Novus Biologicals (12446, MAB7576, 77080). Antibodies were assessed by staining DSS-challenged WT and KO NLRP3 colon sections (601).

For analysing AIM2 expression, we used an antibody from Biosciences that also shows specificity by immunoblot analysis. TLR2 was detected using the Opsona antibody, OPN-301. This antibody is an anti-mouse/ human monoclonal antibody TLR2 antibody created by Opsona Therapeutics. The specificity of this mouse IgG1 antibody has been validated in previous studies (602-604). Preliminary antibody optimisations for IHC staining were performed using DSS-challenged murine colon tissue sections. Subsequently, antibody concentration optimisations were carried out in pL2-IL-1 β /IL-8Tg oesophageal tissue, as this tissue was available for optimisations. This IL-8 expressing phenotype was further described by Münch *et al.* (364). Results show that the AIM2 antibody stained optimally using a 1:1000 dilution and TLR2 stained optimally using a 1:300 dilution in oesophageal tissue (**Figure 5.11**).

5.2.4 AIM2 expression was upregulated at the squamocolumnar junction (SCJ) during a HFD in Barrett's pL2-IL-1 β mice.

Published studies report that AIM2 signalling has functional roles in protecting or driving carcinogenesis (478, 605-607). In Chapter 3, we showed that AIM2 was upregulated in oesophageal cell lines in response to ACM stimulation. Therefore, we hypothesized that AIM2 is upregulated with disease progression to Barrett's and may be further enhanced with a HFD. IHC staining was carried out in SFD- and HFD-fed pL2-IL-1 β mice and this was analysed using a semi-quantitative scoring method by two blinded reviewers (G. Barber, J. Phelan). All staining was performed on the same day, therefore both positivity and intensity were assessed in the metaplastic columnar epithelium and a defined region of the stroma (surrounding the metaplasia) at the SCJ. These scores were subsequently combined to give an overall score of expression (IxP).

Figure 5.12 shows representative images and scores for AIM2 expression in the metaplastic epithelium and stroma of the Barrett's region at 6- and 12-months when pL2-IL-1 β mice were fed a normal chow diet. No differences in epithelial AIM2 expression were observed at 6 and 12 months (**Figure 5.12B-D**). Interestingly, stromal AIM2 positivity scores were increased from 6 to 12 months in SFD-fed mice ($p<0.01$), while intensity scores were relatively high for both groups (**Figure 5.12E, F**). Overall IxP scores showed a significant increase in AIM2 expression ($p<0.01$, **Figure 5.12G**). As the stroma incorporates all connective tissue including infiltrating immune cells, this result may suggest a role for AIM2 activity in the immune microenvironment of Barrett's tissue later in disease.

Figure 5.13 displays representative IHC staining images for AIM2 expression and the associated scores for the epithelium and stroma in HFD-fed pL2-IL-1 β mice. No differences were observed between age groups in the stroma and epithelium. However, it was noted that both positivity and intensity were elevated and strongly expressed in all age groups, suggesting that a HFD had maximally induced AIM2 expression within 6 months in Barrett's mice. This observation was verified when scores for a SFD and HFD were compared in **Figure 5.14**. Here we see that AIM2 expression was significantly upregulated in both the epithelium and stroma at the SCJ during a HFD in 6-month-old mice ($p<0.05$). A trend towards increased epithelial AIM2 was also observed at 12 months, however the significant difference was lost during the later stages of disease (**Figure 5.14B-D**). This was likely due to the increase in AIM2 expression later in disease during a SFD (**Figure 5.12E-G**). These observations agree with our previous results which suggest that the inflammatory effects of a HFD or obesity are most robust during the earlier stages of disease (**Figures 4.24, 5.3**).

Of note, stromal AIM2 levels were decreased, although not significantly, during a HFD at 12 months. This trend was similarly observed for caspase-1 expression during a HFD in 12-month old pL2-IL-1 β mice (**Figure 3.12D**), and may represent preliminary immune-modulatory mechanisms that interfere with anti-tumorigenic immune cell infiltration.

Correlation of histological scores with epithelial AIM2 (**Figure 5.15B-E**) and stromal AIM2 levels (**Figure 5.15E-G**) were carried out and are represented by the Spearman's correlation coefficient, r_s . Representative images of histology and IHC AIM2 staining in

Figure 5.15A. Correlations with inflammation and metaplasia scores were performed but did not provide significance (data not shown). Results in **Figure 5.15** reveal interesting observations supporting a role for AIM2 activity during disease. During a SFD, pL2-IL-1 β mice increasingly expressed both epithelial ($p<0.01$) and stromal ($p<0.05$) AIM2 as dysplasia increased (**Figure 5.15B, F**). To support this finding, AIM2 levels negatively correlated with the GC ratio in these SFD-fed mice ($p<0.05$, **Figure 5D, H**). As mentioned, reduced GC ratio has been associated with increased susceptibility to neoplasia in pL2-IL-1 β mice and is indicative of disease progression (386). In contrast, none of these disease correlations were observed in HFD-fed mice (**Figure 5.15C, E, G, I**). This lack of correlation may be due to elevated AIM2 levels observed within 6 months in HFD-fed mice, which were maintained during disease progression.

5.2.5 No differences in TLR2 expression levels at the SCJ during disease progression in pL2-IL-1 β mice fed a SFD and HFD.

In humans, TLR2 expression has been shown to increase sequentially with disease progression to Barrett's and to OAC (318). Moreover, work by Quante's group have suggested TLR2 involvement during HFD-related Barrett's development (364). In Chapter 4, we showed that TLR2 activity could mediate signalling of IL-8 orthologs, MIP-2 and CXCL1. We therefore hypothesized that oesophageal TLR2 expression would be upregulated during disease progression in the murine Barrett's model, which may be further elevated in mice fed a HFD. Oesophageal sections were stained and scored in a similar manner to AIM2 IHC analysis. Scores were assessed by two blinded reviewers (G. Barber and J. Phelan).

Figure 5.16 shows representative images of IHC staining and associated scores for TLR2 expression levels in SFD-fed pL2-IL-1 β mice at 6 and 12 months. No significant differences in epithelial and stromal expression were observed in the Barrett's tissue (**Figure 5.16B-G**). Additionally, very low levels of epithelial TLR2 were observed overall, indicating poor expression in this tissue (**Figure 5.16A**). TLR2 levels were further determined in Barrett's tissue from mice fed a HFD, aged 6, 9 and 12 months (**Figure 5.17**). Similar to a SFD, no differences were observed between groups and poor

expression overall was observed. Representative images illustrate the low TLR2 staining expression patterns during a HFD (**Figure 5.17A**). These results suggest that TLR2 expression is not enhanced in HFD-fed mice, and contrary to previous observations, is not significantly upregulated during oesophageal disease progression.

Comparisons of TLR2 levels in Barrett's tissue during a SFD and a HFD reveal epithelial TLR2 was poorly expressed overall, at approximately 5-10% positivity. Epithelial TLR2 intensity scores decreased during a HFD at 12 months ($p<0.05$), while there was a trend towards decreased positivity (**Figure 5.18B, C**). However, due to low expression levels this finding may prove to not be meaningful. In stromal tissue surrounding the Barrett's region at the SCJ, there was a similar trend towards reduced TLR2 expression during a HFD at both 6 and 12 months (**Figure 5.18E-G**). These findings do not agree with our observations reported earlier in this chapter, showing elevated TLR2 expression during a HFD in normal oesophageal tissue (**Figure 5.3**). However, these model systems represent different disease stages and cannot be directly compared. Moreover, it is conceivable that there are microbiome dependent elements to these studies that may require consideration, based on recent literature (364, 608).

5.2.6 Summary of the main findings of Chapter 5.

- A HFD in WT mice causes upregulation of inflammatory proteins TLR2, caspase-1 and AIM2 expression, but not IL-1 β expression, in oesophageal tissue.
- The inflammatory secretion profiles of oesophageal explants from SFD- and HFD-fed WT mice are similar, with exception of IL-18, which has lower secretion levels in HFD, compared to SFD, mice.
- Oesophageal explants from SFD- and HFD-fed WT mice are unresponsive to the TLR2 agonist, Pam2 CSK4.
- Inhibition of TLR2 signalling or NLRP3 activity in oesophageal explants from SFD- and HFD-fed mice do not alter inflammatory cytokine secretion profiles.
- Stromal AIM2 expression is upregulated in oesophageal tissue and correlates with disease progression in SFD-fed pL2-IL-1 β mice.
- A HFD causes early upregulation of epithelial and stromal AIM2 at the SCJ of Barrett's mice, compared to those fed a SFD.
- No differences in TLR2 expression were detected in tissue taken from the SCJ in SFD and HFD-fed pL2-IL-1 β mice.

5.3 Discussion

Results from this chapter examine inflammasome involvement during the pre-neoplastic stages of disease by assessing normal oesophageal explant tissue and Barrett's pL2-IL-1 β tissue taken from HFD-fed mice. We demonstrate that AIM2 and caspase-1 levels, but not NLRP3 levels, are elevated in normal oesophageal tissue following a 9-week HFD. This was also observed in the normal oesophageal epithelial cell line, Het-1A, following ACM-stimulation, providing further evidence for adipose-mediated inflammasome priming. During Barrett's metaplasia, we determined that enhanced AIM2 upregulation occurred in HFD-fed mice, compared to SFD. Caspase-1 expression was also shown to be upregulated (Chapter 3) in response to a HFD in the pL2-IL-1 β model. Aside from a HFD, in this study we have shown that both caspase-1 and AIM2 are upregulated during Barrett's disease progression in SFD-fed pL2-IL-1 β mice, and this expression positively correlates with dysplasia scores. Results suggest that the AIM2 inflammasome is involved in early oesophageal disease progression and that a HFD enhances the expression of its components. This chapter therefore provides evidence that a HFD can prime the AIM2 inflammasome in oesophageal tissue.

Inflammatory cytokine secretion levels did not significantly increase in normal oesophageal tissue from HFD-fed mice. This was a surprising finding, as elevated levels of inflammatory cytokines have been reported during obesity (368, 558). However, many of these studies examine cytokine levels within adipose tissue and in circulation, and not directly from oesophageal tissue (368, 558, 609). In contrast, Munch *et al.*'s recent HFD paper commented that they saw no significant differences in cytokine secretion from oesophageal tissue in WT mice fed a SFD and HFD, despite observing differences in the serum of these mice and in the pL2-IL-1 β phenotype (32).

Interestingly, we observed that normal oesophageal tissue secreted less IL-18 in HFD-fed mice. This observation is previously unreported, as very little research has been conducted observing IL-18 levels during the development of Barrett's disease. Findings in Chapter 4 showed reduced IL-18 secretion levels from Barrett's patient explants, when compared to normal oesophageal tissue. Together, these findings allow us to

speculate that there may a protective role for IL-18 in oesophageal disease progression which will be discussed further in the general discussion.

Overall, findings suggest that obesity is priming but not significantly activating the inflammasome in normal oesophageal tissue. A second signal, such as gastric reflux or an infection may be required for inflammasome activation and increased inflammation. This is supported by examining the dietary-related mouse model of Barrett's, where mice develop Barrett's-like features following 88–152 days of a zinc-deficient diet containing hydrophobic bile acid (610).

Oesophageal explants from both SFD- and HFD-fed mice were observed to be largely unresponsive to TLR2 stimulation when stimulated with the TLR2 ligand, Pam2 CSK4. This may be due to an immune tolerogenic environment in the oesophagus as part of this organ's barrier function (611). Mice may have low expression of TLR2 in the oesophagus, leading to less ligand binding and inflammatory signalling. Importantly, one study emphasized that TLR2 expression is poorly expressed in normal oesophageal mouse tissue, while there is considerably more expression in human tissue (316). This may also provide reasoning for the lack of cytokine secretion inhibition observed when explants were incubated with a TLR2 blocking antibody, OPN-301. It should therefore be considered that using a mouse model may not be the most appropriate choice of model for studying the effects of TLR2 during oesophageal disease.

However, we did see a relative increase in TLR2 expression in oesophageal explants from HFD-fed WT mice, and therefore we expected that explants would be more responsive to TLR2 ligands. TLR2 does not homodimerize during signal activation, but rather forms heterodimers with TLR1 or TLR6 (612). Pam2 CSK4 is a synthetic diacylated lipopeptide which functions as an agonist for TLR2/TLR6 heterodimer activity (36, 613). TLR2/TLR6 signalling is typically associated with gram positive bacteria, while TLR2/TLR1 signalling is more associated with a gram negative bacterial response (614). A HFD may influence TLR2 upregulation and possible activity through a different pathway. It therefore would have been interesting to investigate the oesophageal response to a TLR2/TLR1 agonist such as a triacylated lipopeptide, Pam3 CSK4 (615). However, due to the small size of the mouse oesophagus, we had a limited number of oesophageal biopsies available per mouse to investigate different treatments.

Overall, basal oesophageal cytokine secretion levels of IL-1 β , IL-18, IFN- γ , TNF α and IL-1 α were minimal from SFD- and HFD-fed WT mice and no differences between diets were observed (with exception of IL-18). Therefore, the poor inhibition levels observed in the presence of the TLR2 or NLRP3 inhibitors may be due to insufficient levels of inflammation. However, high basal levels of IL-6 and the IL-8 orthologs (CXCL1, MIP-2) secretion were observed, which were also not limited by TLR2 or NLRP3 inhibition. Expression of IL-8 and IL-6 can both be upregulated through TLR2 activation and signalling (616). Therefore, TLR2 may not be mediating basal secretion levels of IL-8 and IL-6 in normal oesophageal tissue. Similarly, use of an NLRP3 inhibitor only resulted in partial, although not significant, inhibition of IL-1 β secretion in oesophageal explants. Additionally, no NLRP3 upregulation or 'priming' occurred in oesophageal tissue from HFD-fed WT mice, suggesting a less important role for NLRP3 during early disease. These results suggest the involvement of another inflammasome pathway, such as AIM2 which will be discussed in more detail later in this discussion.

Another reason for the lack of effect observed with inhibitor incubation may be insufficient inhibitor penetration, as the oesophagus is highly impermeable to drugs (611, 617-619). As a functional barrier, the oesophageal epithelium provides a tough protective layer against passing food bolus, while the outer layer of the oesophagus is surrounded by striated or smooth muscle (617-619). One study in rabbits showed there was little or no permeability to proteins weighing 4 kDa and upwards in normal oesophageal tissue (618). In contrast, in Chapter 4 we observed excellent TLR2 inhibitor efficacy in Barrett's organoids. Penetration may have been facilitated by the multiple budding structure and high epithelial surface area of the organoids. To assess permeability in normal oesophageal explants, immuno-staining of OPN-301 treated explants should be carried out using a fluorescently labelled antibody.

Despite these results, the motivation to study the role of TLR2-associated inflammation during oesophageal disease should not be dismissed. In human cell lines, we have shown that TLR2 is robustly upregulated in a Barrett's cell line and is upregulated in response to ACM stimulation in a normal cell line. Moreover, research from our lab has shown that TLR2 mediates inflammatory crosstalk between human oesophageal cells and primary macrophages. Additionally, other research groups have shown that there

is a stepwise increase in TLR2 expression in human oesophageal tissue during patient disease progression to Barrett's and OAC (316, 318).

In contrast, findings in this chapter showed that TLR2 expression in Barrett's pL2-IL-1 β tissue did not change during disease progression or during a change in diet. Overall, epithelial TLR2 was mostly poorly expressed in Barrett's tissue, which may suggest a non-critical role for TLR2 in Barrett's development. This was a surprising finding taking into consideration we previously showed that significant upregulation of MIP-2 and CXCL1 occurred when Barrett's organoids were stimulated with a TLR2 agonist. We must consider the specificity of the OPN-301 antibody during IHC analysis of Barrett's tissue. However, specificity has been confirmed for the use of this antibody as a therapy in clinical trials and has also previously been used in other research studies for IHC analysis. (533, 616, 620, 621).

This disparity in TLR2-related findings may also be explained by microbiome differences. The mouse facilities at TUM are being moved to an off-campus facility over time and so the pL2-IL-1 β mice were housed in different facilities. The organoids were taken from mice bred on TUM campus in a facility that is considered less clean, compared to the off-campus facility the pL2-IL-1 β mice for IHC analysis were taken from. It is important to consider the microbiome when discussing TLR2 activity as many studies are emerging implicating a microbiome-dependent role for TLR2 in oesophageal and GI tissues (31, 32, 608). TLR2 recognises lipoproteins from gram negative and gram positive bacteria and yeast (622). Huhta *et al.* showed that TLR2 expression in oesophageal tissue was undetectable in germ-free mice, but expression was observed in conventional mice (31). As previously mentioned, Quante's research team have suggested oesophageal TLR2 expression as a mediator of IL-8 in HFD-fed pL2-IL-1 β mice in a microbiome-dependent manner. In this study, they observed increased oesophageal TLR2 mRNA levels in HFD-fed mice compared to SFD-fed mice at 9 months (32). This was not consistent with findings in this chapter. Other studies have also implicated TLR2 signalling in obesity and metabolism. Several studies have reported that TLR2 deficiency protects mice from HFD-induced metabolic syndrome comorbidities in mice, such as insulin resistance, glucose intolerance and systemic inflammation (623-627). Contradicting these studies, Guadagnini *et al.* reported increased weight gain, insulin resistance and glucose

intolerance in TLR2 deficient mice fed a SFD (608). They explained these differences by demonstrating a role for the microbiome. They were able to reverse this phenotype by administering the TLR2 knockout mice antibiotics and reproduce the phenotype in WT mice by microbiota transplantation (608). Changes in TLR2 activity through microbiome signalling are not only altered by diet but can also be induced by drug consumption. Zhang *et al.* showed that morphine tolerance occurred in a microbiome dependent manner in mice, likely mediated by TLR2 (628). Together, these studies demonstrate a role for TLR2 in diet and metabolism, and that expression and signalling can be affected by microbiome conditions.

In the normal chow-fed pL2-IL-1 β model, we show that AIM2 is upregulated from 6 to 12 months in the stroma, but not in the epithelium. Quante's research group have characterised the immune cell populations in the oesophagus of pL2-IL-1 β mice, providing clues as to which cells may be expressing AIM2. They showed elevated populations of infiltrating neutrophils, macrophages, NK cells and Th1 cells (32, 263). There is much literature associating inflammasomes with innate immunity, particularly monocytes and macrophages, and to a lesser extent neutrophils (629-631). Therefore, it may be that AIM2 is mostly expressed from these populations in Barrett's. However, research has also shown Th1 cells can express inflammasomes and mediate inflammation (632). Higher levels of AIM2 expression later in Barrett's metaplasia suggests a more prominent role as disease progresses. To support this, we showed that AIM2, and caspase-1, expression correlated with histological scores for disease progression. Other studies have also implicated a role for AIM2 in gastrointestinal disease. AIM2 knockout mice were reported to have reduced levels of M1-type macrophages in DSS-treated colons, accompanied by reduced secretion levels of IL-1 β , TNF α and IFN- γ . AIM2-deficient mice had increased susceptibility to induced and spontaneous colorectal cancers, indicating a protective function during carcinogenesis (633, 634). Interestingly, these studies demonstrated protection occurred in an inflammasome-independent manner (633, 634). Organoid intestinal stem cells that were taken from AIM2 knockout mice had increased proliferation, when compared to WT mice, which was associated with AKT regulation (633). In agreement with this,

murine fibroblasts and colon cell lines had impaired proliferation and cell cycle arrest when transfected with an AIM2 expressing construct (635, 636). These studies highlight inflammasome-independent functions of AIM2 involving cell proliferative regulation that must be considered.

AIM2 has also been reported to have tumour promoting functions that are inflammasome-dependent. Sorrentino *et al.* documented a tumour promoting function for AIM2 *via* IL-1 α release from DCs during non-small cell lung cancer. This occurred in an inflammasome-dependent manner (637). Zhang *et al.* showed that chronic inflammation induced by low-dose arsenic exposure was mediated through AIM2 inflammasome activity in the skin of mice and in a human keratinocyte cell line (638). Arsenic exposure has been associated with high levels of skin and bladder cancer in the USA (639). Importantly, this identifies a role for AIM2 in chronic inflammation that may drive local tissue fibrosis and the development of cancer in a manner consistent with the chronic inflammation and tissue injury associated with Barrett's. In keeping with this argument, AIM2 knockout mice showed reduced kidney tissue injury, fibrosis and renal failure, compared to WT mice using a mouse model of unilateral urethral obstruction. They identified the AIM2 inflammasome as an important mediator of inflammation instigated by macrophage uptake of DNA from necrotic cells (125). Further, the ascitic fluid of liver cirrhotic patients was reported to have highly activated macrophages mediated through AIM2 inflammasome activity under non-infected conditions. Elevated IL-1 β and IL-18 levels produced by ascitic fluid macrophages correlated with advanced disease scores (640). These studies highlight a disease promoting role for AIM2 during tissue injury, before cancer has developed.

This raises the question whether inhibiting AIM2 may be beneficial early in disease progression when tissue injury is occurring. To our knowledge, no biologic or small molecule inhibitor currently exists that is specific to AIM2 inhibition alone. Although a number of inhibitors exist that indirectly inhibit AIM2 in conjunction with other inflammasomes or DNA sensors, such as synthetic oligodeoxynucleotides (ODNs) which are competitive DNA motifs, ROS inhibitor N-acetylcysteine (NAC) and cGAS small molecule inhibitors (641-646). None of these are currently in use in the clinic and their therapeutic benefit as inhibitors remains elusive.

As a DNA sensor, we know that AIM2 has anti-viral functions (114, 135). Therefore, the new wave of research targeting induction of anti-viral immunity during cancer should be mentioned. Several clinical trials have been initiated in recent years investigating agonists to promote anti-viral activity for immunotherapy. Some of these targets upregulate the AIM2 inflammasome pathway through the type I IFN-mediated pathway. Such targets include STING and cGAS, both of which are cytoplasmic DNA sensors (135, 647, 648). Activation of these pathways is suggestive of a protective role for the AIM2 inflammasome once cancer has developed. Type I IFN signalling was reported to be critical for upregulation and spontaneous activation of CD8⁺ cells and boosting anti-tumour immunity (649, 650). Increased numbers of CD8⁺ T cells was found to be a significant predictor of positive patient prognosis in colorectal cancer (651, 652). The cGAS-STING pathway was identified to be crucial in generating IFNs required for eliciting anti-tumour T cell immunity (648, 653). Interestingly, several cancers were found to be deficient in this pathway which was predictive of poor prognosis, including colon and gastric cancer (654-656). Therefore, instigating an anti-viral response is predicted to kick-start anti-tumour immunity in patients.

In this study we also show that epithelial AIM2 expression is upregulated at the SCJ in HFD-fed pL2-IL-1 β mice. This agrees with our other findings showing increased levels of epithelial caspase-1 in HFD-fed Barrett's mice, and increased AIM2 and caspase-1 expression in normal oesophageal tissue from HFD-fed WT mice. Additionally, stromal AIM2 was increased in 6-month-old HFD-fed pL2-IL-1 β mice. Other studies have also implicated AIM2 signalling during obesity and cancer. One study revealed that there was significant weight gain, glucose intolerance and reduced energy expenditure in AIM2 knockout mice fed a normal chow diet, compared to WT mice. This suggests an important role for AIM2 in modulating metabolism (657). It also agrees with IL-18 and caspase-1 activity during a HFD, where knockout mice showed increased weight gain (658-660). These studies reveal that the AIM2 inflammasome may be important in HFD-associated disease. Certainly, it provides evidence that this inflammasome pathway can be influenced by diet.

The preceding studies refer to systemic roles for inflammasome components during a HFD, but AIM2 may also have important localised roles in tissue. Ratsimandresy *et al.*

observed that AIM2 was an essential regulator of intestinal homeostasis through an IL-18/IL-22-STAT3 pathway and was responsive to faecal microbiota DNA (553). This may be relevant to the increased susceptibility to colorectal cancer reported in AIM2 knockout mice (633). Man *et al.* showed that by co-housing WT and AIM2 knockout mice microbiota exchange helped to alleviate colorectal tumorigenesis in knockout mice (634). These studies suggest the AIM2 inflammasome signalling is influenced by microbial and dietary conditions.

Indeed, it should be considered that inflammation observed during a HFD is not derived from the condition of obesity *per se*, but rather is instigated or partially instigated through diet. This effect may be mediated through sterile diet-based inflammatory triggers or through dysbiosis of the local microbiome caused by an unbalanced diet. To support this, Latz *et al.* have reported that NLRP3 is a principle insult sensor of a Western diet, mediating systemic inflammation and induced trained immunity through myeloid precursor reprogramming (505). Work by Mitroulis *et al.* demonstrates that IL-1 β , generated by NLRP3, is likely the key messenger and mediator of trained immune signalling and myelopoiesis (505, 661). Indeed, speculation that a Western diet may promote adverse immune responses and drive inflammatory disease is not new. Thorburn *et al.* proposed that there is an important immune role for diet- and bacterial-derived metabolites that mediate gut homeostasis through metabolite-sensing receptors present on immune and epithelial cells. They suggest that insufficient intake of 'healthy' metabolites may lead to inflammatory disease development (662).

Several studies have already begun to dissociate IL-18 from weight and obesity, despite clear functional associations with metabolism and diet. For example, a Western diet was shown to induce IL-18-mediated cardiac dysfunction in mice, independent of obesity (663). In patients, circulatory IL-18 was linked to insulin sensitivity, independent of body mass (664). Similarly, Quante's research group proposed that a HFD accelerates disease in pL2-IL-1 β mice, independent of obesity status (32). These studies give us reason to consider the inflammatory potential of dietary factors, independent of weight gain.

Overall, findings from this chapter implicate the inflammasome pathway in disease progression to Barrett's and OAC. We show that caspase-1 and AIM2 expression are upregulated in normal oesophageal tissue during a HFD. While IL-18 secretion is

reduced. We provide evidence that AIM2 expression is further upregulated during disease progression or a HFD in Barrett's pL2-IL-1 β tissue. However, no differences in TLR2 expression were observed in Barrett's tissue.

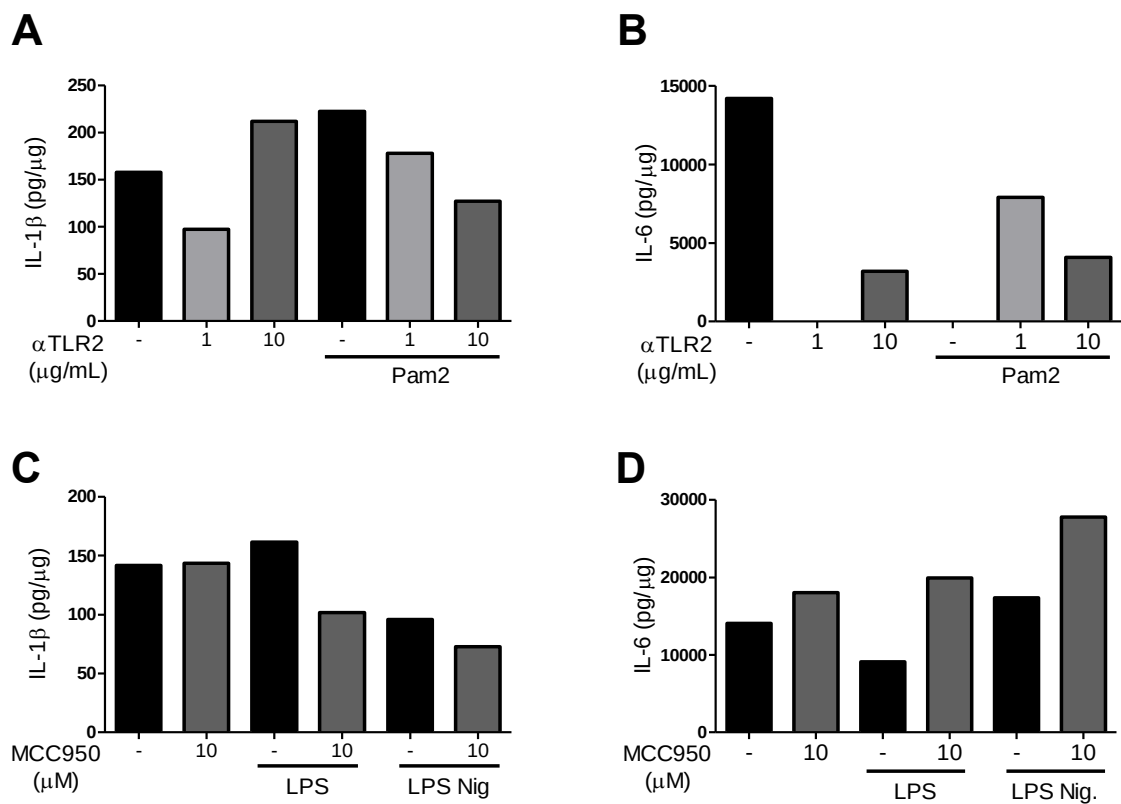


Figure 5.1: Optimisation of conditions for *ex vivo* mouse oesophageal explant experiments.

Mice (WT, standard fat diet, n=2) were IP administered poly I:C (100 μ g in 2:1 PBS/H₂O, 100 μ L) 4 hours prior to sacrifice. Oesophagi were resected, cut longitudinally and divided into 2 mm segments for culture. **A, B**; Explants were pre-treated with OPN-301 (1 and 10 μ g/mL) 1 hour before stimulation with Pam2 CSK4 (500 ng/mL) for 18 hours (200 μ L DMEM). **C, D**; Explants were pre-treated with MCC950 (10 μ M) for 1 hour before stimulation with LPS (1 μ g/mL) and Nigericin (5 μ M, 30 min). Graphs represent **A, C**) IL-1 β and **B, D**) IL-6 secretion levels (pg/ μ g).

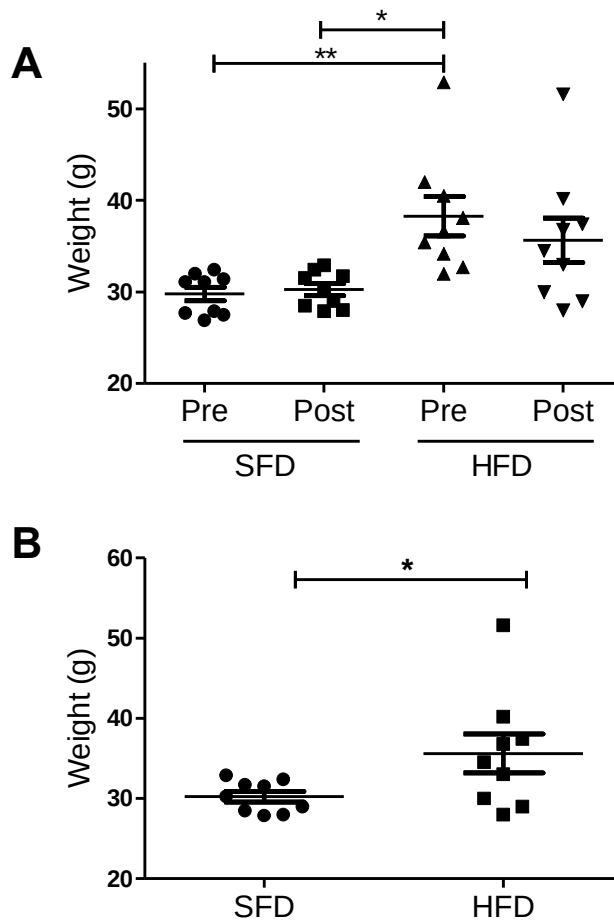
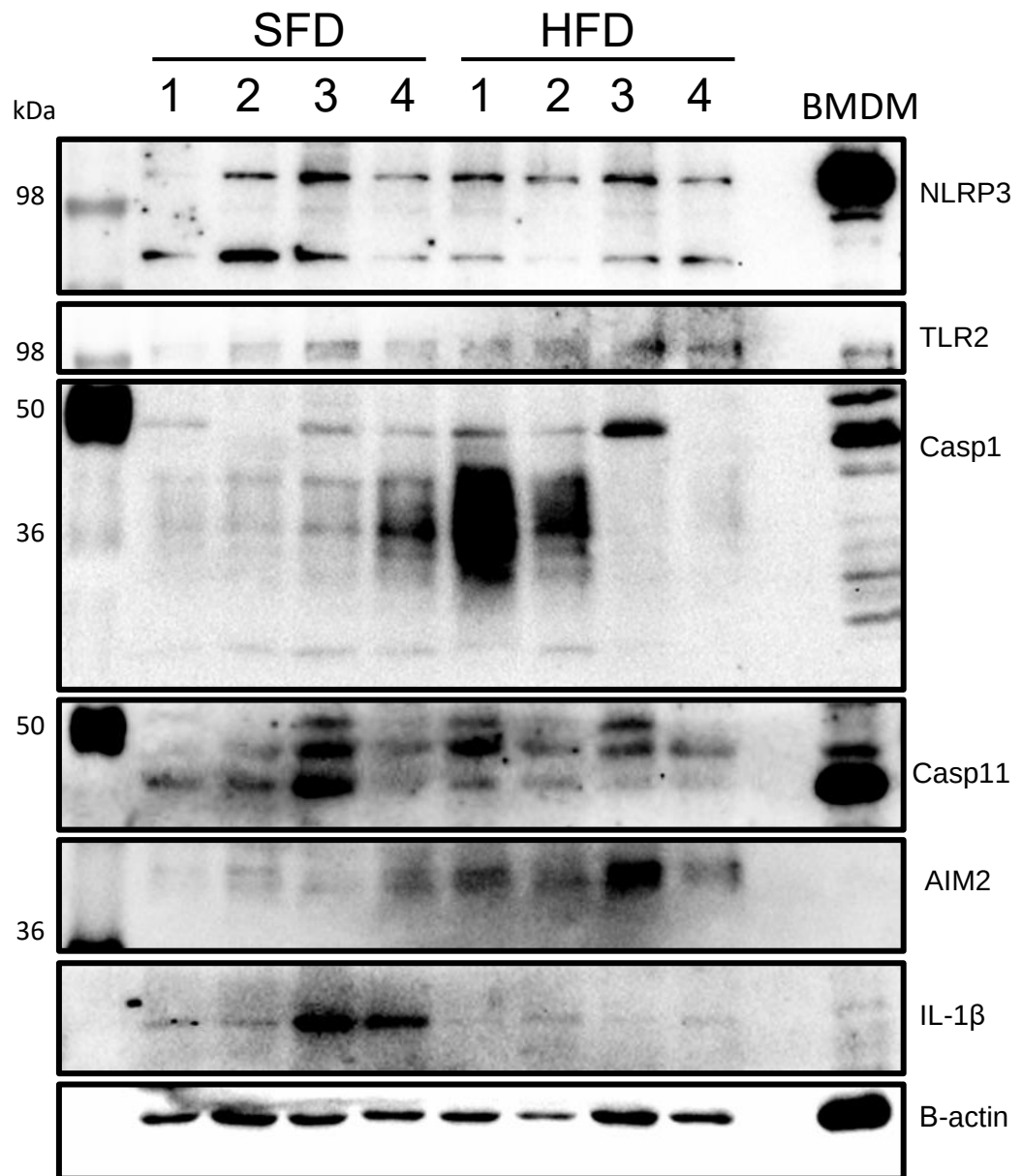


Figure 5.2: Mice fed a 9-week HFD weigh significantly more than SFD-fed controls.

Mice were fed a standard fat diet (SFD) or a 60% lard high fat diet (HFD) for a total of 9 weeks. Seven days prior to sacrifice (week 8), mice were administered daily vehicle (2:1 PBS/DMSO, 100 μ L) IP injections. Four hours prior to sacrifice, mice were IP administered poly I:C (100 μ g in 2:1 PBS/H₂O, 100 μ L). Mice were sacrificed at 15 weeks old. **A**) Mice weights pre- and post-IP injections at 8 and 9 weeks, respectively. One-way ANOVA followed by Tukey's *t* test. **B**) Mice weights at 9 weeks post IP injections. Unpaired Student's *t* test: **p*<0.05, ***p*<0.01. Data represents mean $\pm$ SEM.

A



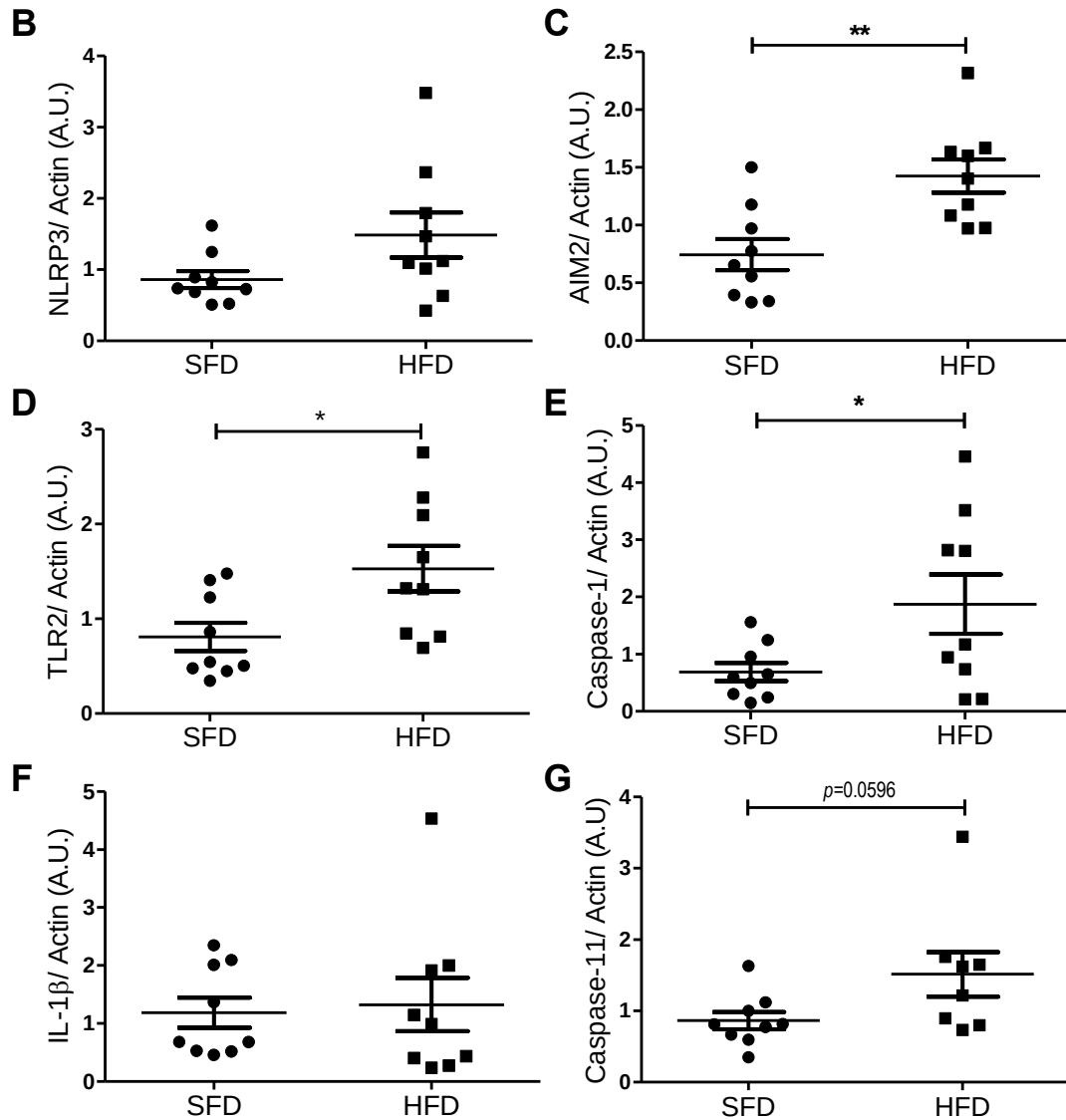


Figure 5.3: Oesophageal tissue from HFD-fed mice express higher levels of AIM2, TLR2 and caspase-1, when compared to tissue from SFD-fed mice.

Oesophageal explants from SFD- and HFD-fed mice were cultured for 18 hours (200 μ L DMEM, 37 $^{\circ}$ C, 5% CO_2). Explants were lysed on ice (220 μ L RIPA buffer) before homogenisation using a bench-top rotor-stator homogeniser. 30 μ g lysate was analysed by Western blot (10% SDS-PAGE). LPS-treated BMDM lysate (15 μ g) was included as a positive control. **A)** Representative immunoblots of the indicated proteins. **B-G)** Subsequent densitometric analysis (arbitrary units) of inflammatory proteins (n=9 mice), **B)** NLRP3 (118, 112, 95.7 kDa), **C)** AIM2 (40.15 kDa), **D)** TLR2 (89.4 kDa), **E)** caspase-1 (45.6 kDa), **F)** IL-1 β (30.9 kDa) and **G)** caspase-11 (42.7 kDa). β -actin (42 kDa) was probed as a loading control. Data represents mean $\pm$ SEM. Unpaired Student's *t* test: * p <0.05, ** p <0.01.

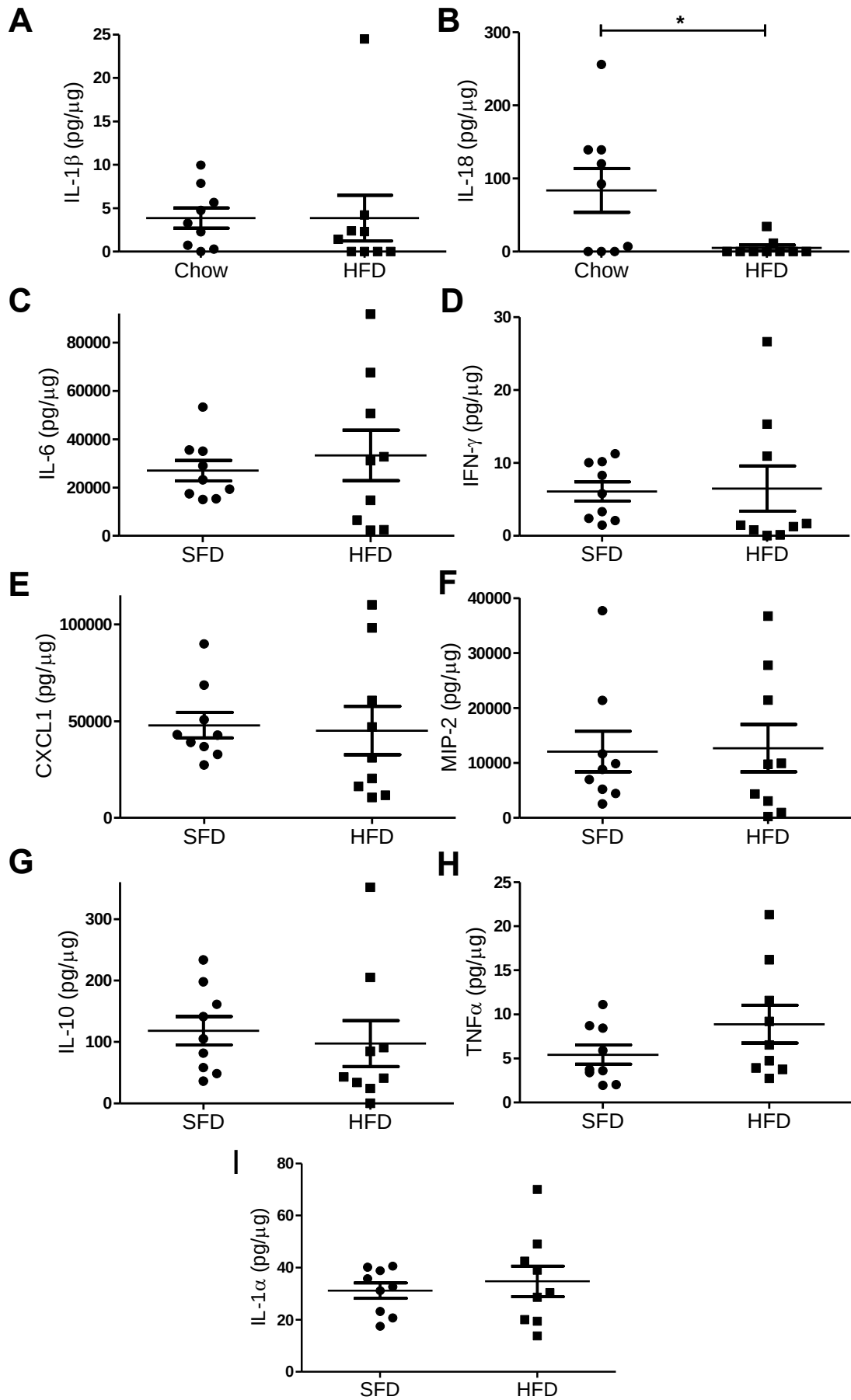


Figure 5.4: Reduced oesophageal IL-18 secretion from HFD-fed mice, when compared to SFD-fed control mice.

Mice were fed a SFD or a 60% lard HFD for 9 weeks. Seven days prior to sacrifice (week 8), mice were administered daily vehicle (2:1 PBS/DMSO, 100 μ L) IP injections. Four hours prior to sacrifice, mice were IP administered poly I:C (100 μ g in 2:1 PBS/H₂O, 100 μ L). Mice were sacrificed at 15 weeks old. The oesophagus was resected, cut longitudinally and explant biopsies were cultured for 18 hours (200 μ L DMEM, 37 °C, 5% CO₂). Supernatants were subsequently measured for levels of secreted inflammatory cytokines and normalised to total protein as determined using the BCA method: **A)** IL-1 β , **B)** IL-18, **C)** IL-6, **D)** IFN- γ , **E)** CXCL1, **F)** MIP-2, **G)** IL-10 **H)** TNF α and **I)** IL-1 α (pg/ μ g). Unpaired Student's *t* test: **p*<0.05.

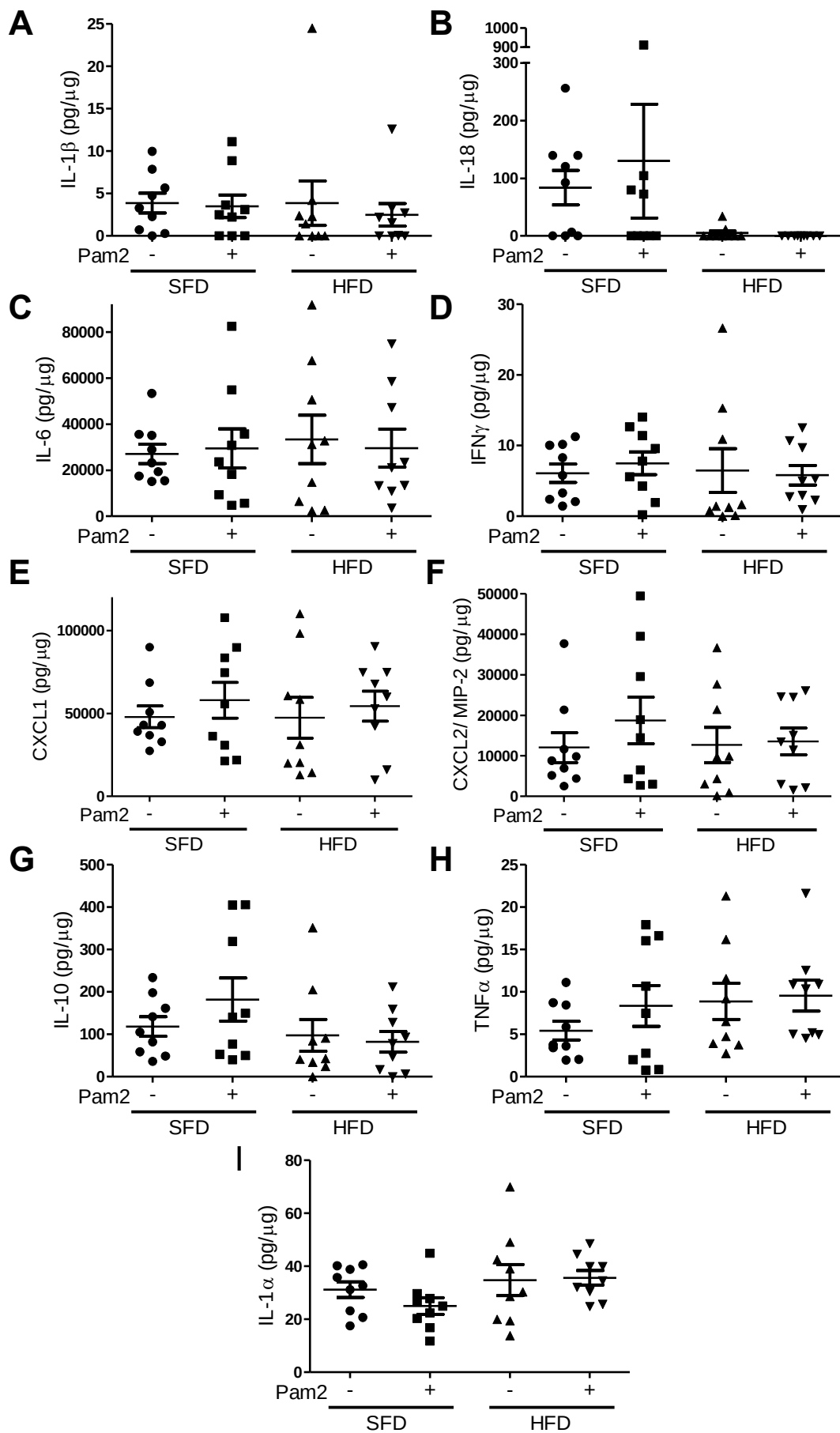


Figure 5.5: Inflammatory cytokine secretion from oesophageal tissue was not affected by the presence of the TLR2 agonist, Pam2 CSK4.

Resected oesophageal tissue from SFD and HFD mice were cultured for 18 hours with or without Pam2 CSK4 ligand (500 ng/mL, 200 μ L DMEM). Supernatants were subsequently measured for levels of secreted cytokines and normalised to total protein as determined using the BCA method: **A)** IL-1 β , **B)** IL-18, **C)** IL-6, **D)** IFN- γ , **E)** CXCL1, **F)** MIP-2, **G)** IL-10 **H)** TNF α and **I)** IL-1 α (pg/ μ g). Unpaired Student's *t* test comparing untreated to treated: non-significant.

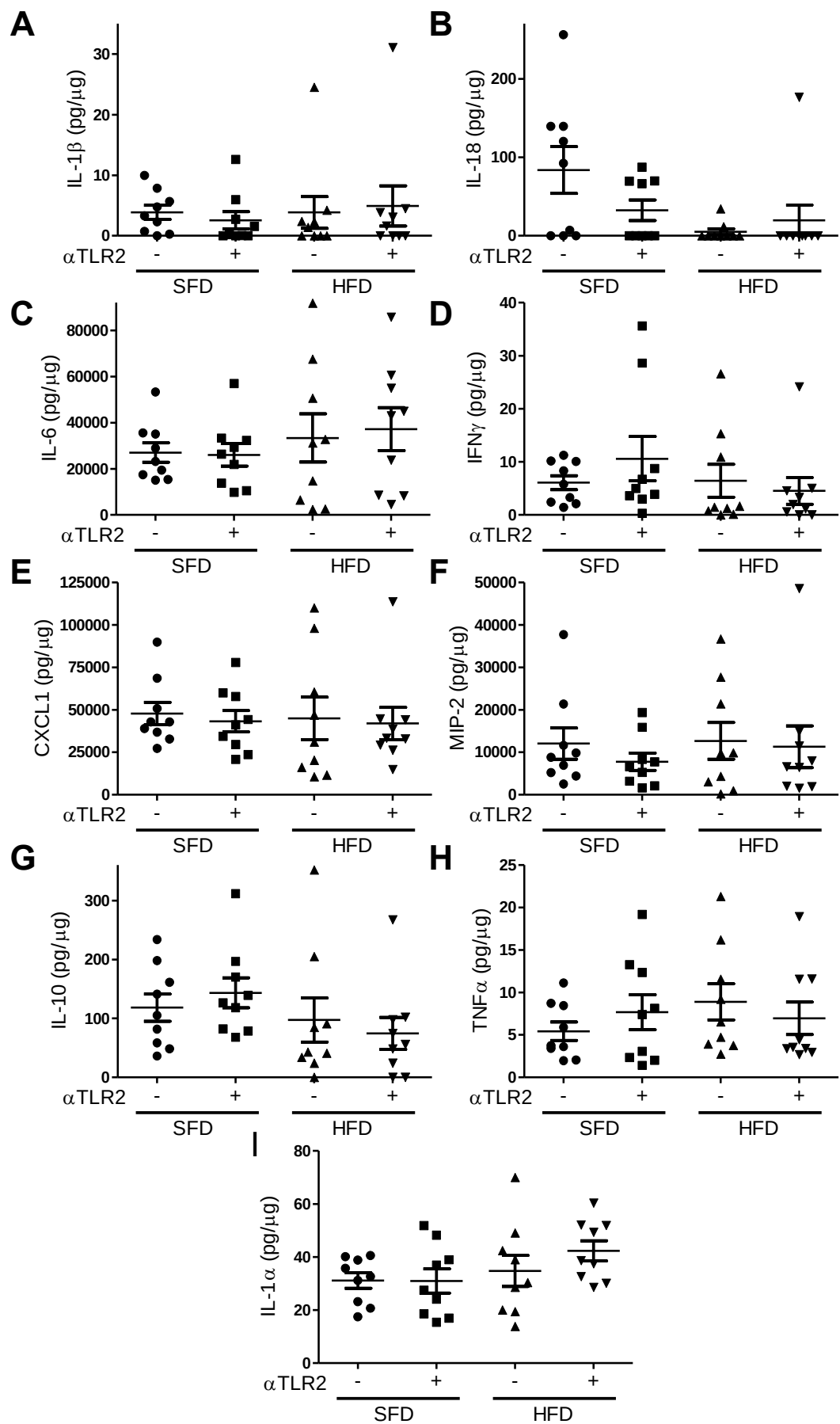


Figure 5.6: Blocking TLR2 activity does not significantly alter cytokine secretion in SFD- and HFD-fed mice.

Resected oesophageal tissue from SFD and HFD mice were cultured for 18 hours with or without anti-TLR2 blocking antibody (OPN-301, 1 $\mu\text{g/mL}$, 200 μL DMEM). Supernatants were subsequently measured for levels of secreted cytokines and normalised to total protein as determined using the BCA method: **A)** IL-1 β , **B)** IL-18, **C)** IL-6, **D)** IFN- γ , **E)** CXCL1, **F)** MIP-2, **G)** IL-10 **H)** TNF α and **I)** IL-1 α (pg/ μg). Unpaired Student's *t* test comparing untreated to treated: non-significant.

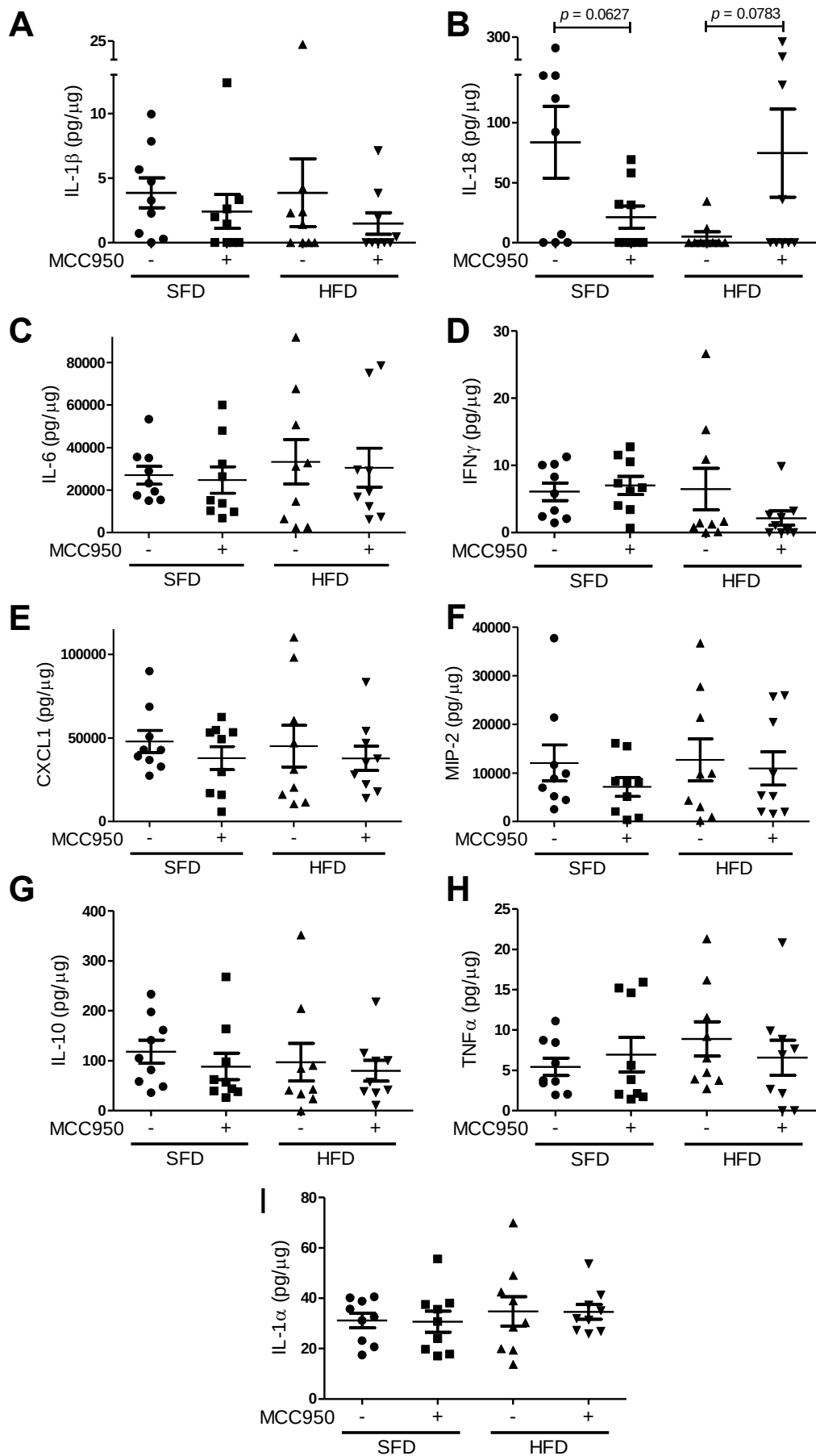


Figure 5.7: The NLRP3 inhibitor, MCC950, affects IL-18 secretion levels from oesophageal explants of SFD and HFD-fed mice.

Resected oesophageal tissue from SFD and HFD mice were cultured for 18 hours with or without NLRP3 inhibitor (MCC950, 10 μ M, 200 μ L DMEM). Supernatants were subsequently measured for levels of secreted cytokines and normalised to total protein as determined using the BCA method: **A)** IL-1 β , **B)** IL-18, **C)** IL-6, **D)** IFN- γ , **E)** CXCL1, **F)** MIP-2, **G)** IL-10 **H)** TNF α and **I)** IL-1 α (pg/ μ g). Unpaired Student's *t* test comparing untreated to treated: non-significant.

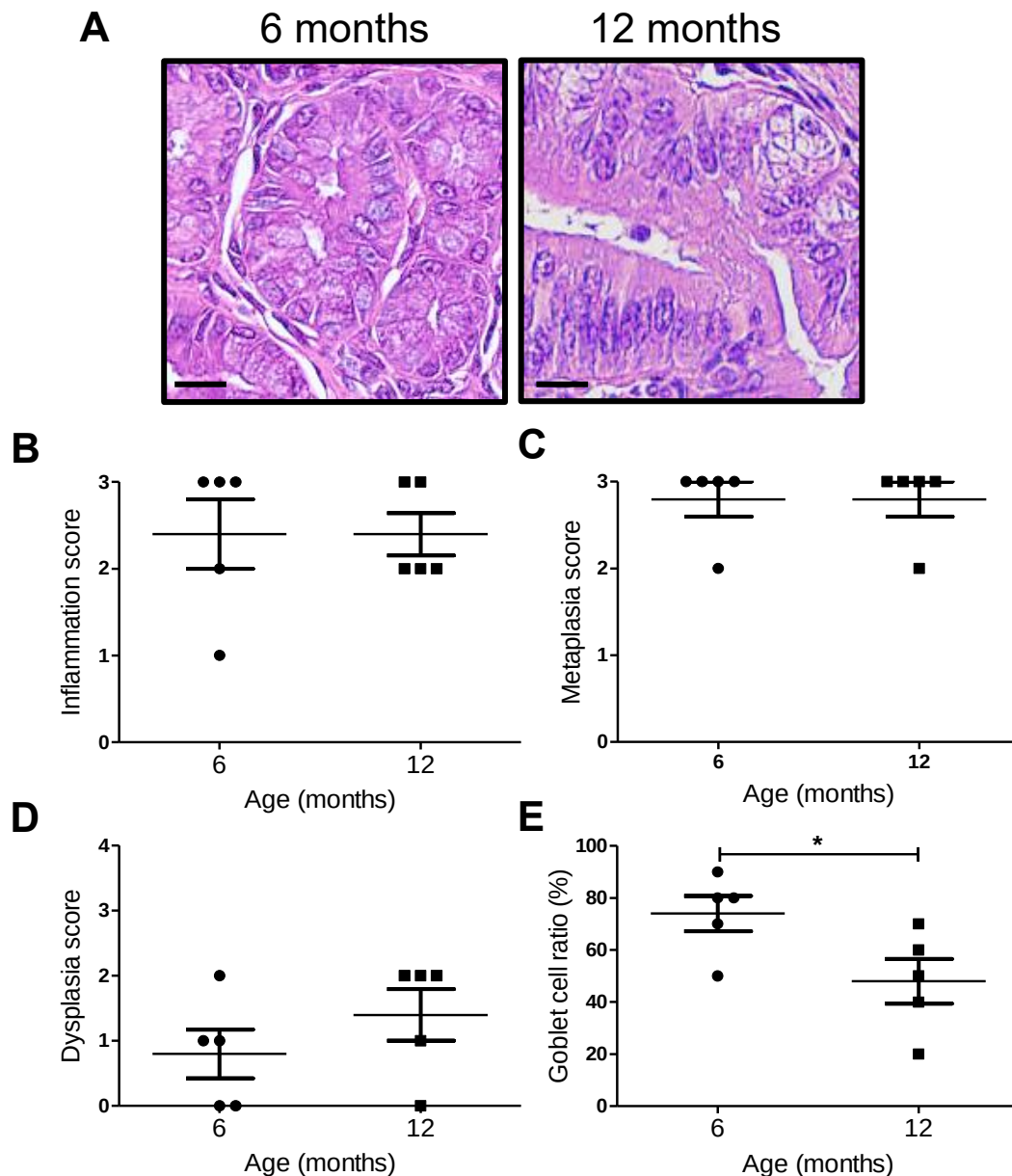


Figure 5.8: Histological scores reveal that twelve-month-old pL2-IL-1 β mice have a more advanced Barrett's phenotype, compared to six-month-old mice.

Disease severity scoring of pL2-IL-1 β SFD-fed mice aged 6 and 12 months (n=5/group). Scoring was performed by M. Quante, Munich. **A)** Representative histological images of metaplasia in 6- and 12-month pL2-IL-1 β mice. **B-E)** Graphs represent histological scores for: **B)** Inflammation; scale: 0 = no inflammation, 1 = mild, 2 = moderate, 3 = severe. **C)** Metaplasia; scale: 0 = no metaplasia, 1 = rare mucous cells, 2 = single metaplastic glands, 3 = multiple metaplastic glands. **D)** Dysplasia; scale: 0 = no dysplasia, 1 = superficial epithelial atypia, 2 = atypia with granular complexity, 3 = low-grade dysplasia, 4 = high-grade dysplasia. **E)** Goblet cell ratio (%) calculated as a percentage of goblet cell positivity within each crypt of the Barrett's region. Magnification 20X, scale bars: 10 μ m. Data represents mean $\pm$ SEM. Unpaired Student's *t* test: **p*<0.05.

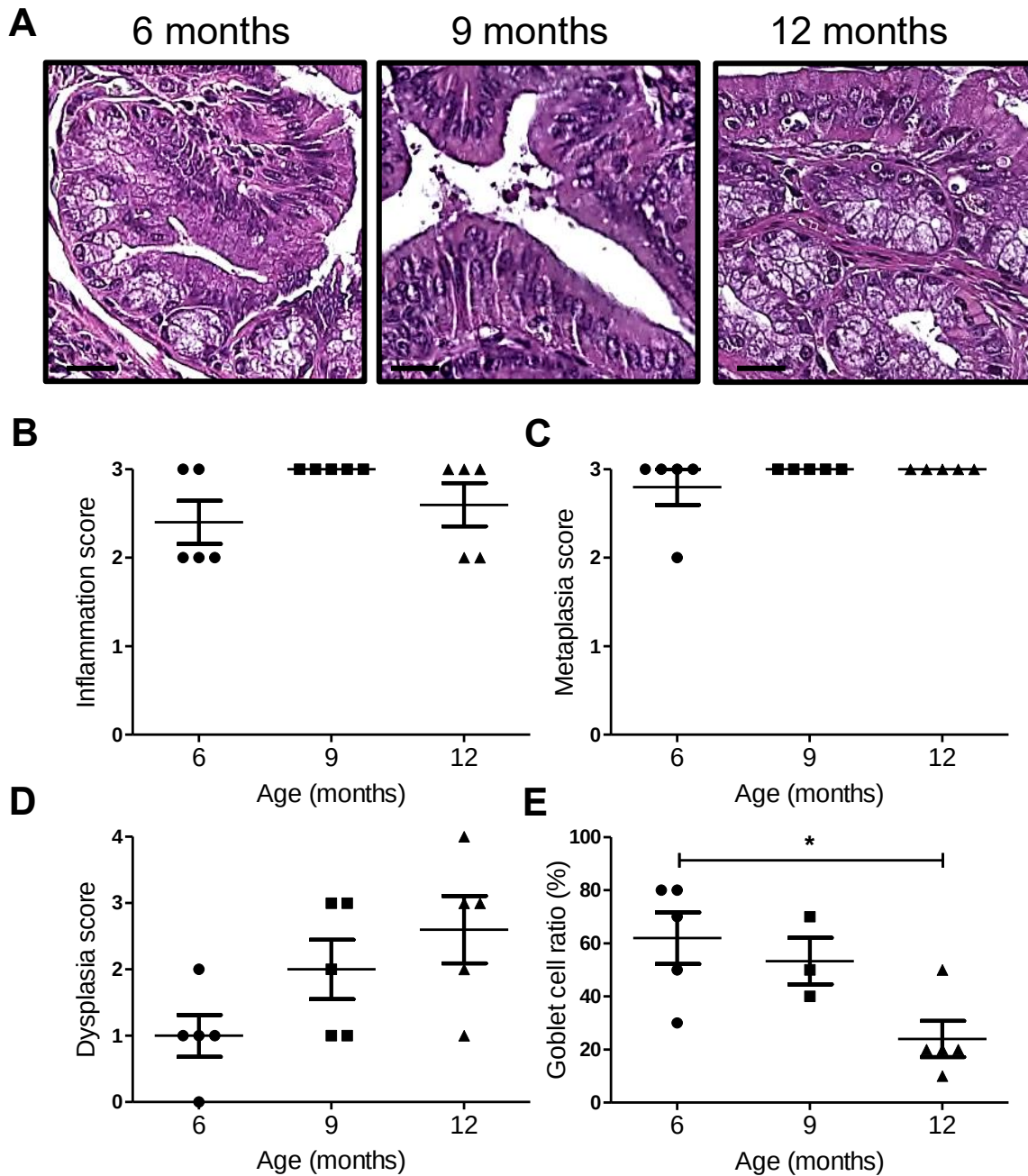


Figure 5.9: Progression of disease severity with age in pL2-IL-1 β mice fed a high fat diet.

Disease severity scoring of pL2-IL-1 β mice of different age groups (n=5/group, M. Quante). **A)** Histological representative images of 6-, 9- and 12-month-old mice. Graphs represent the histological scores for **A)** inflammation **B)** metaplasia **C)** dysplasia and **D)** goblet cell ratio (%). Magnification 20X, scale bars: 10 μ m. Data represents mean $\pm$ SEM. One-way ANOVA followed by Tukey's post hoc test: * p <0.05.

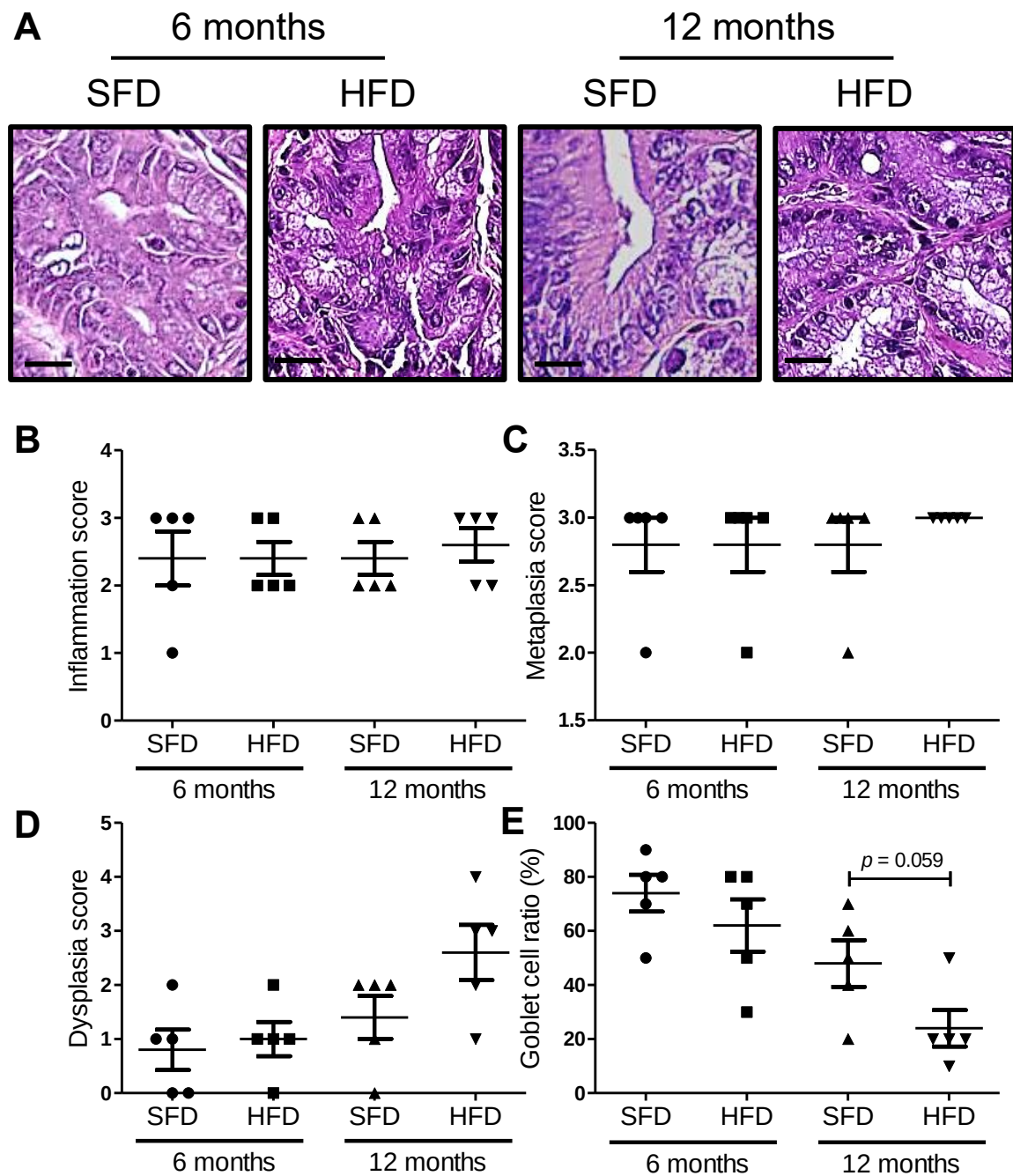


Figure 5.10: Accelerated disease progression in pL2-IL-1 β mice fed a high fat diet.

Comparison of histological scores for 6- and 12-month-old pL2-IL-1 β mice fed a SFD and HFD (n=5/group, M. Quante). **A**) Representative images of SFD- and HFD-fed pL2-IL-1 β mice at 6 and 12 months old. Images taken at 20X magnification, scale bars = 10 μ m. Graphs represent the histological scores for **B**) inflammation **C**) metaplasia **D**) dysplasia and **E**) goblet cell ratio (%). Data represents mean $\pm$ SEM. Unpaired Student's *t* test comparing SFD to HFD: non-significant.

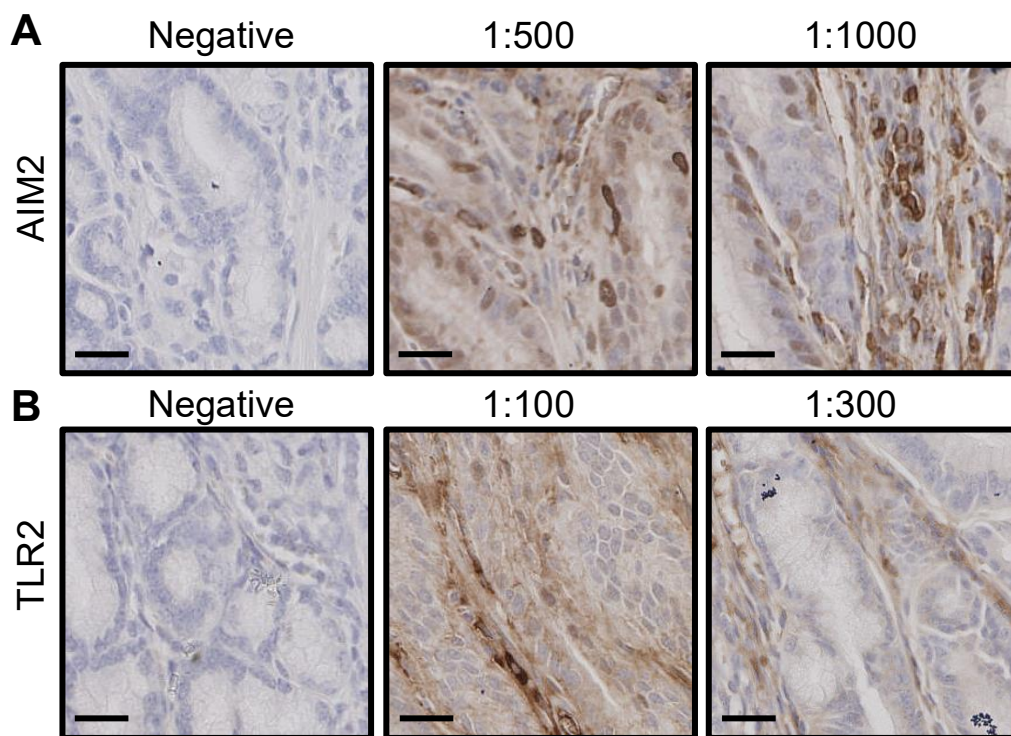


Figure 5.11: Optimisation of AIM2 and TLR2 antibodies for IHC analysis in murine Barrett's tissue sections.

IHC analysis was performed on Barrett's oesophageal tissue sections taken from 12-month-old pL2-IL-1 β /IL8Tg mice. Representative images show optimised antibody concentrations for **A**) anti-AIM2 (Biosciences) at 1:500 and 1:1000 dilutions; and **B**) anti-TLR2 (Opsona) 1:100 and 1:300 dilutions. Negative controls stained with anti-rabbit (AIM2) and anti-mouse (TLR2) secondary antibody alone. Magnification 20X, scale bars: 10 μm .

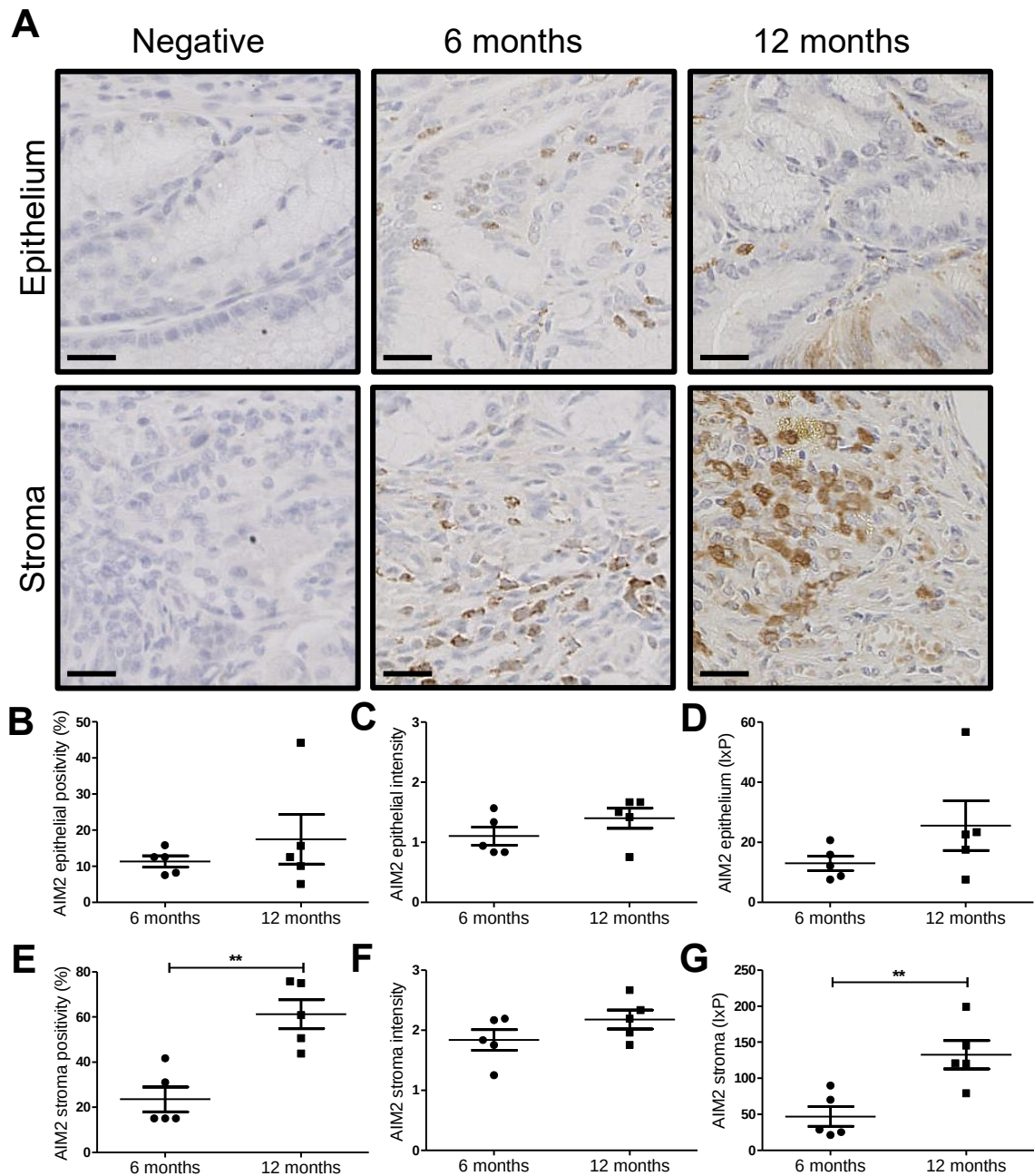


Figure 5.12: IHC analysis reveals upregulation of stromal AIM2 expression at the SCJ in 12-month-old SFD-fed mice.

SFD-fed pL2-IL-1 β mice were sacrificed at 6 and 12 months (n=5/group). The oesophagus and stomach were resected, formalin-fixed, and paraffin embedded (FFPE). IHC staining for AIM2 expression was assessed by two blinded reviewers using a validated semi-quantitative scoring method (G. Barber, J. Phelan). **A**) Representative images of AIM2 expression in 6- and 12-month-old mice. Images taken at 20X magnification, scale bars: 10 μ m. **B-D**) Graphs represent metaplastic epithelial AIM2 scores for **B**) positivity (%), **C**) intensity and **D**) intensity x positivity (IxP) at the SCJ. **E-G**) Graphs represent stromal AIM2 scores for **E**) positivity (%), **F**) intensity and **G**) intensity x positivity (IxP) at the SCJ. Data represents mean $\pm$ SEM. Unpaired Student's *t* test: ***p*<0.01.

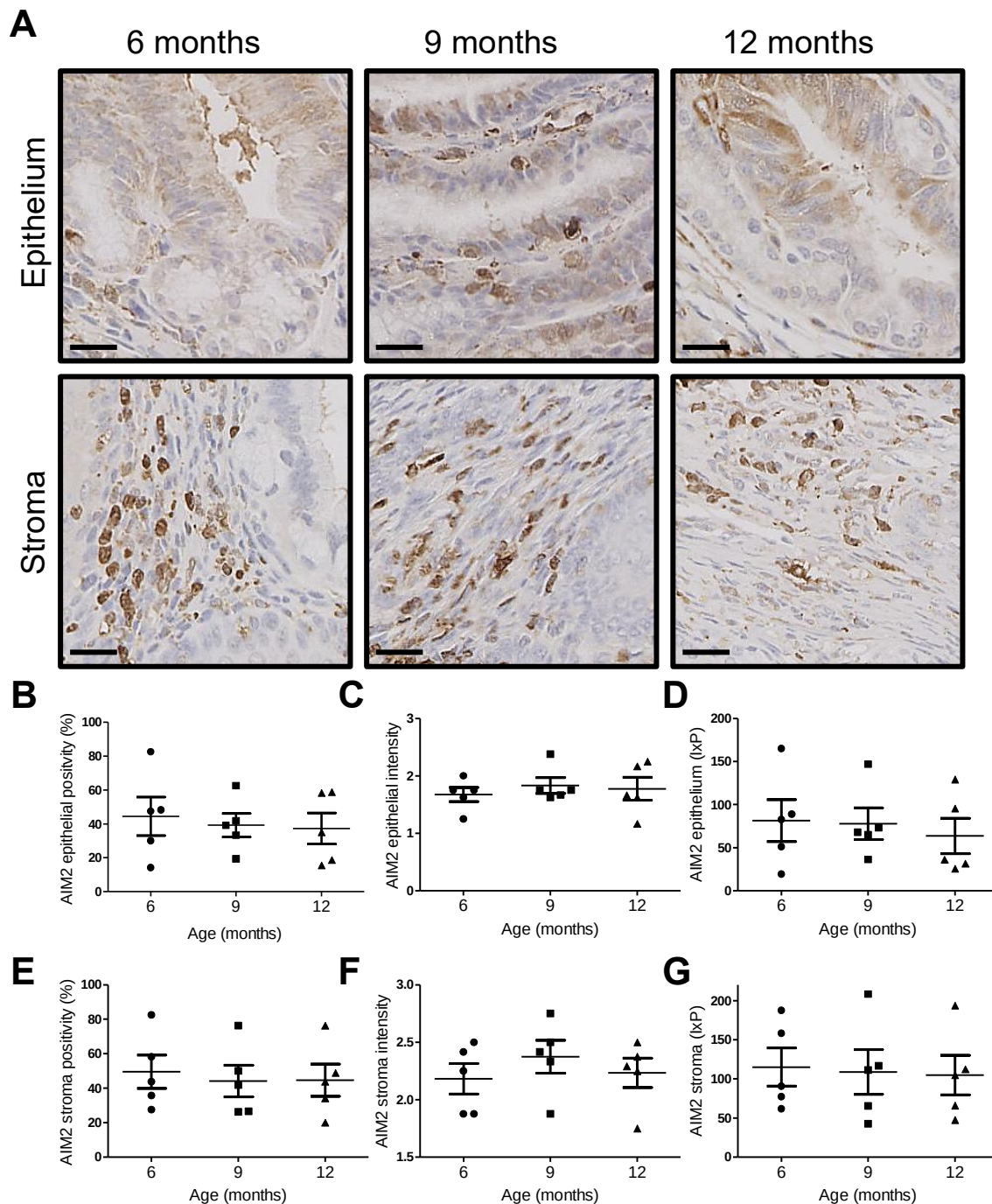


Figure 5.13: No differences in AIM2 expression levels at the SCJ between different age groups of pL2-IL-1 β HFD-fed mice.

HFD-fed pL2-IL-1 β mice were sacrificed at 6, 9 and 12 months (n=5/group). FFPE tissue was IHC stained for AIM2. Expression was assessed by two blinded reviewers using a validated semi-quantitative scoring method. **A)** Representative images of IHC AIM2 expression patterns in 6-, 9- and 12-month-old mice. Images taken at 20X magnification, scale bars: 10 μ m. **B-D)** Graphs represent metaplastic epithelial AIM2 scores for **B)** positivity (%), **C)** intensity and **D)** intensity x positivity (IxP) at the SCJ. **E-G)** Graphs represent stromal AIM2 scores for **E)** positivity (%), **F)** intensity and **G)** intensity x positivity (IxP) at the SCJ. Data represents mean $\pm$ SEM. One-way ANOVA followed by Tukey's post hoc test: non-significant.

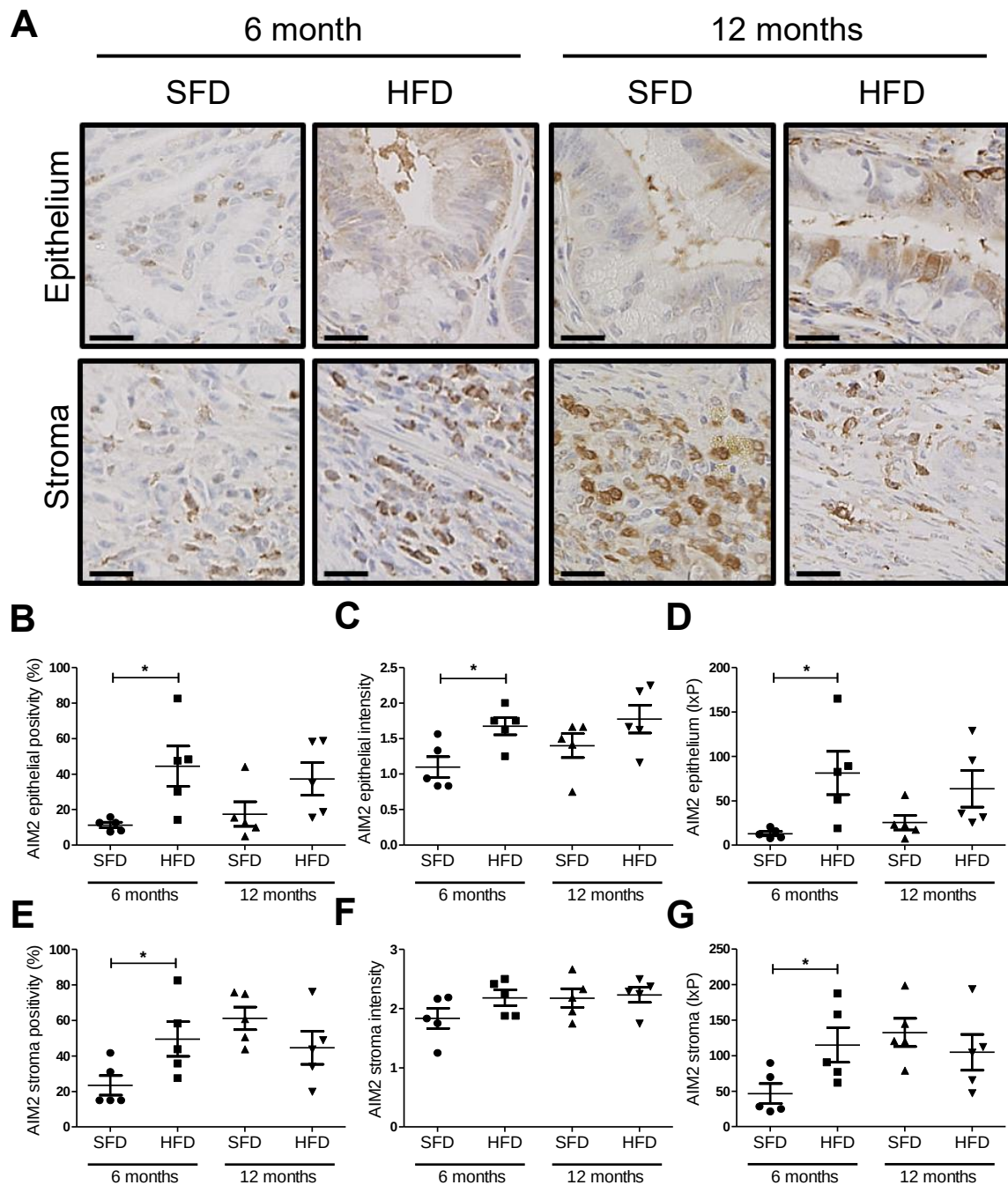
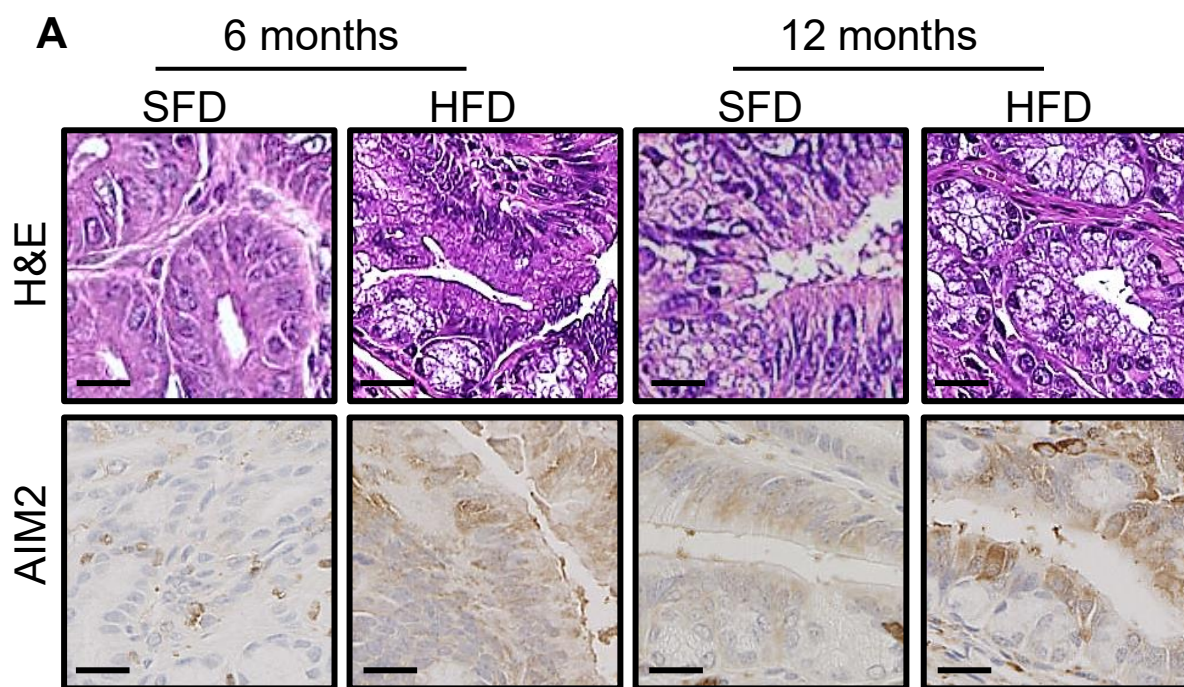
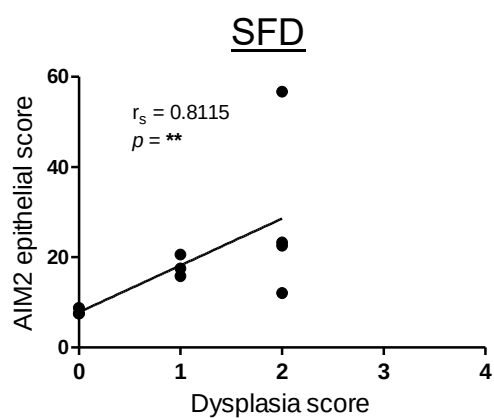


Figure 5.14: Enhanced epithelial expression of AIM2 at 6 and 12 months in HFD-fed, compared to SFD-fed pL2-IL-1 β mice.

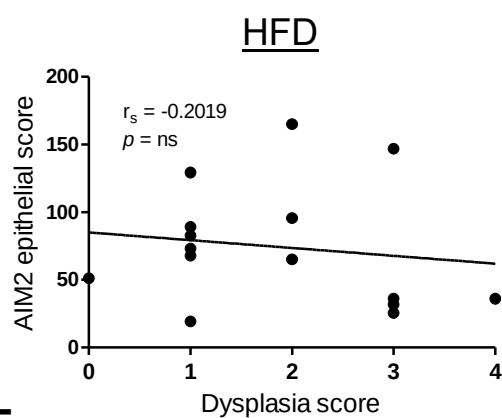
Comparison of AIM2 expression levels in pL2-IL-1 β mice fed a SFD or HFD at 6 and 12 months old (n=5/group). **A**) Representative images of AIM2 IHC expression patterns in SFD and HFD fed mice. Magnification 20X, scale bars: 10 μ m. **B-D**) Graphs represent metaplastic epithelial AIM2 scores for **B**) positivity (%), **C**) intensity and **D**) intensity x positivity (IxP) at the SCJ. **E-G**) Graphs represent stromal AIM2 scores for **E**) positivity (%), **F**) intensity and **G**) intensity x positivity (IxP) at the SCJ. Data represents mean $\pm$ SEM. Unpaired Student's *t* test comparing a SFD to a HFD: **p*<0.05.



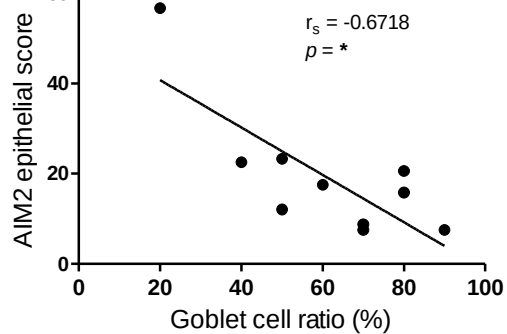
B



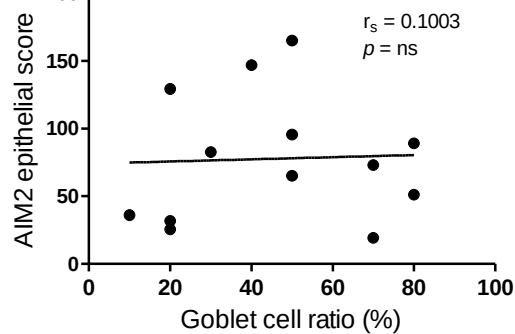
C



D



E



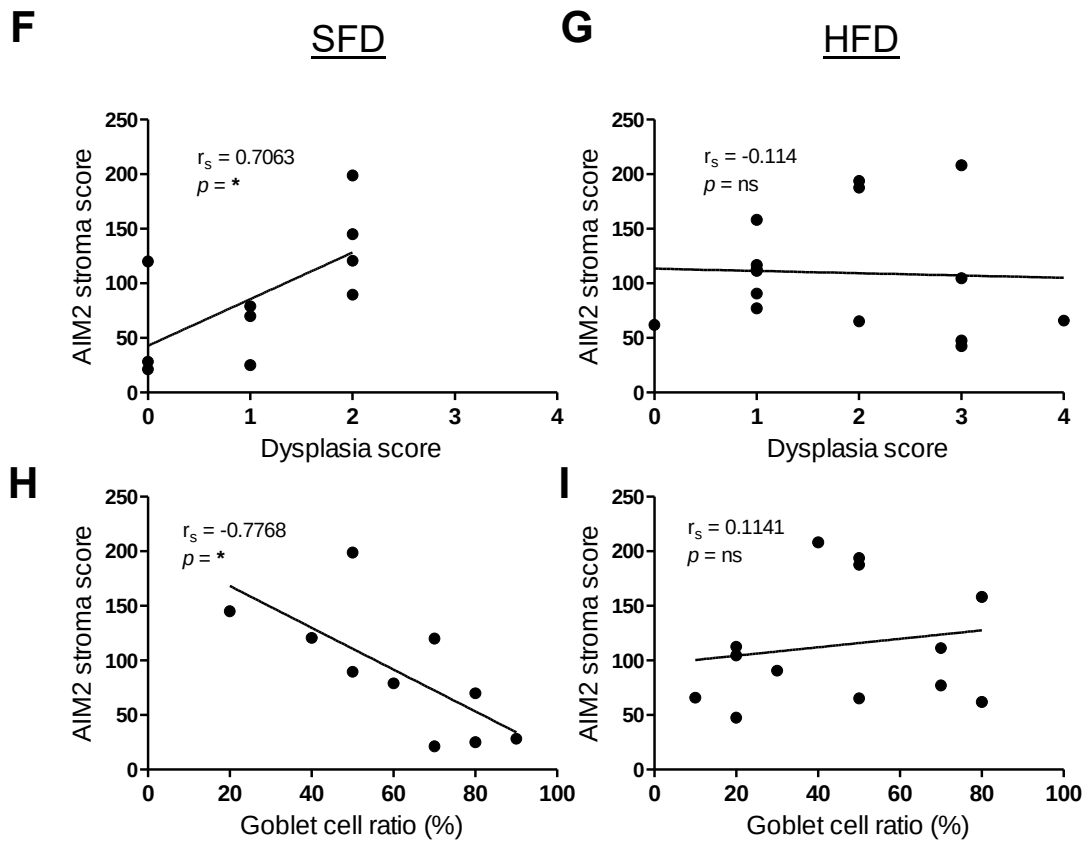


Figure 5.15: AIM2 expression correlates with disease progression scores in SFD-fed, but not HFD-fed, pL2-IL-1 β mice.

A) Representative images of H&E and AIM2 IHC staining from SFD- and HFD-fed pL2-IL-1 β mice at 6- and 12- months old. Images taken at 20X magnification, scale bars = 10 μ m. **B-E)** Correlation analysis of epithelial AIM2 expression (IxP) versus histological scores for dysplasia in **B)** SFD and **C)** HFD mice, and versus goblet cell ratio in **D)** SFD and **E)** HFD mice. **F-I)** Correlation analysis of stromal AIM2 expression (IxP) versus dysplasia scores in **F)** SFD and **G)** HFD mice, and versus goblet cell ratio in **H)** SFD and **I)** HFD mice. r_s = Spearman's correlation coefficient: p = non-significant (ns), $*p < 0.05$, $**p < 0.01$.

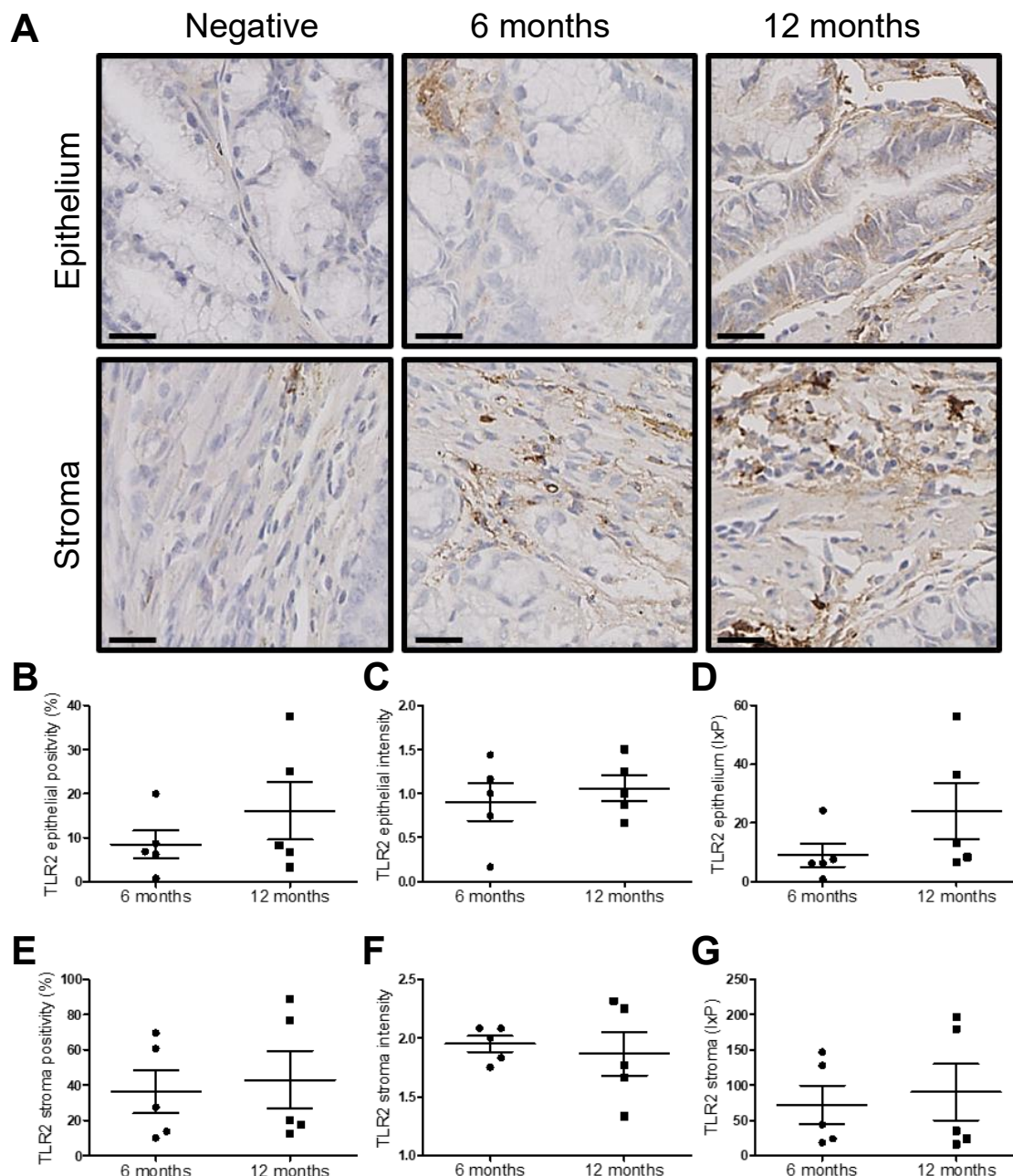


Figure 5.16: No differences in TLR2 expression levels at the SCJ of SFD-fed pL2-IL-1 β mice at 6 and 12 months old.

Barrett's pL2-IL-1 β mice fed a SFD were sacrificed at 6 and 12 months (n=5). FFPE tissue was IHC stained for TLR2 expression. Expression levels were assessed by two blinded reviewers using a validated semi-quantitative scoring method (G. Barber, J. Phelan). **A**) Representative images of TLR2 IHC in 6- and 12-month-old mice. 20X magnification, scale bars: 10 μ m. **B-D**) Graphs represent metaplastic epithelial TLR2 scores for **B**) positivity (%), **C**) intensity and **D**) intensity x positivity (IxP) at the SCJ. **E-G**) Graphs represent stromal TLR2 scores for **E**) positivity (%), **F**) intensity and **G**) intensity x positivity (IxP) at the SCJ. Data represents mean $\pm$ SEM. Unpaired Student's *t* test: non-significant.

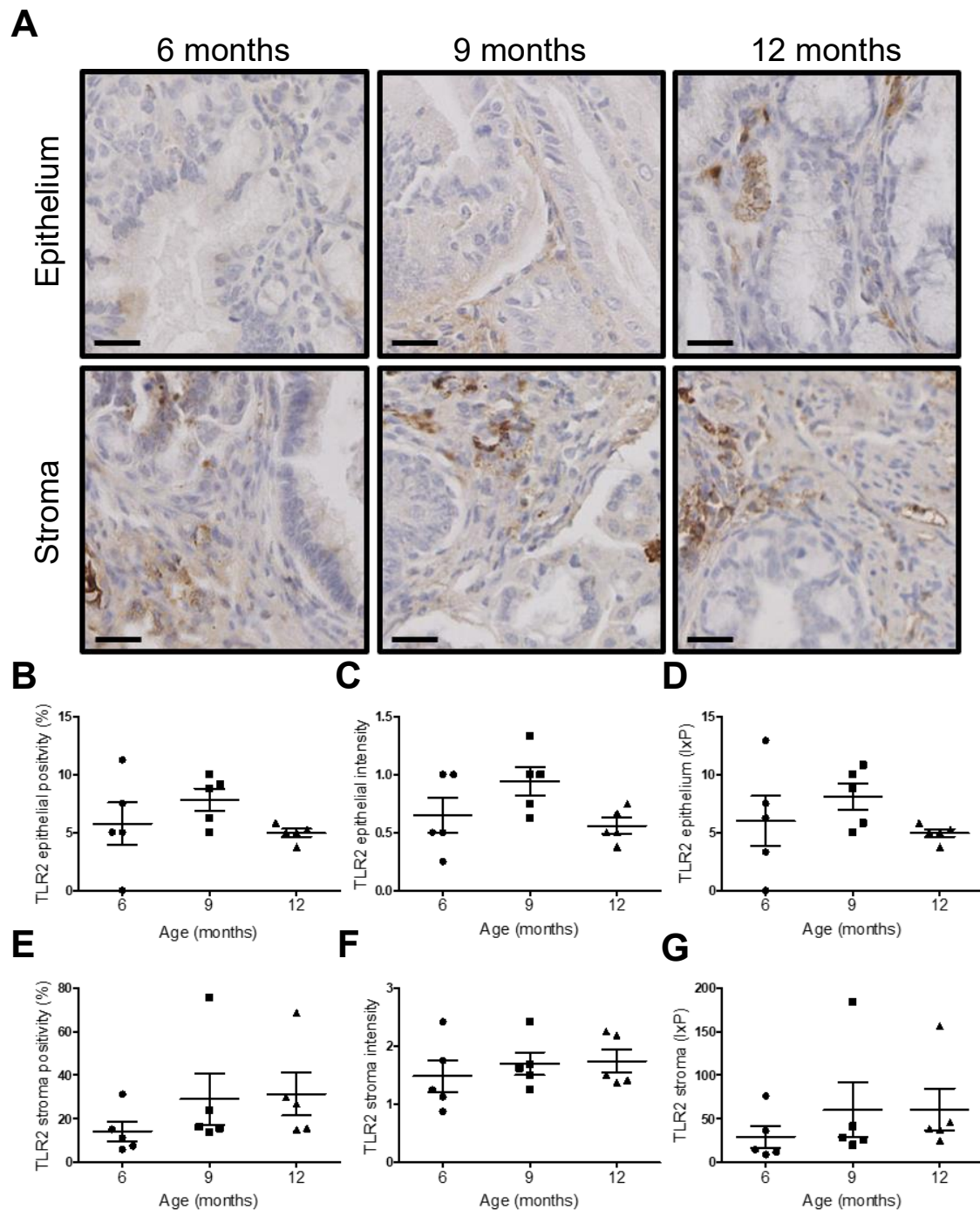


Figure 5.17: No differences in TLR2 expression levels at the SCJ of HFD-fed pL2-IL-1 β mice of different age groups.

Oesophageal tissue (FFPE) from HFD-fed pL2-IL-1 β mice at 6, 9 and 12 months (n=5/group) were IHC stained for TLR2 expression. **A)** Representative IHC images of TLR2 staining at 6, 9 and 12 months (20X magnification, scale bars: 10 μ m). **B-D)** Graphs represent metaplastic epithelial TLR2 scores for **B)** positivity (%), **C)** intensity and **D)** intensity x positivity (IxP) at the SCJ. **E-G)** Graphs represent stromal TLR2 scores for **E)** positivity (%), **F)** intensity and **G)** intensity x positivity (IxP) at the SCJ. Data represents mean $\pm$ SEM. One-way ANOVA followed by Tukey's post hoc test: non-significant.

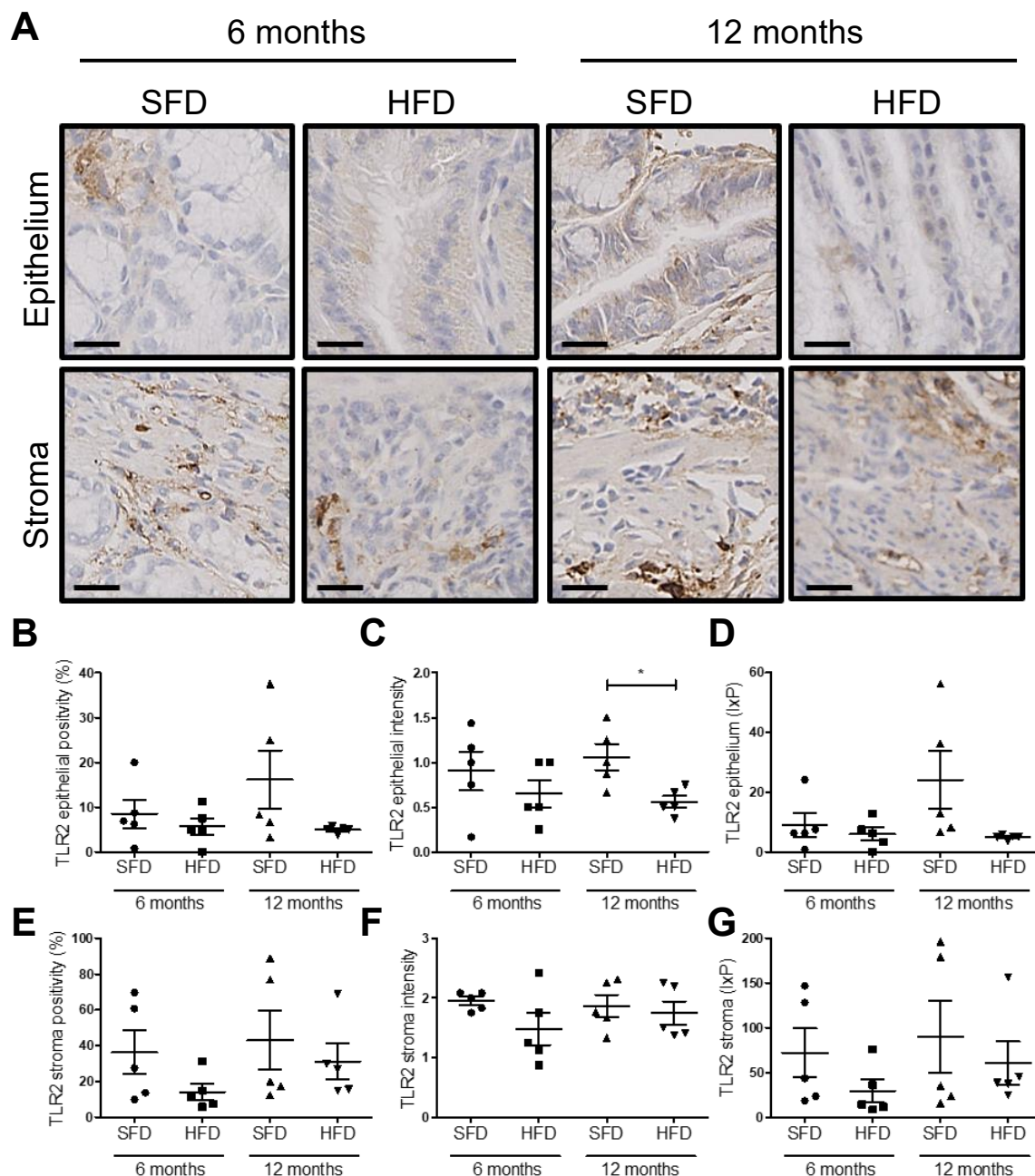


Figure 5.18: Epithelial TLR2 expression levels are downregulated at the SCJ in 12-month-old pL2-IL-1 β HFD-fed mice, compared to SFD-fed mice.

Comparison of pL2-IL-1 β mice fed a SFD or HFD at 6 and 12 months-old (n=5/group).

A) Representative IHC images of TLR2 expression patterns in SFD- and HFD-fed mice (20X magnification, scale bars = 10 μ m). **B-D)** Graphs represent metaplastic epithelial TLR2 scores for **B)** positivity (%), **C)** intensity and **D)** intensity x positivity (IxP) at the SCJ. **E-G)** Graphs represent stromal TLR2 scores for **E)** positivity (%), **F)** intensity and **G)** intensity x positivity (IxP) at the SCJ. Data represents mean $\pm$ SEM. Unpaired Student's *t* test comparing a SFD to a HFD: **p*<0.05.

Chapter 6: General Discussion and Future Directions

6.1 General discussion

In 2000, Hanahan and Weinberg published 'The Hallmarks of cancer', describing the seven essential alterations in cellular and tissue physiology that lead to tumorigenesis (665). In 2011, they added more conditional factors, these being inflammation and immune evasion (157). Within these conditions, the inflammasome pathway plays an essential role in mediating inflammation (82). In a landmark study, Tschopp's research group first described the caspase-1-activating inflammasome platform. Since then, numerous studies have implicated crucial roles for inflammasome signalling during cancer development and survival (82, 85). In this thesis, we described a role for the inflammasome pathway during disease initiation and progression to Barrett's and OAC. Using different *in vitro* and *ex vivo* models originating from both mouse and human tissue, we revealed that inflammasome priming and activation were occurring during Barrett's disease. Additionally, using disease model systems we observed significant inflammasome upregulation during obesity and a HFD, which may be contributing to an increased risk of oesophageal disease (32, 497).

During the course of this project, we employed models derived from mouse and human origins. To reflect on the use of these models, we consider the differences and similarities between the disease phenotypes in these models, how disease criteria are classified and the implications that may arise from differences between species.

In St. James's Hospital, Barrett's patients are histologically confirmed for specialised intestinal metaplasia, defined by well-formed goblet cells (246, 249). Assessment is undertaken using the Vienna classification system which assigns a score based on tissue architecture, cytology, maturation and local inflammation, previously described in Section 1.4.2/3 (243, 244). In St. James's Hospital, no immunoscore is given for Barrett's surveillance. Histological definitions used for the Barrett's mouse model were very similar, which include inflammation, metaplasia and dysplasia (as described in methods). These similar criteria allowed us to make disease comparisons between models (263).

Similarities and conclusions drawn based on defined pathology between species should in theory be reliable when the criteria are similarly assessed. However, features of

dysplasia and neoplasia are easily missed during patient endoscopy and therefore the diagnosed disease stage may not be fully accurate (666). In this circumstance, histological scores for the mouse model which assess the entire Barrett's region may be more informative than the relative patient scores.

Additionally, in this study we reported differences in epithelial caspase-1 expression patterns in patients versus pL2-IL-1 β mice. Where at 12 months, mice show a trend of reduced epithelial caspase-1 which aligns with our findings *in vitro*. However, this reduction was not seen to the same extent in OAC tissue. In Chapter 3, we reason this trend was not followed in part because 13/26 patients had received NAT. However, this difference also highlights some limitations of the random sampling method employed by endoscopists for both diagnosis and research purposes. Core biopsies were sampled from identified areas of Barrett's and OAC from resected oesophageal tissue. Tissue was taken from the top most layer of the oesophagus and scores represent a transverse view of pathology in a small section of the tissue. In contrast, mouse oesophageal tissue was cut and scored in a longitudinal manner, and therefore gives an overview of all the epithelium and stroma across the entire Barrett's region. The human tissue was also patient matched for the purpose of reducing confounding factors that may give false significance. However, it should be considered that adjacent tissue specimens taken from the same patient are influenced by the tumour and the associated tumour microenvironment.

Random biopsy sampling using the Seattle method during endoscopy is highly prone to error as on average only 3.5% of the Barrett's mucosa is assessed (666). Barrett's mapping is performed by sampling four-quadrant biopsies every 1-2 cm apart (Seattle protocol) (249). Acquisition of the Barrett's *ex vivo* explants used in this study was achieved using this method and highlights further limitations for research purposes. Comparisons between caspase inhibitor-treated and -untreated biopsies revealed heterogeneity between samples from individual patients, which hampered experimental read-out. Similar heterogeneity problems arise concerning organoid cultures derived from patient Barrett's tissue (unpublished, Quante's research lab, Munich). In contrast, organoid cultures taken from Barrett's mice were more consistent and less varied. Therefore, using mice may be more useful for initial preliminary

investigative experiments in order to identify molecular pathways involved in disease. However, for the same reasons the mouse model is limited as it is not representative of the variation expected in patients.

Like many murine models, disease in the pL2-IL-1 β model is induced by inflammation and is not generated by the same environmental factors leading to Barrett's in patients. This model is transgenic and assumes Barrett's aetiology is induced through inflammatory signalling alone (186, 263). Barrett's is known to be an inflammation-driven disease, initiated primarily through chronic gastric reflux exposure but also by a conglomeration of other contributing factors such as obesity, a HFD, smoking and hiatus hernia (232, 234, 249, 497, 667). The mouse model skips the initial environmental insult and therefore is not driving all the same pathways or may not involve all the same cell populations associated with Barrett's pathogenesis. Indeed, Quante's characterisation paper of this model assesses the gene expression profiles typically associated with Barrett's in patients against the mouse model. They reveal that the genetic profiles of mice and humans closely resemble each other, however differences were nonetheless evident as well (263). They identified 3832 significantly different genes in the mouse Barrett's mucosa and compared them to Barrett's patient mucosa, which showed 606 differently expressed genes in both mouse and human (263). Additionally, Barrett's patients have been shown to be genetically predisposed to developing Barrett's, a contributing risk factor that is not included in pL2-IL-1 β pathogenesis (668). Taking these factors into consideration, the phenotype in the mouse model is regarded as Barrett's-like features.

The most obvious difference between human and mouse models is the different physiologies of the stomach. In humans, the SCJ occurs at the GOJ where the end of the oesophageal tube meets the stomach. The oesophagus passes through the diaphragm at this region and the phrenoesophageal ligament connects to the outer oesophagus (219). In mice, the SCJ is located distally to the GOJ and mediates a border between a squamous epithelial forestomach and the columnar-lined stomach (186). These distinguishing characteristics need to be considered when deducting conclusions using a mouse model.

During the course of this study we were unable to determine the functional role of the inflammasome pathway during carcinogenesis. Differential expression levels of IL-1 β and IL-18 may contribute to alternating mechanisms of homeostasis and inflammation causing profound disease promoting or protecting effects. To further speculate on the possible functional role of the inflammasome, we looked at genetic studies examining inflammasome-related predispositions in Barrett's and OAC patients which showed IL-1, IL-18 and IL-18RAP polymorphisms were associated with disease susceptibility (443, 444, 669). It was difficult for the authors to derive what functional roles IL-1 β and IL-18 have in disease based on genetic data, however Babar *et al.* showed that the IL-18RAP rs917997C allele was strongly associated with protecting against BO and OAC development. The allele was found at significantly decreased frequencies in patients, compared to control individuals (444). Previous studies reported this allele correlated with elevated levels of IL-18RAP expression on the cell surface of peripheral blood leukocytes during celiac disease (670, 671). Therefore, a functional interpretation of this data might suggest that IL-18 signalling activity is reduced during Barrett's and OAC, causing a disease promoting effect. Indeed, in this study we observed that IL-18 secretion levels and mRNA from Barrett's patient tissue were reduced. Additionally, IL-18 deficiency in mice was reported to promote oesophageal squamous cell carcinoma (SCC) development. Deficient IL-18 oesophageal signalling caused reduced IFN- γ production and NK cell activation required for anti-tumour immunity (672). Interestingly, Quante's research group recently proposed that defective NK cell signalling accelerated Barrett's disease progression in HFD-fed pL2-IL-1 β mice (32). A HFD is known to promote Barrett's and we observed that oesophageal tissue from HFD-fed mice also secreted less IL-18 (32, 334). Together these findings highlight a potential protective role for IL-18 signalling through mediating anti-tumour responses in the oesophagus.

As mentioned, IL-18 is important for promoting epithelial barrier function, integrity and homeostasis. It has been shown to play an important protective role in colitis and CAC, which may also be relevant in the oesophageal mucosa (99, 100, 173, 475). IL-18 has been suggested as a regulator of goblet cell development as part of its role in gut homeostasis. To support this, one study observed increased levels of IL-18 inhibited

goblet cell maturation in the intestine in a concentration dependent manner (673). Similarly, Iyer *et al.* showed reduced IL-18 signalling led to increased goblet cell differentiation and mucous layer thickening (674). As Barrett's metaplasia is defined by the presence of goblet cells (245), we hypothesized that reduced IL-18 levels during Barrett's and obesity may pre-condition the oesophagus for spontaneous goblet cell maturation.

Barrett's is associated with altered epithelial barrier function and injury, which is proposed to be caused by factors including acid reflux and obesity (611). However, given the central role for IL-18 in epithelial barrier function, we propose that reduced levels of IL-18 in metaplastic epithelial cells represents a contributing factor to the decreased mucosal impedance observed in Barrett's epithelium (150, 675). Reduced impedance reflects the mucosal integrity in oesophageal tissue and has been shown to increase sensitivity and severity of reflux exposure (676, 677).

Similarly in obese mice, increased epithelial permeability has been reported in the oesophagus (506). Obesity has also been shown to impair epithelial permeability throughout the oesophagus of patients with and without reflux (506). The authors proposed that impaired epithelial barrier function potentially promotes inflammation and metaplasia. Indeed, other inflammatory oesophageal conditions such as GORD and eosinophilic oesophagitis have all been associated with impaired barrier function (678-682). IL-18 is important for epithelial homeostasis and barrier integrity, however a balance is required (98, 150, 683). Elevated levels of IL-18 signalling have also been associated with mediating harmful inflammation in the oesophagus and colon, therefore complicating its role in disease (673, 684, 685). Studies investigating the role of IL-18 during colitis have conflicting findings (673, 683), making it difficult to extrapolate a functional role of IL-18 that may be relevant in the oesophagus. Some of these studies have speculated that any beneficial or disease promoting effects of IL-18 are due to site specific expression in tissue (98, 150). This may also be the case in the oesophagus, however further studies are necessary before making any assumptions. Therefore, therapeutic administration of IL-18 should be considered during early oesophageal disease, but with caution.

An increasing number of studies have identified an important role for IL-18 in intestinal homeostasis, which is influenced by microbiome and dietary derived metabolites (96, 686, 687). A study by Macia *et al.* showed that dietary fibre mediated gut homeostasis and protected against DSS-colitis in mice through epithelial inflammasome-mediated IL-18 production (686). A zero-fibre diet given to DSS-colitis mice yielded very low to undetectable levels of cleaved caspase-1 and IL-18 expression in colon epithelial cells, compared to a high-fibre diet (686). A Westernised diet is typically characterised by a high fat, low fibre diet. This suggests a protective role for epithelial IL-18 expression in the oesophagus that is diet related (688). Epithelial IL-18 signalling has also been associated with responses to dietary nutrients, such as vitamin A. Using a mouse model that has specific retinoic acid receptor deficiency in intestinal epithelial cells, one study observed abrogated IL-18 signalling and reduced IFN- γ response (674). They showed that retinoic acid receptor-dependent IL-18 signalling was required for resistance to Salmonella infection in the gut, increased epithelial shedding and cell death of infected cells. Feedback of reconstituted IL-18 rescued the mice from early infection, however rescue did not occur with recombinant IFN- γ feedback (674). This further supports a diet-associated function of IL-18 in epithelial homeostasis of the gut, which may be relevant during oesophageal epithelium maintenance.

IL-1 β has been linked to the development of obesity-associated comorbidities, although paradoxically, IL-18 has been linked to alleviating obesity development and related disease (658, 659, 689, 690). By considering research regarding the systemic role of IL-18, we observe IL-18 functions in metabolism and obesity. IL-18 knockout mice were reported to have increased weight gain, which was reduced with administration of recombinant IL-18 (659). NLRP1-mediated IL-18 production has also been associated with preventing obesity and metabolic syndrome in mice (658). Spontaneous obesity, glucose intolerance and reduced IL-18 production occurred in NLRP1-deficient SFD-fed mice, which was further exacerbated by a HFD. Supporting this, NLRP1 mutant activation in mice caused reduced weight, cachexia and eventual death during a HFD, which was dependent on IL-18 signalling. The authors proposed that NLRP1 functions as a negative regulator of obesity and metabolic syndrome by mediating IL-18 signalling

following high energy intake (658). Together, these studies suggest that IL-18 acts as a regulator of weight gain and obesity in mice.

Contradicting these findings, elevated IL-18 circulating levels have been associated with overweight adults (691). Further, one study found that obese women had increased circulating plasma levels of IL-18, which were reduced following weight loss (692). These studies highlight differences observed between mice and humans. Notably, one study reported that elevated IL-18 levels were associated with metabolic syndrome and were independent of obesity, suggesting other factors may be influencing study findings in humans (693).

In opposition to the suggested protective role of IL-18, we speculate a disease promoting role for IL-1 β . Genetic evidence to support this is unclear and conflicting at this time, despite evident involvement of IL1 haplotypes in disease (443). As mentioned previously, during the CANTOS trial, blocking of IL-1 resulted in over 50% reductions in all cancers, increasing to over 70% reduction in lung cancer fatality (535). In the oesophagus, studies have reported that IL-1 β and IL-6 can alter and reduce oesophageal muscle contractility during oesophagitis, instigated by gastric juice exposure (694, 695). Additionally, during oesophageal SCC elevated IL-1 β levels were significantly correlated with poorer patient prognosis and advanced cancer stage (696). While IL-1 β -mediated inflammation alone is enough to induce the Barrett's-like phenotype in pL2-IL-1 β mice (263).

Opposing roles of IL-1 β versus IL-18 secretion during oesophageal disease has not been clearly reported before. However, a study by Zhu *et al.* has shown that IL-18 and IL-1 β have differential regulation. Type I IFN signalling and STAT1 were required for full IL-18 secretion, but not for IL-1 β secretion in BMDMs (697). This has interesting implications due to the ability of type I IFNs to inhibit both transcription and translation of IL-1 β and IL-1 α *in vivo* and *in vitro* (698-700). This relationship was first described in the 1990's and numerous studies have since confirmed this in different cell types (701-703). Indeed, this antagonistic relationship has also been described with type II and type III IFNs and likely occurs due to the divergent roles IFNs and IL-1s play during infections (399, 704, 705). Additionally, IL-18 was first described as an inducer of IFN- γ in NK cells, and is often associated with Th1 cell-type responses (706, 707). In contrast, Barrett's

inflammation has predominantly been characterised by humoral immunity and Th2 cell responses (95, 308). Furthermore, in HFD-fed mice we observed lower levels of IL-18 in oesophageal tissue during this study, while Quante's group observed reduced NK cells in the pL2-IL-1 β model (32). Together these findings reveal a humoral inflammatory phenotype that may contribute to carcinogenesis.

Notably the AIM2 inflammasome pathway can also be activated through type I IFN signalling and can positively regulate IL-1 β and IL-18 induction during certain infections and disease (69, 135). We have shown AIM2 is highly expressed during Barrett's progression and during a HFD, indicating inflammasome priming. However, we were unable to determine what inflammasome pathway IL-1 β and IL-18 secretion were dependent on. Based on our study and other research, AIM2 inflammasome activity may be important for regulating inflammation, however future work is needed to confirm this.

To conclude, this study has identified the inflammasome pathway as a mediator of inflammation during Barrett's metaplasia. We show this inflammation is further enhanced by a HFD and obesity, which may be contributing to disease progression. During this study, we used both *in vitro* and *ex vivo* models to assess inflammasome involvement, including a cell line model representing disease progression, a murine model of Barrett's oesophagus, Barrett's organoid cultures and patient tissue taken from both Barrett's and OAC patients. We observed that caspase-1 and AIM2 expression are highly elevated during Barrett's and expression is further enhanced during a HFD. IL-1 β secretion was elevated in Barrett's, while IL-18 secretion was reduced. TLR2 was also reported to mediate MIP-2 and CXCL1 signalling and may be important during disease progression. We propose that inflammasome signalling is important for driving inflammation during the early stages of oesophageal disease but may not be as active during cancer.

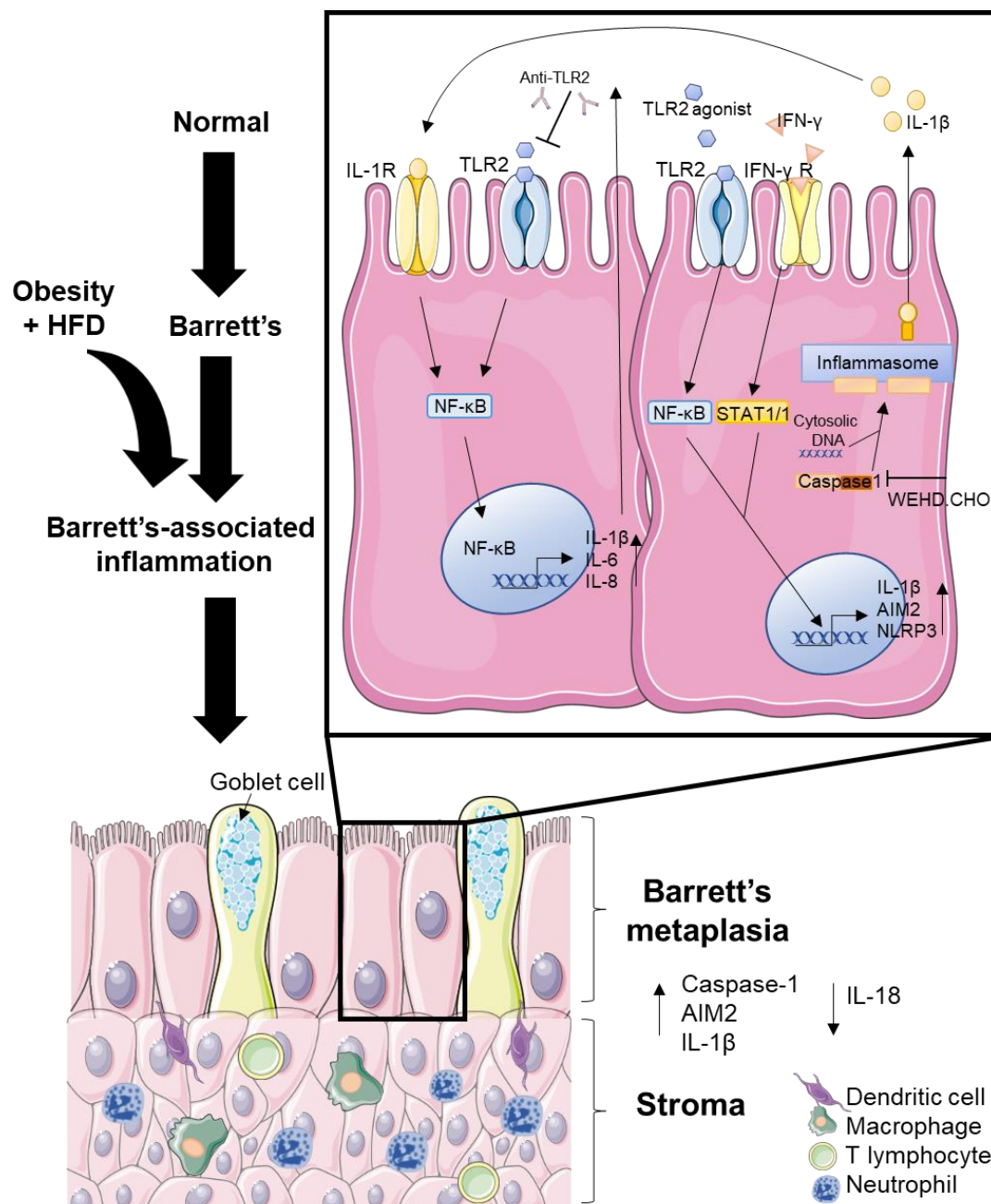


Figure 6.1: Schematic representing the main findings of this study.

Barrett's metaplastic tissue is characterised by elevated levels of inflammation, including increased caspase-1 and AIM2 expression. This inflammation is further enhanced by a HFD and obesity. Signalling through TLR2 and IFN receptors leads to activation of transcription factors, NF-κB and STAT1 homodimer, resulting in transcriptional upregulation of inflammasome components and inflammatory cytokines. Detection of cytosolic DNA and other DAMPs leads to the activation of the inflammasome and IL-1β release. Further induction of inflammatory processes subsequently occurs.

Table 6.1: Summarised results of key findings

Model		Molecule of interest			
		AIM2	Caspase-1	IL-1 β	IL-18
1.	Cell lines	Increased *	Increased **	Increased **	N/A
2.	Datasets	NS	NS	Increased ***	Decreased **
3.	Mouse				
i.	Normal tissue (HFD)	Increased **	Increased *	NS	Decreased *
ii.	Barrett's tissue (epithelium)	SFD: NS HFD: Increased *	SFD: Increased *** HFD: Increased *	N/A	N/A
4.	Patient tissue (epithelium)	N/A	Increased **	Increased **	Decreased **

Key: NS; non-significant, N/A; not available, SFD; standard fat diet, HFD; high fat diet.
Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

6.2 Future directions.

Findings from this study have shown that caspase-1 is upregulated and mediating inflammation during Barrett's. In attempt to identify which inflammasome pathway is important for mediating inflammation, we report that AIM2 expression is upregulated during disease progression and further during obese/HFD conditions in a Barrett's cell line, WT mouse oesophageal explants and in Barrett's pL2-IL-1 β tissue. It would be interesting to further investigate the role of AIM2 in oesophageal pathogenesis, as AIM2 has been reported to have inflammasome dependent and independent functions (135, 633).

The pL2-IL-1 β model functions by overexpressing mature human IL-1 β selectively in the normal squamous epithelium of the oral cavity, oesophagus and forestomach (263). Crossbreeding pL2-IL-1 β mice with AIM2^{-/-} KO mice could provide interesting revelations into the need for AIM2 for the spontaneous development of Barrett's metaplasia. Alternatively, selective conditional knockout of AIM2 expression in Lgr5⁺ cells using the Cre-Lox recombination system may provide further mechanistic insights. This method is tissue-specific and inducible. Therefore, knockout of AIM2 in columnar epithelial cells at a selected timepoint during disease would be possible. This would also not interfere with inflammasome-associated inflammatory signalling occurring in the normal squamous epithelium of the oesophagus. Additionally, as we showed that AIM2 expression is upregulated during a HFD in pL2-IL-1 β mice, further organoid culture would ideally include mice fed a SFD and HFD.

IL-18-dependent NLRP1 inflammasome signalling has been reported to prevent obesity and metabolic syndrome in mice (658). Using The Protein Atlas Repository, we have shown NLRP1 is expressed in patient oesophageal tissue (494). Therefore, it would be interesting to assess NLRP1 involvement during Barrett's and obesity-associated inflammation in a similar manner to how AIM2 and NLRP3 were assessed in this project.

During this study, we also demonstrated that TLR2 activity mediates signalling of IL-8 orthologs, MIP-2 and CXCL1 in Barrett's organoids. Further studies involving administration of the TLR2 blocking antibody, OPN301, throughout Barrett's disease progression in pL2-IL-1 β mice would reveal the therapeutic advantage of using this drug

as a therapy. Other ongoing research in our lab has shown TLR2 is important in mediating inflammatory cross talk between OAC cell lines and macrophages. Therefore, administration of OPN301 in mice that have developed OAC tumours could provide insights into the usefulness of this drug for preventing cancer. This would be carried out using a OAC model currently in development in Quante's research lab, involving pL2-IL-1 β mice with RB + P53 gene knock outs, KRAS gene knock in.

Similarly, it would be interesting to administer recombinant IL-18 to pL2-IL-1 β mice either for a long or short period of time *in vivo*, or by continuous administration during organoid culture. We show that IL-18 is reduced in Barrett's, therefore administration of IL-18 may rescue the mice from disease. Differences in associated inflammation and epithelial proliferation or integrity could be assessed through analysis of explant secretion profiles, transepithelial resistance, tissue histology, epithelial proliferation and cell death. An *ex vivo* culture of Barrett's patient biopsies with recombinant IL-18 could be similarly assessed.

In Chapter 5, we showed that TLR2 expression was very low in pL2-IL-1 β Barrett's tissue and did not increase with disease progression or a HFD. This was surprising considering we observed TLR2 activity through MIP-2 and CXCL1 secretion in pL2-IL-1 β mice organoid culture, indicating TLR2 expression. Additionally, published literature shows increasing TLR2 expression during disease progression in oesophageal patient tissue (316). Although these disparities may be due to the mice being bred in different facilities, we also consider the specificity of the OPN301 TLR2 antibody for use during IHC analysis. It would therefore be insightful to IHC stain pL2-IL-1 β Barrett's tissue with an alternative specific antibody, to confirm this.

Further findings from this study revealed that obesity is priming the inflammasome in oesophageal tissue during the early stages of disease. Future work investigating the possibility of a synergistic inflammatory relationship between obesity and gastric reflux that may cause enhanced activation of the inflammasome than either alone. The effect of bile acids alone on inflammasome activation and signalling may first be investigated, as this has not yet been documented in the literature. This would be followed by investigating the effect of combining both bile acid exposure and obesity. Preliminary experiments involving the stimulation of normal (Het-1A) and Barrett's (QH) cell lines

with bile acids and ACM should reveal whether the combination leads to enhanced inflammation and inflammasome activation.

References

1. Brubaker SW, Bonham KS, Zanoni I, Kagan JC. 2015. Innate Immune Pattern Recognition: A Cell Biological Perspective. *Annual review of immunology* 33: 257-90
2. Iwasaki A, Medzhitov R. 2010. Regulation of adaptive immunity by the innate immune system. *Science* 327: 291-5
3. Turvey SE, Broide DH. 2010. Innate immunity. *The Journal of allergy and clinical immunology* 125: S24-S32
4. Kieser KJ, Kagan JC. 2017. Multi-receptor detection of individual bacterial products by the innate immune system. *Nat Rev Immunol* 17: 376-90
5. Land WG. 2015. The Role of Damage-Associated Molecular Patterns (DAMPs) in Human Diseases: Part II: DAMPs as diagnostics, prognostics and therapeutics in clinical medicine. *Sultan Qaboos University Medical Journal* 15: e157-e70
6. Doria A, Zen M, Bettio S, Gatto M, Bassi N, Nalotto L, Ghirardello A, Iaccarino L, Punzi L. 2012. Autoinflammation and autoimmunity: bridging the divide. *Autoimmun Rev* 12: 22-30
7. Chiba T, Marusawa H, Ushijima T. 2012. Inflammation-associated cancer development in digestive organs: mechanisms and roles for genetic and epigenetic modulation. *Gastroenterology* 143: 550-63
8. Takeuchi O, Akira S. 2010. Pattern recognition receptors and inflammation. *Cell* 140: 805-20
9. Kawai T, Akira S. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 11: 373-84
10. Chiffolleau E. 2018. C-Type Lectin-Like Receptors As Emerging Orchestrators of Sterile Inflammation Represent Potential Therapeutic Targets. *Frontiers in immunology* 9: 227-
11. Takeda K, Kaisho T, Akira S. 2003. Toll-like receptors. *Annu Rev Immunol* 21: 335-76
12. Botos I, Segal DM, Davies DR. 2011. The structural biology of Toll-like receptors. *Structure (London, England : 1993)* 19: 447-59
13. Jin MS, Lee JO. 2008. Structures of the toll-like receptor family and its ligand complexes. *Immunity* 29: 182-91
14. Medzhitov R, Preston-Hurlburt P, Janeway CA. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388: 394-7
15. McClure R, Massari P. 2014. TLR-Dependent Human Mucosal Epithelial Cell Responses to Microbial Pathogens. *Frontiers in Immunology* 5: 386
16. Vidya MK, Kumar VG, Sejian V, Bagath M, Krishnan G, Bhatta R. 2018. Toll-like receptors: Significance, ligands, signaling pathways, and functions in mammals. *Int Rev Immunol* 37: 20-36
17. Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, Janeway CA, Jr. 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol Cell* 2: 253-8
18. Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies CA, Mansell AS, Brady G, Brint E, Dunne A, Gray P, Harte MT, McMurray D, Smith DE, Sims JE, Bird TA, O'Neill LA. 2001. Mal (MyD88-adaptor-like) is required for Toll-like receptor-4 signal transduction. *Nature* 413: 78-83
19. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S. 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 301: 640-3

20. Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, Monks B, Pitha PM, Golenbock DT. 2003. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med* 198: 1043-55
21. Bagchi A, Herrup EA, Warren HS, Trigilio J, Shin H-S, Valentine C, Hellman J. 2007. MyD88-Dependent and MyD88-Independent Pathways in Synergy, Priming, and Tolerance between TLR Agonists. *The Journal of Immunology* 178: 1164
22. Lin SC, Lo YC, Wu H. 2010. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. *Nature* 465: 885-90
23. Chen ZJ. 2005. Ubiquitin signalling in the NF-kappaB pathway. *Nat Cell Biol* 7: 758-65
24. Gao Q, Qi L, Wu T, Wang J. 2012. Clostridium butyricum activates TLR2-mediated MyD88-independent signaling pathway in HT-29 cells. *Mol Cell Biochem* 361: 31-7
25. Nilsen NJ, Vladimer GI, Stenvik J, Orning MPA, Zeid-Kilani MV, Bugge M, Bergstroem B, Conlon J, Husebye H, Hise AG, Fitzgerald KA, Espevik T, Lien E. 2015. A Role for the Adaptor Proteins TRAM and TRIF in Toll-like Receptor 2 Signaling. *Journal of Biological Chemistry* 290: 3209-22
26. Kawai T, Takeuchi O, Fujita T, Inoue J-i, Mühlradt PF, Sato S, Hoshino K, Akira S. 2001. Lipopolysaccharide Stimulates the MyD88-Independent Pathway and Results in Activation of IFN-Regulatory Factor 3 and the Expression of a Subset of Lipopolysaccharide-Inducible Genes. *The Journal of Immunology* 167: 5887
27. Doyle S, Vaidya S, O'Connell R, Dadgostar H, Dempsey P, Wu T, Rao G, Sun R, Haberland M, Modlin R, Cheng G. 2002. IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* 17: 251-63
28. Cusson-Hermance N, Khurana S, Lee TH, Fitzgerald KA, Kelliher MA. 2005. Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation. *J Biol Chem* 280: 36560-6
29. Kawai T, Akira S. 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 34: 637-50
30. Hug H, Mohajeri MH, La Fata G. 2018. Toll-Like Receptors: Regulators of the Immune Response in the Human Gut. *Nutrients* 10: 203
31. Huhta H, Helminen O, Kauppila JH, Salo T, Porvari K, Saarnio J, Lehenkari PP, Karttunen TJ. 2016. The Expression of Toll-like Receptors in Normal Human and Murine Gastrointestinal Organs and the Effect of Microbiome and Cancer. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* 64: 470-82
32. Munch NS, Fang HY, Ingermann J, Maurer HC, Anand A, Kellner V, Sahm V, Wiethaler M, Baumeister T, Wein F, Einwachter H, Bolze F, Klingenspor M, Haller D, Kavanagh M, Lysaght J, Friedman R, Dannenberg AJ, Pollak M, Holt PR, Muthupalani S, Fox JG, Whary MT, Lee Y, Ren TY, Elliot R, Fitzgerald R, Steiger K, Schmid RM, Wang TC, Quante M. 2019. High-Fat Diet Accelerates Carcinogenesis in a Mouse Model of Barrett's Esophagus via Interleukin 8 and Alterations to the Gut Microbiome. *Gastroenterology* 157: 492-506.e2
33. Kim HM, Park BS, Kim JI, Kim SE, Lee J, Oh SC, Enkhbayar P, Matsushima N, Lee H, Yoo OJ, Lee JO. 2007. Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. *Cell* 130: 906-17
34. Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. 2009. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* 458: 1191-5
35. Akashi-Takamura S, Miyake K. 2008. TLR accessory molecules. *Curr Opin Immunol* 20: 420-5

36. Kang JY, Nan X, Jin MS, Youn SJ, Ryu YH, Mah S, Han SH, Lee H, Paik SG, Lee JO. 2009. Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. *Immunity* 31: 873-84
37. Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, Lee H, Lee JO. 2007. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell* 130: 1071-82
38. van Bergenhenegouwen J, Plantinga TS, Joosten LA, Netea MG, Folkerts G, Kraneveld AD, Garssen J, Vos AP. 2013. TLR2 & Co: a critical analysis of the complex interactions between TLR2 and coreceptors. *J Leukoc Biol* 94: 885-902
39. Triantafilou M, Gamper FG, Haston RM, Mouratis MA, Morath S, Hartung T, Triantafilou K. 2006. Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting. *J Biol Chem* 281: 31002-11
40. Erridge C. 2010. Endogenous ligands of TLR2 and TLR4: agonists or assistants? *Journal of Leukocyte Biology* 87: 989-99
41. Gao B, Tsan MF. 2003. Recombinant human heat shock protein 60 does not induce the release of tumor necrosis factor alpha from murine macrophages. *J Biol Chem* 278: 22523-9
42. Bausinger H, Lipsker D, Ziylan U, Manie S, Briand JP, Cazenave JP, Muller S, Haeuw JF, Ravanat C, de la Salle H, Hanau D. 2002. Endotoxin-free heat-shock protein 70 fails to induce APC activation. *Eur J Immunol* 32: 3708-13
43. Youn JH, Oh YJ, Kim ES, Choi JE, Shin JS. 2008. High mobility group box 1 protein binding to lipopolysaccharide facilitates transfer of lipopolysaccharide to CD14 and enhances lipopolysaccharide-mediated TNF-alpha production in human monocytes. *J Immunol* 180: 5067-74
44. Hreggvidsdottir HS, Ostberg T, Wahamoa H, Schierbeck H, Aveberger AC, Klevenvall L, Palmblad K, Ottosson L, Andersson U, Harris HE. 2009. The alarmin HMGB1 acts in synergy with endogenous and exogenous danger signals to promote inflammation. *J Leukoc Biol* 86: 655-62
45. Lotz M, Ebert S, Esselmann H, Iliev AI, Prinz M, Wiazewicz N, Wiltfang J, Gerber J, Nau R. 2005. Amyloid beta peptide 1-40 enhances the action of Toll-like receptor-2 and -4 agonists but antagonizes Toll-like receptor-9-induced inflammation in primary mouse microglial cell cultures. *J Neurochem* 94: 289-98
46. Gasic-Milenkovic J, Dukic-Stefanovic S, Deuther-Conrad W, Gartner U, Munch G. 2003. Beta-amyloid peptide potentiates inflammatory responses induced by lipopolysaccharide, interferon -gamma and 'advanced glycation endproducts' in a murine microglia cell line. *Eur J Neurosci* 17: 813-21
47. Krejcova D, Pekarova M, Safrankova B, Kubala L. 2009. The effect of different molecular weight hyaluronan on macrophage physiology. *Neuro Endocrinol Lett* 30 Suppl 1: 106-11
48. Nadatani Y, Huo X, Zhang X, Yu C, Cheng E, Zhang Q, Dunbar KB, Theiss A, Pham TH, Wang DH, Watanabe T, Fujiwara Y, Arakawa T, Spechler SJ, Souza RF. 2016. NOD-like receptor protein 3 Inflammasome Priming and Activation In Barrett's Epithelial Cells. *Cellular and molecular gastroenterology and hepatology* 2: 439-53
49. Schroder K, Sagulenko V, Zamoshnikova A, Richards AA, Cridland JA, Irvine KM, Stacey KJ, Sweet MJ. 2012. Acute lipopolysaccharide priming boosts inflammasome activation independently of inflammasome sensor induction. *Immunobiology* 217: 1325-9
50. Wang J, Li R, Peng Z, Hu B, Rao X, Li J. 2019. HMGB1 participates in LPS-induced acute lung injury by activating the AIM2 inflammasome in macrophages and inducing polarization of M1 macrophages via TLR2, TLR4, and RAGE/NFkappaB signaling pathways. *Int J Mol Med*

51. Latz E, Xiao TS, Stutz A. 2013. Activation and regulation of the inflammasomes. *Nature reviews. Immunology* 13: 10.1038/nri3452
52. Ting JPY, Lovering RC, Alnemri ESP, Bertin J, Boss JM, Davis B, Flavell RA, Girardin SE, Godzik A, Harton JA, Hoffman HM, Hugot J-P, Inohara N, MacKenzie A, Maltais LJ, Nunez G, Ogura Y, Otten L, Reed JC, Reith W, Schreiber S, Steimle V, Ward PA. 2008. The NLR gene family: An official nomenclature. *Immunity* 28: 285-7
53. Koonin EV, Aravind L. 2000. The NACHT family - a new group of predicted NTPases implicated in apoptosis and MHC transcription activation. In *Trends Biochem Sci*, pp. 223-4. England
54. Motta V, Soares F, Sun T, Philpott DJ. 2015. NOD-like receptors: versatile cytosolic sentinels. *Physiol Rev* 95: 149-78
55. Kim YK, Shin J-S, Nahm MH. 2016. NOD-Like Receptors in Infection, Immunity, and Diseases. *Yonsei Medical Journal* 57: 5-14
56. Kobayashi K, Inohara N, Hernandez LD, Galan JE, Nunez G, Janeway CA, Medzhitov R, Flavell RA. 2002. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 416: 194-9
57. Girardin SE, Tournebise R, Mavris M, Page AL, Li X, Stark GR, Bertin J, DiStefano PS, Yaniv M, Sansonetti PJ, Philpott DJ. 2001. CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive *Shigella flexneri*. *EMBO Rep* 2: 736-42
58. Inohara N, Ogura Y, Fontalba A, Gutierrez O, Pons F, Crespo J, Fukase K, Inamura S, Kusumoto S, Hashimoto M, Foster SJ, Moran AP, Fernandez-Luna JL, Nunez G. 2003. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem* 278: 5509-12
59. Sabbah A, Chang TH, Harnack R, Frohlich V, Tominaga K, Dube PH, Xiang Y, Bose S. 2009. Activation of innate immune antiviral responses by Nod2. *Nat Immunol* 10: 1073-80
60. Shi J, Zhao Y, Wang K, Shi X, Wang Y, Huang H, Zhuang Y, Cai T, Wang F, Shao F. 2015. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* 526: 660-5
61. Connolly DJ, Bowie AG. 2014. The emerging role of human PYHIN proteins in innate immunity: implications for health and disease. *Biochem Pharmacol* 92: 405-14
62. Ludlow LE, Johnstone RW, Clarke CJ. 2005. The HIN-200 family: more than interferon-inducible genes? *Exp Cell Res* 308: 1-17
63. Albrecht M, Choubey D, Lengauer T. 2005. The HIN domain of IFI-200 proteins consists of two OB folds. *Biochem Biophys Res Commun* 327: 679-87
64. Khare S, Ratsimandresy RA, de Almeida L, Cuda CM, Rellick SL, Misharin AV, Wallin MC, Gangopadhyay A, Forte E, Gottwein E, Perlman H, Reed JC, Greaves DR, Dorfleutner A, Stehlik C. 2014. The PYRIN domain-only protein POP3 inhibits AIM2-like receptor inflammasomes and regulates responses to DNA virus infections. *Nature immunology* 15: 343-53
65. Hornung V, Latz E. 2010. Intracellular DNA recognition. *Nat Rev Immunol* 10: 123-30
66. Unterholzner L, Keating SE, Baran M, Horan KA, Jensen SB, Sharma S, Sirois CM, Jin T, Latz E, Xiao TS, Fitzgerald KA, Paludan SR, Bowie AG. 2010. IFI16 is an innate immune sensor for intracellular DNA. *Nat Immunol* 11: 997-1004
67. Kerur N, Veettil MV, Sharma-Walia N, Bottero V, Sadagopan S, Otageri P, Chandran B. 2011. IFI16 acts as a nuclear pathogen sensor to induce the inflammasome in response to Kaposi Sarcoma-associated herpesvirus infection. *Cell Host Microbe* 9: 363-75
68. Ansari MA, Singh VV, Dutta S, Veettil MV, Dutta D, Chikoti L, Lu J, Everly D, Chandran B. 2013. Constitutive Interferon-Inducible Protein 16-Inflammasome Activation during Epstein-Barr Virus Latency I, II, and III in B and Epithelial Cells. *Journal of Virology* 87: 8606

69. Rathinam VA, Jiang Z, Waggoner SN, Sharma S, Cole LE, Waggoner L, Vanaja SK, Monks BG, Ganesan S, Latz E, Hornung V, Vogel SN, Szomolanyi-Tsuda E, Fitzgerald KA. 2010. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol* 11: 395-402
70. Kim S, Bauernfeind F, Ablasser A, Hartmann G, Fitzgerald KA, Latz E, Hornung V. 2010. *Listeria monocytogenes* is sensed by the NLRP3 and AIM2 inflammasome. *Eur J Immunol* 40: 1545-51
71. Fernandes-Alnemri T, Yu JW, Juliana C, Solorzano L, Kang S, Wu J, Datta P, McCormick M, Huang L, McDermott E, Eisenlohr L, Landel CP, Alnemri ES. 2010. The AIM2 inflammasome is critical for innate immunity to *Francisella tularensis*. *Nat Immunol* 11: 385-93
72. Shalini S, Dorstyn L, Dawar S, Kumar S. 2015. Old, new and emerging functions of caspases. *Cell Death and Differentiation* 22: 526-39
73. Kumar S. 2007. Caspase function in programmed cell death. *Cell Death Differ* 14: 32-43
74. Creagh EM. 2014. Caspase crosstalk: integration of apoptotic and innate immune signalling pathways. *Trends in Immunology* 35: 631-40
75. Mascarenhas DPA, Cerqueira DM, Pereira MSF, Castanheira FVS, Fernandes TD, Manin GZ, Cunha LD, Zamboni DS. 2017. Inhibition of caspase-1 or gasdermin-D enable caspase-8 activation in the Naip5/NLRC4/ASC inflammasome. *PLoS Pathog* 13: e1006502
76. Gurung P, Kanneganti T-D. 2015. Novel Roles for Caspase-8 in IL-1 β and Inflammasome Regulation. *The American Journal of Pathology* 185: 17-25
77. Man SM, Karki R, Kanneganti T-D. 2017. Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases. *Immunological reviews* 277: 61-75
78. Kostura MJ, Tocci MJ, Limjuco G, Chin J, Cameron P, Hillman AG, Chartrain NA, Schmidt JA. 1989. Identification of a monocyte specific pre-interleukin 1 beta convertase activity. *Proceedings of the National Academy of Sciences of the United States of America* 86: 5227-31
79. Miller DK, Ayala JM, Egger LA, Raju SM, Yamin TT, Ding GJ, Gaffney EP, Howard AD, Palyha OC, Rolando AM, et al. 1993. Purification and characterization of active human interleukin-1 beta-converting enzyme from THP.1 monocytic cells. *J Biol Chem* 268: 18062-9
80. Yang Y, Wang H, Kouadir M, Song H, Shi F. 2019. Recent advances in the mechanisms of NLRP3 inflammasome activation and its inhibitors. *Cell Death & Disease* 10: 128
81. Feltham R, Vince JE, Lawlor KE. 2017. Caspase-8: not so silently deadly. *Clin Trans Immunol* 6: e124
82. Karan D. 2018. Inflammasomes: Emerging Central Players in Cancer Immunology and Immunotherapy. *Frontiers in immunology* 9: 3028-
83. Jin T, Perry A, Smith P, Jiang J, Xiao TS. 2013. Structure of the absent in melanoma 2 (AIM2) pyrin domain provides insights into the mechanisms of AIM2 autoinhibition and inflammasome assembly. *J Biol Chem* 288: 13225-35
84. Hu Z, Zhou Q, Zhang C, Fan S, Cheng W, Zhao Y, Shao F, Wang H-W, Sui S-F, Chai J. 2015. Structural and biochemical basis for induced self-propagation of NLRC4. *Science* 350: 399
85. Martinon F, Burns K, Tschopp J. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 10: 417-26
86. Jin T, Perry A, Jiang J, Smith P, Curry James A, Unterholzner L, Jiang Z, Horvath G, Rathinam Vijay A, Johnstone Ricky W, Hornung V, Latz E, Bowie Andrew G, Fitzgerald Katherine A, Xiao TS. 2012. Structures of the HIN Domain:DNA Complexes Reveal

- Ligand Binding and Activation Mechanisms of the AIM2 Inflammasome and IFI16 Receptor. *Immunity* 36: 561-71
87. Salvesen GS, Dixit VM. 1999. Caspase activation: The induced-proximity model. *Proceedings of the National Academy of Sciences* 96: 10964
 88. Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J, et al. 1992. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature* 356: 768-74
 89. Datta D, McClendon CL, Jacobson MP, Wells JA. 2013. Substrate and inhibitor-induced dimerization and cooperativity in caspase-1 but not caspase-3. *J Biol Chem* 288: 9971-81
 90. Walsh JG, Logue SE, Luthi AU, Martin SJ. 2011. Caspase-1 promiscuity is counterbalanced by rapid inactivation of processed enzyme. *J Biol Chem* 286: 32513-24
 91. Boucher D, Monteleone M, Coll RC, Chen KW, Ross CM, Teo JL, Gomez GA, Holley CL, Bierschenk D, Stacey KJ, Yap AS, Bezbradica JS, Schroder K. 2018. Caspase-1 self-cleavage is an intrinsic mechanism to terminate inflammasome activity. *J Exp Med* 215: 827-40
 92. Afonina IS, Muller C, Martin SJ, Beyaert R. 2015. Proteolytic Processing of Interleukin-1 Family Cytokines: Variations on a Common Theme. *Immunity* 42: 991-1004
 93. Fitzgerald RC, Abdalla S, Onwuegbusi BA, Sirieix P, Saeed IT, Burnham WR, Farthing MJG. 2002. Inflammatory gradient in Barrett's oesophagus: implications for disease complications. *Gut* 51: 316-22
 94. Vounotrypidis P, Kouklakis G, Anagnostopoulos K, Zazos P, Polychronidis A, Maltezos E, Efremidou E, Pitiakoudis M, Lyratzopoulos N. 2013. Interleukin-1 associations in inflammatory bowel disease and the enteropathic seronegative spondylarthritis. *Auto-Immunity Highlights* 4: 87-94
 95. Kavanagh ME, Conroy MJ, Clarke NE, Gilmartin NT, O'Sullivan KE, Feighery R, MacCarthy F, O'Toole D, Ravi N, Reynolds JV, O'Sullivan J, Lysaght J. 2016. Impact of the inflammatory microenvironment on T-cell phenotype in the progression from reflux oesophagitis to Barrett oesophagus and oesophageal adenocarcinoma. *Cancer Lett* 370: 117-24
 96. Yasuda K, Nakanishi K, Tsutsui H. 2019. Interleukin-18 in Health and Disease. *International journal of molecular sciences* 20: 649
 97. Baker KJ, Houston A, Brint E. 2019. IL-1 Family Members in Cancer; Two Sides to Every Story. *Frontiers in Immunology* 10: 1197
 98. Siegmund B. 2010. Interleukin-18 in intestinal inflammation: friend and foe? *Immunity* 32: 300-2
 99. Fabbi M, Carbotti G, Ferrini S. 2014. Context-dependent role of IL-18 in cancer biology and counter-regulation by IL-18BP. *Journal of Leukocyte Biology* 97: 665-75
 100. Dinarello CA. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol* 27: 519-50
 101. He WT, Wan H, Hu L, Chen P, Wang X, Huang Z, Yang ZH, Zhong CQ, Han J. 2015. Gasdermin D is an executor of pyroptosis and required for interleukin-1beta secretion. *Cell Res* 25: 1285-98
 102. Sollberger G, Strittmatter GE, Garstkiewicz M, Sand J, Beer H-D. 2013. Caspase-1: The inflammasome and beyond. *Innate Immunity* 20: 115-25
 103. Sharma D, Kanneganti T-D. 2016. The cell biology of inflammasomes: Mechanisms of inflammasome activation and regulation. *The Journal of Cell Biology* 213: 617

104. Elliott EI, Miller AN, Banoth B, Iyer SS, Stotland A, Weiss JP, Gottlieb RA, Sutterwala FS, Cassel SL. 2018. Cutting Edge: Mitochondrial Assembly of the NLRP3 Inflammasome Complex Is Initiated at Priming. *J Immunol* 200: 3047-52
105. Elliott EI, Sutterwala FS. 2015. Initiation and perpetuation of NLRP3 inflammasome activation and assembly. *Immunol Rev* 265: 35-52
106. Song N, Liu Z-S, Xue W, Bai Z-F, Wang Q-Y, Dai J, Liu X, Huang Y-J, Cai H, Zhan X-Y, Han Q-Y, Wang H, Chen Y, Li H-Y, Li A-L, Zhang X-M, Zhou T, Li T. 2017. NLRP3 Phosphorylation Is an Essential Priming Event for Inflammasome Activation. *Molecular Cell* 68: 185-97.e6
107. Abderrazak A, Syrovets T, Couchie D, El Hadri K, Friguet B, Simmet T, Rouis M. 2015. NLRP3 inflammasome: From a danger signal sensor to a regulatory node of oxidative stress and inflammatory diseases. *Redox Biology* 4: 296-307
108. Carty M, Kearney J, Shanahan KA, Hams E, Sugisawa R, Connolly D, Doran CG, Munoz-Wolf N, Gurtler C, Fitzgerald KA, Lavelle EC, Fallon PG, Bowie AG. 2019. Cell Survival and Cytokine Release after Inflammasome Activation Is Regulated by the Toll-IL-1R Protein SARM. *Immunity* 50: 1412-24.e6
109. Munoz-Planillo R, Kuffa P, Martinez-Colon G, Smith BL, Rajendiran TM, Nunez G. 2013. K(+) efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* 38: 1142-53
110. He Y, Zeng MY, Yang D, Motro B, Nunez G. 2016. NEK7 is an essential mediator of NLRP3 activation downstream of potassium efflux. *Nature* 530: 354-7
111. Shi H, Wang Y, Li X, Zhan X, Tang M, Fina M, Su L, Pratt D, Bu CH, Hildebrand S, Lyon S, Scott L, Quan J, Sun Q, Russell J, Arnett S, Jurek P, Chen D, Kravchenko VV, Mathison JC, Moresco EMY, Monson NL, Ulevitch RJ, Beutler B. 2016. NLRP3 activation and mitosis are mutually exclusive events coordinated by NEK7, a new inflammasome component. *Nature Immunology* 17: 250-8
112. Wolf AJ, Reyes CN, Liang W, Becker C, Shimada K, Wheeler ML, Cho HC, Popescu NI, Coggeshall KM, Arditi M, Underhill DM. 2016. Hexokinase Is an Innate Immune Receptor for the Detection of Bacterial Peptidoglycan. *Cell* 166: 624-36
113. Gaidt MM, Ebert TS, Chauhan D, Schmidt T, Schmid-Burgk JL, Rapino F, Robertson AA, Cooper MA, Graf T, Hornung V. 2016. Human Monocytes Engage an Alternative Inflammasome Pathway. *Immunity* 44: 833-46
114. Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, Latz E, Fitzgerald KA. 2009. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* 458: 514-8
115. Dombrowski Y, Peric M, Koglin S, Kammerbauer C, Göss C, Anz D, Simanski M, Gläser R, Harder J, Hornung V, Gallo RL, Ruzicka T, Besch R, Schaubert J. 2011. Cytosolic DNA triggers inflammasome activation in keratinocytes in psoriatic lesions. *Science translational medicine* 3: 82ra38-82ra38
116. Di Micco A, Frera G, Lugrin J, Jamilloux Y, Hsu E-T, Tardivel A, De Gassart A, Zaffalon L, Bujisic B, Siegert S, Quadroni M, Broz P, Henry T, Hrycyna CA, Martinon F. 2016. AIM2 inflammasome is activated by pharmacological disruption of nuclear envelope integrity. *Proceedings of the National Academy of Sciences* 113: E4671
117. Kawane K, Motani K, Nagata S. 2014. DNA degradation and its defects. *Cold Spring Harb Perspect Biol* 6
118. Sharma BR, Karki R, Kanneganti T-D. 2019. Role of AIM2 inflammasome in inflammatory diseases, cancer and infection. *European Journal of Immunology* 49: 1998-2011
119. DeYoung KL, Ray ME, Su YA, Anzick SL, Johnstone RW, Trapani JA, Meltzer PS, Trent JM. 1997. Cloning a novel member of the human interferon-inducible gene family

- associated with control of tumorigenicity in a model of human melanoma. *Oncogene* 15: 453-7
120. Lugrin J, Martinon F. 2018. The AIM2 inflammasome: Sensor of pathogens and cellular perturbations. *Immunological Reviews* 281: 99-114
 121. Hakimi M, Peters A, Becker A, Böckler D, Dihlmann S. 2014. Inflammation-related induction of absent in melanoma 2 (AIM2) in vascular cells and atherosclerotic lesions suggests a role in vascular pathogenesis. *Journal of Vascular Surgery* 59: 794-803.e2
 122. Pan J, Han L, Guo J, Wang X, Liu D, Tian J, Zhang M, An F. 2018. AIM2 accelerates the atherosclerotic plaque progressions in ApoE^{-/-} mice. *Biochemical and Biophysical Research Communications* 498: 487-94
 123. Brunette RL, Young JM, Whitley DG, Brodsky IE, Malik HS, Stetson DB. 2012. Extensive evolutionary and functional diversity among mammalian AIM2-like receptors. *J Exp Med* 209: 1969-83
 124. Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES. 2009. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature* 458: 509-13
 125. Komada T, Chung H, Lau A, Platnich JM, Beck PL, Benediktsson H, Duff HJ, Jenne CN, Muruve DA. 2018. Macrophage Uptake of Necrotic Cell DNA Activates the AIM2 Inflammasome to Regulate a Proinflammatory Phenotype in CKD. *J Am Soc Nephrol* 29: 1165-81
 126. Paulin N, Viola Joana R, Maas Sanne L, de Jong R, Fernandes-Alnemri T, Weber C, Drechsler M, Döring Y, Soehnlein O. 2018. Double-Strand DNA Sensing Aim2 Inflammasome Regulates Atherosclerotic Plaque Vulnerability. *Circulation* 138: 321-3
 127. Hu B, Jin C, Li H-B, Tong J, Ouyang X, Cetinbas NM, Zhu S, Strowig T, Lam FC, Zhao C, Henao-Mejia J, Yilmaz O, Fitzgerald KA, Eisenbarth SC, Elinav E, Flavell RA. 2016. The DNA-sensing AIM2 inflammasome controls radiation-induced cell death and tissue injury. *Science* 354: 765
 128. Cresswell KS, Clarke CJP, Jackson JT, Darcy PK, Trapani JA, Johnstone RW. 2005. Biochemical and growth regulatory activities of the HIN-200 family member and putative tumor suppressor protein, AIM2. *Biochemical and Biophysical Research Communications* 326: 417-24
 129. Yeung CY, Tso AW, Xu A, Wang Y, Woo YC, Lam TH, Lo SV, Fong CH, Wat NM, Woo J, Cheung BM, Lam KS. 2013. Pro-inflammatory adipokines as predictors of incident cancers in a Chinese cohort of low obesity prevalence in Hong Kong. *PLoS One* 8: e78594
 130. Sun L, Wu J, Du F, Chen X, Chen ZJ. 2013. Cyclic GMP-AMP Synthase Is a Cytosolic DNA Sensor That Activates the Type I Interferon Pathway. *Science* 339: 786
 131. Luecke S, Holleufer A, Christensen MH, Jønsson KL, Boni GA, Sørensen LK, Johannsen M, Jakobsen MR, Hartmann R, Paludan SR. 2017. cGAS is activated by DNA in a length-dependent manner. *EMBO reports* 18: 1707-15
 132. Ishikawa H, Barber GN. 2008. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* 455: 674-8
 133. Ishii KJ, Coban C, Kato H, Takahashi K, Torii Y, Takeshita F, Ludwig H, Sutter G, Suzuki K, Hemmi H, Sato S, Yamamoto M, Uematsu S, Kawai T, Takeuchi O, Akira S. 2006. A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. *Nature Immunology* 7: 40-8
 134. Stetson DB, Medzhitov R. 2006. Recognition of Cytosolic DNA Activates an IRF3-Dependent Innate Immune Response. *Immunity* 24: 93-103
 135. Man SM, Karki R, Kanneganti T-D. 2016. AIM2 inflammasome in infection, cancer, and autoimmunity: Role in DNA sensing, inflammation, and innate immunity. *European journal of immunology* 46: 269-80

136. Cheon H, Holvey-Bates EG, Schoggins JW, Forster S, Hertzog P, Imanaka N, Rice CM, Jackson MW, Junk DJ, Stark GR. 2013. IFN β -dependent increases in STAT1, STAT2, and IRF9 mediate resistance to viruses and DNA damage. *Embo j* 32: 2751-63
137. Ivashkiv LB, Donlin LT. 2014. Regulation of type I interferon responses. *Nat Rev Immunol* 14: 36-49
138. Meunier E, Wallet P, Dreier RF, Costanzo S, Anton L, Ruhl S, Dussurgey S, Dick MS, Kistner A, Rigard M, Degrandi D, Pfeffer K, Yamamoto M, Henry T, Broz P. 2015. Guanylate-binding proteins promote activation of the AIM2 inflammasome during infection with *Francisella novicida*. *Nat Immunol* 16: 476-84
139. Man SM, Karki R, Malireddi RK, Neale G, Vogel P, Yamamoto M, Lamkanfi M, Kanneganti TD. 2015. The transcription factor IRF1 and guanylate-binding proteins target activation of the AIM2 inflammasome by *Francisella* infection. *Nat Immunol* 16: 467-75
140. Shi J, Zhao Y, Wang Y, Gao W, Ding J, Li P, Hu L, Shao F. 2014. Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature* 514: 187-92
141. Yi YS. 2017. Caspase-11 non-canonical inflammasome: a critical sensor of intracellular lipopolysaccharide in macrophage-mediated inflammatory responses. *Immunology* 152: 207-17
142. Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, Newton K, Qu Y, Liu J, Heldens S, Zhang J, Lee WP, Roose-Girma M, Dixit VM. 2011. Non-canonical inflammasome activation targets caspase-11. *Nature* 479: 117-21
143. Kayagaki N, Stowe IB, Lee BL, O'Rourke K, Anderson K, Warming S, Cuellar T, Haley B, Roose-Girma M, Phung QT, Liu PS, Lill JR, Li H, Wu J, Kummerfeld S, Zhang J, Lee WP, Snipas SJ, Salvesen GS, Morris LX, Fitzgerald L, Zhang Y, Bertram EM, Goodnow CC, Dixit VM. 2015. Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature* 526: 666
144. Yang D, He Y, Munoz-Planillo R, Liu Q, Nunez G. 2015. Caspase-11 Requires the Pannexin-1 Channel and the Purinergic P2X7 Pore to Mediate Pyroptosis and Endotoxic Shock. *Immunity* 43: 923-32
145. Pilla DM, Hagar JA, Halder AK, Mason AK, Degrandi D, Pfeffer K, Ernst RK, Yamamoto M, Miao EA, Coers J. 2014. Guanylate binding proteins promote caspase-11-dependent pyroptosis in response to cytoplasmic LPS. *Proc Natl Acad Sci U S A* 111: 6046-51
146. Yang J, Zhao Y, Shao F. 2015. Non-canonical activation of inflammatory caspases by cytosolic LPS in innate immunity. *Curr Opin Immunol* 32: 78-83
147. Shin HJ, Lee H, Park JD, Hyun HC, Sohn HO, Lee DW, Kim YS. 2007. Kinetics of binding of LPS to recombinant CD14, TLR4, and MD-2 proteins. *Mol Cells* 24: 119-24
148. Broz P, Ruby T, Belhocine K, Bouley DM, Kayagaki N, Dixit VM, Monack DM. 2012. Caspase-11 increases susceptibility to *Salmonella* infection in the absence of caspase-1. *Nature* 490: 288-91
149. Rathinam VAK, Vanaja SK, Waggoner L, Sokolovska A, Becker C, Stuart LM, Leong JM, Fitzgerald KA. 2012. TRIF Licenses Caspase-11-Dependent NLRP3 Inflammasome Activation by Gram-Negative Bacteria. *Cell* 150: 606-19
150. Oficjalska K, Raverdeau M, Aviello G, Wade SC, Hickey A, Sheehan KM, Corr SC, Kay EW, O'Neill LA, Mills KHG, Creagh EM. 2014. Protective Role for Caspase-11 during Acute Experimental Murine Colitis. *The Journal of Immunology*
151. Chen R, Zeng L, Zhu S, Liu J, Zeh HJ, Kroemer G, Wang H, Billiar TR, Jiang J, Tang D, Kang R. 2019. cAMP metabolism controls caspase-11 inflammasome activation and pyroptosis in sepsis. *Science Advances* 5: eaav5562

152. Vigano E, Diamond CE, Spreafico R, Balachander A, Sobota RM, Mortellaro A. 2015. Human caspase-4 and caspase-5 regulate the one-step non-canonical inflammasome activation in monocytes. *Nat Commun* 6: 8761
153. Casson CN, Yu J, Reyes VM, Taschuk FO, Yadav A, Copenhaver AM, Nguyen HT, Collman RG, Shin S. 2015. Human caspase-4 mediates noncanonical inflammasome activation against gram-negative bacterial pathogens. *Proc Natl Acad Sci U S A* 112: 6688-93
154. Lin XY, Choi MS, Porter AG. 2000. Expression analysis of the human caspase-1 subfamily reveals specific regulation of the CASP5 gene by lipopolysaccharide and interferon-gamma. *J Biol Chem* 275: 39920-6
155. Sollberger G, Strittmatter GE, Kistowska M, French LE, Beer HD. 2012. Caspase-4 is required for activation of inflammasomes. *J Immunol* 188: 1992-2000
156. Tschoop J, Martinon F, Burns K. 2003. NALPs: a novel protein family involved in inflammation. *Nat Rev Mol Cell Biol* 4: 95-104
157. Hanahan D, Weinberg Robert A. 2011. Hallmarks of Cancer: The Next Generation. *Cell* 144: 646-74
158. Dvorak HF. 2015. Tumors: Wounds that do not heal--Redux. *Cancer immunology research* 3: 1-11
159. Dvorak HF. 1986. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 315: 1650-9
160. Long AG, Lundsmith ET, Hamilton KE. 2017. Inflammation and Colorectal Cancer. *Current colorectal cancer reports* 13: 341-51
161. Askling J, Dickman PW, Karlen P, Brostrom O, Lapidus A, Lofberg R, Ekblom A. 2001. Family history as a risk factor for colorectal cancer in inflammatory bowel disease. *Gastroenterology* 120: 1356-62
162. Choi C-HR, Al Bakir I, Ding N-S, Lee G-H, Askari A, Warusavitarne J, Moorghen M, Humphries A, Ignjatovic-Wilson A, Thomas-Gibson S, Saunders BP, Rutter MD, Graham TA, Hart AL. 2019. Cumulative burden of inflammation predicts colorectal neoplasia risk in ulcerative colitis: a large single-centre study. *Gut* 68: 414
163. Gillen CD, Walmsley RS, Prior P, Andrews HA, Allan RN. 1994. Ulcerative colitis and Crohn's disease: a comparison of the colorectal cancer risk in extensive colitis. *Gut* 35: 1590-2
164. Meira LB, Bugni JM, Green SL, Lee CW, Pang B, Borenshtein D, Rickman BH, Rogers AB, Moroski-Erkul CA, McFaline JL, Schauer DB, Dedon PC, Fox JG, Samson LD. 2008. DNA damage induced by chronic inflammation contributes to colon carcinogenesis in mice. *J Clin Invest* 118: 2516-25
165. Terzić J, Grivennikov S, Karin E, Karin M. 2010. Inflammation and Colon Cancer. *Gastroenterology* 138: 2101-14.e5
166. Shaked H, Hofseth LJ, Chumanevich A, Chumanevich AA, Wang J, Wang Y, Taniguchi K, Guma M, Shenouda S, Clevers H, Harris CC, Karin M. 2012. Chronic epithelial NF-kappaB activation accelerates APC loss and intestinal tumor initiation through iNOS up-regulation. *Proc Natl Acad Sci U S A* 109: 14007-12
167. Tervaniemi MH, Katayama S, Skoog T, Siitonen HA, Vuola J, Nuutila K, Sormunen R, Johnsson A, Linnarsson S, Suomela S, Kankuri E, Kere J, Elomaa O. 2016. NOD-like receptor signaling and inflammasome-related pathways are highlighted in psoriatic epidermis. *Scientific Reports* 6: 22745
168. Mortimer L, Moreau F, MacDonald JA, Chadee K. 2016. NLRP3 inflammasome inhibition is disrupted in a group of auto-inflammatory disease CAPS mutations. *Nat Immunol* 17: 1176-86

169. Kopalli SR, Kang TB, Lee KH, Koppula S. 2018. NLRP3 Inflammasome Activation Inhibitors in Inflammation-Associated Cancer Immunotherapy: An Update on the Recent Patents. *Recent Pat Anticancer Drug Discov* 13: 106-17
170. Harrison OJ, Srinivasan N, Pott J, Schiering C, Krausgruber T, Ilott NE, Maloy KJ. 2015. Epithelial-derived IL-18 regulates Th17 cell differentiation and Foxp3(+) Treg cell function in the intestine. *Mucosal Immunol* 8: 1226-36
171. Grivennikov SI. 2013. Inflammation and colorectal cancer: colitis-associated neoplasia. *Seminars in immunopathology* 35: 229-44
172. Allen IC, TeKippe EM, Woodford RM, Uronis JM, Holl EK, Rogers AB, Herfarth HH, Jobin C, Ting JP. 2010. The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. *J Exp Med* 207: 1045-56
173. Zaki MH, Boyd KL, Vogel P, Kastan MB, Lamkanfi M, Kanneganti TD. 2010. The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. *Immunity* 32: 379-91
174. Flood B, Manils J, Nulty C, Flis E, Kenealy S, Barber G, Fay J, Mills KHG, Kay EW, Creagh EM. 2018. Caspase-11 regulates the tumour suppressor function of STAT1 in a murine model of colitis-associated carcinogenesis. *Oncogene*
175. Hu B, Elinav E, Huber S, Booth CJ, Strowig T, Jin C, Eisenbarth SC, Flavell RA. 2010. Inflammation-induced tumorigenesis in the colon is regulated by caspase-1 and NLRC4. *Proc Natl Acad Sci U S A* 107: 21635-40
176. Drexler SK, Bonsignore L, Masin M, Tardivel A, Jackstadt R, Hermeking H, Schneider P, Gross O, Tschopp J, Yazdi AS. 2012. Tissue-specific opposing functions of the inflammasome adaptor ASC in the regulation of epithelial skin carcinogenesis. *Proc Natl Acad Sci U S A* 109: 18384-9
177. Chow MT, Tschopp J, Moller A, Smyth MJ. 2012. NLRP3 promotes inflammation-induced skin cancer but is dispensable for asbestos-induced mesothelioma. *Immunol Cell Biol* 90: 983-6
178. Kolb R, Phan L, Borchering N, Liu Y, Yuan F, Janowski AM, Xie Q, Markan KR, Li W, Potthoff MJ, Fuentes-Mattei E, Ellies LG, Knudson CM, Lee MH, Yeung SJ, Cassel SL, Sutterwala FS, Zhang W. 2016. Obesity-associated NLRC4 inflammasome activation drives breast cancer progression. *Nat Commun* 7: 13007
179. Lin C, Zhang J. 2017. Inflammasomes in Inflammation-Induced Cancer. *Frontiers in Immunology* 8: 271
180. Resende C, Regalo G, Durães C, Pinto MT, Wen X, Figueiredo C, Carneiro F, Machado JC. 2016. Interleukin-1B signalling leads to increased survival of gastric carcinoma cells through a CREB-C/EBPβ-associated mechanism. *Gastric Cancer* 19: 74-84
181. Tu S, Bhagat G, Cui G, Takaishi S, Kurt-Jones EA, Rickman B, Betz KS, Penz-Oesterreicher M, Bjorkdahl O, Fox JG, Wang TC. 2008. Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. *Cancer Cell* 14: 408-19
182. Shimada M, Ando T, Peek RM, Watanabe O, Ishiguro K, Maeda O, Ishikawa D, Hasegawa M, Ina K, Ohmiya N, Niwa Y, Goto H. 2008. Helicobacter pylori infection upregulates interleukin-18 production from gastric epithelial cells. *Eur J Gastroenterol Hepatol* 20: 1144-50
183. El-Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF, Jr., Rabkin CS. 2000. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 404: 398-402
184. El-Omar EM. 2001. The importance of interleukin 1beta in Helicobacter pylori associated disease. *Gut* 48: 743-7

185. Kavanagh ME, O'Sullivan KE, O'Hanlon C, O'Sullivan JN, Lysaght J, Reynolds JV. 2014. The esophagitis to adenocarcinoma sequence; the role of inflammation. *Cancer Letters* 345: 182-9
186. Quante M, Abrams JA, Lee Y, Wang TC. 2012. Barrett esophagus: what a mouse model can teach us about human disease. *Cell Cycle* 11: 4328-38
187. Siegel RL, Miller KD, Jemal A. 2017. Cancer statistics, 2017. *CA: A Cancer Journal for Clinicians* 67: 7-30
188. Brown LM, Devesa SS, Chow WH. 2008. Incidence of adenocarcinoma of the esophagus among white Americans by sex, stage, and age. *J Natl Cancer Inst* 100: 1184-7
189. Thrumurthy SG, Chaudry MA, Thrumurthy SSD, Mughal M. 2019. Oesophageal cancer: risks, prevention, and diagnosis. *BMJ* 366: l4373
190. Offman J, Pesola F, Sasieni P. 2018. Trends and projections in adenocarcinoma and squamous cell carcinoma of the oesophagus in England from 1971 to 2037. *British journal of cancer* 118: 1391-8
191. Castellsague X, Munoz N, De Stefani E, Victora CG, Castelletto R, Rolon PA, Quintana MJ. 1999. Independent and joint effects of tobacco smoking and alcohol drinking on the risk of esophageal cancer in men and women. *Int J Cancer* 82: 657-64
192. NHS D. 2013. *Statistics on Smoking*, The Health and Social Care Information Centre, England
193. Ireland. NCR. 2011. *Cancer trends, no 8: cancers of the oesophagus and stomach.*, National Cancer Registry Ireland.
194. Xie SH, Lagergren J. 2016. A global assessment of the male predominance in esophageal adenocarcinoma. *Oncotarget* 7: 38876-83
195. Xie S-H, Lagergren J. 2016. The Male Predominance in Esophageal Adenocarcinoma. *Clinical Gastroenterology and Hepatology* 14: 338-47.e1
196. Cronin-Fenton DP, Murray LJ, Whiteman DC, Cardwell C, Webb PM, Jordan SJ, Corley DA, Sharp L, Lagergren J. 2010. Reproductive and sex hormonal factors and oesophageal and gastric junction adenocarcinoma: a pooled analysis. *Eur J Cancer* 46: 2067-76
197. Reynolds JV, Preston SR, O'Neill B, Baeksgaard L, Griffin SM, Mariette C, Cuffe S, Cunningham M, Crosby T, Parker I, Hofland K, Hanna G, Svendsen LB, Donohoe CL, Muldoon C, O'Toole D, Johnson C, Ravi N, Jones G, Corkhill AK, Illsley M, Mellor J, Lee K, Dib M, Marchesin V, Cunnane M, Scott K, Lawner P, Warren S, O'Reilly S, O'Dowd G, Leonard G, Hennessy B, Dermott RM. 2017. ICORG 10-14: NEOadjuvant trial in Adenocarcinoma of the oEsophagus and oesophagoGastric junction International Study (Neo-AEGIS). *BMC Cancer* 17: 401
198. Rice TW, Blackstone EH, Rusch VW. 2010. 7th Edition of the AJCC Cancer Staging Manual: Esophagus and Esophagogastric Junction. *Annals of Surgical Oncology* 17: 1721-4
199. Napier KJ, Scheerer M, Misra S. 2014. Esophageal cancer: A Review of epidemiology, pathogenesis, staging workup and treatment modalities. *World Journal of Gastrointestinal Oncology* 6: 112-20
200. Edge SB, Compton CC. 2010. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. *Ann Surg Oncol* 17: 1471-4
201. Podell ER, Harrington DJ, Taatjes DJ, Koch TH. 1999. Crystal structure of epidoxorubicin-formaldehyde virtual crosslink of DNA and evidence for its formation in human breast-cancer cells. *Acta Crystallogr D Biol Crystallogr* 55: 1516-23
202. Siddik ZH. 2003. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* 22: 7265-79

203. Longley DB, Harkin DP, Johnston PG. 2003. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* 3: 330-8
204. Cunningham D, Allum WH, Stenning SP, Thompson JN, Van de Velde CJ, Nicolson M, Scarffe JH, Lofts FJ, Falk SJ, Iveson TJ, Smith DB, Langley RE, Verma M, Weeden S, Chua YJ, Participants MT. 2006. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. *N Engl J Med* 355: 11-20
205. Baskar R, Dai J, Wenlong N, Yeo R, Yeoh K-W. 2014. Biological response of cancer cells to radiation treatment. *Frontiers in molecular biosciences* 1: 24-
206. Dasari S, Tchounwou PB. 2014. Cisplatin in cancer therapy: molecular mechanisms of action. *European journal of pharmacology* 740: 364-78
207. Weaver BA. 2014. How Taxol/paclitaxel kills cancer cells. *Molecular biology of the cell* 25: 2677-81
208. Oppedijk V, van der Gaast A, van Lanschot JJ, van Hagen P, van Os R, van Rij CM, van der Sagen MJ, Beukema JC, Rutten H, Spruit PH, Reinders JG, Richel DJ, van Berge Henegouwen MI, Hulshof MC. 2014. Patterns of recurrence after surgery alone versus preoperative chemoradiotherapy and surgery in the CROSS trials. *J Clin Oncol* 32: 385-91
209. Shapiro J, van Lanschot JJB, Hulshof M, van Hagen P, van Berge Henegouwen MI, Wijnhoven BPL, van Laarhoven HWM, Nieuwenhuijzen GAP, Hospers GAP, Bonenkamp JJ, Cuesta MA, Blaisse RJB, Busch ORC, Ten Kate FJW, Creemers GM, Punt CJA, Plukker JTM, Verheul HMW, Bilgen EJS, van Dekken H, van der Sagen MJC, Rozema T, Biermann K, Beukema JC, Piet AHM, van Rij CM, Reinders JG, Tilanus HW, Steyerberg EW, van der Gaast A. 2015. Neoadjuvant chemoradiotherapy plus surgery versus surgery alone for oesophageal or junctional cancer (CROSS): long-term results of a randomised controlled trial. *Lancet Oncol* 16: 1090-8
210. Holmberg D, Ness-Jensen E, Mattsson F, El-Serag HB, Lagergren J. 2017. Risk of oesophageal adenocarcinoma in individuals with Barrett's oesophagus. *European Journal of Cancer* 75: 41-6
211. de Jonge PJ, van Blankenstein M, Looman CW, Casparie MK, Meijer GA, Kuipers EJ. 2010. Risk of malignant progression in patients with Barrett's oesophagus: a Dutch nationwide cohort study. *Gut* 59: 1030-6
212. Lagergren J, Mattsson F. 2012. Diverging trends in recent population-based survival rates in oesophageal and gastric cancer. *PLoS One* 7: e41352
213. Souza RF. 2016. From Reflux Esophagitis to Esophageal Adenocarcinoma. *Digestive diseases (Basel, Switzerland)* 34: 483-90
214. Thrift AP. 2016. Determination of risk for Barrett's esophagus and esophageal adenocarcinoma. *Curr Opin Gastroenterol* 32: 319-24
215. van Soest EM, Dieleman JP, Siersema PD, Sturkenboom MC, Kuipers EJ. 2005. Increasing incidence of Barrett's oesophagus in the general population. *Gut* 54: 1062-6
216. Ronkainen J, Aro P, Storskrubb T, Johansson SE, Lind T, Bolling-Sternevald E, Vieth M, Stolte M, Talley NJ, Agreus L. 2005. Prevalence of Barrett's esophagus in the general population: an endoscopic study. *Gastroenterology* 129: 1825-31
217. Zagari RM, Fuccio L, Wallander MA, Johansson S, Fiocca R, Casanova S, Farahmand BY, Winchester CC, Roda E, Bazzoli F. 2008. Gastro-oesophageal reflux symptoms, oesophagitis and Barrett's oesophagus in the general population: the Loiano-Monghidoro study. *Gut* 57: 1354-9
218. Burnat G, Majka J, Konturek PC. 2010. Bile acids are multifunctional modulators of the Barrett's carcinogenesis. *J Physiol Pharmacol* 61: 185-92
219. DeMeester SR, DeMeester TR. 2000. Columnar mucosa and intestinal metaplasia of the esophagus: fifty years of controversy. *Ann Surg* 231: 303-21

220. Chandrasoma P, DeMeester T. 2016. A New Pathologic Assessment of Gastroesophageal Reflux Disease: The Squamo-Oxyntic Gap. *Adv Exp Med Biol* 908: 41-78
221. Burke ZD, Tosh D. 2012. Barrett's metaplasia as a paradigm for understanding the development of cancer. *Current Opinion in Genetics & Development* 22: 494-9
222. Spechler SJ, Souza RF. 2014. Barrett's Esophagus. *New England Journal of Medicine* 371: 836-45
223. Huo X, Juergens S, Zhang X, Rezaei D, Yu C, Strauch ED, Wang JY, Cheng E, Meyer F, Wang DH, Zhang Q, Spechler SJ, Souza RF. 2011. Deoxycholic acid causes DNA damage while inducing apoptotic resistance through NF-kappaB activation in benign Barrett's epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 301: G278-86
224. Bus P, Siersema PD, van Baal JWPM. 2012. Cell culture models for studying the development of Barrett's esophagus: a systematic review. *Cellular oncology (Dordrecht)* 35: 149-61
225. Shirvani VN, Ouatu-Lascar R, Kaur BS, Omary MB, Triadafilopoulos G. 2000. Cyclooxygenase 2 expression in Barrett's esophagus and adenocarcinoma: Ex vivo induction by bile salts and acid exposure. *Gastroenterology* 118: 487-96
226. Fitzgerald RC, Abdalla S, Onwuegbusi BA, Sirieix P, Saeed IT, Burnham WR, Farthing MJ. 2002. Inflammatory gradient in Barrett's oesophagus: implications for disease complications. *Gut* 51: 316-22
227. Gadaleta RM, van Erpecum KJ, Oldenburg B, Willemsen EC, Renooij W, Murzilli S, Klomp LW, Siersema PD, Schipper ME, Danese S, Penna G, Laverny G, Adorini L, Moschetta A, van Mil SW. 2011. Farnesoid X receptor activation inhibits inflammation and preserves the intestinal barrier in inflammatory bowel disease. *Gut* 60: 463-72
228. Hollman DA, Milona A, van Erpecum KJ, van Mil SW. 2012. Anti-inflammatory and metabolic actions of FXR: insights into molecular mechanisms. *Biochim Biophys Acta* 1821: 1443-52
229. Iijima K, Henry E, Moriya A, Wirz A, Kelman AW, McColl KE. 2002. Dietary nitrate generates potentially mutagenic concentrations of nitric oxide at the gastroesophageal junction. *Gastroenterology* 122: 1248-57
230. Kusaka G, Uno K, Iijima K, Endo H, Asano N, Koike T, Imatani A, Shimosegawa T. 2012. The role of nitric oxide in the induction of caudal-type homeobox 2 through epidermal growth factor receptor in the development of Barrett's esophagus. *Scand J Gastroenterol* 47: 1148-58
231. Sharma N, Ho KY. 2016. Risk Factors for Barrett's Oesophagus. *Gastrointestinal tumors* 3: 103-8
232. Andrici J, Tio M, Cox MR, Eslick GD. 2013. Hiatal hernia and the risk of Barrett's esophagus. *J Gastroenterol Hepatol* 28: 415-31
233. Tan WK, di Pietro M, Fitzgerald RC. 2017. Past, present and future of Barrett's oesophagus. *European Journal of Surgical Oncology (EJSO)* 43: 1148-60
234. Cook MB, Shaheen NJ, Anderson LA, Giffen C, Chow WH, Vaughan TL, Whitman DC, Corley DA. 2012. Cigarette smoking increases risk of Barrett's esophagus: an analysis of the Barrett's and Esophageal Adenocarcinoma Consortium. *Gastroenterology* 142: 744-53
235. Matsuo T, Ito M, Takata S, Tanaka S, Yoshihara M, Chayama K. 2011. Low prevalence of Helicobacter pylori-negative gastric cancer among Japanese. *Helicobacter* 16: 415-9
236. Fischbach LA, Graham DY, Kramer JR, Rugge M, Verstovsek G, Parente P, Alsarraj A, Fitzgerald S, Shaib Y, Abraham NS, Kolpachi A, Gupta S, Vela MF, Velez M, Cole R, Anand B, El Serag HB. 2014. Association between Helicobacter pylori and Barrett's esophagus: a case-control study. *Am J Gastroenterol* 109: 357-68

237. Thrift AP, Shaheen NJ, Gammon MD, Bernstein L, Reid BJ, Onstad L, Risch HA, Liu G, Bird NC, Wu AH, Corley DA, Romero Y, Chanock SJ, Chow WH, Casson AG, Levine DM, Zhang R, Ek WE, MacGregor S, Ye W, Hardie LJ, Vaughan TL, Whiteman DC. 2014. Obesity and risk of esophageal adenocarcinoma and Barrett's esophagus: a Mendelian randomization study. *J Natl Cancer Inst* 106
238. Kastelein F, van Olphen SH, Steyerberg EW, Spaander MC, Bruno MJ. 2016. Impact of surveillance for Barrett's oesophagus on tumour stage and survival of patients with neoplastic progression. *Gut* 65: 548-54
239. Estores D, Velanovich V. 2013. Barrett esophagus: epidemiology, pathogenesis, diagnosis, and management. *Current Problems in Surgery* 50: 192-226
240. Sharma P, Morales TG, Sampliner RE. 1998. Short segment Barrett's esophagus--the need for standardization of the definition and of endoscopic criteria. *Am J Gastroenterol* 93: 1033-6
241. Anaparthi R, Gaddam S, Kanakadandi V, Alsop BR, Gupta N, Higbee AD, Wani SB, Singh M, Rastogi A, Bansal A, Cash BD, Young PE, Lieberman DA, Falk GW, Vargo JJ, Thota P, Sampliner RE, Sharma P. 2013. Association between length of Barrett's esophagus and risk of high-grade dysplasia or adenocarcinoma in patients without dysplasia. *Clin Gastroenterol Hepatol* 11: 1430-6
242. Sharma P, Dent J, Armstrong D, Bergman JJ, Gossner L, Hoshihara Y, Jankowski JA, Junghard O, Lundell L, Tytgat GN, Vieth M. 2006. The development and validation of an endoscopic grading system for Barrett's esophagus: the Prague C & M criteria. *Gastroenterology* 131: 1392-9
243. Booth CL, Thompson KS. 2012. Barrett's esophagus: A review of diagnostic criteria, clinical surveillance practices and new developments. *Journal of Gastrointestinal Oncology* 3: 232-42
244. Schlemper RJ, Riddell RH, Kato Y, Borchard F, Cooper HS, Dawsey SM, Dixon MF, Fenoglio-Preiser CM, Flejou JF, Geboes K, Hattori T, Hirota T, Itabashi M, Iwafuchi M, Iwashita A, Kim YI, Kirchner T, Klimpfinger M, Koike M, Lauwers GY, Lewin KJ, Oberhuber G, Offner F, Price AB, Rubio CA, Shimizu M, Shimoda T, Sipponen P, Solcia E, Stolte M, Watanabe H, Yamabe H. 2000. The Vienna classification of gastrointestinal epithelial neoplasia. *Gut* 47: 251-5
245. Amadi C, Gatenby P. 2017. Barrett's oesophagus: Current controversies. *World J Gastroenterol* 23: 5051-67
246. Spechler SJ, Sharma P, Souza RF, Inadomi JM, Shaheen NJ. 2011. American Gastroenterological Association technical review on the management of Barrett's esophagus. *Gastroenterology* 140: e18-52; quiz e13
247. Evans JA, Early DS, Fukami N, Ben-Menachem T, Chandrasekhara V, Chathadi KV, Decker GA, Fanelli RD, Fisher DA, Foley KQ, Hwang JH, Jain R, Jue TL, Khan KM, Lightdale J, Malpas PM, Maple JT, Pasha SF, Saltzman JR, Sharaf RN, Shergill A, Dornitz JA, Cash BD. 2012. The role of endoscopy in Barrett's esophagus and other premalignant conditions of the esophagus. *Gastrointest Endosc* 76: 1087-94
248. Fitzgerald RC, di Pietro M, Ragunath K, Ang Y, Kang J-Y, Watson P, Trudgill N, Patel P, Kaye PV, Sanders S, Donovan M, Bird-Lieberman E, Bhandari P, Jankowski JA, Attwood S, Parsons SL, Loft D, Lagergren J, Moayyedi P, Lyratzopoulos G, de Caestecker J. 2014. British Society of Gastroenterology guidelines on the diagnosis and management of Barrett's oesophagus. *Gut* 63: 7
249. Picardo SL, O'Brien MP, Feighery R, O'Toole D, Ravi N, O'Farrell NJ, O'Sullivan JN, Reynolds JV. 2015. A Barrett's esophagus registry of over 1000 patients from a specialist center highlights greater risk of progression than population-based registries and high risk of low grade dysplasia. *Diseases of the Esophagus* 28: 121-6

250. Chaves P, Crespo M, Ribeiro C, Laranjeira C, Pereira AD, Suspiro A, Cardoso P, Leitao CN, Soares J. 2007. Chromosomal analysis of Barrett's cells: demonstration of instability and detection of the metaplastic lineage involved. *Mod Pathol* 20: 788-96
251. Liu W, Hahn H, Odze RD, Goyal RK. 2009. Metaplastic esophageal columnar epithelium without goblet cells shows DNA content abnormalities similar to goblet cell-containing epithelium. *Am J Gastroenterol* 104: 816-24
252. Srivastava A, Golden KL, Sanchez CA, Liu K, Fong PY, Li X, Cowan DS, Rabinovitch PS, Reid BJ, Blount PL, Odze RD. 2015. High Goblet Cell Count Is Inversely Associated with Ploidy Abnormalities and Risk of Adenocarcinoma in Barrett's Esophagus. *PLoS ONE* 10: e0133403
253. Falk GW. 2017. Current Management of Low-Grade Dysplasia in Barrett Esophagus. *Gastroenterology & Hepatology* 13: 221-5
254. Hvid-Jensen F, Pedersen L, Drewes AM, Sorensen HT, Funch-Jensen P. 2011. Incidence of adenocarcinoma among patients with Barrett's esophagus. *N Engl J Med* 365: 1375-83
255. Peters Y, Al-Kaabi A, Shaheen NJ, Chak A, Blum A, Souza RF, Di Pietro M, Iyer PG, Pech O, Fitzgerald RC, Siersema PD. 2019. Barrett oesophagus. *Nature Reviews Disease Primers* 5: 35
256. Wang KK, Sampliner RE. 2008. Updated guidelines 2008 for the diagnosis, surveillance and therapy of Barrett's esophagus. *Am J Gastroenterol* 103: 788-97
257. Zhuang L, Fitzgerald RC. 2017. Origins in the oesophagus. *Nature* 550: 463
258. Yu WY, Slack JM, Tosh D. 2005. Conversion of columnar to stratified squamous epithelium in the developing mouse oesophagus. *Dev Biol* 284: 157-70
259. McDonald SAC, Lavery D, Wright NA, Jansen M. 2015. Barrett oesophagus: lessons on its origins from the lesion itself. *Nature Reviews Gastroenterology & Hepatology* 12: 50-60
260. Berenson MM, Johnson TD, Markowitz NR, Buchi KN, Samowitz WS. 1993. Restoration of squamous mucosa after ablation of Barrett's esophageal epithelium. *Gastroenterology* 104: 1686-91
261. Hamilton SR, Yardley JH. 1977. Regenerative of cardiac type mucosa and acquisition of Barrett mucosa after esophagogastrostomy. *Gastroenterology* 72: 669-75
262. Koak Y, Winslet M. 2002. Changing role of in vivo models in columnar-lined lower esophagus. *Dis Esophagus* 15: 271-7
263. Quante M, Bhagat G, Abrams JA, Marache F, Good P, Lee MD, Lee Y, Friedman R, Asfaha S, Dubeykovskaya Z, Mahmood U, Figueiredo JL, Kitajewski J, Shawber C, Lightdale CJ, Rustgi AK, Wang TC. 2012. Bile acid and inflammation activate gastric cardia stem cells in a mouse model of Barrett-like metaplasia. *Cancer Cell* 21: 36-51
264. Wang X, Ouyang H, Yamamoto Y, Kumar PA, Wei TS, Dagher R, Vincent M, Lu X, Bellizzi AM, Ho KY, Crum CP, Xian W, McKeon F. 2011. Residual embryonic cells as precursors of a Barrett's-like metaplasia. *Cell* 145: 1023-35
265. Sarosi G, Brown G, Jaiswal K, Feagins LA, Lee E, Crook TW, Souza RF, Zou YS, Shay JW, Spechler SJ. 2008. Bone marrow progenitor cells contribute to esophageal regeneration and metaplasia in a rat model of Barrett's esophagus. *Dis Esophagus* 21: 43-50
266. Moss A, Clarke E, Crowe J, Lennon J, MacMathuna P. 2003. Management of Barrett's oesophagus in 2001 in Ireland. *Irish Journal of Medical Science* 172: 174-6
267. 2011. American Gastroenterological Association Medical Position Statement on the Management of Barrett's Esophagus. *Gastroenterology* 140: 1084-91
268. Old O, Moayyedi P, Love S, Roberts C, Hapeshi J, Foy C, Stokes C, Briggs A, Jankowski J, Barr H. 2015. Barrett's Oesophagus Surveillance versus endoscopy at need Study

- (BOSS): protocol and analysis plan for a multicentre randomized controlled trial. *J Med Screen* 22: 158-64
269. Ramus JR, Gatenby PA, Caygill CP, Winslet MC, Watson A. 2009. Surveillance of Barrett's columnar-lined oesophagus in the UK: endoscopic intervals and frequency of detection of dysplasia. *Eur J Gastroenterol Hepatol* 21: 636-41
 270. Picardo SL, Maher SG, O'Sullivan JN, Reynolds JV. 2012. Barrett's to oesophageal cancer sequence: a model of inflammatory-driven upper gastrointestinal cancer. *Dig Surg* 29: 251-60
 271. Castell DO, Kahrilas PJ, Richter JE, Vakil NB, Johnson DA, Zuckerman S, Skammer W, Levine JG. 2002. Esomeprazole (40 mg) compared with lansoprazole (30 mg) in the treatment of erosive esophagitis. *Am J Gastroenterol* 97: 575-83
 272. Singh S, Garg SK, Singh PP, Iyer PG, El-Serag HB. 2014. Acid-suppressive medications and risk of oesophageal adenocarcinoma in patients with Barrett's oesophagus: a systematic review and meta-analysis. *Gut* 63: 1229-37
 273. Hu Q, Sun T-T, Hong J, Fang J-Y, Xiong H, Meltzer SJ. 2017. Proton Pump Inhibitors Do Not Reduce the Risk of Esophageal Adenocarcinoma in Patients with Barrett's Esophagus: A Systematic Review and Meta-Analysis. *PloS one* 12: e0169691-e
 274. Brusselaers N, Engstrand L, Lagergren J. 2018. Maintenance proton pump inhibition therapy and risk of oesophageal cancer. *Cancer Epidemiology* 53: 172-7
 275. Jovani M, Cao Y, Feskanich D, Drew DA, Hur C, Fuchs CS, Jacobson BC, Chan AT. 2017. Aspirin Use is Associated With Lower Risk of Barrett's Esophagus in Women. *Clinical And Translational Gastroenterology* 8: e131
 276. Jankowski JAZ, de Caestecker J, Love SB, Reilly G, Watson P, Sanders S, Ang Y, Morris D, Bhandari P, Brooks C, Attwood S, Harrison R, Barr H, Moayyedi P. 2018. Esomeprazole and aspirin in Barrett's oesophagus (AspECT): a randomised factorial trial. *Lancet* 392: 400-8
 277. Weil J, Colin-Jones D, Langman M, Lawson D, Logan R, Murphy M, Rawlins M, Vessey M, Wainwright P. 1995. Prophylactic aspirin and risk of peptic ulcer bleeding. *BMJ* 310: 827
 278. Lai KC, Lam SK, Chu KM, Wong BCY, Hui WM, Hu WHC, Lau GKK, Wong WM, Yuen MF, Chan AOO, Lai CL, Wong J. 2002. Lansoprazole for the Prevention of Recurrences of Ulcer Complications from Long-Term Low-Dose Aspirin Use. *New England Journal of Medicine* 346: 2033-8
 279. Hawkey CJ, Karrasch JA, Szczepański L, Walker DG, Barkun A, Swannell AJ, Yeomans ND. 1998. Omeprazole Compared with Misoprostol for Ulcers Associated with Nonsteroidal Antiinflammatory Drugs. *New England Journal of Medicine* 338: 727-34
 280. Lai K-C, Lam S-K, Hui W-M, Wong C-Y, Chu K-M, Hu W, Wong W-M, Chan O-O, Lai C-L, Lau K-K, Yuen M-F, Chan C-K. 2000. Lansoprazole reduces ulcer relapse after eradication of helicobacter pylori in NSAIDS users. *Gastroenterology* 118: A251
 281. Belghazi K, Bergman J, Pouw RE. 2016. Endoscopic Resection and Radiofrequency Ablation for Early Esophageal Neoplasia. *Digestive diseases (Basel, Switzerland)* 34: 469-75
 282. Peters JH, Watson TA. 2011. Endoscopic Mucosal Resection of Barrett's Esophagus and Early Esophageal Cancer. *Journal of Gastrointestinal Surgery* 15: 1299
 283. O'Farrell NJ, Reynolds JV, Ravi N, Larkin JO, Malik V, Wilson GF, Muldoon C, O'Toole D. 2013. Evolving changes in the management of early oesophageal adenocarcinoma in a tertiary centre. *Ir J Med Sci* 182: 363-9
 284. Haidry RJ, Dunn JM, Butt MA, Burnell MG, Gupta A, Green S, Miah H, Smart HL, Bhandari P, Smith LA, Willert R, Fullarton G, Morris J, Di Pietro M, Gordon C, Penman I, Barr H, Patel P, Boger P, Kapoor N, Mahon B, Hoare J, Narayanasamy R, O'Toole D, Cheong E, Direkze NC, Ang Y, Novelli M, Banks MR, Lovat LB. 2013. Radiofrequency

- Ablation and Endoscopic Mucosal Resection for Dysplastic Barrett's Esophagus and Early Esophageal Adenocarcinoma: Outcomes of the UK National Halo RFA Registry. *Gastroenterology* 145: 87-95
285. Dunkin BJ, Martinez J, Bejarano PA, Smith CD, Chang K, Livingstone AS, Melvin WS. 2006. Thin-layer ablation of human esophageal epithelium using a bipolar radiofrequency balloon device. *Surgical Endoscopy And Other Interventional Techniques* 20: 125-30
 286. Tétreault M-P. 2015. Esophageal Cancer: Insights From Mouse Models. *Cancer growth and metastasis* 8: 37-46
 287. Garman KS, Orlando RC, Chen X. 2012. Review: Experimental models for Barrett's esophagus and esophageal adenocarcinoma. *American journal of physiology. Gastrointestinal and liver physiology* 302: G1231-G43
 288. Senoo M, Pinto F, Crum CP, McKeon F. 2007. p63 is essential for the proliferative potential of stem cells in stratified epithelia. *Cell* 129: 523-36
 289. Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, Tabin C, Sharpe A, Caput D, Crum C, McKeon F. 1999. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 398: 714-8
 290. Fein M, Peters JH, Chandrasoma P, Ireland AP, Oberg S, Ritter MP, Bremner CG, Hagen JA, DeMeester TR. 1998. Duodenoesophageal reflux induces esophageal adenocarcinoma without exogenous carcinogen. *J Gastrointest Surg* 2: 260-8
 291. Choi SY, Lin D, Gout PW, Collins CC, Xu Y, Wang Y. 2014. Lessons from patient-derived xenografts for better in vitro modeling of human cancer. *Adv Drug Deliv Rev* 79-80: 222-37
 292. Shultz LD, Goodwin N, Ishikawa F, Hosur V, Lyons BL, Greiner DL. 2014. Human cancer growth and therapy in immunodeficient mouse models. *Cold Spring Harb Protoc* 2014: 694-708
 293. Liu DS, Duong CP, Phillips WA, Clemons NJ. 2016. Preclinical models of esophageal adenocarcinoma for drug development. *Discov Med* 22: 371-9
 294. Gros SJ, Dohrmann T, Peldschus K, Schurr PG, Kaifi JT, Kalinina T, Reichelt U, Mann O, Strate TG, Adam G, Hoffman RM, Izbicki JR. 2010. Complementary use of fluorescence and magnetic resonance imaging of metastatic esophageal cancer in a novel orthotopic mouse model. *Int J Cancer* 126: 2671-81
 295. Gros SJ. 2011. Orthotopic models of esophageal carcinoma and their use in drug discovery. *Curr Protoc Pharmacol* Chapter 14: Unit14.20
 296. Nakagawa H, Wang TC, Zukerberg L, Odze R, Togawa K, May GHW, Wilson J, Rustgi AK. 1997. The targeting of the cyclin D1 oncogene by an Epstein-Barr virus promoter in transgenic mice causes dysplasia in the tongue, esophagus and forestomach. *Oncogene* 14: 1185
 297. Souza RF, Huo X, Mittal V, Schuler CM, Carmack SW, Zhang HY, Zhang X, Yu C, Hormi-Carver K, Genta RM, Spechler SJ. 2009. Gastroesophageal reflux might cause esophagitis through a cytokine-mediated mechanism rather than caustic acid injury. *Gastroenterology* 137: 1776-84
 298. Zhang HY, Zhang Q, Zhang X, Yu C, Huo X, Cheng E, Wang DH, Spechler SJ, Souza RF. 2011. Cancer-related inflammation and Barrett's carcinogenesis: interleukin-6 and STAT3 mediate apoptotic resistance in transformed Barrett's cells. *Am J Physiol Gastrointest Liver Physiol* 300: G454-60
 299. Dvorakova K, Payne CM, Ramsey L, Holubec H, Sampliner R, Dominguez J, Dvorak B, Bernstein H, Bernstein C, Prasad A, Fass R, Cui H, Garewal H. 2004. Increased expression and secretion of interleukin-6 in patients with Barrett's esophagus. *Clin Cancer Res* 10: 2020-8

300. Dvorak K, Dvorak B. 2013. Role of interleukin-6 in Barrett's esophagus pathogenesis. *World journal of gastroenterology* 19: 2307-12
301. Lukaszewicz-Zajac M, Mroczko B, Kozlowski M, Niklinski J, Laudanski J, Szmitkowski M. 2012. Higher importance of interleukin 6 than classic tumor markers (carcinoembryonic antigen and squamous cell cancer antigen) in the diagnosis of esophageal cancer patients. *Dis Esophagus* 25: 242-9
302. Bannister JR, Khan AL, Eccleston DW, Deol-Poonia RK, Hughes SF. 2012. Interleukin-17 Expression in the Barrett's Metaplasia-Dysplasia-Adenocarcinoma Sequence. *ISRN Inflammation* 2012: 578149
303. Zhang HY, Hormi-Carver K, Zhang X, Spechler SJ, Souza RF. 2009. In Benign Barrett's Epithelial Cells, Acid Exposure Generates Reactive Oxygen Species that Cause DNA Double Strand Breaks. *Cancer research* 69: 9083-9
304. Kandulski A, Malfertheiner P. 2011. Gastroesophageal reflux disease--from reflux episodes to mucosal inflammation. *Nat Rev Gastroenterol Hepatol* 9: 15-22
305. Nam SY, Choi IJ, Ryu KH, Park BJ, Kim YW, Kim HB, Kim JS. 2015. The effect of abdominal visceral fat, circulating inflammatory cytokines, and leptin levels on reflux esophagitis. *J Neurogastroenterol Motil* 21: 247-54
306. Fitzgerald RC, Onwuegbusi BA, Bajaj-Elliott M, Saeed IT, Burnham WR, Farthing MJG. 2002. Diversity in the oesophageal phenotypic response to gastro-oesophageal reflux: immunological determinants. *Gut* 50: 451-9
307. Rodrigo S, Abboud G, Oh D, DeMeester SR, Hagen J, Lipham J, DeMeester TR, Chandrasoma P. 2008. High intraepithelial eosinophil counts in esophageal squamous epithelium are not specific for eosinophilic esophagitis in adults. *Am J Gastroenterol* 103: 435-42
308. Moons LM, Kusters JG, Bultman E, Kuipers EJ, van Dekken H, Tra WM, Kleinjan A, Kwekkeboom J, van Vliet AH, Siersema PD. 2005. Barrett's oesophagus is characterized by a predominantly humoral inflammatory response. *J Pathol* 207: 269-76
309. Bobryshev YV, Tran D, Killingsworth MC, Buckland M, Lord RVN. 2009. Dendritic Cells in Barrett's Esophagus and Esophageal Adenocarcinoma. *Journal of Gastrointestinal Surgery* 13: 44-53
310. Somja J, Demoulin S, Roncarati P, Herfs M, Bletard N, Delvenne P, Hubert P. 2013. Dendritic Cells in Barrett's Esophagus Carcinogenesis: An Inadequate Microenvironment for Antitumor Immunity? *The American Journal of Pathology* 182: 2168-79
311. Dohi T, Fujihashi K, Koga T, Shirai Y, Kawamura YI, Ejima C, Kato R, Saitoh K, McGhee JR. 2003. T helper type-2 cells induce ileal villus atrophy, goblet cell metaplasia, and wasting disease in T cell-deficient mice. *Gastroenterology* 124: 672-82
312. Kohata Y, Fujiwara Y, Machida H, Okazaki H, Yamagami H, Tanigawa T, Watanabe K, Watanabe T, Tominaga K, Wei M, Wanibuchi H, Arakawa T. 2011. Role of Th-2 cytokines in the development of Barrett's esophagus in rats. *Journal of Gastroenterology* 46: 883-93
313. Melo AM, O'Brien AM, Phelan JJ, Kennedy SA, Wood NAW, Veerapen N, Besra GS, Clarke NE, Foley EK, Ravi A, MacCarthy F, O'Toole D, Ravi N, Reynolds JV, Conroy MJ, Hogan AE, O'Sullivan J, Dunne MR. 2019. Mucosal-Associated Invariant T Cells Display Diminished Effector Capacity in Oesophageal Adenocarcinoma. *Frontiers in Immunology* 10: 1580
314. Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H, Fujii H. 2003. Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. *Clin Cancer Res* 9: 4404-8
315. Kawasaki T, Kawai T. 2014. Toll-like receptor signaling pathways. *Frontiers in immunology* 5: 461-

316. Huhta H, Helminen O, Lehenkari PP, Saarnio J, Karttunen TJ, Kauppila JH. 2016. Toll-like receptors 1, 2, 4 and 6 in esophageal epithelium, Barrett's esophagus, dysplasia and adenocarcinoma. *Oncotarget* 7: 23658-67
317. Kohtz PD, Halpern AL, Eldeiry MA, Hazel K, Kalatardi S, Ao L, Meng X, Reece TB, Fullerton DA, Weyant MJ. 2019. Toll-Like Receptor-4 Is a Mediator of Proliferation in Esophageal Adenocarcinoma. *The Annals of Thoracic Surgery* 107: 233-41
318. Verbeek RE, Siersema PD, Vleggaar FP, Ten Kate FJ, Posthuma G, Souza RF, de Haan J, van Baal JW. 2016. Toll-like Receptor 2 Signalling and the Lysosomal Machinery in Barrett's Esophagus. *J Gastrointest Liver Dis* 25: 273-82
319. Macfarlane S, Furrie E, Macfarlane GT, Dillon JF. 2007. Microbial Colonization of the Upper Gastrointestinal Tract in Patients with Barrett's Esophagus. *Clinical Infectious Diseases* 45: 29-38
320. Yang L, Lu X, Nossa CW, Francois F, Peek RM, Pei Z. 2009. Inflammation and Intestinal Metaplasia of the Distal Esophagus Are Associated With Alterations in the Microbiome. *Gastroenterology* 137: 588-97
321. World Health Organisation E. 2013. *Nutrition, Physical Activity and Obesity Ireland*
322. Nuttall FQ. 2015. Body Mass Index: Obesity, BMI, and Health: A Critical Review. *Nutrition today* 50: 117-28
323. 1995. Physical status: the use and interpretation of anthropometry. Report of a WHO Expert Committee. *World Health Organ Tech Rep Ser* 854: 1-452
324. Long E, Beales ILP. 2014. The role of obesity in oesophageal cancer development. *Therapeutic Advances in Gastroenterology* 7: 247-68
325. Alberti KG, Zimmet P, Shaw J. 2006. Metabolic syndrome--a new world-wide definition. A Consensus Statement from the International Diabetes Federation. *Diabet Med* 23: 469-80
326. Doyle SL, Bennett AM, Donohoe CL, Mongan AM, Howard JM, Lithander FE, Pidgeon GP, Reynolds JV, Lysaght J. 2013. Establishing computed tomography-defined visceral fat area thresholds for use in obesity-related cancer research. *Nutr Res* 33: 171-9
327. Stattin P, Lukanova A, Biessy C, Soderberg S, Palmqvist R, Kaaks R, Olsson T, Jellum E. 2004. Obesity and colon cancer: does leptin provide a link? *Int J Cancer* 109: 149-52
328. Wu MH, Chou YC, Chou WY, Hsu GC, Chu CH, Yu CP, Yu JC, Sun CA. 2009. Circulating levels of leptin, adiposity and breast cancer risk. *Br J Cancer* 100: 578-82
329. Kim S, Keku TO, Martin C, Galanko J, Woosley JT, Schroeder JC, Satia JA, Halabi S, Sandler RS. 2008. Circulating levels of inflammatory cytokines and risk of colorectal adenomas. *Cancer Res* 68: 323-8
330. Csak T, Ganz M, Pespisa J, Kodys K, Dolganiuc A, Szabo G. 2011. Fatty acid and endotoxin activate inflammasomes in mouse hepatocytes that release danger signals to stimulate immune cells. *Hepatology* 54: 133-44
331. Wu X, Dong L, Lin X, Li J. 2017. Relevance of the NLRP3 Inflammasome in the Pathogenesis of Chronic Liver Disease. *Frontiers in Immunology* 8: 1728
332. Gong Z, Jiang R, Su KAI, Goetzman E, Dong H, Muzumdar R. 2018. Deficiency in Absent in Melanoma (AIM) 2, an Innate Immunity Protein, Is Associated with Hepatic Steatosis. *Diabetes* 67: 2443-PUB
333. Youm Y-H, Adijiang A, Vandanmagsar B, Burk D, Ravussin A, Dixit VD. 2011. Elimination of the NLRP3-ASC Inflammasome Protects against Chronic Obesity-Induced Pancreatic Damage. *Endocrinology* 152: 4039-45
334. Corley DA, Kubo A, Levin TR, Block G, Habel L, Zhao W, Leighton P, Quesenberry C, Rumore GJ, Buffler PA. 2007. Abdominal obesity and body mass index as risk factors for Barrett's esophagus. *Gastroenterology* 133: 34-41; quiz 311
335. Masci E, Viaggi P, Mangiavillano B, Di Pietro S, Micheletto G, Di Prisco F, Paganelli M, Pontiroli AE, Laneri M, Testoni S, Testoni PA. 2011. No increase in prevalence of

- Barrett's oesophagus in a surgical series of obese patients referred for laparoscopic gastric banding. *Dig Liver Dis* 43: 613-5
336. Gerson LB, Ullah N, Fass R, Green C, Shetler K, Singh G. 2007. Does body mass index differ between patients with Barrett's oesophagus and patients with chronic gastro-oesophageal reflux disease? *Aliment Pharmacol Ther* 25: 1079-86
 337. Jacobson BC, Chan AT, Giovannucci EL, Fuchs CS. 2009. Body mass index and Barrett's oesophagus in women. *Gut* 58: 1460-6
 338. Steevens J, Schouten LJ, Driessen AL, Huysentruyt CJ, Keulemans YC, Goldbohm RA, van den Brandt PA. 2011. A prospective cohort study on overweight, smoking, alcohol consumption, and risk of Barrett's esophagus. *Cancer Epidemiol Biomarkers Prev* 20: 345-58
 339. Anderson LA, Watson RG, Murphy SJ, Johnston BT, Comber H, Mc Guigan J, Reynolds JV, Murray LJ. 2007. Risk factors for Barrett's oesophagus and oesophageal adenocarcinoma: results from the FINBAR study. *World J Gastroenterol* 13: 1585-94
 340. Stein DJ, El-Serag HB, Kuczynski J, Kramer JR, Sampliner RE. 2005. The association of body mass index with Barrett's oesophagus. *Aliment Pharmacol Ther* 22: 1005-10
 341. Smith KJ, O'Brien SM, Smithers BM, Gotley DC, Webb PM, Green AC, Whiteman DC. 2005. Interactions among smoking, obesity, and symptoms of acid reflux in Barrett's esophagus. *Cancer Epidemiol Biomarkers Prev* 14: 2481-6
 342. Bu X, Ma Y, Der R, Demeester T, Bernstein L, Chandrasoma PT. 2006. Body mass index is associated with Barrett esophagus and cardiac mucosal metaplasia. *Dig Dis Sci* 51: 1589-94
 343. Cook MB, Greenwood DC, Hardie LJ, Wild CP, Forman D. 2008. A systematic review and meta-analysis of the risk of increasing adiposity on Barrett's esophagus. *Am J Gastroenterol* 103: 292-300
 344. Kramer JR, Fischbach LA, Richardson P, Alsarraj A, Fitzgerald S, Shaib Y, Abraham NS, Velez M, Cole R, Anand B, Verstovsek G, Rugge M, Parente P, Graham DY, El-Serag HB. 2013. Waist-to-hip ratio, but not body mass index, is associated with an increased risk of Barrett's esophagus in white men. *Clin Gastroenterol Hepatol* 11: 373-81.e1
 345. Kubo A, Cook MB, Shaheen NJ, Vaughan TL, Whiteman DC, Murray L, Corley DA. 2013. Sex-specific associations between body mass index, waist circumference and the risk of Barrett's oesophagus: a pooled analysis from the international BEACON consortium. *Gut* 62: 1684-91
 346. Turati F, Tramacere I, La Vecchia C, Negri E. 2013. A meta-analysis of body mass index and esophageal and gastric cardia adenocarcinoma. *Ann Oncol* 24: 609-17
 347. Corley DA, Kubo A, Zhao W. 2008. Abdominal Obesity and the Risk of Esophageal and Gastric Cardia Carcinomas. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 17: 352-8
 348. Du X, Hidayat K, Shi B-M. 2017. Abdominal obesity and gastroesophageal cancer risk: systematic review and meta-analysis of prospective studies. *Bioscience reports* 37: BSR20160474
 349. Karastergiou K, Smith SR, Greenberg AS, Fried SK. 2012. Sex differences in human adipose tissues – the biology of pear shape. *Biology of Sex Differences* 3: 13-
 350. Pinho CPS, Diniz AdS, Arruda IKGd, Leite APDL, Petribu MdMV, Rodrigues IG. 2018. Waist circumference measurement sites and their association with visceral and subcutaneous fat and cardiometabolic abnormalities. *Archives of Endocrinology and Metabolism* 62: 416-23
 351. Akiyama T, Yoneda M, Inamori M, Iida H, Endo H, Hosono K, Yoneda K, Fujita K, Koide T, Tokoro C, Takahashi H, Goto A, Abe Y, Kirikoshi H, Kobayashi N, Kubota K, Saito S,

- Nakajima A. 2009. Visceral obesity and the risk of Barrett's esophagus in Japanese patients with non-alcoholic fatty liver disease. *BMC Gastroenterol* 9: 56
352. Schoetl T, Fischer IP, Ussar S. 2018. Heterogeneity of adipose tissue in development and metabolic function. *J Exp Biol* 221
 353. El-Serag HB, Tran T, Richardson P, Ergun G. 2006. Anthropometric correlates of intragastric pressure. *Scand J Gastroenterol* 41: 887-91
 354. Jensen MD. 2008. Role of body fat distribution and the metabolic complications of obesity. *J Clin Endocrinol Metab* 93: S57-63
 355. Després J-P, Lemieux I. 2006. Abdominal obesity and metabolic syndrome. *Nature* 444: 881
 356. Rakotoarivelo V, Lacraz G, Mayhue M, Brown C, Rottembourg D, Fradette J, Ilangumaran S, Menendez A, Langlois M-F, Ramanathan S. 2018. Inflammatory Cytokine Profiles in Visceral and Subcutaneous Adipose Tissues of Obese Patients Undergoing Bariatric Surgery Reveal Lack of Correlation With Obesity or Diabetes. *EBioMedicine* 30: 237-47
 357. Coelho M, Oliveira T, Fernandes R. 2013. Biochemistry of adipose tissue: an endocrine organ. *Archives of medical science : AMS* 9: 191-200
 358. Hotamisligil GS. 2006. Inflammation and metabolic disorders. *Nature* 444: 860-7
 359. O'Sullivan J, Lysaght J, Donohoe CL, Reynolds JV. 2018. Obesity and gastrointestinal cancer: the interrelationship of adipose and tumour microenvironments. *Nat Rev Gastroenterol Hepatol* 15: 699-714
 360. Michelet X, Dyck L, Hogan A, Loftus RM, Duquette D, Wei K, Beyaz S, Tavakkoli A, Foley C, Donnelly R, O'Farrelly C, Raverdeau M, Vernon A, Pettee W, O'Shea D, Nikolajczyk BS, Mills KHG, Brenner MB, Finlay D, Lynch L. 2018. Metabolic reprogramming of natural killer cells in obesity limits antitumor responses. *Nature Immunology* 19: 1330-40
 361. Dyck L, Lynch L. 2018. Cancer, obesity and immunometabolism - Connecting the dots. *Cancer Lett* 417: 11-20
 362. Macia L, Delacre M, Abboud G, Ouk TS, Delanoye A, Verwaerde C, Saule P, Wolowczuk I. 2006. Impairment of dendritic cell functionality and steady-state number in obese mice. *J Immunol* 177: 5997-6006
 363. Bahr I, Jahn J, Zipprich A, Pahlow I, Spielmann J, Kielstein H. 2018. Impaired natural killer cell subset phenotypes in human obesity. *Immunol Res* 66: 234-44
 364. Munch NS, Fang HY, Ingermann J, Maurer HC, Anand A, Kellner V, Sahm V, Wiethaler M, Baumeister T, Wein F, Einwachter H, Bolze F, Klingenspor M, Haller D, Kavanagh M, Lysaght J, Friedman R, Dannenberg AJ, Pollak M, Holt PR, Muthupalani S, Fox JG, Whary MT, Lee Y, Ren TY, Elliot R, Fitzgerald R, Steiger K, Schmid RM, Wang TC, Quante M. 2019. High-fat Diet Accelerates Carcinogenesis in a Mouse Model of Barrett's Esophagus via IL8 and Alterations to the Gut Microbiome. *Gastroenterology*
 365. Donohoe CL, Lysaght J, O'Sullivan J, Reynolds JV. 2017. Emerging Concepts Linking Obesity with the Hallmarks of Cancer. *Trends in Endocrinology & Metabolism* 28: 46-62
 366. Alemán JO, Eusebi LH, Ricciardiello L, Patidar K, Sanyal AJ, Holt PR. 2014. Mechanisms of Obesity-induced Gastrointestinal Neoplasia. *Gastroenterology* 146: 357-73
 367. Kolb R, Sutterwala FS, Zhang W. 2016. Obesity and cancer: inflammation bridges the two. *Current opinion in pharmacology* 29: 77-89
 368. Lysaght J, van der Stok EP, Allott EH, Casey R, Donohoe CL, Howard JM, McGarrigle SA, Ravi N, Reynolds JV, Pidgeon GP. 2011. Pro-inflammatory and tumour proliferative properties of excess visceral adipose tissue. *Cancer Lett* 312: 62-72
 369. Bullo M, Garcia-Lorda P, Peinado-Onsurbe J, Hernandez M, Del Castillo D, Argiles JM, Salas-Salvado J. 2002. TNFalpha expression of subcutaneous adipose tissue in obese

- and morbid obese females: relationship to adipocyte LPL activity and leptin synthesis. *Int J Obes Relat Metab Disord* 26: 652-8
370. Tselepis C, Perry I, Dawson C, Hardy R, Darnton SJ, McConkey C, Stuart RC, Wright N, Harrison R, Jankowski JA. 2002. Tumour necrosis factor- α in Barrett's oesophagus: a potential novel mechanism of action. *Oncogene* 21: 6071-81
 371. Young L, Sung J, Stacey G, Masters JR. 2010. Detection of Mycoplasma in cell cultures. *Nature Protocols* 5: 929
 372. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. *Anal Biochem* 150: 76-85
 373. di Pietro M, Lao-Sirieix P, Boyle S, Cassidy A, Castillo D, Saadi A, Eskeland R, Fitzgerald RC. 2012. Evidence for a functional role of epigenetically regulated midcluster HOXB genes in the development of Barrett esophagus. *Proc Natl Acad Sci U S A* 109: 9077-82
 374. Kimchi ET, Posner MC, Park JO, Darga TE, Kocherginsky M, Karrison T, Hart J, Smith KD, Mezhir JJ, Weichselbaum RR, Khodarev NN. 2005. Progression of Barrett's metaplasia to adenocarcinoma is associated with the suppression of the transcriptional programs of epidermal differentiation. *Cancer Res* 65: 3146-54
 375. Fried SK, Moustaid-Moussa N. 2001. Culture of adipose tissue and isolated adipocytes. *Methods Mol Biol* 155: 197-212
 376. Cruz I, Van den Brule AJ, Steenbergen RD, Snijders PJ, Meijer CJ, Walboomers JM, Snow GB, Van der Waal I. 1997. Prevalence of Epstein-Barr virus in oral squamous cell carcinomas, premalignant lesions and normal mucosa--a study using the polymerase chain reaction. *Oral Oncol* 33: 182-8
 377. Nakagawa H, Inomoto T, Rustgi AK. 1997. A CACCC box-like cis-regulatory element of the Epstein-Barr virus ED-L2 promoter interacts with a novel transcriptional factor in tissue-specific squamous epithelia. *J Biol Chem* 272: 16688-99
 378. Tetreault M-P, Yang Y, Travis J, Yu Q-C, Klein-Szanto A, Tobias JW, Katz JP. 2010. Esophageal squamous cell dysplasia and delayed differentiation with deletion of krüppel-like factor 4 in murine esophagus. *Gastroenterology* 139: 171-81.e9
 379. Wilson JB, Weinberg W, Johnson R, Yuspa S, Levine AJ. 1990. Expression of the BNLF-1 oncogene of Epstein-Barr virus in the skin of transgenic mice induces hyperplasia and aberrant expression of keratin 6. *Cell* 61: 1315-27
 380. Opitz OG, Harada H, Suliman Y, Rhoades B, Sharpless NE, Kent R, Kopelovich L, Nakagawa H, Rustgi AK. 2002. A mouse model of human oral-esophageal cancer. *The Journal of Clinical Investigation* 110: 761-9
 381. Wingren AG, Bjorkdahl O, Labuda T, Bjork L, Andersson U, Gullberg U, Hedlund G, Sjogren HO, Kalland T, Widegren B, Dohlsten M. 1996. Fusion of a signal sequence to the interleukin-1 beta gene directs the protein from cytoplasmic accumulation to extracellular release. *Cell Immunol* 169: 226-37
 382. Bjorkdahl O, Akerblad P, Gyorloff-Wingren A, Leanderson T, Dohlsten M. 1999. Lymphoid hyperplasia in transgenic mice over-expressing a secreted form of the human interleukin-1beta gene product. *Immunology* 96: 128-37
 383. Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH, Sato T, Stange DE, Begthel H, van den Born M, Danenberg E, van den Brink S, Korving J, Abo A, Peters PJ, Wright N, Poulsom R, Clevers H. 2010. Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell* 6: 25-36
 384. Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ. 2000. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell* 102: 731-44

385. Fox JG, Beck P, Dangler CA, Whary MT, Wang TC, Shi HN, Nagler-Anderson C. 2000. Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces helicobacter-induced gastric atrophy. *Nat Med* 6: 536-42
386. Schellnegger R, Quante A, Rospleszcz S, Schernhammer M, Höhl B, Tobiasch M, Pastula A, Brandtner A, Abrams JA, Strauch K, Schmid RM, Vieth M, Wang TC, Quante M. 2017. Goblet-cell ratio in combination with differentiation and stem cell markers in Barrett's esophagus allow distinction of patients with and without esophageal adenocarcinoma. *Cancer prevention research (Philadelphia, Pa.)* 10: 55-66
387. Phelan JJ, MacCarthy F, Feighery R, O'Farrell NJ, Lynam-Lennon N, Doyle B, O'Toole D, Ravi N, Reynolds JV, O'Sullivan J. 2014. Differential expression of mitochondrial energy metabolism profiles across the metaplasia-dysplasia-adenocarcinoma disease sequence in Barrett's oesophagus. *Cancer Lett* 354: 122-31
388. O'Farrell NJ, Phelan JJ, Feighery R, Doyle B, Picardo SL, Ravi N, O'Toole D, Reynolds JV, O'Sullivan J. 2019. Differential Expression Profiles of Oxidative Stress Levels, 8-oxo-dG and 4-HNE, in Barrett's Esophagus Compared to Esophageal Adenocarcinoma. *International journal of molecular sciences* 20: 4449
389. Phelan JJ, Feighery R, Eldin OS, Meachair SO, Cannon A, Byrne R, MacCarthy F, O'Toole D, Reynolds JV, O'Sullivan J. 2016. Examining the connectivity between different cellular processes in the Barrett tissue microenvironment. *Cancer Lett* 371: 334-46
390. O'Farrell NJ, Feighery R, Picardo SL, Lynam-Lennon N, Biniecka M, McGarrigle SA, Phelan JJ, MacCarthy F, O'Toole D, Fox EJ, Ravi N, Reynolds JV, O'Sullivan J. 2016. Changes in mitochondrial stability during the progression of the Barrett's esophagus disease sequence. *BMC cancer* 16: 497-
391. Pastula A, Middelhoff M, Brandtner A, Tobiasch M, Hohl B, Nuber AH, Demir IE, Neupert S, Kollmann P, Mazzuoli-Weber G, Quante M. 2016. Three-Dimensional Gastrointestinal Organoid Culture in Combination with Nerves or Fibroblasts: A Method to Characterize the Gastrointestinal Stem Cell Niche. *Stem Cells Int* 2016: 3710836
392. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ, Clevers H. 2009. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 459: 262-5
393. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H. 2007. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 449: 1003-7
394. Kim KA, Kakitani M, Zhao J, Oshima T, Tang T, Binnerts M, Liu Y, Boyle B, Park E, Emtage P, Funk WD, Tomizuka K. 2005. Mitogenic influence of human R-spondin1 on the intestinal epithelium. *Science* 309: 1256-9
395. Pinto D, Gregorieff A, Begthel H, Clevers H. 2003. Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes Dev* 17: 1709-13
396. Kuhnert F, Davis CR, Wang HT, Chu P, Lee M, Yuan J, Nusse R, Kuo CJ. 2004. Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. *Proc Natl Acad Sci U S A* 101: 266-71
397. Dignass AU, Sturm A. 2001. Peptide growth factors in the intestine. *Eur J Gastroenterol Hepatol* 13: 763-70
398. Haramis AP, Begthel H, van den Born M, van Es J, Jonkheer S, Offerhaus GJ, Clevers H. 2004. De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. *Science* 303: 1684-6
399. Zhao CM, Hayakawa Y, Kodama Y, Muthupalani S, Westphalen CB, Andersen GT, Flatberg A, Johannessen H, Friedman RA, Renz BW, Sandvik AK, Beisvag V, Tomita H, Hara A, Quante M, Li Z, Gershon MD, Kaneko K, Fox JG, Wang TC, Chen D. 2014. Denervation suppresses gastric tumorigenesis. *Sci Transl Med* 6: 250ra115

400. Reya T, Clevers H. 2005. Wnt signalling in stem cells and cancer. *Nature* 434: 843-50
401. Merker SR, Weitz J, Stange DE. 2016. Gastrointestinal organoids: How they gut it out. *Developmental Biology* 420: 239-50
402. Bottenstein JE, Sato GH. 1979. Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proceedings of the National Academy of Sciences* 76: 514
403. Yiben G, Jie F, Pang-Kuo L, Wang S, Qian W, Chen H. 2011. The effect of B27 supplement on promoting in vitro propagation of Her2/neu-transformed mammary tumorspheres. *Journal of Biotech Research* 3
404. Kaushik G, Ponnusamy MP, Batra SK. 2018. Concise Review: Current Status of Three-Dimensional Organoids as Preclinical Models. *STEM CELLS* 36: 1329-40
405. Brewer GJ, Torricelli JR, Evege EK, Price PJ. 1993. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res* 35: 567-76
406. Zhang F, Lau SS, Monks TJ. 2010. The Cytoprotective Effect of N-acetyl-L-cysteine against ROS-Induced Cytotoxicity Is Independent of Its Ability to Enhance Glutathione Synthesis. *Toxicological Sciences* 120: 87-97
407. Bartfeld S, Bayram T, van de Wetering M, Huch M, Begthel H, Kujala P, Vries R, Peters PJ, Clevers H. 2015. In Vitro Expansion of Human Gastric Epithelial Stem Cells and Their Responses to Bacterial Infection. *Gastroenterology* 148: 126-36.e6
408. Willert K, Brown JD, Danenberg E, Duncan AW, Weissman IL, Reya T, Yates JR, Nusse R. 2003. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* 423: 448-52
409. Souza RF. 2010. The Role of Acid and Bile Reflux in Esophagitis and Barrett's Metaplasia. *Biochemical Society transactions* 38: 348-52
410. Lind A, Siersema PD, Kusters JG, Van der Linden JA, Knol EF, Koenderman L. 2012. The immune cell composition in Barrett's metaplastic tissue resembles that in normal duodenal tissue. *PLoS One* 7: e33899
411. Garcia JM, Splenser AE, Kramer J, Alsarraj A, Fitzgerald S, Ramsey D, El-Serag HB. 2014. Circulating inflammatory cytokines and adipokines are associated with increased risk of Barrett's esophagus: a case-control study. *Clin Gastroenterol Hepatol* 12: 229-38.e3
412. Borthwick LA. 2016. The IL-1 cytokine family and its role in inflammation and fibrosis in the lung. *Seminars in Immunopathology* 38: 517-34
413. Guo B, Fu S, Zhang J, Liu B, Li Z. 2016. Targeting inflammasome/IL-1 pathways for cancer immunotherapy. *Sci Rep* 6: 36107
414. Salcedo R, Worschech A, Cardone M, Jones Y, Gyulai Z, Dai RM, Wang E, Ma W, Haines D, O'HUigin C, Marincola FM, Trinchieri G. 2010. MyD88-mediated signaling prevents development of adenocarcinomas of the colon: role of interleukin 18. *J Exp Med* 207: 1625-36
415. Flood B, Oficjalska K, Laukens D, Fay J, O'Grady A, Caiazza F, Heetun Z, Mills KH, Sheahan K, Ryan EJ, Doherty GA, Kay E, Creagh EM. 2015. Altered expression of caspases-4 and -5 during inflammatory bowel disease and colorectal cancer: Diagnostic and therapeutic potential. *Clin Exp Immunol* 181: 39-50
416. Vyas D, Laput G, Vyas AK. 2014. Chemotherapy-enhanced inflammation may lead to the failure of therapy and metastasis. *OncoTargets and therapy* 7: 1015-23
417. Singh A, DeMars C, Buttar NS. 2010. Chapter 3 - Molecular Determinants of Metaplastic and Neoplastic Transformation in the Esophageal Epithelium. In *Progress in Molecular Biology and Translational Science*, ed. KH Kaestner, pp. 63-92: Academic Press
418. Stojic ZM, Stevanovic RM, Stojanovic MM, Stanojevic AD, Bacetic DT. 2011. Histological features of gastric cardia in adults: an autopsy study. *J Gastrointest Liver Dis* 20: 13-8

419. Chandrasoma P, Wijetunge S, DeMeester S, Ma Y, Hagen J, Zamis L, DeMeester T. 2012. Columnar-lined esophagus without intestinal metaplasia has no proven risk of adenocarcinoma. *Am J Surg Pathol* 36: 1-7
420. Glickman JN, Fox V, Antonioli DA, Wang HH, Odze RD. 2002. Morphology of the cardia and significance of carditis in pediatric patients. *Am J Surg Pathol* 26: 1032-9
421. Chandrasoma P. 1997. Pathophysiology of Barrett's esophagus. *Semin Thorac Cardiovasc Surg* 9: 270-8
422. di Pietro M, Chan D, Fitzgerald RC, Wang KK. 2015. Screening for Barrett's Esophagus. *Gastroenterology* 148: 912-23
423. Conroy MJ, Galvin KC, Doyle SL, Kavanagh ME, Mongan AM, Cannon A, Moore GY, Reynolds JV, Lysaght J. 2016. Parallel Profiles of Inflammatory and Effector Memory T Cells in Visceral Fat and Liver of Obesity-Associated Cancer Patients. *Inflammation* 39: 1729-36
424. Gillen P, Keeling P, Byrne PJ, Healy M, O'Moore RR, Hennessy TP. 1988. Implication of duodenogastric reflux in the pathogenesis of Barrett's oesophagus. *Br J Surg* 75: 540-3
425. Nehra D, Howell P, Williams CP, Pye JK, Beynon J. 1999. Toxic bile acids in gastro-oesophageal reflux disease: influence of gastric acidity. *Gut* 44: 598-602
426. Stein HJ, Feussner H, Kauer W, DeMeester TR, Siewert JR. 1994. Alkaline gastroesophageal reflux: assessment by ambulatory esophageal aspiration and pH monitoring. *Am J Surg* 167: 163-8
427. Bos JL, Fearon ER, Hamilton SR, Verlaan-de Vries M, van Boom JH, van der Eb AJ, Vogelstein B. 1987. Prevalence of ras gene mutations in human colorectal cancers. *Nature* 327: 293-7
428. Laghi L, Orbetegli O, Bianchi P, Zerbi A, Di Carlo V, Boland CR, Malesci A. 2002. Common occurrence of multiple K-RAS mutations in pancreatic cancers with associated precursor lesions and in biliary cancers. *Oncogene* 21: 4301-6
429. Miller CT, Moy JR, Lin L, Schipper M, Normolle D, Brenner DE, Iannettoni MD, Orringer MB, Beer DG. 2003. Gene amplification in esophageal adenocarcinomas and Barrett's with high-grade dysplasia. *Clin Cancer Res* 9: 4819-25
430. Jiang W, Zhang YJ, Kahn SM, Hollstein MC, Santella RM, Lu SH, Harris CC, Montesano R, Weinstein IB. 1993. Altered expression of the cyclin D1 and retinoblastoma genes in human esophageal cancer. *Proc Natl Acad Sci U S A* 90: 9026-30
431. Boynton RF, Huang Y, Blount PL, Reid BJ, Raskind WH, Haggitt RC, Newkirk C, Resau JH, Yin J, McDaniel T, Meltzer SJ. 1991. Frequent loss of heterozygosity at the retinoblastoma locus in human esophageal cancers. *Cancer Res* 51: 5766-9
432. Sun Y, Guo Y. 2018. Expression of Caspase-1 in breast cancer tissues and its effects on cell proliferation, apoptosis and invasion. *Oncol Lett* 15: 6431-5
433. Winter RN, Kramer A, Borkowski A, Kyprianou N. 2001. Loss of caspase-1 and caspase-3 protein expression in human prostate cancer. *Cancer Res* 61: 1227-32
434. Lopez-Castejon G, Brough D. 2011. Understanding the mechanism of IL-1 β secretion. *Cytokine & Growth Factor Reviews* 22: 189-95
435. Bruchard M, Mignot G, Derangere V, Chalmin F, Chevriaux A, Vegran F, Boireau W, Simon B, Ryffel B, Connat JL, Kanellopoulos J, Martin F, Rebe C, Apetoh L, Ghiringhelli F. 2013. Chemotherapy-triggered cathepsin B release in myeloid-derived suppressor cells activates the Nlrp3 inflammasome and promotes tumor growth. *Nat Med* 19: 57-64
436. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, Vermaelen K, Panaretakis T, Mignot G, Ullrich E, Perfettini JL, Schlemmer F, Tasdemir E, Uhl M, Genin P, Civas A, Ryffel B, Kanellopoulos J, Tschopp J, Andre F, Lidereau R, McLaughlin NM, Haynes NM, Smyth MJ, Kroemer G, Zitvogel L. 2009. Activation of the NLRP3 inflammasome in

- dendritic cells induces IL-1 β -dependent adaptive immunity against tumors. *Nat Med* 15: 1170-8
437. Zitvogel L, Kepp O, Galluzzi L, Kroemer G. 2012. Inflammasomes in carcinogenesis and anticancer immune responses. *Nat Immunol* 13: 343-51
 438. Son S, Shim D-W, Hwang I, Park J-H, Yu J-W. 2019. Chemotherapeutic Agent Paclitaxel Mediates Priming of NLRP3 Inflammasome Activation. *Frontiers in immunology* 10: 1108-
 439. Westbom C, Thompson JK, Leggett A, MacPherson M, Beuschel S, Pass H, Vacek P, Shukla A. 2015. Inflammasome Modulation by Chemotherapeutics in Malignant Mesothelioma. *PLoS one* 10: e0145404-e
 440. Buckley AM, Lynam-Lennon N, Kennedy SA, Dunne MR, Aird JJ, Foley EK, Clarke N, Ravi N, O'Toole D, Reynolds JV, Kennedy BN, O'Sullivan J. 2018. Leukaemia inhibitory factor is associated with treatment resistance in oesophageal adenocarcinoma. *Oncotarget* 9: 33634-47
 441. Maher SG, McDowell DT, Collins BC, Muldoon C, Gallagher WM, Reynolds JV. 2011. Serum proteomic profiling reveals that pretreatment complement protein levels are predictive of esophageal cancer patient response to neoadjuvant chemoradiation. *Ann Surg* 254: 809-16; discussion 16-7
 442. Barlow WJ, Orlando RC. 2005. The pathogenesis of heartburn in nonerosive reflux disease: a unifying hypothesis. *Gastroenterology* 128: 771-8
 443. Izakovicova Holla L, Borilova Linhartova P, Hrdlickova B, Marek F, Dolina J, Rihak V, Kala Z. 2013. Haplotypes of the IL-1 gene cluster are associated with gastroesophageal reflux disease and Barrett's esophagus. *Hum Immunol* 74: 1161-9
 444. Babar M, Ryan AW, Anderson LA, Segurado R, Turner G, Murray LJ, Murphy SJ, Johnston BT, Comber H, Reynolds JV, McManus R. 2012. Genes of the interleukin-18 pathway are associated with susceptibility to Barrett's esophagus and esophageal adenocarcinoma. *Am J Gastroenterol* 107: 1331-41
 445. Farhadi A, Fields J, Banan A, Keshavarzian A. 2002. Reactive oxygen species: are they involved in the pathogenesis of GERD, barrett's esophagus, and the latter's progression toward esophageal cancer? *American Journal Of Gastroenterology* 97: 22
 446. Wetscher GJ, Hinder RA, Bagchi D, Hinder PR, Bagchi M, Perdakis G, McGinn T. 1995. Reflux esophagitis in humans is mediated by oxygen-derived free radicals. *Am J Surg* 170: 552-6; discussion 6-7
 447. He Y, Hara H, Núñez G. 2016. Mechanism and Regulation of NLRP3 Inflammasome Activation. *Trends in Biochemical Sciences* 41: 1012-21
 448. Feagins LA, Zhang HY, Zhang X, Hormi-Carver K, Thomas T, Terada LS, Spechler SJ, Souza RF. 2008. Mechanisms of oxidant production in esophageal squamous cell and Barrett's cell lines. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 294: G411-G7
 449. Song S, Guha S, Liu K, Buttar NS, Bresalier RS. 2007. COX-2 induction by unconjugated bile acids involves reactive oxygen species-mediated signalling pathways in Barrett's oesophagus and oesophageal adenocarcinoma. *Gut* 56: 1512-21
 450. Tulotta C, Ottewill P. 2018. The role of IL-1 β in breast cancer bone metastasis. *Endocrine-Related Cancer* 25: R421-R34
 451. Nutter F, Holen I, Brown HK, Cross SS, Evans CA, Walker M, Coleman RE, Westbrook JA, Selby PJ, Brown JE, Ottewill PD. 2014. Different molecular profiles are associated with breast cancer cell homing compared with colonisation of bone: evidence using a novel bone-seeking cell line. *Endocr Relat Cancer* 21: 327-41
 452. Coleman RE, Marshall H, Cameron D, Dodwell D, Burkinshaw R, Keane M, Gil M, Houston SJ, Grieve RJ, Barrett-Lee PJ, Ritchie D, Pugh J, Gaunt C, Rea U, Peterson J,

- Davies C, Hiley V, Gregory W, Bell R. 2011. Breast-cancer adjuvant therapy with zoledronic acid. *N Engl J Med* 365: 1396-405
453. Kuschner WG, D'Alessandro A, Wong H, Blanc PD. 1996. Dose-dependent cigarette smoking-related inflammatory responses in healthy adults. *Eur Respir J* 9: 1989-94
454. Lee A, Fox JG, Otto G, Murphy J. 1990. A small animal model of human *Helicobacter pylori* active chronic gastritis. *Gastroenterology* 99: 1315-23
455. Odze R. 2018. Histology of Barrett's Metaplasia: Do Goblet Cells Matter? *Digestive Diseases and Sciences* 63: 2042-51
456. Ross-Innes CS, Debiram-Beecham I, O'Donovan M, Walker E, Varghese S, Lao-Sirieix P, Lovat L, Griffin M, Ragunath K, Haidry R, Sami SS, Kaye P, Novelli M, Disep B, Ostler R, Aigret B, North BV, Bhandari P, Haycock A, Morris D, Attwood S, Dhar A, Rees C, Rutter MD, Sasieni PD, Fitzgerald RC. 2015. Evaluation of a minimally invasive cell sampling device coupled with assessment of trefoil factor 3 expression for diagnosing Barrett's esophagus: a multi-center case-control study. *PLoS Med* 12: e1001780
457. Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, Danilova L, Douville C, Javed AA, Wong F, Mattox A, Hruban RH, Wolfgang CL, Goggins MG, Dal Molin M, Wang TL, Roden R, Klein AP, Ptak J, Dobbys L, Schaefer J, Silliman N, Popoli M, Vogelstein JT, Browne JD, Schoen RE, Brand RE, Tie J, Gibbs P, Wong HL, Mansfield AS, Jen J, Hanash SM, Falconi M, Allen PJ, Zhou S, Bettgowda C, Diaz LA, Jr., Tomasetti C, Kinzler KW, Vogelstein B, Lennon AM, Papadopoulos N. 2018. Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science* 359: 926-30
458. Cook MB, Barnett MJ, Bock CH, Cross AJ, Goodman PJ, Goodman GE, Haiman CA, Khaw K-T, McCullough ML, Newton CC, Boutron-Ruault M-C, Lund E, Rutegård M, Thornquist MD, Spriggs M, Giffen C, Freedman ND, Kemp T, Kroenke CH, Le Marchand L, Park JY, Simon M, Wilkens LR, Pinto L, Hildesheim A, Campbell PT. 2018. Prediagnostic circulating markers of inflammation and risk of oesophageal adenocarcinoma: a study within the National Cancer Institute Cohort Consortium. *Gut*: gutjnl-2018-316678
459. Deans DAC, Wigmore SJ, Gilmour H, Paterson-Brown S, Ross JA, Fearon KCH. 2006. Elevated tumour interleukin-1 β is associated with systemic inflammation: a marker of reduced survival in gastro-oesophageal cancer. *British Journal Of Cancer* 95: 1568
460. Falconer JS, Fearon KC, Ross JA, Elton R, Wigmore SJ, Garden OJ, Carter DC. 1995. Acute-phase protein response and survival duration of patients with pancreatic cancer. *Cancer* 75: 2077-82
461. Crumley AB, McMillan DC, McKernan M, McDonald AC, Stuart RC. 2006. Evaluation of an inflammation-based prognostic score in patients with inoperable gastro-oesophageal cancer. *Br J Cancer* 94: 637-41
462. Nozoe T, Saeki H, Sugimachi K. 2001. Significance of preoperative elevation of serum C-reactive protein as an indicator of prognosis in esophageal carcinoma. *Am J Surg* 182: 197-201
463. Vandanmagsar B, Youm YH, Ravussin A, Galgani JE, Stadler K, Mynatt RL, Ravussin E, Stephens JM, Dixit VD. 2011. The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nat Med* 17: 179-88
464. Stienstra R, Joosten LAB, Koenen T, van Tits B, van Diepen JA, van den Berg SAA, Rensen PCN, Voshol PJ, Fantuzzi G, Hijmans A, Kersten S, Müller M, van den Berg WB, van Rooijen N, Wabitsch M, Kullberg B-J, van der Meer JWM, Kanneganti T, Tack CJ, Netea MG. 2010. The Inflammasome-Mediated Caspase-1 Activation Controls Adipocyte Differentiation and Insulin Sensitivity. *Cell metabolism* 12: 593-605
465. Stienstra R, van Diepen JA, Tack CJ, Zaki MH, van de Veerdonk FL, Perera D, Neale GA, Hooiveld GJ, Hijmans A, Vroegrijk I, van den Berg S, Romijn J, Rensen PC, Joosten LA, Netea MG, Kanneganti TD. 2011. Inflammasome is a central player in the induction of obesity and insulin resistance. *Proc Natl Acad Sci U S A* 108: 15324-9

466. Coll RC, Robertson AAB, Chae JJ, Higgins SC, Muñoz-Planillo R, Inerra MC, Vetter I, Dungan LS, Monks BG, Stutz A, Croker DE, Butler MS, Haneklaus M, Sutton CE, Núñez G, Latz E, Kastner DL, Mills KHG, Masters SL, Schroder K, Cooper MA, O'Neill LAJ. 2015. A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nature medicine* 21: 248-55
467. Xu S, Li X, Liu Y, Xia Y, Chang R, Zhang C. 2019. Inflammasome inhibitors: promising therapeutic approaches against cancer. *Journal of Hematology & Oncology* 12: 64
468. El-Serag HB, Hashmi A, Garcia J, Richardson P, Alsarraj A, Fitzgerald S, Vela M, Shaib Y, Abraham NS, Velez M, Cole R, Rodriguez MB, Anand B, Graham DY, Kramer JR. 2014. Visceral abdominal obesity measured by CT scan is associated with an increased risk of Barrett's oesophagus: a case-control study. *Gut* 63: 220-9
469. Fernandes-Alnemri T, Kang S, Anderson C, Sagara J, Fitzgerald KA, Alnemri ES. 2013. Cutting edge: TLR signaling licenses IRAK1 for rapid activation of the NLRP3 inflammasome. *J Immunol* 191: 3995-9
470. Broz P, Dixit VM. 2016. Inflammasomes: mechanism of assembly, regulation and signalling. *Nat Rev Immunol* 16: 407-20
471. Malik A, Kanneganti T-D. 2017. Inflammasome activation and assembly at a glance. *Journal of Cell Science* 130: 3955
472. Janowski AM, Colegio OR, Hornick EE, McNiff JM, Martin MD, Badovinac VP, Norian LA, Zhang W, Cassel SL, Sutterwala FS. 2016. NLRC4 suppresses melanoma tumor progression independently of inflammasome activation. *J Clin Invest* 126: 3917-28
473. Chai D, Liu N, Li H, Wang G, Song J, Fang L, Lu Z, Yao H, Zheng J. 2018. H1/pAIM2 nanoparticles exert anti-tumour effects that is associated with the inflammasome activation in renal carcinoma. *J Cell Mol Med* 22: 5670-81
474. Xu Z, Wang H, Wei S, Wang Z, Ji G. 2018. Inhibition of ER stress-related IRE1alpha/CREB/NLRP1 pathway promotes the apoptosis of human chronic myelogenous leukemia cell. *Mol Immunol* 101: 377-85
475. Zaki MH, Vogel P, Body-Malapel M, Lamkanfi M, Kanneganti TD. 2010. IL-18 production downstream of the Nlrp3 inflammasome confers protection against colorectal tumor formation. *J Immunol* 185: 4912-20
476. Williams TM, Leeth RA, Rothschild DE, Coutermarsh-Ott SL, McDaniel DK, Simmons AE, Heid B, Cecere TE, Allen IC. 2015. The NLRP1 inflammasome attenuates colitis and colitis-associated tumorigenesis. *J Immunol* 194: 3369-80
477. Daley D, Mani VR, Mohan N, Akkad N, Pandian G, Savadkar S, Lee KB, Torres-Hernandez A, Aykut B, Diskin B, Wang W, Farooq MS, Mahmud AI, Werba G, Morales EJ, Lall S, Wadowski BJ, Rubin AG, Berman ME, Narayanan R, Hundeyin M, Miller G. 2017. NLRP3 signaling drives macrophage-induced adaptive immune suppression in pancreatic carcinoma. *J Exp Med* 214: 1711-24
478. Zhang M, Jin C, Yang Y, Wang K, Zhou Y, Zhou Y, Wang R, Li T, Hu R. 2019. AIM2 promotes non-small-cell lung cancer cell growth through inflammasome-dependent pathway. *Journal of Cellular Physiology* 234: 20161-73
479. Runge TM, Abrams JA, Shaheen NJ. 2015. Epidemiology of Barrett's Esophagus and Esophageal Adenocarcinoma. *Gastroenterology clinics of North America* 44: 203-31
480. Hruby A, Hu FB. 2015. The Epidemiology of Obesity: A Big Picture. *Pharmacoeconomics* 33: 673-89
481. Health Do. 2015. *Healthy Ireland survey 2015 - Summary of findings*, Ireland
482. Lean ME, Han TS, Morrison CE. 1995. Waist circumference as a measure for indicating need for weight management. *BMJ (Clinical research ed.)* 311: 158-61
483. Ibrahim MM. 2010. Subcutaneous and visceral adipose tissue: structural and functional differences. *Obes Rev* 11: 11-8

484. MacKenzie SH, Schipper JL, Clark AC. 2010. The potential for caspases in drug discovery. *Current opinion in drug discovery & development* 13: 568-76
485. Rano TA, Timkey T, Peterson EP, Rotonda J, Nicholson DW, Becker JW, Chapman KT, Thornberry NA. 1997. A combinatorial approach for determining protease specificities: application to interleukin-1 β converting enzyme (ICE). *Chemistry & Biology* 4: 149-55
486. Benkova B, Lozanov V, Ivanov IP, Mitev V. 2009. Evaluation of recombinant caspase specificity by competitive substrates. *Anal Biochem* 394: 68-74
487. Rozman-Pungercar J, Kopitar-Jerala N, Bogyo M, Turk D, Vasiljeva O, Stefe I, Vandenabeele P, Bromme D, Puizdar V, Fonovic M, Trstenjak-Prebenda M, Dolenc I, Turk V, Turk B. 2003. Inhibition of papain-like cysteine proteases and legumain by caspase-specific inhibitors: when reaction mechanism is more important than specificity. *Cell Death Differ* 10: 881-8
488. Shi XY, Bhagwande B, Leong AS. 2008. CDX2 and villin are useful markers of intestinal metaplasia in the diagnosis of Barrett esophagus. *Am J Clin Pathol* 129: 571-7
489. Kerkhof M, Bax DA, Moons LM, van Vuuren AJ, van Dekken H, Steyerberg EW, Kuipers EJ, Kusters JG, Siersema PD. 2006. Does CDX2 expression predict Barrett's metaplasia in oesophageal columnar epithelium without goblet cells? *Aliment Pharmacol Ther* 24: 1613-21
490. Ko MS, Fung KZ, Shi Y, Espaldon R, Shergill A, Walter LC. 2016. Barrett's Esophagus Commonly Diagnosed in Elderly Men with Limited Life Expectancy. *Journal of the American Geriatrics Society* 64: e109-e11
491. Franceschi C, Campisi J. 2014. Chronic Inflammation (Inflammaging) and Its Potential Contribution to Age-Associated Diseases. *The Journals of Gerontology: Series A* 69: S4-S9
492. Jones CL, Weiss DS. 2011. TLR2 signaling contributes to rapid inflammasome activation during *F. novicida* infection. *PloS one* 6: e20609-e
493. Yu HB, Finlay BB. 2008. The Caspase-1 Inflammasome: A Pilot of Innate Immune Responses. *Cell Host & Microbe* 4: 198-208
494. Ponten F, Jirstrom K, Uhlen M. 2008. The Human Protein Atlas--a tool for pathology. *J Pathol* 216: 387-93
495. Ponomareva L, Liu H, Duan X, Dickerson E, Shen H, Panchanathan R, Choubey D. 2013. AIM2, an IFN-inducible cytosolic DNA sensor, in the development of benign prostate hyperplasia and prostate cancer. *Mol Cancer Res* 11: 1193-202
496. Algaba-Chueca F, de-Madaria E, Lozano-Ruiz B, Martinez-Cardona C, Quesada-Vazquez N, Bachiller V, Tarin F, Such J, Frances R, Zapater P, Gonzalez-Navajas JM. 2017. The expression and activation of the AIM2 inflammasome correlates with inflammation and disease severity in patients with acute pancreatitis. *Pancreatology* 17: 364-71
497. Singh S, Sharma AN, Murad MH, Buttar NS, El-Serag HB, Katzka DA, Iyer PG. 2013. Central Adiposity Is Associated With Increased Risk of Esophageal Inflammation, Metaplasia, and Adenocarcinoma: A Systematic Review and Meta-analysis. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association* 11: 10.1016/j.cgh.2013.05.009
498. Allott EH, Morine MJ, Lysaght J, McGarrigle SA, Donohoe CL, Reynolds JV, Roche HM, Pidgeon GP. 2012. Elevated Tumor Expression of PAI-1 and SNAI2 in Obese Esophageal Adenocarcinoma Patients and Impact on Prognosis. *Clinical And Translational Gastroenterology* 3: e12
499. Haneklaus M, Gerlic M, Kurowska-Stolarska M, Rainey AA, Pich D, McInnes IB, Hammerschmidt W, O'Neill LA, Masters SL. 2012. Cutting edge: miR-223 and EBV miR-BART15 regulate the NLRP3 inflammasome and IL-1 β production. *J Immunol* 189: 3795-9

500. Meng X-F, Tan L, Tan M-S, Jiang T, Tan C-C, Li M-M, Wang H-F, Yu J-T. 2014. Inhibition of the NLRP3 inflammasome provides neuroprotection in rats following amygdala kindling-induced status epilepticus. *Journal of Neuroinflammation* 11: 212
501. Yang J, Liu Z, Xiao TS. 2017. Post-translational regulation of inflammasomes. *Cellular & molecular immunology* 14: 65-79
502. Gros Lambert M, Py BF. 2018. Spotlight on the NLRP3 inflammasome pathway. *Journal of inflammation research* 11: 359-74
503. Fettelschoss A, Kistowska M, LeibundGut-Landmann S, Beer HD, Johansen P, Senti G, Contassot E, Bachmann MF, French LE, Oxenius A, Kundig TM. 2011. Inflammasome activation and IL-1 β target IL-1 α for secretion as opposed to surface expression. *Proc Natl Acad Sci U S A* 108: 18055-60
504. Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. 2001. Interleukin-18 regulates both Th1 and Th2 responses. *Annu Rev Immunol* 19: 423-74
505. Christ A, Gunther P, Lauterbach MAR, Duewell P, Biswas D, Pelka K, Scholz CJ, Oosting M, Haendler K, Bassler K, Klee K, Schulte-Schrepping J, Ulas T, Moorlag S, Kumar V, Park MH, Joosten LAB, Groh LA, Riksen NP, Espevik T, Schlitzer A, Li Y, Fitzgerald ML, Netea MG, Schultze JL, Latz E. 2018. Western Diet Triggers NLRP3-Dependent Innate Immune Reprogramming. *Cell* 172: 162-75.e14
506. Blevins CH, Dierkhising RA, Geno DM, Johnson ML, Vela MF, Ravi K, Iyer PG, Katzka DA. 2018. Obesity and GERD impair esophageal epithelial permeability through 2 distinct mechanisms. *Neurogastroenterology & Motility* 30: e13403
507. Dinarello CA. 1996. Biologic basis for interleukin-1 in disease. *Blood* 87: 2095-147
508. Hoffman HM, Rosengren S, Boyle DL, Cho JY, Nayar J, Mueller JL, Anderson JP, Wanderer AA, Firestein GS. 2004. Prevention of cold-associated acute inflammation in familial cold autoinflammatory syndrome by interleukin-1 receptor antagonist. *The Lancet* 364: 1779-85
509. Labrousse D, Perret M, Hayez D, Da Silva S, Badiou C, Couzon F, Bes M, Chavanet P, Lina G, Vandenesch F, Croisier-Bertin D, Henry T. 2014. Kineret(R)/IL-1ra blocks the IL-1/IL-8 inflammatory cascade during recombinant Panton Valentine Leukocidin-triggered pneumonia but not during *S. aureus* infection. *PLoS One* 9: e97546
510. Eskin MA, Benakanakere MR, Rose BG, Zhang P, Zhao J, Stathopoulou P, Fujioka D, Kinane DF. 2008. Interleukin-1 β modulates proinflammatory cytokine production in human epithelial cells. *Infect Immun* 76: 2080-9
511. Beales ILP, Calam J. 1997. STIMULATION OF IL-8 PRODUCTION IN HUMAN GASTRIC EPITHELIAL CELLS BY HELICOBACTER PYLORI, IL-1 β AND TNF- α REQUIRES TYROSINE KINASE ACTIVITY, BUT NOT PROTEIN KINASE C. *Cytokine* 9: 514-20
512. Mora-Buch R, Dotti I, Planell N, Calderón-Gómez E, Jung P, Masamunt MC, Llach J, Ricart E, Batlle E, Panés J, Salas A. 2016. Epithelial IL-1R2 acts as a homeostatic regulator during remission of ulcerative colitis. *Mucosal Immunology* 9: 950-9
513. Brabcova E, Kolesar L, Thorburn E, Striz I. 2014. Chemokines induced in human respiratory epithelial cells by IL-1 family of cytokines. *Folia Biol (Praha)* 60: 180-6
514. Grivennikov S, Karin M. 2008. Autocrine IL-6 signaling: a key event in tumorigenesis? *Cancer Cell* 13: 7-9
515. Lu Y-C, Yeh W-C, Ohashi PS. 2008. LPS/TLR4 signal transduction pathway. *Cytokine* 42: 145-51
516. Verbeek RE, Siersema PD, Ten Kate FJ, Fluiter K, Souza RF, Vleggaar FP, Bus P, van Baal JW. 2014. Toll-like receptor 4 activation in Barrett's esophagus results in a strong increase in COX-2 expression. *J Gastroenterol* 49: 1121-34
517. Jenkins GJS, Harries K, Doak SH, Wilmes A, Griffiths AP, Baxter JN, Parry JM. 2004. The bile acid deoxycholic acid (DCA) at neutral pH activates NF- κ B and induces IL-8 expression in oesophageal cells in vitro. *Carcinogenesis* 25: 317-23

518. Yoshida N, Imamoto E, Uchiyama K, Kuroda M, Naito Y, Mukaida N, Kawabe A, Shimada Y, Yoshikawa T, Okanoue T. 2006. Molecular mechanisms involved in interleukin-8 production by normal human oesophageal epithelial cells. *Alimentary Pharmacology & Therapeutics* 24: 219-26
519. Asfaha S, Dubeykovskiy AN, Tomita H, Yang X, Stokes S, Shibata W, Friedman RA, Ariyama H, Dubeykovskaya ZA, Muthupalani S, Ericksen R, Frucht H, Fox JG, Wang TC. 2013. Mice that express human interleukin-8 have increased mobilization of immature myeloid cells, which exacerbates inflammation and accelerates colon carcinogenesis. *Gastroenterology* 144: 155-66
520. David JM, Dominguez C, Hamilton DH, Palena C. 2016. The IL-8/IL-8R Axis: A Double Agent in Tumor Immune Resistance. *Vaccines* 4: 22
521. Egesten A, Eliasson M, Olin AI, Erjefalt JS, Bjartell A, Sangfelt P, Carlson M. 2007. The proinflammatory CXC-chemokines GRO- α /CXCL1 and MIG/CXCL9 are concomitantly expressed in ulcerative colitis and decrease during treatment with topical corticosteroids. *Int J Colorectal Dis* 22: 1421-7
522. Yoshida N, Uchiyama K, Kuroda M, Sakuma K, Kokura S, Ichikawa H, Naito Y, Takemura T, Yoshikawa T, Okanoue T. 2004. Interleukin-8 expression in the esophageal mucosa of patients with gastroesophageal reflux disease. *Scand J Gastroenterol* 39: 816-22
523. Thrift AP, Garcia JM, El-Serag HB. 2014. A multibiomarker risk score helps predict risk for Barrett's esophagus. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association* 12: 1267-71
524. Mishima Y, Oka A, Liu B, Herzog JW, Eun CS, Fan T-J, Bulik-Sullivan E, Carroll IM, Hansen JJ, Chen L, Wilson JE, Fisher NC, Ting JPY, Nochi T, Wahl A, Garcia JV, Karp CL, Sartor RB. 2019. Microbiota maintain colonic homeostasis by activating TLR2/MyD88/PI3K signaling in IL-10-producing regulatory B cells. *The Journal of Clinical Investigation* 129: 3702-16
525. Kuo P-L, Shen K-H, Hung S-H, Hsu Y-L. 2012. CXCL1/GRO α increases cell migration and invasion of prostate cancer by decreasing fibulin-1 expression through NF- κ B/HDAC1 epigenetic regulation. *Carcinogenesis* 33: 2477-87
526. Christine B, Michael AH, Robert CB, Deborah JM. 2010. The chemokine CXCL1 induces proliferation in epithelial ovarian cancer cells by transactivation of the epidermal growth factor receptor. *Endocrine-Related Cancer* 17: 929-40
527. Miyake M, Goodison S, Gomes E, Rizwani W, Ross S, Zhang G, Rosser CJ. 2013. Induction of endothelial proliferation and angiogenesis through activating the ERK1/2/EGF pathway mediate by CXC chemokine receptor 2 by chemokine (C-X-C motif) ligand 1. *Journal of Clinical Oncology* 31: 138-
528. De Filippo K, Henderson RB, Laschinger M, Hogg N. 2008. Neutrophil chemokines KC and macrophage-inflammatory protein-2 are newly synthesized by tissue macrophages using distinct TLR signaling pathways. *J Immunol* 180: 4308-15
529. Moles A, Murphy L, Wilson CL, Chakraborty JB, Fox C, Park EJ, Mann J, Oakley F, Howarth R, Brain J, Masson S, Karin M, Seki E, Mann DA. 2014. A TLR2/S100A9/CXCL-2 signaling network is necessary for neutrophil recruitment in acute and chronic liver injury in the mouse. *Journal of hepatology* 60: 782-91
530. Andrews K, Abdelsamed H, Yi AK, Miller MA, Fitzpatrick EA. 2013. TLR2 regulates neutrophil recruitment and cytokine production with minor contributions from TLR9 during hypersensitivity pneumonitis. *PLoS One* 8: e73143
531. Gillette-Ferguson I, Daehnel K, Hise AG, Sun Y, Carlson E, Diaconu E, McGarry HF, Taylor MJ, Pearlman E. 2007. Toll-Like Receptor 2 Regulates CXC Chemokine Production and Neutrophil Recruitment to the Cornea in *Onchocerca volvulus*/ *Wolbachia*-Induced Keratitis. *Infection and Immunity* 75: 5908

532. Rosales C. 2018. Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? *Frontiers in physiology* 9: 113-
533. Tye H, Kennedy Catherine L, Najdovska M, McLeod L, McCormack W, Hughes N, Dev A, Sievert W, Ooi Chia H, Ishikawa T-o, Oshima H, Bhathal Prithi S, Parker Andrew E, Oshima M, Tan P, Jenkins Brendan J. 2012. STAT3-Driven Upregulation of TLR2 Promotes Gastric Tumorigenesis Independent of Tumor Inflammation. *Cancer Cell* 22: 466-78
534. Dinarello CA, Simon A, van der Meer JWM. 2012. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nature reviews. Drug discovery* 11: 633-52
535. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, Fonseca F, Nicolau J, Koenig W, Anker SD, Kastelein JJP, Cornel JH, Pais P, Pella D, Genest J, Cifkova R, Lorenzatti A, Forster T, Kobalava Z, Vida-Simiti L, Flather M, Shimokawa H, Ogawa H, Dellborg M, Rossi PRF, Troquay RPT, Libby P, Glynn RJ. 2017. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *New England Journal of Medicine* 377: 1119-31
536. Belghazi K, Weusten BL, Meijer SL, Bergman J, Pouw RE. 2017. Tu1155 Seven-Year Prospective Follow-Up Results of Radiofrequency Ablation for Barrett's Esophagus With High-Grade Dysplasia and Early Cancer. *Gastrointestinal Endoscopy* 85: AB560
537. Kudelova J, Fleischmannova J, Adamova E, Matalova E. 2015. Pharmacological caspase inhibitors: research towards therapeutic perspectives. *J Physiol Pharmacol* 66: 473-82
538. Rudolphi K, Gerwin N, Verzijl N, van der Kraan P, van den Berg W. 2003. Pralnacasan, an inhibitor of interleukin-1beta converting enzyme, reduces joint damage in two murine models of osteoarthritis. *Osteoarthritis Cartilage* 11: 738-46
539. Bialer M, Johannessen SI, Levy RH, Perucca E, Tomson T, White HS. 2013. Progress report on new antiepileptic drugs: a summary of the Eleventh Eilat Conference (EILAT XI). *Epilepsy Res* 103: 2-30
540. Wannamaker W, Davies R, Namchuk M, Pollard J, Ford P, Ku G, Decker C, Charifson P, Weber P, Germann UA, Kuida K, Randle JC. 2007. (S)-1-((S)-2-[[1-(4-amino-3-chloro-phenyl)-methanoyl]-amino]-3,3-dimethyl-butanoyl)-pyrrolidine-2-carboxylic acid ((2R,3S)-2-ethoxy-5-oxo-tetrahydro-furan-3-yl)-amide (VX-765), an orally available selective interleukin (IL)-converting enzyme/caspase-1 inhibitor, exhibits potent anti-inflammatory activities by inhibiting the release of IL-1beta and IL-18. *J Pharmacol Exp Ther* 321: 509-16
541. Mantovani A, Allavena P, Sica A, Balkwill F. 2008. Cancer-related inflammation. *Nature* 454: 436-44
542. Pera M, Cameron AJ, Trastek VF, Carpenter HA, Zinsmeister AR. 1993. Increasing incidence of adenocarcinoma of the esophagus and esophagogastric junction. *Gastroenterology* 104: 510-3
543. Blot WJ, Devesa SS, Fraumeni JF, Jr. 1993. Continuing climb in rates of esophageal adenocarcinoma: an update. *Jama* 270: 1320
544. Celardo I, Grespi F, Antonov A, Bernassola F, Garabadgiu AV, Melino G, Amelio I. 2013. Caspase-1 is a novel target of p63 in tumor suppression. *Cell Death & Disease* 4: e645
545. Haabeth OAW, Bogen B, Corthay A. 2012. A model for cancer-suppressive inflammation. *Oncoimmunology* 1: 1146-55
546. North RJ, Neubauer RH, Huang JJ, Newton RC, Loveless SE. 1988. Interleukin 1-induced, T cell-mediated regression of immunogenic murine tumors. Requirement for an adequate level of already acquired host concomitant immunity. *J Exp Med* 168: 2031-43

547. Gasparoto TH, de Oliveira CE, de Freitas LT, Pinheiro CR, Hori JI, Garlet GP, Cavassani KA, Schillaci R, da Silva JS, Zamboni DS, Campanelli AP. 2014. Inflammasome Activation Is Critical to the Protective Immune Response during Chemically Induced Squamous Cell Carcinoma. *PLoS ONE* 9: e107170
548. Voloshin T, Alishekevitz D, Kaneti L, Miller V, Isakov E, Kaplanov I, Voronov E, Fremder E, Benhar M, Machluf M, Apte RN, Shaked Y. 2015. Blocking IL1beta Pathway Following Paclitaxel Chemotherapy Slightly Inhibits Primary Tumor Growth but Promotes Spontaneous Metastasis. *Mol Cancer Ther* 14: 1385-94
549. Alfonso L, Ai G, Spitale RC, Bhat GJ. 2014. Molecular targets of aspirin and cancer prevention. *British Journal of Cancer* 111: 61-7
550. Nguyen T, Tang Z, Younes M, Alsarraj A, Ramsey D, Fitzgerald S, Kramer JR, El-Serag HB. 2015. Esophageal COX-2 expression is increased in Barrett's esophagus, obesity, and smoking. *Dig Dis Sci* 60: 65-73
551. Majka J, Rembiasz K, Migaczewski M, Budzynski A, Ptak-Belowska A, Pabianczyk R, Urbanczyk K, Zub-Pokrowiecka A, Matlok M, Brzozowski T. 2010. Cyclooxygenase-2 (COX-2) is the key event in pathophysiology of Barrett's esophagus. Lesson from experimental animal model and human subjects. *J Physiol Pharmacol* 61: 409-18
552. Hua KF, Chou JC, Ka SM, Tasi YL, Chen A, Wu SH, Chiu HW, Wong WT, Wang YF, Tsai CL, Ho CL, Lin CH. 2015. Cyclooxygenase-2 regulates NLRP3 inflammasome-derived IL-1beta production. *J Cell Physiol* 230: 863-74
553. Ratsimandresy RA, Indramohan M, Dorfleutner A, Stehlik C. 2017. The AIM2 inflammasome is a central regulator of intestinal homeostasis through the IL-18/IL-22/STAT3 pathway. *Cellular & molecular immunology* 14: 127-42
554. Hu S, Peng L, Kwak Y-T, Tekippe EM, Pasare C, Malter JS, Hooper LV, Zaki MH. 2015. The DNA Sensor AIM2 Maintains Intestinal Homeostasis via Regulation of Epithelial Antimicrobial Host Defense. *Cell reports* 13: 1922-36
555. Hirota SA, Ng J, Lueng A, Khajah M, Parhar K, Li Y, Lam V, Potentier MS, Ng K, Bawa M, McCafferty D-M, Rioux KP, Ghosh S, Xavier RJ, Colgan SP, Tschopp J, Muruve D, MacDonald JA, Beck PL. 2011. NLRP3 inflammasome plays a key role in the regulation of intestinal homeostasis. *Inflammatory bowel diseases* 17: 1359-72
556. Hoque R, Sohail M, Malik A, Sarwar S, Luo Y, Shah A, Barrat F, Flavell R, Gorelick F, Husain S, Mehal W. 2011. TLR9 and the NLRP3 Inflammasome Link Acinar Cell Death With Inflammation in Acute Pancreatitis. *Gastroenterology* 141: 358-69
557. Kang R, Chen R, Xie M, Cao L, Lotze MT, Tang D, Zeh HJ. 2016. The Receptor for Advanced Glycation End Products Activates the AIM2 Inflammasome in Acute Pancreatitis. *The Journal of Immunology* 196: 4331
558. Lysaght J, Allott EH, Donohoe CL, Howard JM, Pidgeon GP, Reynolds JV. 2011. T lymphocyte activation in visceral adipose tissue of patients with oesophageal adenocarcinoma. *Br J Surg* 98: 964-74
559. Dinarello CA. 2018. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunological reviews* 281: 8-27
560. Lust JA, Lacy MQ, Zeldenrust SR, Dispenzieri A, Gertz MA, Witzig TE, Kumar S, Hayman SR, Russell SJ, Buadi FK, Geyer SM, Campbell ME, Kyle RA, Rajkumar SV, Greipp PR, Kline MP, Xiong Y, Moon-Tasson LL, Donovan KA. 2009. Induction of a chronic disease state in patients with smoldering or indolent multiple myeloma by targeting interleukin 1{beta}-induced interleukin 6 production and the myeloma proliferative component. *Mayo Clin Proc* 84: 114-22
561. Ferrara N. 2009. VEGF-A: a critical regulator of blood vessel growth. *Eur Cytokine Netw* 20: 158-63
562. Carmeliet P. 2005. VEGF as a key mediator of angiogenesis in cancer. *Oncology* 69 Suppl 3: 4-10

563. Nong S, Zhang Y, Zhou S, Li W, Ma L, Wu Y. 2010. Relationship between serum IL-18 and VEGF levels in patients with prostate cancer. *The Chinese-German Journal of Clinical Oncology* 9: 643-7
564. Shen J, Choy DF, Yoshida T, Iwase T, Hafiz G, Xie B, Hackett SF, Arron JR, Campochiaro PA. 2014. Interleukin-18 has antipermeability and antiangiogenic activities in the eye: reciprocal suppression with VEGF. *Journal of cellular physiology* 229: 974-83
565. Cho M-L, Jung YO, Moon Y-M, Min S-Y, Yoon C-H, Lee S-H, Park S-H, Cho C-S, Jue D-M, Kim H-Y. 2006. Interleukin-18 induces the production of vascular endothelial growth factor (VEGF) in rheumatoid arthritis synovial fibroblasts via AP-1-dependent pathways. *Immunology Letters* 103: 159-66
566. Yang S, Chen X-q, Lu H-s, Li Z-y, Lin T-y. 2010. Interleukin-18 suppresses angiogenesis and lymphangiogenesis in implanted Lewis lung cancer. *Chinese Journal of Cancer Research* 22: 303-9
567. Kang JS, Bae SY, Kim HR, Kim YS, Kim DJ, Cho BJ, Yang HK, Hwang YI, Kim KJ, Park HS, Hwang DH, Cho DJ, Lee WJ. 2009. Interleukin-18 increases metastasis and immune escape of stomach cancer via the downregulation of CD70 and maintenance of CD44. *Carcinogenesis* 30: 1987-96
568. Kim KE, Song H, Kim TS, Yoon D, Kim CW, Bang SI, Hur DY, Park H, Cho DH. 2007. Interleukin-18 is a critical factor for vascular endothelial growth factor-enhanced migration in human gastric cancer cell lines. *Oncogene* 26: 1468-76
569. Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, Abela GS, Franchi L, Nunez G, Schnurr M, Espevik T, Lien E, Fitzgerald KA, Rock KL, Moore KJ, Wright SD, Hornung V, Latz E. 2010. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature* 464: 1357-61
570. Karasawa T, Kawashima A, Usui-Kawanishi F, Watanabe S, Kimura H, Kamata R, Shirasuna K, Koyama Y, Sato-Tomita A, Matsuzaka T, Tomoda H, Park SY, Shibayama N, Shimano H, Kasahara T, Takahashi M. 2018. Saturated Fatty Acids Undergo Intracellular Crystallization and Activate the NLRP3 Inflammasome in Macrophages. *Arterioscler Thromb Vasc Biol* 38: 744-56
571. Catano Canizales YG, Uresti Rivera EE, Garcia Jacobo RE, Portales Perez DP, Yadira B, Rodriguez Rivera JG, Amaro RG, Enciso Moreno JA, Garcia Hernandez MH. 2018. Increased Levels of AIM2 and Circulating Mitochondrial DNA in Type 2 Diabetes. *Iran J Immunol* 15: 142-55
572. Datta R, Podolsky MJ, Atabai K. 2018. Fat fibrosis: friend or foe? *JCI insight* 3: e122289
573. Kim Y, Chang Y, Cho YK, Ahn J, Shin H, Ryu S. 2019. Obesity and Weight Gain Are Associated With Progression of Fibrosis in Patients With Nonalcoholic Fatty Liver Disease. *Clinical Gastroenterology and Hepatology* 17: 543-50.e2
574. Hotamisligil GS. 2017. Inflammation, metaflammation and immunometabolic disorders. *Nature* 542: 177
575. Miura K, Yang L, van Rooijen N, Brenner DA, Ohnishi H, Seki E. 2013. Toll-like receptor 2 and palmitic acid cooperatively contribute to the development of nonalcoholic steatohepatitis through inflammasome activation in mice. *Hepatology (Baltimore, Md.)* 57: 577-89
576. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP. 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor- α from cells. *Nature* 385: 729-33
577. Nguyen MT, Favelyukis S, Nguyen AK, Reichart D, Scott PA, Jenn A, Liu-Bryan R, Glass CK, Neels JG, Olefsky JM. 2007. A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. *J Biol Chem* 282: 35279-92

578. Dupaul-Chicoine J, Arabzadeh A, Dagenais M, Douglas T, Champagne C, Morizot A, Rodrigue-Gervais Ian G, Breton V, Colpitts Sara L, Beauchemin N, Saleh M. 2015. The Nlrp3 Inflammasome Suppresses Colorectal Cancer Metastatic Growth in the Liver by Promoting Natural Killer Cell Tumorcidal Activity. *Immunity* 43: 751-63
579. Mongan AM, Lynam-Lennon N, Doyle SL, Casey R, Carr E, Cannon A, Conroy MJ, Pidgeon GP, Brennan L, Lysaght J, Reynolds JV, O'Sullivan J. 2019. Visceral Adipose Tissue Modulates Radiosensitivity in Oesophageal Adenocarcinoma. *International journal of medical sciences* 16: 519-28
580. Wang Z, Aguilar EG, Luna JJ, Dunai C, Khuat LT, Le CT, Mirsoian A, Minnar CM, Stoffel KM, Sturgill IR, Grossenbacher SK, Withers SS, Rebhun RB, Hartigan-O'Connor DJ, Mendez-Lagares G, Tarantal AF, Isseroff RR, Griffith TS, Schalper KA, Merleev A, Saha A, Maverakis E, Kelly K, Aljumaily R, Ibrahimi S, Mukherjee S, Machiorlatti M, Vesely SK, Longo DL, Blazar BR, Canter RJ, Murphy WJ, Monjazebe AM. 2019. Paradoxical effects of obesity on T cell function during tumor progression and PD-1 checkpoint blockade. *Nat Med* 25: 141-51
581. Hoss F, Mueller JL, Rojas Ringeling F, Rodriguez-Alcazar JF, Brinkschulte R, Seifert G, Stahl R, Broderick L, Putnam CD, Kolodner RD, Canzar S, Geyer M, Hoffman HM, Latz E. 2019. Alternative splicing regulates stochastic NLRP3 activity. *Nature communications* 10: 3238-
582. MacFarlane B. 2018. Management of gastroesophageal reflux disease in adults: a pharmacist's perspective. *Integrated pharmacy research & practice* 7: 41-52
583. Fullard M, Kang JY, Neild P, Poullis A, Maxwell JD. 2006. Systematic review: does gastro-oesophageal reflux disease progress? *Aliment Pharmacol Ther* 24: 33-45
584. Savarino E, de Bortoli N, De Cassan C, Della Coletta M, Bartolo O, Furnari M, Ottonello A, Marabotto E, Bodini G, Savarino V. 2017. The natural history of gastro-esophageal reflux disease: a comprehensive review. *Dis Esophagus* 30: 1-9
585. Dodds WJ, Dent J, Hogan WJ, Helm JF, Hauser R, Patel GK, Egide MS. 1982. Mechanisms of Gastroesophageal Reflux in Patients with Reflux Esophagitis. *New England Journal of Medicine* 307: 1547-52
586. Nebel OT, Fornes MF, Castell DO. 1976. Symptomatic gastroesophageal reflux: Incidence and precipitating factors. *The American Journal of Digestive Diseases* 21: 953-6
587. Nam SY, Choi IJ, Ryu KH, Park BJ, Kim HB, Nam BH. 2010. Abdominal visceral adipose tissue volume is associated with increased risk of erosive esophagitis in men and women. *Gastroenterology* 139: 1902-11.e2
588. Pandolfino JE, El-Serag HB, Zhang Q, Shah N, Ghosh SK, Kahrilas PJ. 2006. Obesity: a challenge to esophagogastric junction integrity. *Gastroenterology* 130: 639-49
589. Isomoto H, Wang A, Mizuta Y, Akazawa Y, Ohba K, Omagari K, Miyazaki M, Murase K, Hayashi T, Inoue K, Murata I, Kohno S. 2003. Elevated levels of chemokines in esophageal mucosa of patients with reflux esophagitis. *Am J Gastroenterol* 98: 551-6
590. Isomoto H, Saenko VA, Kanazawa Y, Nishi Y, Ohtsuru A, Inoue K, Akazawa Y, Takeshima F, Omagari K, Miyazaki M, Mizuta Y, Murata I, Yamashita S, Kohno S. 2004. Enhanced expression of interleukin-8 and activation of nuclear factor kappa-B in endoscopy-negative gastroesophageal reflux disease. *Am J Gastroenterol* 99: 589-97
591. Kahrilas PJ, Shaheen NJ, Vaezi MF. 2008. American Gastroenterological Association Institute Technical Review on the Management of Gastroesophageal Reflux Disease. *Gastroenterology* 135: 1392-413.e5
592. Freedberg DE, Kim LS, Yang YX. 2017. The Risks and Benefits of Long-term Use of Proton Pump Inhibitors: Expert Review and Best Practice Advice From the American Gastroenterological Association. *Gastroenterology* 152: 706-15

593. Eusebi LH, Rabitti S, Artesiani ML, Gelli D, Montagnani M, Zagari RM, Bazzoli F. 2017. Proton pump inhibitors: Risks of long-term use. *J Gastroenterol Hepatol* 32: 1295-302
594. El-Serag HB, Satia JA, Rabeneck L. 2005. Dietary intake and the risk of gastro-oesophageal reflux disease: a cross sectional study in volunteers. *Gut* 54: 11
595. Yates M, Luben R, Cheong E, Igali L, Fitzgerald R, Khaw KT, Hart A. 2011. Dietary fat intake in the aetiology of barrett's oesophagus and oesophageal adenocarcinoma: data from a prospective cohort study (EPIC-Norfolk) using 7-day food diary data. *Gut* 60: A179
596. O'Doherty MG, Cantwell MM, Murray LJ, Anderson LA, Abnet CC, Group FS. 2011. Dietary fat and meat intakes and risk of reflux esophagitis, Barrett's esophagus and esophageal adenocarcinoma. *International journal of cancer* 129: 1493-502
597. Li N, Petrick JL, Steck SE, Bradshaw PT, McClain KM, Niehoff NM, Engel LS, Shaheen NJ, Corley DA, Vaughan TL, Gammon MD. 2017. Dietary sugar/starches intake and Barrett's esophagus: a pooled analysis. *Eur J Epidemiol* 32: 1007-17
598. Stoner GD, Kaighn ME, Reddel RR, Resau JH, Bowman D, Naito Z, Matsukura N, You M, Galati AJ, Harris CC. 1991. Establishment and characterization of SV40 T-antigen immortalized human esophageal epithelial cells. *Cancer Res* 51: 365-71
599. Hilakivi-Clarke L, Cho E, Onojafe I. 1996. High-fat diet induces aggressive behavior in male mice and rats. *Life Sci* 58: 1653-60
600. Fisher-Wellman KH, Ryan TE, Smith CD, Gilliam LAA, Lin C-T, Reese LR, Torres MJ, Neuffer PD. 2016. A Direct Comparison of Metabolic Responses to High-Fat Diet in C57BL/6J and C57BL/6NJ Mice. *Diabetes* 65: 3249
601. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440: 237-41
602. Farrar CA, Keogh B, McCormack W, O'Shaughnessy A, Parker A, Reilly M, Sacks SH. 2012. Inhibition of TLR2 promotes graft function in a murine model of renal transplant ischemia-reperfusion injury. *Faseb j* 26: 799-807
603. Meng G, Rutz M, Schiemann M, Metzger J, Grabiec A, Schwandner R, Luppa PB, Ebel F, Busch DH, Bauer S, Wagner H, Kirschning CJ. 2004. Antagonistic antibody prevents toll-like receptor 2-driven lethal shock-like syndromes. *J Clin Invest* 113: 1473-81
604. Arslan F, Smeets MB, O'Neill LA, Keogh B, McGuirk P, Timmers L, Tersteeg C, Hoefer IE, Doevendans PA, Pasterkamp G, de Kleijn DP. 2010. Myocardial ischemia/reperfusion injury is mediated by leukocytic toll-like receptor-2 and reduced by systemic administration of a novel anti-toll-like receptor-2 antibody. *Circulation* 121: 80-90
605. Chen IF, Ou-Yang F, Hung JY, Liu JC, Wang H, Wang SC, Hou MF, Hortobagyi GN, Hung MC. 2006. AIM2 suppresses human breast cancer cell proliferation in vitro and mammary tumor growth in a mouse model. *Mol Cancer Ther* 5: 1-7
606. Dihlmann S, Tao S, Echterdiek F, Herpel E, Jansen L, Chang-Claude J, Brenner H, Hoffmeister M, Kloor M. 2014. Lack of Absent in Melanoma 2 (AIM2) expression in tumor cells is closely associated with poor survival in colorectal cancer patients. *Int J Cancer* 135: 2387-96
607. Farshchian M, Nissinen L, Siljamaki E, Riihila P, Piipponen M, Kivisaari A, Kallajoki M, Grenman R, Peltonen J, Peltonen S, Quint KD, Bavinck JNB, Kahari VM. 2017. Tumor cell-specific AIM2 regulates growth and invasion of cutaneous squamous cell carcinoma. *Oncotarget* 8: 45825-36
608. Guadagnini D, Rocha GZ, Santos A, Assalin HB, Hirabara SM, Curi R, Oliveira AG, Prada PO, Saad MJA. 2019. Microbiota determines insulin sensitivity in TLR2-KO mice. *Life Sci* 234: 116793
609. Ellulu MS, Patimah I, Khaza'ai H, Rahmat A, Abed Y. 2017. Obesity and inflammation: the linking mechanism and the complications. *Archives of medical science : AMS* 13: 851-63

610. Guy NC, Garewal H, Holubec H, Bernstein H, Payne CM, Bernstein C, Bhattacharyya AK, Dvorak K. 2007. A novel dietary-related model of esophagitis and Barrett's esophagus, a premalignant lesion. *Nutr Cancer* 59: 217-27
611. Blevins CH, Iyer PG, Vela MF, Katzka DA. 2018. The Esophageal Epithelial Barrier in Health and Disease. *Clin Gastroenterol Hepatol* 16: 608-17
612. Farhat K, Riekenberg S, Heine H, Debarry J, Lang R, Mages J, Buwitt-Beckmann U, Röschmann K, Jung G, Wiesmüller K-H, Ulmer AJ. 2008. Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. *Journal of Leukocyte Biology* 83: 692-701
613. Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, Takeda K, Akira S. 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int Immunol* 13: 933-40
614. Robichon C, Vidal-Ingigliardi D, Pugsley AP. 2005. Depletion of apolipoprotein N-acyltransferase causes mislocalization of outer membrane lipoproteins in *Escherichia coli*. *J Biol Chem* 280: 974-83
615. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, Modlin RL, Akira S. 2002. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol* 169: 10-4
616. Nic An Ultaigh S, Saber TP, McCormick J, Connolly M, Dellacasagrande J, Keogh B, McCormack W, Reilly M, O'Neill LA, McGuirk P, Fearon U, Veale DJ. 2011. Blockade of Toll-like receptor 2 prevents spontaneous cytokine release from rheumatoid arthritis ex vivo synovial explant cultures. *Arthritis Research & Therapy* 13: R33
617. Tobey NA, Carson JL, Alkiek RA, Orlando RC. 1996. Dilated intercellular spaces: a morphological feature of acid reflux--damaged human esophageal epithelium. *Gastroenterology* 111: 1200-5
618. Tobey NA, Hosseini SS, Argote CM, Dobrucali AM, Awayda MS, Orlando RC. 2004. Dilated intercellular spaces and shunt permeability in nonerosive acid-damaged esophageal epithelium. *Am J Gastroenterol* 99: 13-22
619. Batchelor H. 2005. Bioadhesive dosage forms for esophageal drug delivery. *Pharm Res* 22: 175-81
620. Reilly M, Miller RM, Thomson MH, Patris V, Ryle P, McLoughlin L, Mutch P, Gilboy P, Miller C, Broekema M, Keogh B, McCormack W, van de Wetering de Rooij J. 2013. Randomized, double-blind, placebo-controlled, dose-escalating phase I, healthy subjects study of intravenous OPN-305, a humanized anti-TLR2 antibody. *Clin Pharmacol Ther* 94: 593-600
621. Garcia-Manero G, Montalban-Bravo G, Yang H, Wei Y, Alvarado Y, DiNardo CD, Daver NG, Konopleva M, Hearn KP, Miller R, Arbe-Barnes S, McGuirk P, Kearney T, Keogh B, Kantarjian HM, Reilly M. 2016. A Clinical Study of OPN-305, a Toll-like Receptor 2 (TLR-2) Antibody, in Patients with Lower Risk Myelodysplastic Syndromes (MDS) That Have Received Prior Hypomethylating Agent (HMA) Therapy. *Blood* 128: 227-
622. Valentini M, Piermattei A, Di Sante G, Migliara G, Delogu G, Ria F. 2014. Immunomodulation by gut microbiota: role of Toll-like receptor expressed by T cells. *Journal of immunology research* 2014: 586939-
623. Davis JE, Braucher DR, Walker-Daniels J, Spurlock ME. 2011. Absence of Tlr2 protects against high-fat diet-induced inflammation and results in greater insulin-stimulated glucose transport in cultured adipocytes. *J Nutr Biochem* 22: 136-41
624. Ehses JA, Meier DT, Wueest S, Rytka J, Boller S, Wielinga PY, Schraenen A, Lemaire K, Debray S, Van Lommel L, Pospisilik JA, Tschopp O, Schultze SM, Malipiero U, Esterbauer H, Ellingsgaard H, Rütli S, Schuit FC, Lutz TA, Böni-Schnetzler M, Konrad D, Donath MY. 2010. Toll-like receptor 2-deficient mice are protected from insulin

- resistance and beta cell dysfunction induced by a high-fat diet. *Diabetologia* 53: 1795-806
625. Jang HJ, Kim HS, Hwang DH, Quon MJ, Kim JA. 2013. Toll-like receptor 2 mediates high-fat diet-induced impairment of vasodilator actions of insulin. *Am J Physiol Endocrinol Metab* 304: E1077-88
 626. Himes RW, Smith CW. 2010. Tlr2 is critical for diet-induced metabolic syndrome in a murine model. *Faseb j* 24: 731-9
 627. Kuo LH, Tsai PJ, Jiang MJ, Chuang YL, Yu L, Lai KTA, Tsai YS. 2011. Toll-like receptor 2 deficiency improves insulin sensitivity and hepatic insulin signalling in the mouse. *Diabetologia* 54: 168-79
 628. Zhang L, Meng J, Ban Y, Jalodia R, Chupikova I, Fernandez I, Brito N, Sharma U, Abreu MT, Ramakrishnan S, Roy S. 2019. Morphine tolerance is attenuated in germfree mice and reversed by probiotics, implicating the role of gut microbiome. *Proc Natl Acad Sci U S A* 116: 13523-32
 629. Jha S, Brickey WJ, Ting JP-Y. 2017. Inflammasomes in Myeloid Cells: Warriors Within. *Microbiology spectrum* 5: 10.1128/microbiolspec.MCHD-0049-2016
 630. Kanneganti T-D. 2015. The inflammasome: firing up innate immunity. *Immunological reviews* 265: 1-5
 631. Bakele M, Joos M, Burdi S, Allgaier N, Poschel S, Fehrenbacher B, Schaller M, Marcos V, Kummerle-Deschner J, Rieber N, Borregaard N, Yazdi A, Hector A, Hartl D. 2014. Localization and functionality of the inflammasome in neutrophils. *J Biol Chem* 289: 5320-9
 632. Arbore G, West EE, Spolski R, Robertson AAB, Klos A, Rheinheimer C, Dutow P, Woodruff TM, Yu ZX, O'Neill LA, Coll RC, Sher A, Leonard WJ, Köhl J, Monk P, Cooper MA, Arno M, Afzali B, Lachmann HJ, Cope AP, Mayer-Barber KD, Kemper C. 2016. T helper 1 immunity requires complement-driven NLRP3 inflammasome activity in CD4⁺ T cells. *Science (New York, N.Y.)* 352: aad1210-aad
 633. Wilson JE, Petrucelli AS, Chen L, Koblansky AA, Truax AD, Oyama Y, Rogers AB, Brickey WJ, Wang Y, Schneider M, Muhlbauer M, Chou WC, Barker BR, Jobin C, Allbritton NL, Ramsden DA, Davis BK, Ting JP. 2015. Inflammasome-independent role of AIM2 in suppressing colon tumorigenesis via DNA-PK and Akt. *Nat Med* 21: 906-13
 634. Man SM, Zhu Q, Zhu L, Liu Z, Karki R, Malik A, Sharma D, Li L, Malireddi RK, Gurung P, Neale G, Olsen SR, Carter RA, McGoldrick DJ, Wu G, Finkelstein D, Vogel P, Gilbertson RJ, Kanneganti TD. 2015. Critical Role for the DNA Sensor AIM2 in Stem Cell Proliferation and Cancer. *Cell* 162: 45-58
 635. Choubey D, Walter S, Geng Y, Xin H. 2000. Cytoplasmic localization of the interferon-inducible protein that is encoded by the AIM2 (absent in melanoma) gene from the 200-gene family. *FEBS Lett* 474: 38-42
 636. Patsos G, Germann A, Gebert J, Dihlmann S. 2010. Restoration of absent in melanoma 2 (AIM2) induces G2/M cell cycle arrest and promotes invasion of colorectal cancer cells. *Int J Cancer* 126: 1838-49
 637. Sorrentino R, Terlizzi M, Di Crescenzo VG, Popolo A, Pecoraro M, Perillo G, Galderisi A, Pinto A. 2015. Human lung cancer-derived immunosuppressive plasmacytoid dendritic cells release IL-1alpha in an AIM2 inflammasome-dependent manner. *Am J Pathol* 185: 3115-24
 638. Zhang M, Qi Y, Li H, Cui J, Dai L, Frank JA, Chen J, Xu W, Chen G. 2016. AIM2 inflammasome mediates Arsenic-induced secretion of IL-1 β and IL-18. *Oncolimmunology* 5: e1160182
 639. Karagas MR, Stukel TA, Tosteson TD. 2002. Assessment of cancer risk and environmental levels of arsenic in New Hampshire. *Int J Hyg Environ Health* 205: 85-94

640. Lozano-Ruiz B, Bachiller V, Garcia-Martinez I, Zapater P, Gomez-Hurtado I, Moratalla A, Gimenez P, Bellot P, Frances R, Such J, Gonzalez-Navajas JM. 2015. Absent in melanoma 2 triggers a heightened inflammasome response in ascitic fluid macrophages of patients with cirrhosis. *J Hepatol* 62: 64-71
641. Kaminski JJ, Schattgen SA, Tzeng TC, Bode C, Klinman DM, Fitzgerald KA. 2013. Synthetic oligodeoxynucleotides containing suppressive TTAGGG motifs inhibit AIM2 inflammasome activation. *J Immunol* 191: 3876-83
642. Crane DD, Bauler TJ, Wehrly TD, Bosio CM. 2014. Mitochondrial ROS potentiates indirect activation of the AIM2 inflammasome. *Frontiers in Microbiology* 5: 438
643. Zorman J, Sušjan P, Hafner-Bratkovič I. 2016. Shikonin Suppresses NLRP3 and AIM2 Inflammasomes by Direct Inhibition of Caspase-1. *PLOS ONE* 11: e0159826
644. Ahn H, Kang SG, Yoon S-i, Ko H-J, Kim P-H, Hong E-J, An B-S, Lee E, Lee G-S. 2017. Methylene blue inhibits NLRP3, NLRC4, AIM2, and non-canonical inflammasome activation. *Scientific Reports* 7: 12409
645. Kwak SB, Koppula S, In EJ, Sun X, Kim YK, Kim MK, Lee KH, Kang TB. 2018. Artemisia Extract Suppresses NLRP3 and AIM2 Inflammasome Activation by Inhibition of ASC Phosphorylation. *Mediators Inflamm* 2018: 6054069
646. Lama L, Adura C, Xie W, Tomita D, Kamei T, Kuryavyi V, Gogakos T, Steinberg JI, Miller M, Ramos-Espiritu L, Asano Y, Hashizume S, Aida J, Imaeda T, Okamoto R, Jennings AJ, Michino M, Kuroita T, Stamford A, Gao P, Meinke P, Glickman JF, Patel DJ, Tuschl T. 2019. Development of human cGAS-specific small-molecule inhibitors for repression of dsDNA-triggered interferon expression. *Nature Communications* 10: 2261
647. Rivera Vargas T, Benoit-Lizon I, Apetoh L. 2017. Rationale for stimulator of interferon genes-targeted cancer immunotherapy. *European journal of cancer (Oxford, England : 1990)* 75: 86-97
648. Li A, Yi M, Qin S, Song Y, Chu Q, Wu K. 2019. Activating cGAS-STING pathway for the optimal effect of cancer immunotherapy. *Journal of Hematology & Oncology* 12: 35
649. Diamond MS, Kinder M, Matsushita H, Mashayekhi M, Dunn GP, Archambault JM, Lee H, Arthur CD, White JM, Kalinke U, Murphy KM, Schreiber RD. 2011. Type I interferon is selectively required by dendritic cells for immune rejection of tumors. *J Exp Med* 208: 1989-2003
650. Fuertes MB, Kacha AK, Kline J, Woo SR, Kranz DM, Murphy KM, Gajewski TF. 2011. Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8 α + dendritic cells. *J Exp Med* 208: 2005-16
651. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pages F. 2006. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 313: 1960-4
652. Galon J, Mlecnik B, Bindea G, Angell HK, Berger A, Lagorce C, Lugli A, Zlobec I, Hartmann A, Bifulco C, Nagtegaal ID, Palmqvist R, Masucci GV, Botti G, Tatangelo F, Delrio P, Maio M, Laghi L, Grizzi F, Asslaber M, D'Arrigo C, Vidal-Vanaclocha F, Zavadova E, Chouchane L, Ohashi PS, Hafezi-Bakhtiari S, Wouters BG, Roehrl M, Nguyen L, Kawakami Y, Hazama S, Okuno K, Ogino S, Gibbs P, Waring P, Sato N, Torigoe T, Itoh K, Patel PS, Shukla SN, Wang Y, Kopetz S, Sinicrope FA, Scripcariu V, Ascierto PA, Marincola FM, Fox BA, Pages F. 2014. Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *J Pathol* 232: 199-209
653. Woo SR, Fuertes MB, Corrales L, Spranger S, Furdyna MJ, Leung MY, Duggan R, Wang Y, Barber GN, Fitzgerald KA, Alegre ML, Gajewski TF. 2014. STING-dependent cytosolic DNA sensing mediates innate immune recognition of immunogenic tumors. *Immunity* 41: 830-42

654. Xia T, Konno H, Ahn J, Barber GN. 2016. Deregulation of STING Signaling in Colorectal Carcinoma Constrains DNA Damage Responses and Correlates With Tumorigenesis. *Cell Rep* 14: 282-97
655. Ahn J, Konno H, Barber GN. 2015. Diverse roles of STING-dependent signaling on the development of cancer. *Oncogene* 34: 5302-8
656. Song S, Peng P, Tang Z, Zhao J, Wu W, Li H, Shao M, Li L, Yang C, Duan F, Zhang M, Zhang J, Wu H, Li C, Wang X, Wang H, Ruan Y, Gu J. 2017. Decreased expression of STING predicts poor prognosis in patients with gastric cancer. *Scientific reports* 7: 39858-
657. Gong Z, Zhang X, Su K, Jiang R, Sun Z, Chen W, Forno E, Goetzman ES, Wang J, Dong HH, Dutta P, Muzumdar R. 2019. Deficiency in AIM2 induces inflammation and adipogenesis in white adipose tissue leading to obesity and insulin resistance. *Diabetologia*
658. Murphy AJ, Kraakman MJ, Kammoun HL, Dragoljevic D, Lee MK, Lawlor KE, Wentworth JM, Vasanthakumar A, Gerlic M, Whitehead LW, DiRago L, Cengia L, Lane RM, Metcalf D, Vince JE, Harrison LC, Kallies A, Kile BT, Croker BA, Febbraio MA, Masters SL. 2016. IL-18 Production from the NLRP1 Inflammasome Prevents Obesity and Metabolic Syndrome. *Cell Metab* 23: 155-64
659. Netea MG, Joosten LA, Lewis E, Jensen DR, Voshol PJ, Kullberg BJ, Tack CJ, van Krieken H, Kim SH, Stalenhoef AF, van de Loo FA, Verschueren I, Pulawa L, Akira S, Eckel RH, Dinarello CA, van den Berg W, van der Meer JW. 2006. Deficiency of interleukin-18 in mice leads to hyperphagia, obesity and insulin resistance. *Nat Med* 12: 650-6
660. Wang H, Capell W, Yoon JH, Faubel S, Eckel RH. 2014. Obesity development in caspase-1-deficient mice. *Int J Obes (Lond)* 38: 152-5
661. Mitroulis I, Ruppova K, Wang B, Chen LS, Grzybek M, Grinenko T, Eugster A, Troullinaki M, Palladini A, Kourtzelis I, Chatzigeorgiou A, Schlitzer A, Beyer M, Joosten LAB, Isermann B, Lesche M, Petzold A, Simons K, Henry I, Dahl A, Schultze JL, Wielockx B, Zamboni N, Mirtschink P, Coskun U, Hajishengallis G, Netea MG, Chavakis T. 2018. Modulation of Myelopoiesis Progenitors Is an Integral Component of Trained Immunity. *Cell* 172: 147-61.e12
662. Thorburn AN, Macia L, Mackay CR. 2014. Diet, metabolites, and "western-lifestyle" inflammatory diseases. *Immunity* 40: 833-42
663. Carbone S, Lee PJH, Mauro AG, Mezzaroma E, Buzzetti R, Van Tassell B, Abbate A, Toldo S. 2017. Interleukin-18 mediates cardiac dysfunction induced by western diet independent of obesity and hyperglycemia in the mouse. *Nutrition & Diabetes* 7: e258-e
664. Herder C, Baumert J, Kolb H, Koenig W, Martin S, Thorand B. 2006. Circulating levels of interleukin-18 independent of body fat and fat-free mass: results from the MONICA/KORA study. In *Diabetes Care*, pp. 174-5. United States
665. Hanahan D, Weinberg RA. 2000. The Hallmarks of Cancer. *Cell* 100: 57-70
666. Tschanz ER. 2005. Do 40% of patients resected for barrett esophagus with high-grade dysplasia have unsuspected adenocarcinoma? *Arch Pathol Lab Med* 129: 177-80
667. Souza RF. 2017. Reflux esophagitis and its role in the pathogenesis of Barrett's metaplasia. *Journal of gastroenterology* 52: 767-76
668. Findlay JM, Middleton MR, Tomlinson I. 2016. Genetic susceptibility to Barrett's oesophagus: Lessons from early studies. *United European Gastroenterol J* 4: 485-92
669. Gough MD, Ackroyd R, Majeed AW, Bird NC. 2005. Prediction of malignant potential in reflux disease: are cytokine polymorphisms important? *Am J Gastroenterol* 100: 1012-8
670. Hrdlickova B, Westra HJ, Franke L, Wijmenga C. 2011. Celiac disease: moving from genetic associations to causal variants. *Clin Genet* 80: 203-313

671. Hunt KA, Zhernakova A, Turner G, Heap GA, Franke L, Bruinenberg M, Romanos J, Dinesen LC, Ryan AW, Panesar D, Gwilliam R, Takeuchi F, McLaren WM, Holmes GK, Howdle PD, Walters JR, Sanders DS, Playford RJ, Trynka G, Mulder CJ, Mearin ML, Verbeek WH, Trimble V, Stevens FM, O'Morain C, Kennedy NP, Kelleher D, Pennington DJ, Strachan DP, McArdle WL, Mein CA, Wapenaar MC, Deloukas P, McGinnis R, McManus R, Wijmenga C, van Heel DA. 2008. Newly identified genetic risk variants for celiac disease related to the immune response. *Nat Genet* 40: 395-402
672. Li J, Qiu G, Fang B, Dai X, Cai J. 2018. Deficiency of IL-18 Aggravates Esophageal Carcinoma Through Inhibiting IFN-gamma Production by CD8(+)T Cells and NK Cells. *Inflammation* 41: 667-76
673. Nowarski R, Jackson R, Gagliani N, de Zoete MR, Palm NW, Bailis W, Low JS, Harman CC, Graham M, Elinav E, Flavell RA. 2015. Epithelial IL-18 Equilibrium Controls Barrier Function in Colitis. *Cell* 163: 1444-56
674. Iyer N, Grizotte-Lake M, Gordon SR, Palmer ACS, Calvin C, Vaishnav S. 2019. Epithelium intrinsic vitamin A signaling co-ordinates pathogen clearance in the gut via IL-18. *bioRxiv*: 667998
675. Gonzalez-Correa CA, Brown BH, Smallwood RH, Stephenson TJ, Stoddard CJ, Bardhan KD. 2003. Low frequency electrical bioimpedance for the detection of inflammation and dysplasia in Barrett's oesophagus. *Physiol Meas* 24: 291-6
676. Xie C, Sifrim D, Li Y, Chen M, Xiao Y. 2018. Esophageal Baseline Impedance Reflects Mucosal Integrity and Predicts Symptomatic Outcome With Proton Pump Inhibitor Treatment. *Journal of neurogastroenterology and motility* 24: 43-50
677. Zhong C, Duan L, Wang K, Xu Z, Ge Y, Yang C, Han Y. 2013. Esophageal intraluminal baseline impedance is associated with severity of acid reflux and epithelial structural abnormalities in patients with gastroesophageal reflux disease. *Journal of gastroenterology* 48: 601-10
678. Katzka DA, Ravi K, Geno DM, Smyrk TC, Iyer PG, Alexander JA, Mabary JE, Camilleri M, Vaezi MF. 2015. Endoscopic Mucosal Impedance Measurements Correlate With Eosinophilia and Dilation of Intercellular Spaces in Patients With Eosinophilic Esophagitis. *Clin Gastroenterol Hepatol* 13: 1242-8.e1
679. Ates F, Yuksel ES, Higginbotham T, Slaughter JC, Mabary J, Kavitt RT, Garrett CG, Francis D, Vaezi MF. 2015. Mucosal impedance discriminates GERD from non-GERD conditions. *Gastroenterology* 148: 334-43
680. Kessing BF, Bredenoord AJ, Weijenberg PW, Hemmink GJ, Loots CM, Smout AJ. 2011. Esophageal acid exposure decreases intraluminal baseline impedance levels. *Am J Gastroenterol* 106: 2093-7
681. Farre R, Blondeau K, Clement D, Vicario M, Cardozo L, Vieth M, Mertens V, Pauwels A, Silny J, Jimenez M, Tack J, Sifrim D. 2011. Evaluation of oesophageal mucosa integrity by the intraluminal impedance technique. *Gut* 60: 885-92
682. Mullin JM, Valenzano MC, Trembeth S, Allegretti PD, Verrecchio JJ, Schmidt JD, Jain V, Meddings JB, Mercogliano G, Thornton JJ. 2006. Transepithelial leak in Barrett's esophagus. *Dig Dis Sci* 51: 2326-36
683. Takagi H, Kanai T, Okazawa A, Kishi Y, Sato T, Takaishi H, Inoue N, Ogata H, Iwao Y, Hoshino K, Takeda K, Akira S, Watanabe M, Ishii H, Hibi T. 2003. Contrasting action of IL-12 and IL-18 in the development of dextran sodium sulphate colitis in mice. *Scand J Gastroenterol* 38: 837-44
684. Niranjana R, Rajavelu P, Ventateshaiah SU, Shukla JS, Zaidi A, Mariswamy SJ, Mattner J, Fortgang I, Kowalczyk M, Balart L, Shukla A, Mishra A. 2015. Involvement of interleukin-18 in the pathogenesis of human eosinophilic esophagitis. *Clin Immunol* 157: 103-13

685. Wang F, Li G, Ning J, Chen L, Xu H, Kong X, Bu J, Zhao W, Li Z, Wang X, Li X, Ma J. 2018. Alcohol accumulation promotes esophagitis via pyroptosis activation. *International Journal of Biological Sciences* 14: 1245-55
686. Macia L, Tan J, Vieira AT, Leach K, Stanley D, Luong S, Maruya M, Ian McKenzie C, Hijikata A, Wong C, Binge L, Thorburn AN, Chevalier N, Ang C, Marino E, Robert R, Offermanns S, Teixeira MM, Moore RJ, Flavell RA, Fagarasan S, Mackay CR. 2015. Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. *Nat Commun* 6: 6734
687. Dupaul-Chicoine J, Yeretssian G, Doiron K, Bergstrom KS, McIntire CR, LeBlanc PM, Meunier C, Turbide C, Gros P, Beauchemin N, Vallance BA, Saleh M. 2010. Control of intestinal homeostasis, colitis, and colitis-associated colorectal cancer by the inflammatory caspases. *Immunity* 32: 367-78
688. Statovci D, Aguilera M, MacSharry J, Melgar S. 2017. The Impact of Western Diet and Nutrients on the Microbiota and Immune Response at Mucosal Interfaces. *Frontiers in immunology* 8: 838-
689. Nov O, Shapiro H, Ovadia H, Tarnovscki T, Dvir I, Shemesh E, Kovsan J, Shelef I, Carmi Y, Voronov E, Apte RN, Lewis E, Haim Y, Konrad D, Bashan N, Rudich A. 2013. Interleukin-1 β regulates fat-liver crosstalk in obesity by auto-paracrine modulation of adipose tissue inflammation and expandability. *PLoS one* 8: e53626-e
690. McGillicuddy FC, Harford KA, Reynolds CM, Oliver E, Claessens M, Mills KHG, Roche HM. 2011. Lack of interleukin-1 receptor I (IL-1RI) protects mice from high-fat diet-induced adipose tissue inflammation coincident with improved glucose homeostasis. *Diabetes* 60: 1688-98
691. Jung C, Gerdes N, Fritzenwanger M, Figulla HR. 2010. Circulating levels of interleukin-1 family cytokines in overweight adolescents. *Mediators of inflammation* 2010: 958403-
692. Esposito K, Pontillo A, Ciotola M, Di Palo C, Grella E, Nicoletti G, Giugliano D. 2002. Weight loss reduces interleukin-18 levels in obese women. *J Clin Endocrinol Metab* 87: 3864-6
693. Hung J, McQuillan BM, Chapman CM, Thompson PL, Beilby JP. 2005. Elevated interleukin-18 levels are associated with the metabolic syndrome independent of obesity and insulin resistance. *Arterioscler Thromb Vasc Biol* 25: 1268-73
694. Rieder F, Cheng L, Harnett KM, Chak A, Cooper GS, Isenberg G, Ray M, Katz JA, Catanzaro A, O'Shea R, Post AB, Wong R, Sivak MV, McCormick T, Phillips M, West GA, Willis JE, Biancani P, Fiocchi C. 2007. Gastroesophageal reflux disease-associated esophagitis induces endogenous cytokine production leading to motor abnormalities. *Gastroenterology* 132: 154-65
695. Cao W, Cheng L, Behar J, Fiocchi C, Biancani P, Harnett KM. 2004. Proinflammatory cytokines alter/reduce esophageal circular muscle contraction in experimental cat esophagitis. *Am J Physiol Gastrointest Liver Physiol* 287: G1131-9
696. Chen M-F, Lu M-S, Chen P-T, Chen W-C, Lin P-Y, Lee K-D. 2012. Role of interleukin 1 beta in esophageal squamous cell carcinoma. *Journal of Molecular Medicine* 90: 89-100
697. Zhu Q, Kanneganti T-D. 2017. Cutting Edge: Distinct Regulatory Mechanisms Control Proinflammatory Cytokines IL-18 and IL-1 β . *Journal of immunology (Baltimore, Md. : 1950)* 198: 4210-5
698. Huang Y, Blatt LM, Taylor MW. 1995. Type 1 interferon as an antiinflammatory agent: inhibition of lipopolysaccharide-induced interleukin-1 beta and induction of interleukin-1 receptor antagonist. *J Interferon Cytokine Res* 15: 317-21
699. Reznikov LL, Puren AJ, Fantuzzi G, Muhl H, Shapiro L, Yoon DY, Cutler DL, Dinarello CA. 1998. Spontaneous and inducible cytokine responses in healthy humans receiving a

- single dose of IFN- α 2b: increased production of interleukin-1 receptor antagonist and suppression of IL-1-induced IL-8. *J Interferon Cytokine Res* 18: 897-903
700. Coclet-Ninin J, Dayer JM, Burger D. 1997. Interferon-beta not only inhibits interleukin-1beta and tumor necrosis factor-alpha but stimulates interleukin-1 receptor antagonist production in human peripheral blood mononuclear cells. *Eur Cytokine Netw* 8: 345-9
 701. Mayer-Barber KD, Andrade BB, Barber DL, Hieny S, Feng CG, Caspar P, Oland S, Gordon S, Sher A. 2011. Innate and adaptive interferons suppress IL-1alpha and IL-1beta production by distinct pulmonary myeloid subsets during Mycobacterium tuberculosis infection. *Immunity* 35: 1023-34
 702. Novikov A, Cardone M, Thompson R, Shenderov K, Kirschman KD, Mayer-Barber KD, Myers TG, Rabin RL, Trinchieri G, Sher A, Feng CG. 2011. Mycobacterium tuberculosis triggers host type I IFN signaling to regulate IL-1beta production in human macrophages. *J Immunol* 187: 2540-7
 703. Mayer-Barber KD, Yan B. 2017. Clash of the Cytokine Titans: counter-regulation of interleukin-1 and type I interferon-mediated inflammatory responses. *Cellular & Molecular Immunology* 14: 22-35
 704. Schindler R, Ghezzi P, Dinarello CA. 1990. IL-1 induces IL-1. IV. IFN-gamma suppresses IL-1 but not lipopolysaccharide-induced transcription of IL-1. *J Immunol* 144: 2216-22
 705. Hara K, Shirasuna K, Usui F, Karasawa T, Mizushima Y, Kimura H, Kawashima A, Ohkuchi A, Matsuyama S, Kimura K, Takahashi M. 2014. Interferon-tau attenuates uptake of nanoparticles and secretion of interleukin-1beta in macrophages. *PLoS One* 9: e113974
 706. Okamura H, Nagata K, Komatsu T, Tanimoto T, Nukata Y, Tanabe F, Akita K, Torigoe K, Okura T, Fukuda S. 1995. A novel costimulatory factor for gamma interferon induction found in the livers of mice causes endotoxic shock. *Infection and Immunity* 63: 3966
 707. Dinarello C, Novick D, Kim S, Kaplanski G. 2013. Interleukin-18 and IL-18 Binding Protein. *Frontiers in Immunology* 4: 289