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REMOTE ISCHAEMIC CONDITIONING AS A NOVEL THERAPY FOR NECROTISING ENTEROCOLITIS

by

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ABSTRACT

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Ian Howard Jones

Necrotising enterocolitis (NEC) is a devastating disease that afflicts neonates leading to high mortality and morbidity. The majority of infants affected are born prematurely but the disease is also seen in other groups of babies – pre-eminently those born with congenital cardiac conditions. There is a broad range of clinical manifestations of the disease; in some cases babies recover completely with only conservative management. In more severe cases, babies require surgery and bowel resection. These infants also develop systemic disease and multi-organ failure.

A systematic review I conducted showed that in the 21st century, whilst the overall outcomes for babies born prematurely continue to improve, NEC still confers significant mortality and morbidity. Even the most premature babies born today have an expected survival of around 90%. Conversely, NEC carries an average mortality of one in four and for the smallest babies (< 1000g) who require surgery for NEC, the mortality is over 50%. Similarly, in the most severely ill babies, the majority will have life-long disability due to the injury to the developing brain resulting in neurodevelopmental delay.

The pathophysiology of NEC is complicated and involves multiple pathways leading to bowel necrosis. The cumulative evidence shows that key risk factors including enteral feeds, prematurity, and colonisation by potentially pathogenic bacteria are all critical to the development of NEC. In recent years, much interest has focused on the immunological pathways involved but ultimately, NEC is a disease characterised by ischaemia-reperfusion injury (IRI) to the bowel. Ischaemic conditioning is a phenomenon whereby brief episodes of non-injurious ischaemia and reperfusion 'condition' tissue and organs to be resistant to IRI. Remote ischaemic conditioning (RIC) refers to the fact that the conditioning can be done to one organ or tissue and provide a protective effect

on a different organ. The fact that the conditioning can be done on skeletal muscle with a simple blood pressure cuff inflated to above systolic pressure for short periods of time means that this has the potential to be easily translated to clinical practice.

In this work, I used a rat model of NEC based on creating an ischaemia-reperfusion injury to the bowel that produces a similar injury pattern and systemic effects to human NEC.

Animals that underwent RIC immediately prior to injury had significant decreases in both the extent and severity of the bowel injury. Similarly, animals that underwent RIC following the ischaemic insult demonstrated reduced injury although the effect was not as pronounced as those who had the RIC prior to injury. Furthermore, a protective effect was demonstrated in animals that had the RIC upto 48 hours prior to injury. An additive effect was seen if the animals underwent two separate cycles of RIC 48 hours apart.

Analysis of serum cytokine levels across the various protocols supports a role for IFN-γ, IL-1β and IL-4 in the transmission of RIC: All three may be important in the pathway of delivering a protective effect in a distant organ.

Whole transcriptome analysis performed on the intestine showed that multiple pathways are involved in this process including the down-regulation of NF-κβ which is known to be a central regulator of multiple pro-inflammatory pathways. This is particularly appealing as NF-κβ is important for the pathogenesis of NEC at several stages in the process.

NEC remains a devastating disease, very much in need of novel therapeutic options. These results collectively show the potential for using RIC as that novel therapeutic option. The potential protective effect of RIC even 48 hours later as well as the additive effect of more than one application provides a good starting point for designing a protocol for a clinical study. The data on the mechanisms of RIC also deserve further study.

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1. R script for meta-analysis of proportions
   - ***/Meta_Analysis_of_NEC_mortality.R***
2. Video demonstrating the Ischaemia-Reperfusion-Injury model of NEC it rat pups (MP4 file)
   - ***/IRI_model_video.mp4***
   - http://doctor-jones.co.uk/download/IRI%20model%20video.mp4
3. Illumina NGS sequence by synthesis – explanation of RNAseq performed by Qiagen Labs.
   - https://www.youtube.com/watch?v=fCd6B5HRaZ8&feature=youtu.be
4. R scripts used for analysis of differential gene expression
   - ***/R_code_gene_expression.R***
   - ***/Gene_Names.R***
   - ***/Targeted_Gene_Expression_(t-Tst).R***
5. Data from RNA analysis
   - **/filtered_matrix_counts_per_million.csv**
   - **/IRI_vs_SHAM_DE.csv**
   - **/RIC_vs_SHAM_DE.csv**
   - **/RIC+IRI_vs_IRI_DE.csv**

University of Southampton Data Repository DOI: 112869439

Academic Thesis: Declaration Of Authorship

I, Ian Howard Jones

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

REMOTE ISCHAEMIC CONDITIONING AS A NOVEL THERAPY FOR NECROTISING ENTEROCOLITIS

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

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Definitions and Abbreviations

'3-R's' - Replace, Reduce, Refine – principals of animal research ethics

Apgar Score - Perinatal assessment of newborn

AREDF - Absent or reversed end-diastolic flow (in umbilical artery)

ASPA - Animals (Scientific Procedures) Act (1986) and revisions

ATP - Adenosine triphosphate

ATP - Adenosine triphosphate

BAD - Bcl-2-associated death promoter

Bell (1-3) - Bell stages of necrotising enterocolitis (1a, 1b, 2a, 2b, 3a and 3b)

BHT - 2,6-Di-tert-butyl-4-methylphenol

BSID - Bayley Score of Infant Development

CD11b - Cluster of differentiation 11b

CD55 - Cluster of differentiation 55

cDNA - Complementary DNA (synthesised from RNA)

CGRP - Calcitonin gene-related peptide

CHD - Congenital heart disease

CI - Confidence Interval

Cochrane - The Cochrane Library (https://www.cochranelibrary.com/)

DBM - Donor breast milk

DNA - Deoxyribonucleaic acid

EDTA - Ethylenediaminetetraacetic

ELBW - Extremely low birth weight ([1000g)

eNOS - endothelial nitric oxide synthase

EPRC - Endothelial cell Protein C Receptor

ERK - Extracellular signal-regulated kinase

ERK1/2 - Extracellular signal-regulated kinase 1/2

ET-1 - Endothelin 1

Glut-1 - Glucose transporter 1

GSK-β - glycogen synthase kinase-3β

HIF-1α / HIF-1β - Hypoxia-inducible factor 1-alpha / Hypoxia-inducible factor 1-beta

HSP - Heat shock protein

hsPDA - Heamodynamically-significant PDA

hsPDA - Haemodynamically significant PDA

ICAM-1 - Intercellular adhesion molecule

IF - Intestinal failure

IFN-γ - Interferon gamma

IgA - Immunologlobin A

IL-10 - Interleukin 10

IL-13 - Interleukin 13

IL-18 - Interleukin 18

IL-1β - Interleukin 1-beta

IL-4 - Interleukin 4

IL-5 - Interleukin 5

IL-6 - Interleukin 6

IL-8 - Interleukin 8

IRI - Ischaemia reperfusion injury

IUGR - Intrauterine growth restriction

KATP - Adenosine triphosphate - sensitive potassium channel

Definitions and Abbreviations

LBW - Low birth weight (< 2500g)

LPS - Lipopolysaccharide

MAP3K8 - Mitogen-activated protein kinase kinase kinase

MAPK - Mitogen-activated protein kinase

MBM - Maternal breast milk

MDA - Malondialdehyde

MDI - Mental development index (part of BSID)

MEK 1/2 - MAPK / ERK 1/2

MMP - Metalloproteinase

mOsm/kg - Milliosmoles per kilogram of water

MPO - Myeloperoxidase

MPTP - Mitochondrial permeability transition pore

mRNA - Messenger RNA

MyD88 - Myeloid differentiation factor 88

NACWO - Named Animal Care and Welfare Officer

NDD - Neurodevelopmental delay

NEC - Necrotising enterocolitis

NEDD - N-1-(naphthyl)ethylenediamine

NF-κβ - nuclear factor kappa-light-chain-enhancer of activated B cells

NICU - Neonatal intensive care unit

NNT - Number needed to treat

NO - Nitric oxide

NSAID - Non-steroidal anti-inflammatory drug

NVS - Named Ventinary Surgeon

OECD - Organisation for Economic Co-operation and Development

OR - Odds Ratio

PAF - Platelet activating factor

PAF-AH - PAF-acetylhydrolase

PAMP(s) - Pathogen-associated microbial patterns

PBS - Phospate buffered saline

PCR - Polymerase Chain Reaction

PDA - Patent ductus arteriosus

PI3K - Phosphoinositide 3-kinase / Phosphatidylinositol 3-kinase

PI3K-Akt - Phosphatidylinositol 3-kinase - AK-thymoma

PIL - Procedure Individual Licence (Licence to perform regulated procedures under APSA(1986)

PKC - Protein Kinase C

PN - Parenteral nutrition

PPL - Procedure Project Licence (Licence for a project of work under APSA (1986)

Procr - Protein C Receptor (see EPRC)

PRR(s) - Pattern recognition receptor(s)

RIC - Remote ischaemic condition

RISK - Reperfusion injury salvage kinase

RISK pathway - Reperfusion injury salvage kinase pathway

RNA - Ribonucleaic acid

ROS - Reactive oxygen species

RR - Relative risk

SAFE pathway - Survivor-activating factor enhancement pathway

SGA - Small for gestational age

SMA - Superior mesenteric artery

Smad7 - SMAD Family member 7

SOD2 - Superoxide dismutase 2

STAT3 - Signal transducer and activator of transcription 3

TANEC - Transfusion-associated necrotising enterocolitis

TBA - thiobarbituric acid

TBS - Tris buffered saline

TCR - T-cell receptor (T-lymphocyte receptor)

TIMP1 - Tissue Inhibitor of Metalloproteinase 1

TLR(s) - Toll-like receptor(s)

TLR2 - Toll-like receptor 2

TLR4 - Toll-like receptor 4

TNF-α - Tumour necrosis factor alpha

Usp36 - Ubiquitin-specific Protease 36

VLBW - Very low birth weight (< 1500g)

:

Chapter 1 Necrotising Enterocolitis

1.1 Introduction

Necrotising enterocolitis (NEC) is a devastating disease causing both severe bowel pathology and systemic inflammation. NEC is primarily a disease of prematurity and it is rare in term babies. Most term babies who do contract NEC have an underlying condition, most commonly congenital cardiac disease. NEC has a high mortality and significant morbidity for survivors. In an era that has seen year-on-year progress in the care of premature babies - such that now, the majority of even very premature neonates survive[1] - NEC treatment has remained, for the most part, unchanged. Intensive research into the pathophysiology of NEC has elucidated a complex interplay of various factors in the pathogenesis but, as yet, has not yielded much in the way of clinically useful treatments.

1.2 Definition

Necrotising enterocolitis is an acquired condition seen in neonates that is characterised by bowel inflammation and necrosis and translocation of gas-forming bacteria into the bowel wall. The bowel injury can range from mucosal alone to full thickness necrosis and perforation.[2] It can be present in an isolated area of both the small and large bowel or affect large segments of bowel or even the entire intestine (NEC Totalis). NEC can also result in damage to multiple other organ systems as it can trigger a large systemic inflammatory response.

1.2.1 Definitional challenges and controversies

Defining NEC precisely remains a challenge as what is termed “NEC” may represent a range of pathophysiology[3] and a consensus does not exist on what does and does not constitute NEC.[4] Clinically, NEC typically begins with bilious gastric aspirates, abdominal distension and systemic signs such as temperature instability and bradycardia.[5] If the disease progresses, the most severe cases require respiratory support and inotropes. Radiologically, the presence of pneumatosis (gas within the bowel wall), progressing to gas in the portal venous system are cardinal signs but are also transient signs of NEC.[6] Histologically, NEC is typically defined as ischaemic necrosis[7] of the bowel but this inevitably only constitutes the most severe extent of NEC.

1.2.2 The history of necrotising enterocolitis

In what is arguably the first case-report of necrotising enterocolitis, Charles Billard in Paris in 1823 described 'gangrenous enterocolitis' in a 'weak infant with infection, inflammation and necrosis of the gastrointestinal tract.'[8] However, the clinical entity of NEC began to truly emerge with the development of special care nurseries in the early 20th century. In 1965 Mizrahi *et al.* first used the term 'necrotising enterocolitis' to describe a clinical syndrome consisting of vomiting, abdominal distention, gastrointestinal bleeding, and shock in premature infants.[9] At that time, the mortality of NEC was considered almost universal, but the often-insidious nature of the onset of NEC in clinical practice meant accurate assessment of the disease prevalence and prognosis was difficult.

1.3 Diagnosing Necrotising enterocolitis

In 1978, Bell *et al.* [10] proposed what came to be known as the Bell classification of NEC, later modified by Walsh and Kleigman. The (modified) Bell Classification[11] remains the most widely-used diagnostic criteria for NEC. The modified Bell Classification is summarised in Table 1-1

Other classification systems exist but all suffer from the same issue that it remains difficult to define precisely what constitutes a diagnosis of NEC. There is a lack of a unifying cause and multiple variations in presentation.[12] As Bell noted, the early stages of NEC are often indistinguishable from other conditions prevalent in the neonatal population. [10] Feeding intolerance is very common in premature babies.[13] Indeed, Neu *et al.*[3] argue that the majority of extremely premature infants will meet the criteria for Bell stage 1 without progressing to more significant disease.

The diagnosis of NEC is made based on some or all of the following signs: temperature instability, apnoea, bradycardia, lethargy, poor handling, increased nasogastric aspirates, vomiting (bilious or non-bilious), bright red blood per rectum, abdominal distension and abdominal tenderness. The classic radiological sign of pneumotosis intestinalis (Figure 1-1) which reflects gas (a large proportion of which is hydrogen),[14] in the abdominal wall is considered pathognomonic in this context. Blood tests often reveal a rise in C-reactive protein, leucocytosis and thrombocytopenia.[5] From a clinical perspective, this uncertainty is less important as the initial, conservative management is straightforward. However, from a research point-of-view this can be problematic as it makes for very heterogeneous datasets.[3]

Stage	Systemic signs	Abdominal signs	Radiographic signs	Treatment
1a Suspected	Temperature instability, apnea, bradycardia, lethargy	Gastric retention, abdominal distention, emesis, heme-positive stool	Normal or intestinal dilation, mild ileus	NPO, antibiotics x 3 days and review progress
1b Suspected	As 1a	Grossly bloody stool	As 1a	As 1a
2a Definite, mildly unwell	As above	Same as above, plus absent bowel sounds with or without abdominal tenderness	Intestinal dilation, ileus, pneumatosis intestinalis	NPO, antibiotics x 7 to 10 days
2b Definite, moderately unwell	Same as above, plus mild metabolic acidosis and thrombocytopenia	Same as above, plus absent bowel sounds, definite tenderness, with or without abdominal cellulitis or right lower quadrant mass	As 2a, plus ascites	NPO, antibiotics x 14 days
3a Advanced, severely ill, intact bowel	Same as 2a, plus hypotension, bradycardia, severe apnea, combined respiratory and metabolic acidosis, DIC, and neutropenia	Same as above, plus signs of peritonitis, marked tenderness, and abdominal distention	As 2a, plus ascites	NPO, antibiotics x 14 days, fluid resuscitation, inotropic support, ventilator therapy, paracentesis
3b Advanced, severely ill, perforated bowel	As 3a	As 3a	Same as above, plus pneumoperitoneum	Same as 3a, plus surgery

Table 1-1 Modified Bell Classification of NEC [Walsh and Kleigman (1986)[11]]

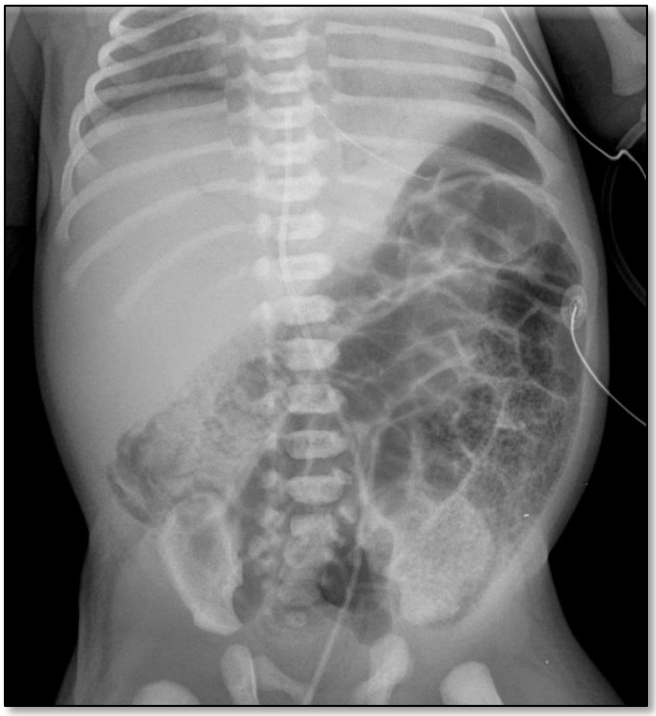


Figure 1-1 Abdominal Radiograph showing pneumotosis intestinalis

The 'bubbly' appearance, most notable in the patient's left lower quadrant is seen on plain radiograph when gas is present in the bowel wall. This feature, when associated with clinical signs, such as feed intolerance, abdominal distension, etc. (Table 1-1.) is diagnostic of NEC.

1.4 Epidemiology

Given the insidious nature of 'early' NEC (Bell stage 1 being often indistinguishable from other pathologies), it is unsurprisingly that estimates of the incidence vary. However the true incidence probably lies between 0.5 and 5 per 1000 livebirths.[5] With around 710,000 live births each year in the UK,[15-17] that equates to between 355 and 3550 cases. However, the UK has lagged behind in collecting robust epidemiological data.[18] Holman e*t al.* 2006[19] based on data from a large cohort in US neonatal units, put the incidence at 1.1/1000 live births (equivalent to ~800 UK cases /year). Extrapolation from prospective UK data based on a survey of neonatal units would support an estimate of up to 1000 UK cases each year.[20]

Incidence varies across the world and ethnic groups with higher frequencies reported in North America, the UK and Ireland and lower frequencies in Japan, Switzerland, and Austria. Whether this variability is due to genetic and/or environmental factors in the population or whether it is influenced by neonatal care strategies or simply reflects differences in data collection methodology, is unknown.[23] A more recent review of NEC incidence in developed countries showed a range of 2% to 7% in babies born before 32 weeks gestation and 5% to 22% among those born weighing less than 1000g.[24] It is likely that genetic predisposition is at least partly responsible for this difference because studies limited to the USA have demonstrated that African American infants have a higher risk than Caucasians.[25] Similarly, twin studies suggest a genetic component of this risk.[23] Currently, there is limited data on which genes may be important for NEC risk but the multiple pathways involved in the pathophysiology of NEC (Section 1.6) means that there are a large number of potential candidate genes.[26]

Neonatal Definitions	
Prematurity	**Born before 37 completed weeks of gestation** ***(defined from first day of last menstrual period)***
- Extremely premature	Less than 28 completed weeks of gestation
- Very premature	28 weeks to 31 weeks, 6 days gestation
- Moderate to late premature	32 weeks to 36 weeks, 6 days gestation
Birth Weight	
Low birth weight (LBW)	< 2500g (1500g – 2499g)
Very low birth weight (VLBW)	< 1500g (1000g – 1499g)
Extremely low birth weight (ELBW)	< 1000g

Table 1-2 World Health Organisation definitions of prematurity[21] and low birth weight.[22]

1.4.1 Necrotising enterocolitis and prematurity

Whilst the overall incidence of NEC is difficult to measure precisely, studies consistently show a very strong correlation with birth weight and gestational age. NEC is primarily a disease of prematurity (Definitions of prematurity: Table 1-2), with around 90% of cases occurring in babies

born before 37 weeks gestation.[27,28] It is well-established that there is an inverse relationship with gestational age: the risk of developing NEC is demonstrably higher for the most premature babies.[29,30] Those born between 28 and 31 weeks have an estimated incidence as high as 6.6%,[31] falling to around 1 in 20,000 at term.[32] Among those born at term, NEC is very rare in the absence of specific risk factors.[33] Moreover, the improved survival of premature neonates overall seems to have led to an increase incidence of NEC. Premature babies who may have died within a few weeks of birth previously are now surviving long enough to develop NEC.[34]

The association between birth weight and NEC is the most widely reported link in the literature. Implicitly, it is difficult to separate birth weight from gestational age but sub-group analysis in at-least two studies has shown that being small for gestational age (SGA) (i.e. a lower birth weight than would be expected for a specific gestational age) is an independent risk factor for NEC.[31,35]

Fitzgibbons *et al.* (2009)[36] provided a quantified analysis of the association between birthweight and NEC (Table 1-3). These data, based on over 71,000 infants in the Vermont Oxford Network, showed the risk of developing NEC is 12% in babies weighing 500-750g dropping to 3.3% in those between 1251g and 1500g. In each group, contracting NEC substantially increased the mortality. The biggest effect on mortality was seen in the largest babies, as those born under 750g have a high background mortality at 34%. In those weighing between 1251g and 1500g, the adjusted odds ratio for mortality is 9.9; reflecting a mortality of 15.9% in those babies who contract NEC compared to 2.6% in those that do not.

Birth Weight (g)	**n**	**NEC**	**NEC Risk (%)**	**Mortality (%)** NEC (non-NEC)	**Adjusted Mortality OR (95% CI)**
501-750	13050	1568	12.0	42.0 (34.0)	1.6 (1.4-1.8)
751-1000	16993	1569	9.2	29.4 (10.7)	3.6 (3.1-4.2)
1001-1250	18794	1063	5.7	21.3 (4.1)	7.5 (6.2-9.1)
1251-1500	22970	758	3.3	15.9 (2.6)	9.9 (7.3-13.4)

Table 1-3 NEC incidence and mortality by birth weight
Mortality for each birth weight is shown and in brackets, for babies born at the same weight who did not contract NEC [*based on* Fitzgibbons *et al.* (2009)[36]].

Several prognostic factors have been implicated in NEC mortality, including: birth weight, sepsis, ethnicity, hypotension, being 'outborn' from a specialist neonatal unit, the need for assisted ventilation, use of surfactant, prolonged rupture of membranes and being small for gestational age.[37]

An example of the challenge in appreciating the epidemiological risk factors for NEC is epitomised in the reported association with surfactant use. Surfactant use has been associated with an increased risk of NEC with an odds ratio of 1.59 [95% CI 1.17,2.16][38] and also with a reported

reduced risk (OR 0.41 [95%CI 0.19,0.90]).[39] These conflicting results may be explained by the fact that the administration of surfactant is both a marker of severity of systematic disease, and potentially a protective treatment (i.e. it improves respiratory function and thus oxygen delivery[40] which in turn impacts on NEC risk[41]). Similarly, the correlation between NEC risk and the need for assisted ventilation (odds ratio 13.1)[30] is arguably best explained by positing that the use of mechanical ventilation is an effective marker of acute illness in a neonate.

1.4.2 Necrotising enterocolitis in term infants

Although NEC primarily affects preterm babies with around 10% of cases occurring in neonates born after 36 weeks gestation.[42] In term infants, other risk factors are invariably present. Christensen *et al.* reported that NEC in term neonates was only seen in those that had already been admitted to the neonatal unit for some other reason.[43] Invariably NEC in term infants is associated with specific risk factors such as low Apgar scores, birth asphyxia, sepsis or congenital defects (such as cardiac or gastrointestinal anomalies).[28] Gastroschisis has long been associated with NEC[44] which may imply that poor perfusion is a key factor for NEC. Late preterm infants are more likely to develop NEC if they have other risk factors including umbilical lines, sepsis, intrauterine growth retardation or polycythemia.[29]

Feature	Developed NEC (n=30)	Did not develop NEC (n=5847)	NEC Risk	p value
Congenital heart disease	27% (8)	5% (270)	2.9%	0.000
Polycythaemia	7% (2)	0.2% (13)	13.3%	0.003
Early-onset bacterial sepsis	13% (4)	2% (131)	3.0%	0.005
Birth weight (g)	2849±581	3180±594		0.010
Hypotension	27% (8)	13% (713)	1.1%	0.025
Endotracheal intubation	60% (18)	41% (2395)	0.7%	0.041

Table 1-4 Risk factors for NEC in term infants
Based on Lambert *et al.* (2007).[28] The risk of NEC in a term neonate admitted to the neonatal unit was 30/5877 (0.51%). However, RR's could not be calculated as the data did not report NEC risk in neonates without each specific feature. p-values (apart from birth weight) calculated using Fisher's Exact Test.

The association between NEC and congenital heart disease (CHD) is well established.[45] Indeed, babies born with CHD are the second largest group to develop NEC after those born prematurely.[42] Whilst, some other embryological explanation is possible, this is strongly suggestive that gut hypoperfusion and thus intestinal reperfusion injury (IRI) is the key pathogenic step in this group who develop NEC. This hypothesis is further supported by the fact that NEC is seen most commonly in the infants with CHD that have a low-output state and poor systemic perfusion.[46] Unlike NEC seen in preterm infants, the most common site of bowel injury in CHD

babies is the colon[42] (in contrast to the terminal ileum in 'classical' NEC.) although there is no clear explanation for why the colon should be more susceptible in this group.

A multicentre series by Lambert *et al.* reported on nearly 6000 term infants; of which only 30 developed NEC.[28] This highlights how rare NEC is in the term infant but also highlights some important risk factors, including CHD, polycythaemia, early-onset bacteria sepsis, and hypotension (as well as low birthweight).[28] Within this cohort, there was a much higher rate of Patent ductus arteriosus (PDA) in those that developed NEC (compared to the controls) but this was not statistically significant. The most statistically significant factors identified are shown in Table 1-4.

1.5 Pathophysiology

As discussed in section 1.4, there are multiple risk factors involved in the epidemiology of NEC, prematurity (and low birth weight) being foremost among them. As well as being important in understanding the disease burden, these epidemiological data provide important clues to the pathophysiology of NEC. Indeed, a full understanding of the pathogenesis of NEC would account for these various, diverse risk factors.

Ballance *et al.* (1990) reported histological analysis of a series of 84 patients with NEC.[7] Ischaemic necrosis, inflammation and bacterial overgrowth are all present in the majority of patients. They noted that the histological picture of NEC was "inescapably similar to other bowel diseases known to be ischaemic in origin." However, the degree of bacterial overgrowth seen in NEC is not found in other ischaemic conditions. Similarly, the pathognomonic feature of pneumotosis intestalis is reported as containing a high proportion of hydrogen gas[14] which is a bacterial product. Conversely, the histological features of NEC are not in keeping with infective enterocolitis. More than two thirds of these patients showed reparative changes (epithelial regeneration, granulation tissue fibrosis, vilious atrophy) indicating that NEC is an on-going disease process rather than a single event. In the following sub-sections I will discuss various factors known to contribute to the pathogenesis of NEC. There is significant cross-over between these which overlap and interact with each other. A schematic summary is shown in Figure 1-2 which is a putative way to assimilate the evidence of the diverse factors into a single diagram with a common final pathway.

1.5.1 Prematurity

With around 90% of cases of NEC occurring in premature neonates,[5] there is no doubt that prematurity is a key factor in the pathogenesis. Conceptually, it is not surprisingly that the premature gut is not well-adapted to ex-utero life. By 20 weeks gestation the anatomical

arrangement of the gut is very similar to the term infant but the functional capability is significantly underdeveloped.[47] Hence, there are multiple specific factors that make it vulnerable.

Both the stomach and pancreas play a role in innate immunity by reducing the bacterial load on the small intestine as gastric hydrochloric acid and pancreatic secretions are bactericidal.[48] In the premature neonate, the production of gastric acid[49] and pancreaticobiliary secretions[50] are impaired compared to the term infant. Similarly, normal gut mucus prevents adherence of bacteria. Mucin – a component of gut mucus – does not reach full production levels until 27 weeks of gestation.[51] Paneth cells in the intestinal crypts produce multiple peptides, among them α-defensin and lysozyme-C[52] which have antimicrobial properties. Both the number of Paneth cells, and the production of α-defensin are reduced in human premature infants.[53,54] The whole range of peptides produced by Paneth cells limit pathogen colonisation and invasion in the gut.[54] The premature gut also has reduced motility, which only achieves the mature pattern of peristalsis around 35 weeks post-conception age.[55] This reduced motility may well contribute to bacterial overgrowth.

1.5.2 Enteral feeding

The digestive and absorptive capacity of the immature gut is poor compared to that of a full term infant. Moreover, this capacity has been shown to progress with each week of gestation.[47] NEC is very rarely seen in infants who have never had enteral feed supporting the notion that enteral feeding is an important risk factor for developing NEC.[56] It is not only enteral feeding *per se* that confers an NEC Risk, the choice of feed is important. Essentially there are three options for enteral feeding in the preterm infant; maternal breast milk, donor breast milk or cow's milk-based formula feeds (or a combination of these three). The imperative for optimising nutrition is due to the fact that there is a clear association between poor growth in the neonatal period and poor neurodevelopmental outcomes[57] and there is some evidence that improving nutrition in the neonatal period reduces neurodevelopment impairment in preterm infants.[58]

The risk of NEC is much lower in infants fed maternal breast milk (MBM) compared to formula feeds. Sisk *et al.* (2007) reported a 6-fold reduction in the risk of NEC in infants fed on at least 50% maternal milk. Similarly, there does appear to be a dose effect. In ELBW infants, in a secondary analysis of a large cohort of 1272 infants, Meinzen-Derr *et al.* (2009) showed a reduction in the composite outcome of NEC or death for each 10% increase in the proportion of feeding that was human milk. Risk ratio 0.83 (95% CI 0.72-0.96).[59]

However, breast milk does not contain sufficient nutrients (especially protein) to meet the growth requirement in premature babies.[60] Unsurprisingly this 'nutritional requirement gap' is inversely proportional to gestational age.[61]

If MBM is not available, donor breast milk (DBM) is an option for feeding neonates. A Cochrane review of formula feeding vs donor breast milk for preterm infants shows that formula feed is better in terms of achieving growth but at the cost of a higher NEC risk; risk ratio 2.77 (95% CI 1.4 – 5.46).[62,63] It is not surprising that DBM appears to be less protective than MBM as safety requirements mean that DBM in the UK is pasteurised[64] which alters the protein and nutrient content. DBM has been shown to contain less immunoprotective factors than MBM.[65]

The compromise approach is to use breast milk feeds with fortifiers. However, fortifiers may confer a risk for NEC; An early trial was suggestive with NEC rates of 5.8% compared with 2.2% in controls but this was not statistically significant.[66] However, a systematic review showed only limited evidence of an increased risk of NEC.[67] Given the importance of nutrition for long term outcomes, The European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) guidelines recommend the use of human milk with nutrition fortifiers as 'standard practice.'[60]

The volume of feed given was also thought to be important for NEC risk. A randomised control trial of feed volumes had to be stopped early due to the number of babies who developed NEC in the 'progressive' group;[68] 141 infants randomly assigned to either 20ml/kg/day of feeds for the first 10 days of life or 20ml/kg/day on day one, increasing by 20ml/kg/day to a maximum of 140ml/kg/day. The 'static' group had a rate of NEC of 1.4% compared to 10% in the 'progressive' group. This finding was not supported by a subsequent Cochrane review. The ten studies included did not show a statistically significant difference in terms of NEC risk but did show that delayed advancement in feeds lead to poorer growth with concerns about the potential impact on neurodevelopment in the long term.[69,70] Given the limitations of these studies, The Speed of Increasing Milk Feeds Trial (SIFT) was performed to compare faster (30 ml per kilogram) with slower (18 ml per kilogram) daily increments in milk feeding volumes. This showed no difference in the NEC risk between the two groups (risk ratio 0.90 (95% CI 0.66 to 1.24)).[71]

An obvious strategy to avoid the risk would be to not give enteral feeds at all in the first few weeks of life to babies born prematurely. However, this in itself carries significant risks and negative sequelae. Delayed enteral feeding leads to more central catheter days, higher rates of bloodstream infections and delayed gut development.[29] Indeed, animal models suggest that delaying feeding for only three days leads to gut villi atrophy.[72] Parenteral nutrition is key part of the care of premature neonates. Yet providing adequate nutrition for optimal growth via the

parenteral route is challenging[73] (especially in terms of energy and protein content of the feed). Overall the negative effects of withholding enteral feeds significantly outweigh the potential benefit of reducing the NEC risk.[74]

The mechanism by which feeding confers a risk of NEC is not entirely established, although the association with immaturity is clearly important. At least in part, the relationship between feeding and NEC is likely to be due to the increase in metabolic demand of the gut due to digestion and absorption of nutrients.[75-77] Indeed, in healthy infants, blood flow in the superior mesenteric artery (SMA) is increased in response to feeds. This increase is greater in response to formula feeds than human milk, suggesting that formula feed confers a greater metabolic demand on the intestine than breast milk.[75] An inability of the intestine to respond to this need appears to be a key part of the pathogenesis of NEC.[78] This will be discussed in detail in section 1.5.7.

Other factors that may be important include the incomplete digestion and absorption of carbohydrates which leads to bacterial overgrowth.[79] The relative lactase deficiency of the premature infant means that lactose may be converted to short chain fatty acids by bacterial fermentation in the colon.[80] Over production of short chain fatty acids can cause mucosal injury in experimental animals.[81] The osmolality of feeds has long been a cause for concern. Human milk has an osmolality of approximately 300 mOsm/kg. This can be increased to more than 400 mOsm/kg with the addition of nutritional fortifiers.[82] Formula feeds can have an even higher osmolality but a recent systematic review showed there is no evidence of an increase in NEC risk with high osmolality feeds.[83] Although very high osmolality may be associated with delayed gastric emptying.

The effect of formula feeds on mucosal immunity seems to be significant both in terms of the pro-inflammatory effects of formula feeds and the absence of the immunological benefits of breast milk.[79,84,85] This will be discussed further in section 1.5.4.

1.5.3 Bacterial colonisation

The role of bacteria in NEC has long been established but the significance, timing and pathway remains unclear. Bacterial overgrowth is seen in NEC and not in other bowel conditions considered to be of ischaemic origin.[7] Moreover the pathognomonic feature of pneumatosis intestinalis contains hydrogen gas – which is not a product of human metabolism.[14] However the histological changes of NEC are distinctly different from that seen in infective enterocolitis.[7] Similarly, the fact that NEC does not occur in utero and classically occurs around two weeks of age (as the neonatal gut is colonised), suggests that the presence of bacteria in the bowel is necessary

for the pathogenesis of NEC.[86] Several species of bacteria have been implicated in both human and experimental NEC but they are also found in healthy matched individuals.[87]

A prospective study that collected bacteriological and clinical data on all neonates less than 36 weeks of post conception age admitted to a single centre, revealed that all patients were colonised with potentially pathogenic bacteria in the week before contracting NEC.[88] Importantly, 79% of controls also had potentially pathogenic bacteria in their stools. This suggests that bacterial colonisation is a prerequisite for developing NEC but not on its own sufficient. Similarly, clusters of cases of NEC within neonatal units have been associated with the isolation of specific organisms.[89]

There are multiple reports of bacterial, viral and fungal infection in association with NEC[90] based on both culture and molecular techniques to identify potential pathogens. Several studies have isolated specific organisms that seem to play some role in causing NEC, these include the bacteria *Escherichia coli, Pseudomonas aeruginosa, Klebsiella, Clostridium*, as well as Rotavirus, A*denovirus*, CMV and fungal organisms including *Candida.*[91]

The normal microbiome of the neonate is simple but also diverse and fluid. It has been described as having four distinct phases as it becomes more like that seen in older children and adults.[86] Phase 1 (birth to 2 weeks): *Streptococci* and coliforms predominate; Phase 2: Gram positive and non-spore-forming anaerobes emerge. Phase 3 coincides with the change of diet to solid food. The relative paucity of anerobes during Phase 1 allows pathogenic bacteria to colonize the neonatal gut more easily. The temporal association with Phase 1 and the typical timing of the onset of NEC supports the idea that this is an important factor.

Bacterial colonisation with a range of potentially pathogenic organisms (especially Enterobacteriae) is necessary but not in itself sufficient for the development of NEC.

Remon *et al*.[92] found that the depth of infection in resected specimens (i.e. whether the bacterial invasion was only in the mucosa, or had progressed to the submucosa, muscularis or serosa) correlated with the extent and depth of necrosis and with the risk of mortality.

Given that the colonisation by potentially pathogenic organisms is a key-step in the pathogenesis of NEC and given that the normal colonisation of the neonatal gut is disrupted by preterm birth, it is logical to suggest that the use of prophylactic enteral probiotics (live microbial supplements) could be protective against NEC. There have been several studies examining the potential of probiotic preparations and a Cochrane Review in 2014 concluded that enteral probiotics should be used for the prevention of NEC (Bell stage 2 or more) with an estimated relative risk (RR) of 0.43 (95% CI 0.33 to 0.56).[93] In simple terms, the administration of probiotic provides competitive

exclusion of pathogenic bacteria. Detailed studies suggest that providing commensal bacteria to the preterm gut has multiple positive effects, including; strengthening of the intestinal barrier, improved peristalsis and by stimulating mucin production.[94] These findings are consistent with the known physiology of the preterm intestine (section 1.5.1). However, probiotics are not universally accepted. A large double-blind, randomized control trial conducted since the Cochrane review showed no clear benefit.[95] Although follow up work showed that controls were highly colonised with the probiotic bacterial species as well, potentially diluting the effect.[96] This finding is particularly intriguing as studies have tended to produce conflicting results which may be explained by this one particular factor which is peculiar to randomised trials of probiotics in neonatal units.[96] Other reasons for caution include the fact that there has been no consistency in the choice of bacterial species used for these trials (Most commonly *Lactobacillus* species but also *Bifidobacterium* species and combinations)[97] thus there is no clear consensus as to which probiotics should be used. Moreover, in most countries (including the UK), probiotics are regulated as a food product rather than a medicine and thus the quantity, quality and purity of the agents used is not clear. Whilst there have been no reported invasive infections in the major trials, routine use of probiotics would mean exposing a relatively large number of neonates to probiotics to prevent a minority of them developing NEC. The number needed to treat (NNT) is large and variable – because the incidence of NEC is very variable between units and countries. Hence, the routine use of probiotics remains controversial[97] but the role of bacteria in the pathogenesis of NEC is agreed upon.

1.5.4 Immunological Mechanisms

There is a complex interaction of each of the various factors involved in the pathogenesis of NEC such that discussing immunology separately from bacterial colonisation is overly simplistic and potentially misleading. It is well known that colonisation of the intestinal tract plays a role in the development and education of the immune system[98] and hence the colonisation of the preterm neonate could be very significant in modulating the inflammatory response seen in NEC. Nevertheless, there has been considerable research interest in recent years in the specific role of the immune system in NEC pathology. Cho *et al.* (2016) argue that NEC results from a profoundly dysregulated inflammatory response as a common endpoint of the various precipitating factors.[99] This is supported by the cumulative evidence that shows marked increases in certain pro-inflammatory cytokines, such as TNF-α, IL-18 and IL-6 in intestinal tissue and the development of NEC. Various animal studies suggest that the Toll-like receptors (TLRs) play an important role here, especially TLR4.[100] TLR4-knockout mice do not develop NEC.[101]

The TLRs are a group of transmembrane receptors that are a key part of the innate immune system.[102] They are found on both haemopoetic cells and non-haemopoetic cell types including enterocytes. TLRs function by binding to structurally conserved molecules derived from bacteria, viruses and other microorganisms.[103] These highly conserved structural motifs are known as pathogen-associated microbial patterns (PAMPs) and are critical to the innate immune system being able to recognise invading organisms.[104] Receptors that bind PAMPs are collectively known as pattern recognition receptors (PRRs).[102] Once this binding occurs, TLRs dimerise and recruit myeloid differentiation factor 88 (MyD88), which triggers down-stream signalling of the nuclear factor kappa beta pathway (NF-κβ).[105] NF-κβ is a transcription factor that triggers an inflammatory response, bringing effector cells to the site of injury/invasion. This activation of TLR4 has been shown to result in increased enterocyte apoptosis, reduced enterocyte proliferation and migration and ultimately, breakdown of the intestinal epithelium. Interestingly, TLR4 expression levels are higher in the premature gut compared to term controls thus providing further explanation as to why the premature bowel is much more vulnerable to NEC.[100,102,106]

Lipopolysaccharide (LPS) is a major surface molecule of Gram-negative bacteria and is a potent stimulator of the innate immune response; it is the archetypal PAMP molecule.[107] LPS is a potent activator of TLR4[108] and it has been shown that this triggers the NF-κβ inflammatory cascade.[109] Therefore the importance of TLR4 in the pathogenesis of NEC is consistent with the association with Gram-negative bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Klebsiella* as discussed in section 1.5.3.

Murine studies have shown that breast milk attenuates TLR4 signalling[84] thus the reduced activation of the TLR4 pathway could (at-least in part) explain the protective effect of breast milk.

As discussed in 1.5.2, breast milk confers a lower risk of NEC compared to cows' milk-based formula feeds. It is challenging to untangle the various factors involved: Is it the presence of various factors in formula that are not found in human milk that increase risk or does breast milk contain protective factors? Cumulative evidence would support both hypotheses. Recent work has shown that maternal Immunoglobulin A (IgA) is important in making this protective effect conferred by breast milk. Analysis of faecal cultures showed a decrease in IgA-bound bacteria preceding the onset on NEC in preterm babies.[85] Moreover in a mouse model of NEC, maternal milk is protective but this protective effect is abolished in IgA-deficient mothers. The importance of maternal IgA here seems to be in shaping the development of the neonatal microbiota and the absence of IgA in the gut allows the predominance of potentially pathogenic bacteria described in section 1.5.3 to develop.

As discussed in 1.5.1., Paneth cells are reduced in number and have reduced function in the premature bowel. Paneth cells are an important contributor to intestinal epithelial integrity.[102] In the small bowel, they secrete multiple anti-bacterial peptides (such as α-defensin and lysozyme C) in response to PAMPs. The significance in NEC is not clearly established but the reduced function in the immature bowel would suggest that the epithelial barrier which is critical to innate immunity in the bowel is compromised in the premature infant.

Platelet activating factor (PAF) is another inflammatory mediator that has been implicated as being important in the pathophysiology of NEC. Human studies have shown that plasma levels of PAF are higher in infants with NEC than healthy controls.[110] PAF is found in multiple tissues and activation leads to epithelial cell damage and apoptosis, as well as affecting tight junctions, leukocyte activation, platelet aggregation and vasoconstriction.[94] PAF-acetylhydrolase (PAF-AH) is the enzyme responsible for PAF degradation. In a rat model, PAF infusion leads to ischaemic bowel necrosis.[111] This intestinal injury is prevented if the animals are pre-treated with dexamethasone and medroxyprogesterone which increase PAF-AH levels.[112] In neonatal rats, the use of a PAF-antagonist significantly reduced the incidence of NEC.[113,114] Similarly in a mouse model, PAF-AH knockout mice were far more susceptible to disease. Maternal breast milk has detectable levels of PAF-AH.[115] Quantitative real-time PCR studies have shown that PAF induces expression of TLR4 in the intestine.[116]

It has long been thought that neutrophils play an important role in the pathogenesis of NEC. In one animal model, the depletion of neutrophils with vinblastine prior to inducing disease, resulted in a reduced bowel necrosis.[117] Conversely, work in a different animal model showed that neutrophil depletion resulted in an increase in pro-inflammatory cytokines and enterocyte apoptosis.[118] A human study in SGA infants (small for gestational age) found that neutropenia was associated with an increased risk of developing NEC.[119] Neutrophils are a key part of the innate immune system, thus it is not surprising that they seem to be important in NEC but these conflicting data suggest that they may be both protective and play a role in promoting intestinal injury.[102] As such the exact role(s) of neutrophils are not well-understood. However it is known that PAF activates neutrophils,[120] so given the importance of PAF it would be logical that neutrophils do play a role in the pathophysiology of NEC but it might be that the effect of PAF is unrelated to platelets and any such action is by a different pathway completely.

Macrophages have multiple functions including phagocytosis of invading organisms. As bacterial infection is clearly important in NEC, intestinal macrophages are likely to be important. Indeed intestinal macrophages are very potent phagocytes.[121] Macrophages are also important producers of a range of cytokines, necessary for an effective immune response. Detailed analysis of

intestinal macrophages suggests that pro-inflammatory macrophages are a different sub-population from the resident macrophages that have a phagocytic activity against invading organisms.[122] Indeed resident macrophages are profoundly inactive in terms of producing an inflammatory response in normal circumstances.[123] Macrophage-rich infiltrates in both the inflamed and non-inflamed mucosa are found in NEC.[92,124] These macrophages contrast with the normal intestinal macrophage in that they have a pro-inflammatory profile.[125] Tumour growth factor beta (TGF-β) suppresses the inflammatory profile of macrophages. Maheshwari *et al.* showed that intestinal macrophages acquire a non-inflammatory profile during maturation of the premature gut. This maturation is mediated by the expression of TGF-β.[126] Subsequently, MohanKumar *et al.* demonstrated that expression of Smad7 (SMAD family member 7) in response to bacterial products interrupts the TGF-β -mediated inflammatory downregulation of macrophages and promotes NF-κβ activation.[125] Taken together these findings provide a pathway by which bacteria induce a profound inflammatory response that is seen in NEC and provide some explanation as to why premature babies would be at greater risk.

T-lymphocytes are known to be important in NEC.[102] In adults the majority of T-lymphocytes are referred to as αβ T-cells because these are the two subunits that make up the T-cell receptor (TCR). There is another, much smaller population of T-lymphocytes with different subunits making up the TCR, these are known as γδ T-cells. In the premature neonate the γδ T-cells in the intestine are far more active and produce higher levels of cytokines including IFN-γ and IL-10.[127] Weitkamp *et al.* discovered that these γδ lymphocytes are depleted in NEC compared to controls.[128] The fact that γδ cell are much more populous in the preterm neonatal gut and that the population changes in NEC is good evidence that they are important in the pathogenesis but the exact role is less clear, although it is thought that they may be critical in maintaining barrier integrity.[102] Regulatory T-cell populations are reduced in human NEC specimens.[128] CD-17+ T-helper cells (a recognised subgroup of T-helper cells) produce a range of cytokines that cause intestinal inflammation and Egan *et al.* demonstrated in a mouse model that regulatory T-cells are reduced whilst these pro-inflammatory CD17+ T-helper cells are increased.[129] These T-cells produce high levels of IL-17A that results in a loss of tight-junctions between epithelial cells, reduced enterocyte proliferation and increased apoptosis in intestinal crypts. Moreover they found that TLR-4 caused the influx of these CD17+ lymphocytes.

1.5.5 Genetic factors

As noted in section 1.4, the incidence of NEC varies considerably between different populations. NEC is less common in Austria, Japan and Switzerland and more common in Ireland, North America and the UK.[23] There are several potential explanations for these differences, including

differences in neonatal care, but studies limited to the USA demonstrated that there were differences linked to race alone. African-American infants are at greater risk of NEC than Caucasians.[25,130] Furthermore, twin studies have demonstrated significant concordance.[131] Hence, whilst NEC is undoubtedly an acquired condition, there may be multiple genetic factors that may predispose an infant to NEC. Due to the apparent importance of TLR4 in the inflammatory pathways of NEC, it has been suspected that some TLR4 polymorphisms might confer risk but thus far no such predisposing alleles have been identified.[26] Similarly, research into genetic variations of pro-inflammatory cytokines, the NF-κ β pathway and PAF have yet to yield conclusive results.[26]

1.5.6 The pathologenesis of Necrotising enterocolitis in term infants – is it the same disease?

As discussed in section 1.4.2., around 10% of NEC cases occur in term infants. The significance of this is that the specific risk factors for preterm infants (section 1.5.1) cannot explain the onset of disease in this minority of cases. Some argue that NEC should not be considered a single disease entity because of the multifactorial pathogenic pathways involved.[132] This subgroup who develop NEC suggest that IRI is a critical part of the pathophysiology.

An important counter argument to this complete separation is that when the same risk factors are seen in preterm infants, they have an increased risk of developing NEC.[29] This suggests that whilst it is not a simple, single pathway, there is clearly some cross-over of these pathophysiological factors, which is an argument against considering NEC as truly separate diseases. In essence, NEC in term infants is not related to the risk factors peculiar to preterm infants but the corollary is that the risk factors for NEC in term infants are also associated with an increased risk in preterm babies.

1.5.7 Ischaemia-reperfusion injury

1.5.7.1 Evidence that demonstrates Ischaemia-reperfusion injury is part of necrotising enterocolitis

The role of Ischaemia-reperfusion injury (IRI) in the pathogenesis of NEC was first suspected due to the macroscopic appearance of the bowel injury.[7] Moreover the association with low-cardiac-output states (or other risk factors) in term babies that develop NEC is consistent with an ischaemic process. Similarly, a recent Cochrane review showed that targeting lower oxygen saturation levels in premature neonates (to reduce the risk of retinopathy) results in higher rates of NEC.[41]

Initially it was thought that the so-called 'diving reflex' may be the explanation for these findings.[133] The diving reflex diverts blood flow away from the intestine, allowing for sustained perfusion to the brain and heart, but ultimately resulting in intestinal injury[134] and was thought to be induced by various stresses in the perinatal period. More recently, the evidence has very much undermined that theory with much more focus on the immunological and microbiological aspects.[135] It is certainly incorrect to think of NEC as a purely ischaemic disease[135] but the histopathology of NEC is one of ischaemic necrosis so the real question is one of the sequence of causative factors. Chen *et al.* (2016)[136] investigated the ischaemic markers hypoxia-inducible factor-1 (HIF-1) and glucose transporter 1 (Glut1) in histological specimens of patients who underwent resection for NEC. HIF-1 is a transcription factor made of two subunits (HIF-1α and HIF-1β) and is a key part of the cell's response to hypoxia. Hence it is a marker of hypoxic insult at the cellular level. Under normoxic conditions HIF-1α has very high turnover, being constitutively expressed and broken down by proteasomal degradation. Hypoxia inhibits the breakdown of HIF-1α thus leading to its nuclear accumulation.[137] The HIF-1 molecule is a transcription factor that activates multiple genes, including Glut-1. Chen *et al.* showed that both HIF-1α and Glut-1 are expressed in the epithelial layer of bowel exposed to ischaemia (i.e. from small bowel volvulus or incarcerated hernia), confirming the role of HIF-1α and Glut-1 as a reliable marker in gut tissue. Bowel specimens from patients with severe NEC (requiring multiple resections) had an 'ischaemic-type' pattern of HIF-1α and Glut-1 expression.[136]

1.5.7.2 Gut immaturity and ischaemia-reperfusion injury

The immature bowel is not able to regulate its blood flow as the term neonate's bowel does and the increased metabolic demand of feeding or abnormal bacterial colonisation or inflammation may provide an explanation with a common pathway of the various causative factors. As discussed in 1.5.2, there is a strong imperative to give enteral feeds to neonates. This inevitably means an increased metabolic demand in the gut circulation.[75-77] The regulation of blood flow in the intestinal circulation of the newborn is controlled by the antagonistic actions of endothelin-1 (ET-1) and nitric oxide (NO) which regulate intestinal vascular resistance. In pathological states, endothelial dysfunction alters this balance towards ET-1, causing vasoconstriction and consequential intestinal ischaemia.[135] Downard *et al.* (2011) showed in an animal model that altered microcirculation is a critical event in the pathogenesis of NEC.[78] Using real-time observation of the microvasculature they observed arteriole diameters were significantly smaller in animals who developed NEC. Similarly, it has been shown that early tolerance of enteral feeds correlates with an increase in superior-mesenteric artery (SMA) blood flow velocity, and high SMA resistance demonstrated on ultrasonography predicts the development of NEC.[138,139] The occurrence of NEC in term infants with congenital heart disease(CHD), especially low-output

states similarly supports the notion that under-perfusion is a trigger for NEC; infants who suffer poor perfusion or shock are the ones who subsequently develop NEC.[140] In this context, NEC seems to be very much an IRI disease with the subsequent bacterial infection of the bowel wall resulting from the initial ischaemic injury. The unanswered question has always been what causes such an ischemia-reperfusion in the preterm infant who has normal vascular anatomy and no clear global under-perfusion like the child with CHD. The increased metabolic demand of feeding, the insult of infection and the inflammatory injuries seen in preterm babies, especially those fed on formula – coupled with the immature gut's inability to maintain perfusion haemostasis leads to mucosal ischaemia and hence may provide a common pathway for NEC pathogenesis. This kind of synergistic relationship between tissue hypoxia and formula feeding has been demonstrated in a mouse model.[141] Moreover, the role of TLR4 discussed in 1.6.4 is not separate from this ischaemic process as TLR4 impairs intestinal perfusion by inhibiting endothelial nitric oxide synthase (eNOS) which is part of the NO pathway for regulating the intestinal microcirculation.[142] This reduction in microcirculation causes localised ischaemia.[103] The importance of eNOS is further demonstrated by the fact that the administration of sildenafil (a phosphodiesterase-5 inhibitor) to maintain intraluminal nitric oxide activity also markedly reduces NEC severity in a murine model whilst eNOS-deficient mice have much greater disease severity.[142] Given that (as previously discussed, in Section 1.5.4) the expression levels of TLR4 are higher in immature gut compared to full-term controls,[103] this pathway connects the inflammatory process with the ischaemia-reperfusion injury which is observed histologically.[7] This understanding of NEC with IRI at the mucosal level as a common pathway from multiple factors, is further supported by the fact that systemic factors (such as hypoxia) increase the risk of NEC.[41]

1.5.7.3 Ischaemia-reperfusion injury and NEC risk-factors

As previously noted, in term infants, NEC is virtually never seen without specific risk factors[28] and the same risk factors increase the likelihood of NEC in premature infants as well.[29] This is important as it suggests a significant commonality between the disease in term babies and pre-term. There is clearly significant heterogeneity in NEC such that some argue it should be considered as multiple disease entities.[3] This may well be a correct approach, however this overlap in risk-factors between term and preterm infants strongly suggests some sort of common pathway as well. Moreover, this common pathway is very attractive as a therapeutic target as it would remain valid, regardless of the specific aetiology of NEC.

1.5.7.4 Intrauterine growth restriction

Maternal pre-eclampsia and intrauterine growth restriction (IUGR) are associated with foetal risks.[143] In the second trimester, a third of the cardiac output of the foetus will go to the placenta.

In IUGR this fraction is reduced, in extreme cases to one-tenth.[144] Foetal Doppler assessment of the umbilical arteries detects absent or reversed end-diastolic flow (AREDF) velocity which corresponds to this extreme under perfusion. A Cochrane review from 2010 recommended the use of Doppler ultrasound in high risk pregnancies but has been subsequently withdrawn, pending an update.[145] The presence of AREDF has been shown to be associated with a higher risk of NEC.[146] The mechanism here is not simple but in essence, the intestinal perfusion is reduced due to the fact that under-stress there is preferential circulation to the brain, heart and liver.[144] This increased risk led to the logical hypothesis that delaying feeds in such high-risk infants would be a wise precaution to reduce the NEC risk. However, a trial of early vs late feeding showed no reduced risk of NEC and an increase in the need for parenteral nutrition.[147]

1.5.7.5 Transfusion-associated necrotising enterocolitis

It has long been suspected that red blood cell (RBC) transfusions may be a risk factor for NEC such that the term TANEC (transfusion-associated necrotising enterocolitis)[148] has been used. A recent trial by Janjindamai *et al.*(2019) with over 400 VLBW infants showed that after controlling for confounders, no association with RBC transfusions and NEC was found.[149] Indeed a previous review by Hay *et al.* that applied the GRADE (Grading of recommendations assessment, development and evaluation) system to the literature on TANEC found that the overall quality of the evidence that RBC transfusion was a risk for NEC was 'very low.'[148] One of the great problems with all the studies in this area is the potential for confounders. The most obvious such confounder is anaemia. Patel *et al.* in an observational study, report that severe anaemia (defined as a haemoglobin level of less than 8g/dL) is associated with a 6-fold increase in NEC risk in the following week (independent of transfusion).[150] Although the reported cause-specific hazard ratio (HR) of 5.99 had a very wide 95% confidence interval (2.00-18.0). Importantly, additional analysis showed that each 1g/dL decrease in the nadir of haemoglobin was associated with a 65% increase in the risk of NEC. These data support the notion that anaemia rather than RBC transfusion is the more likely risk factor for NEC. However, this does not exclude reverse-causation. Anaemia is significant for the development of NEC in that global anaemia will lead to (relative) intestinal hypoxia.

It has been shown that there is an association between NEC and the activation of Thomsen-Friedenreich cryptic T antigen on RBCs which causes haemolosis.[151] Similarly Le *et al.* in a case-crossover study reported no association between RBC transfusion and NEC. Sub-group analysis did suggest that anaemia however, did confer an increased risk, but these data did not reach statistical significance. (OR 6, 95% CI 0.7-50, p = 0.19).[152] It has been common practice to withhold feeding whilst transfusions are being given, however several studies have shown that there is no

increased risk with continuing feeding whilst giving RBC transfusions.[151] Both anaemia and transfusion could plausibly cause or augment injury. It has been proposed that RBC transfusion could cause a TRALI-like (transfusion-related lung injury) reaction in the gut causing inflammation, triggering NEC.[153] Anaemia is known to impair splanchnic perfusion resulting in tissue hypoxia and anaerobic metabolism.[154] If anaemia is indeed the true association with NEC (rather than RBC transfusion) that tends towards an IRI explanation rather than an inflammatory one. Although, as previously discussed an inflammatory response due to transfusion increases the metabolic demand thus creating hypoxia.

1.5.7.6 Patent ductus arteriosus

In utero, the ductus arteriosus is a key component of the foetal circulation that (along with the foramen ovale) allows the majority of oxygenated blood from the placenta to bypass the lungs and enter the systemic circulation. In a term infant, the duct will normally close within 72 hours of birth.[155] Failure of closure and hence a patent ductus arteriosus (PDA) beyond 72 hours occurs in the majority of preterm infants (>70% <28wks;[156] >80% 24-25wks[157]). Ex-utero, the difference in pressure between the systemic and pulmonary circulation means that a PDA will usually lead to a left-to-right (systemic to pulmonary) shunt. There is little consensus as what constitutes a haemodynamically significant PDA (hsPDA)[155] however, it has been shown that a hsPDA (by whichever definition) results in lower splanchnic oxygenation.[158] Therefore, it has long been suspected that an hsPDA would increase the risk of NEC. However, some recent data shows no clear link between hsPDA and developing NEC.[159] In recent years, several studies have reported that there is no benefit in terms of reduced mortality or NEC from treating even an hsPDA.[160]Conversely, a detailed review and meta-analysis of PDA treatments did show that high-dose ibuprofen treatment did reduce the risk of developing NEC,[161] implying that the left-to-right shunt of a PDA and consequential reduction if splanchnic oxygenation is indeed a risk-factor for NEC. In part, demonstrating that a PDA is an independent risk-factor for NEC might be challenging because of the near-ubiquity of PDAs in preterm infants.[156,157]

1.5.7.7 Ischaemia-reperfusion injury as a common end-pathway for necrotising enterocolitis pathophysiology

As discussed in section 1.4 (and throughout this section), several predisposing factors have been implicated in NEC, especially in the term infant[28] but also in the pre-term infant.[29] The commonality of these factors (i.e sepsis, polycythaemia and hypotension) is that they result in compromised bowel perfusion.

Global mild hypoxia may be well tolerated by the bowel. Similarly, the increased metabolic demand of absorbing feeds may, on its own not result in intestinal injury. Inflammation triggered by bacterial overgrowth may result in only minimal injury. However when one or more of these factors is combined, it is highly likely to result in a localised IRI resulting in tissue necrosis. IRI itself triggers a pronounced inflammatory response.[162] Compromise of the bowel wall enables bacterial invasion, a cardinal sign of NEC.[7,14]

This understanding makes IRI a secondary event rather than the primary cause.[3,163] However, it is arguably more accurate to describe this as a vicious cycle as IRI triggers further inflammation[164] and necrosis is a major driver of inflammation leading to further ischaemia.[165] This is consistent with the evidence that NEC is an evolving disease process rather than a singular event: inflammation leads to further damage[166] and histologically, resected human specimens show repair processes indicating that the disease is progressive over time rather than a single event.[7] In this sense, it is very different to some IRI diseases but potentially more amenable to intervention. A potential way to assimilate this interaction of various factors leading to necrosis is summarised in Figure 1-2.

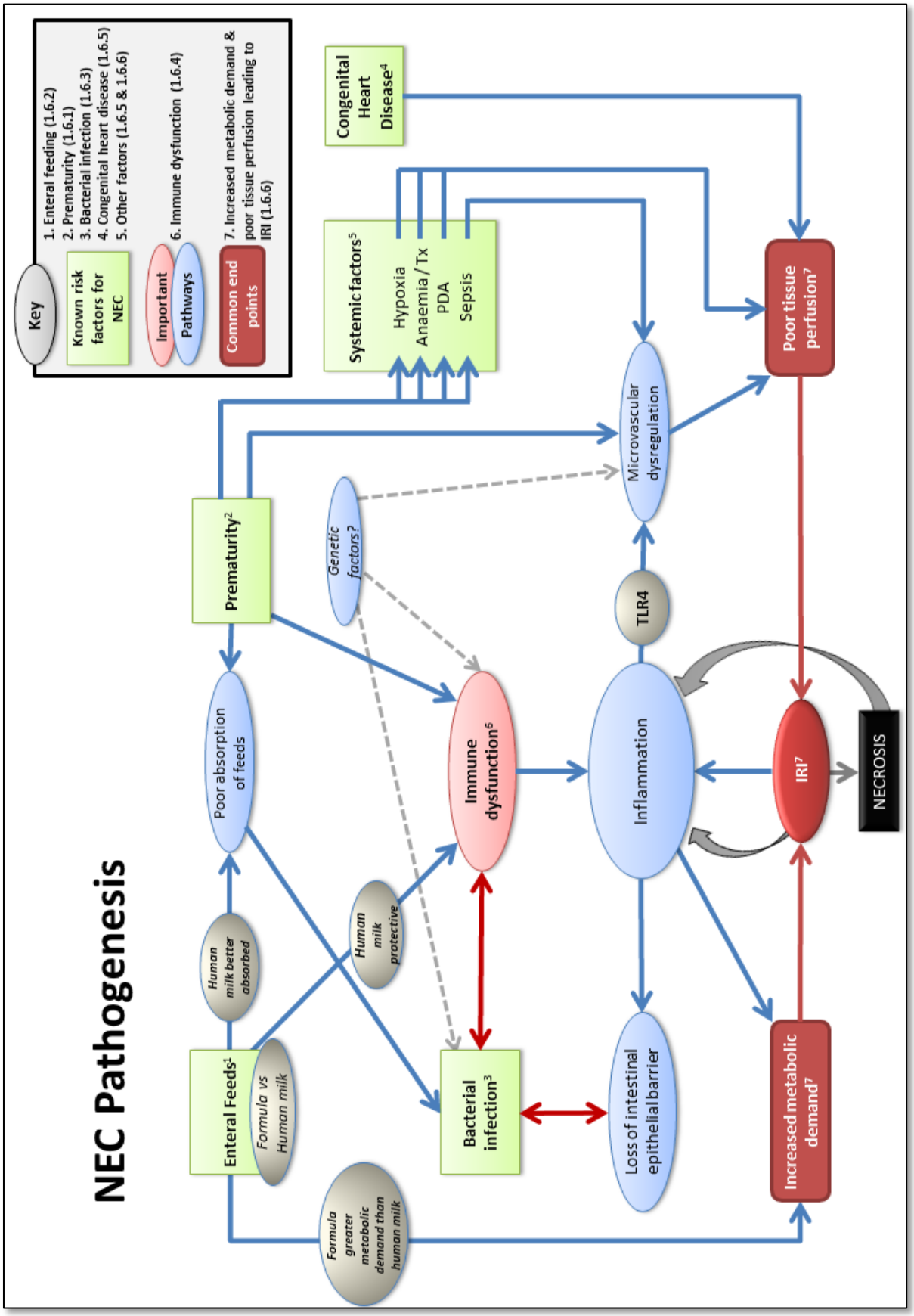


Figure 1-2 NEC Pathophysiology:
Schematic illustrating the interactions of multiple factors in NEC pathophysiology.

1.6 Current Clinical Treatments

Treatment of the different stages of NEC are outlined in Table 1-1, The mainstay of NEC treatment (especially early-stage disease (Bell stage 1)) is supportative. This consists of gut rest (withholding enteral feeding and gastrointestinal decompression with a nasogastric (or orogastric) tube) and the administration of broad-spectrum intravenous antibiotics and intravenous fluids.[167] If the disease progresses, additional systemic support is often needed: typically this may include ventilator support and/or ionotropic medication (pharmaceuticals that increase heart contractility and support cardiac output and therefore organ perfusion).[27] A Scandinavian study involving over 36,000 infants showed that the use of inotropic drugs was rare in the neonatal population at 2.7% of those admitted to the Neonatal intensive care unit (NICU).[168] However, 72.4% of babies with a diagnosis of NEC required inotropic support (within the context of NICU care – i.e. the most severely unwell neonates).

A subset of patients require some sort of surgical intervention and these patients are undoubtedly ones with a higher burden of disease and consequentially a higher mortality and morbidity.[169] NEC remains the most common surgical emergency in the neonatal population. The primary aim of surgery is the removal of necrotic bowel and peritoneal decontamination. A laparotomy for NEC with perforation is the standard treatment.[170] However, in the most unwell babies, where a laparotomy may not be tolerated, the use of a simple peritoneal drain (PD) as a temporising measure has been considered for several decades.[171] Placement of a drain allows drainage of air, pus and stool from the abdominal cavity. Initially, the drain placement was considered simply as a means to stabilise the patient until they were well enough to undergo definitive surgery. Subsequently it emerged that some of these babies will recover fully without the need for a laparotomy.[172] The evidence remains limited. A Cochrane review in 2011 concluded that no recommendation could be made as the only two randomised studies involved very small numbers[170] (there are several non-randomised studies but selection bias makes interpretation difficult). What these two studies did show was that approximately 50% of the babies treated with a PD did not require a subsequent laparotomy and there was no significant difference in short-term outcomes.[173,174]

Surgical decision-making in NEC is not straight-forward. Enterostomy formation is considered a safe surgical option but does confer a risk of impaired growth and that these infants will become significantly underweight.[175] There is certainly a place for a damage-control approach whereby the initial laparotomy is aimed at treating the deadly triad of acidosis, hypothermia and coagulopathy[176] with an expectation of a second procedure around 48 hours later.[177] The evidence for this is limited by the difficulty in performing a randomised-control trial but mortality

appears to be the similar but with the advantage that surgical resection is minimised and enterostomy is often avoided and hence the expectation that in terms of long-term morbidity, there may be a benefit with drainage.

1.7 Novel and potential future treatments

1.7.1 Stem cell therapy

In recent years, there has been some work with animal models that show the potential benefit of stem cell therapy. This has been done with placentally-derived stem cells and with stem cells derived from amniotic fluid, as well as stem cells from the bone-marrow. These all show a significant protective effect on the intestine. A similar benefit can also be achieved using exosomes derived from the stem cells.[178] The key mechanisms here appear to be anti-inflammatory rather than dependent on the successful grafting of stem cells which would explain why the exosomes are also effective.[179] The potential for stem cell technology to promote intestinal healing is clear but currently this work has not moved beyond the basic science stage.

1.7.2 Sildenafil

As discussed in Section 1.5.7.1, eNOS-mediation of mucosal blood flow is known to be important in the developing injury in NEC.[142] Sidenafil is known to increase the levels of eNOS in the intestine. It is also used in neonatal practice for other indications, such as treating pulmonary hypertension in the context of a congenital diaphragmatic hernia.[180]

In animal models, sildenafil has been shown to reduce intestinal injury and more recent work suggests this is not simply as a result of increasing eNOS but that there are other mechanism(s) involved as well.[181] As yet, there is no data on the use of Sidenafil for NEC in clinical practice.

1.7.3 Therapeutic hypothermia

For some time now, therapeutic hypothermia has been considered standard treatment for babies born with signs of hypoxic ischaemic encephalopathy.[182] This involves cooling the infant to between $33^{O}C$ and $35^{O}C$ for approximately 72 hours to prevent further neuronal loss following hypoxic injury. Logically, hypothermia could also be beneficial in NEC. Based on experimental animal work, a safety and efficacy study on the use of therapeutic hypothermia for NEC showed it to be a safe treatment.[183] Essentially, work in this area then stalled for a decade, partly because of the logisitical challenges of delivering therapeutic hypothermia making further research challenging and expensive. However, more recently, one study suggests that there may be a

measurable benefit in terms of reduced severity of disease, reduced need for surgery and indeed reduced mortality.[184] Though only involving small numbers, the reduction in disease severity here is encouraging.

1.8 Long term outcomes

Prematurity itself, as well as conferring a notable mortality risk, confers significant morbidity on the survivors. Such morbidity is often far-reaching and lifelong.[185,186] Consistently, studies have shown that NEC is associated with neonatal outcomes that are worse than anticipated among equivalent premature infants who did not suffer NEC.[27,169,187,188] Any putative treatment for NEC needs to measured against these long-term outcomes.

Neurodevelomental disability is the most far-reaching long term morbidity, with profound life-long consequences.

Intestinal failure (IF) is well-recognised as another long-term sequelae of NEC.[189] The risk of IF (to some extent) correlates with the amount of bowel resection that is required[190] – or more correctly – with the amount of residual bowel after resection.

Moreover, whilst mortality of NEC is most often considered at 30 days, there is also a late mortality. Allin *et al.* (2018)[191] reported a UK cohort of 236 infants who underwent surgery for NEC: 203 were followed up to 1 year, 59 were known to have died, 43 within 30 days and the remaining 16 died beyond 30 days but before 1 year. This represents a 30 day mortality rate of 21% and a 1 year mortality rate of 29%. In a large US cohort, around 5% of the babies who did not survive to 20 months, died after initial discharge.[169] So, long-term mortality must also be considered.

1.8.1 Neurodevelopmental disability

The reported rate of severe disability among surviving extremely-low birthweight (ELBW) infants (<1000g) is very high; 34% in one large series.[192] However, over and above this, there is an increased risk of neurodevelopmental disability (NDD) for infants who contract NEC during the neonatal period. The Vermont-Oxford Network evaluated a cohort of nearly 10,000 ELBW infants to assess the difference in outcomes at 20 months corrected age between those who contracted NEC and those who did not.[169] The cohort of 9926 who were evaluated represents 49% of the originally eligible cohort of infants born within the network in the time period studied. Such a rate of loss-to-follow-up is not uncommon and does mean there is a potential of bias in these data but whether this would indicate that the data constitutes overestimates or underestimates of

morbidity is unclear. ELBW infants have high rates of morbidity and the infants who suffered NEC show consistently higher rates. The infants who had suffered NEC (Bell stage 2 or 3) in the neonatal period were sub-divided into 'medical NEC' – NEC treated with antibiotics and supportative care only - and surgical NEC – those that underwent a laparotomy or primary peritoneal drainage. Clinical treatments are discussed in Section 1.6. Infants who had NEC had higher rates of neurodevelopment disability (as measured by a composite score, including the Bayley Scales of Infant Development (BSID). BSID-II and BSID-III were both used as BSID-III was released during the study period). Similarly, NEC conferred a higher risk of further surgery and the need for post discharge naso-gastric tube feeding. Babies who had surgical NEC were at higher risk of 'medical' rehospitalisation as well.

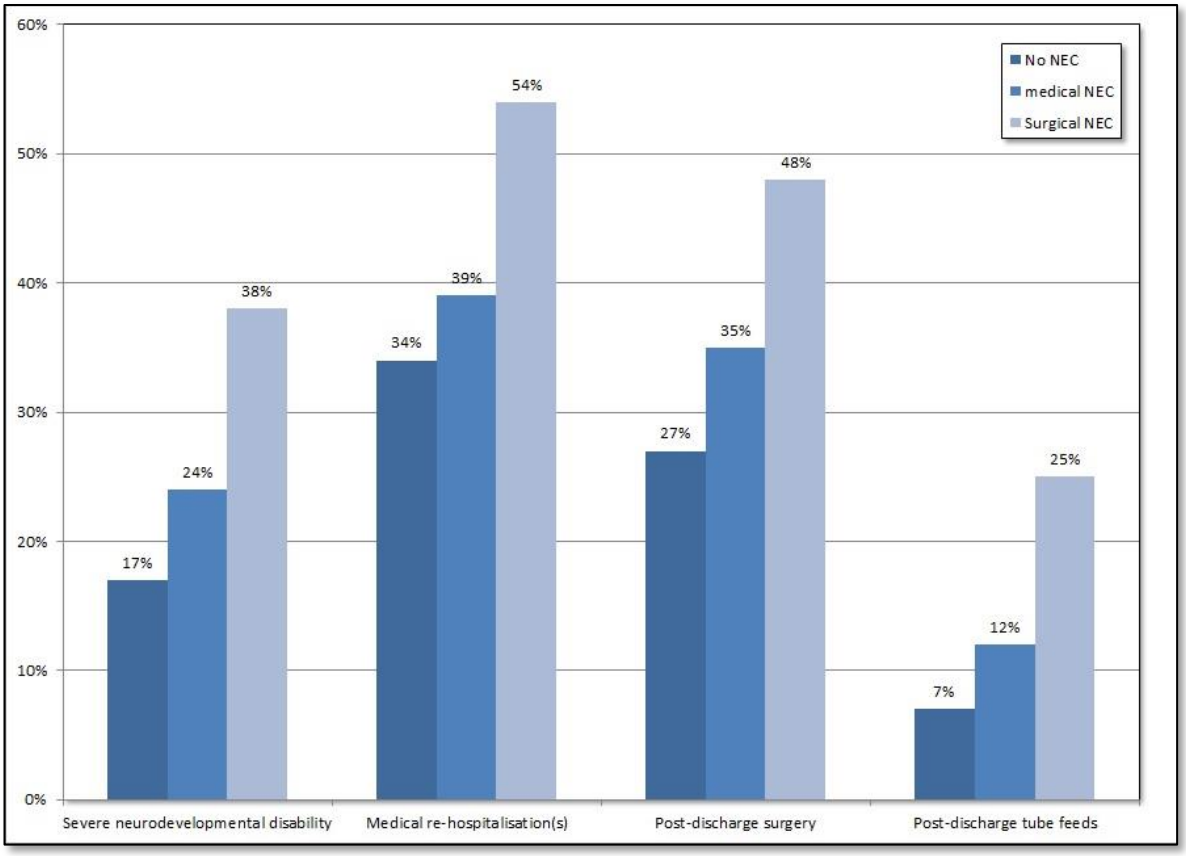


Figure 1-3 Outcomes for ELBW infants at 20 months corrected age
Rates of neurodevelopmental disability, rehospitalisation, further surgery and feeding support post discharge of infants born at less than 1000g that survived to follow-up (20 months). Infants are reported in three groups: No NEC, NEC managed with medical therapy only, and infants requiring surgery for NEC. [*Based on* Fullerton *et al.* (2017)[169]

Figure 1-3 summarises this graphically. Overall, 38% of infants weighing less than 1000g at birth who contract NEC requiring surgery will not survive to 20 months corrected age. Of those that survive to 20 months, 38% will have severe neurodevelopmental disability (defined as a BSID-II/-III score two standard-deviations below the mean or other clear diagnosis such as cerebral palsy, hearing impairment or visual impairment). Importantly these data show the increased risk of

mortality and morbidity that is conferred by having NEC on top of the base-line risk seen in these very premature babies. The adjusted risk ratio for severe neurodevelopmental disability is 1.32 for medical NEC (95% confidence interval 1.03-1.69) and 1.86 (2.59-2.20) for surgical NEC. A meta-analysis in 2007 reported similar results based on a total of 7843 children, the majority of which were under 1000g.[187] Overall, 45% of children who had NEC in the neonatal period were neurodevelopmentally impaired. This corresponds to an odds ratio of 1.6 (95% CI 1.2 to 2.0) compared to infants of the same gestation who did not develop NEC. They also reported that infants who underwent surgery for NEC had a further increase in risk of neurodevelopmental impairment with an odds ratio of 2.3 (95% CI 1.5-3.6). How precisely the BSID-II and –III score measure neurodevelopmental impairment and disease severity is the subject of some discussion.[193] However, Rees *et al.*[20] examined multiple measures of neurodevelopment and Fullerton *et al.*[169] opted for an inclusive definition of impairment. Due to the high loss to follow-up in these children, these data could be both an under- or over estimate; this loss to follow-up could affect these estimates in either direction – it is reasonable to assume that parents of children with greater needs are more likely to seek medical help. However, it is not clear if cognitive deficits will always be evident in children not assessed. It has also been shown that the BSID at 2-2.5 years does not capture all of the children at risk of poor neurodevelopmental outcomes. Impaired motor function at 4.5 years[194] and impaired cognition at 6.5 years[195] are not always predicted by the toddler assessment. If the children are not followed-up in childhood it is impossible to know for sure the burden of disease and more importantly that children are receiving any extra support they might need.

1.8.2 Intestinal failure

Intestinal failure (IF) in children can be defined as *a reduction in functional intestine below that which is necessary for adequate digestion and absorption of fluid and nutrients required for healthy growth.*[196] Given that surgery for NEC often necessitates the resection of variable lengths of both small and large bowel, it is not surprisingly that NEC is a leading cause of IF in children. It has been reported that NEC is responsible for 28% of IF in children in one study.[197] Even with little or no bowel resection, NEC is associated with poor feed tolerance for a variable amount of time. Hence, bowel length alone is not a predictor of long-term function. Whilst NEC confers a better prognosis than other aetiologies, with more than half achieving enteral autonomy, there is a significant on-going disease burden from NEC in the medium-long term if significant resection is needed.[197]

Several studies report parenteral-nutrition (PN) -dependence at 90 days but a significant number of these children obtain enteral-autonomy and hence the number that are PN-dependent at 90

days does not give a full picture. More recently, Sjoberg Bexelius *et al.*[198] reported IF in NEC at 2 years compared to matched controls of patients born at the same gestation. This shows an IF rate of 6% which is 15 times more common than children matched for gestational age. Khan *et al.*[197] showed that 43% of children with IF (multiple aetiologies) will achieve enteral autonomy at 72 months; this rises to over 60% in children whose IF is secondary to NEC.

1.8.3 Necrotising enterocolitis mortality

Estimates of NEC mortality vary enormously. These variations are due to multiple factors. The lack-of-agreement for a clear definition of NEC is one factor, another being that the mortality varies between different subgroups. Table 1-3 summarises the data published by Fitzgibbons *et al.* (2009)[36] looking at NEC incidence and mortality by birthweight. In these data, the mortality of infants born at less that 1000g with NEC is approximately a third falling to one in five in those with a birth weight over 1000g. What is especially noteworthy here is the odds ratios for each subgroup. The smallest babies have a much higher background mortality and hence the increased risk conferred by NEC is much less. For the smallest group, the risk of death increases by around 60% if they contract NEC. For the largest babies in this study (all VLBW or ELBW) the odds ratio suggests that the mortality increases more than 9-fold.[36]

As discussed in 1.8.2, work published by the Vermont-Oxford Network describe a cohort of ELBW infants followed up for neurodevelopmental outcomes (Figure 1-3). These data also reveal a mortality rate of around 16% for infants who contracted NEC but did not undergo surgery, rising to 38% in those that underwent surgery.[169]

Contemporary data on the outcomes of NEC are incomplete, therefore I conducted a systematic review to obtain accurate estimates of mortality and significant morbidity in NEC (Chapter 2).

1.9 Conclusion

Despite over four decades of research that has yielded a great deal of knowledge about the pathophysiology of NEC, effective treatments remain elusive. In an era when more and more preterm neonates are surviving, it is possible that there will be an increase in the incidence of NEC going forward. Data from large prospective studies show that for each gestational age or weight, NEC confers a markedly higher mortality and for the survivors, significant and often life-long morbidity compared to infants who do not contract NEC. There is no doubt that new therapies for this devastating disease are needed. Whilst the role and timing of an ischaemia-reperfusion injury is controversial, it is part of the pathogenesis of NEC, such that a putative intervention that acts

on IRI pathways offers the hope of a new approach to this disease for both treatment and prevention.

It is also the case that the current outcomes, especially beyond the primary admission, are not clearly known. In order to justify this research effort and the use of animals in research, more up to date outcomes from NEC should be clarified. For this reason, I conducted a systematic review of current outcomes for NEC (Chapter 2). This is designed to test the following hypothesis:

Hypothesis: Despite improvements in neonatal outcomes overall, Necrotising enterocolitis remains a cause of significant mortality and morbidity.

The broader aims of this thesis follow-on from this analysis and the main hypothesis is described at the end of Chapter 3.

Chapter 2 Contemporary Outcomes of Necrotising Enterocolitis

This work has been published in the *Journal of Pediatrics* as Jones, I.H. and Hall N.J., Contemporary Outcomes for Infants with Necrotizing Enterocolitis-A Systematic Review. J Pediatr 2020;220:86-92.[199] The *Journal of paediatrics* also published an accompanying commentary as Flahive *et al.* J Pediatr 2020; 220:7-9.[200]

2.1 Abstract

2.1.1 Background

In Section 1.8, I discussed the long-term outcomes for infants who contract Necrotising enterocolitis (NEC). These data are incomplete and whilst there seems to be a significant disease burden from NEC in terms of both mortality and life-long morbidity, this is not clearly elucidated.

2.1.2 Objective

To develop an accurate understanding of contemporary outcomes for necrotizing enterocolitis to inform parental counselling, clinical care, and research agendas; specifically to justify this research effort and the use of animals herein. The intent is to establish current knowledge with this disease and answer the recurrent clinical question: *This baby has NEC, what is the prognosis?*

2.1.3 Study design

A systematic review of recent (January 2010-January 2018) large cohort studies reporting outcomes of infants who developed NEC. Only studies reporting national, regional, or multicenter outcomes of NEC in high-income countries were included. Outcomes assessed were mortality, neurodevelopmental outcome, and intestinal failure. Meta-analyses were used to generate summary statistics for these outcomes.

2.1.4 Results

Of 1375 abstracts, 38 articles were included. Overall mortality was 23.5% in all neonates with confirmed necrotizing enterocolitis (Bell 2a+) NEC (95% CI 18.5%-28.8%), 34.5% (30.1%-39.2%) for neonates that underwent surgery for NEC, 40.5% (37.2%-43.8%) for extremely low birthweight infants (<1000 g), and 50.9% (38.1%-63.5%) for extremely low birthweight infants with surgical

NEC (Table 2-4). Studies examining causes of neonatal mortality showed NEC is responsible for around 1 in 10 of all neonatal deaths. Neurodevelopmental disability was reported in four studies at between 24.8% and 61.1% (1209 total NEC cases). Three studies reported intestinal failure with an incidence of 15.2%-35.0% (n = 1370). The main limitation of this review is the lack of agreed definition for diagnosing NEC and the differences in the way that outcomes are reported.

2.1.5 Conclusions

Mortality following NEC remains high. These contemporary data inform clinical care and justify ongoing research efforts. All infants with NEC should have long-term neurodevelopmental assessment. Data on the long-term risk of intestinal failure are limited.

Trial registration CRD42018094791.

2.1.6 Journal of Pediatrics accompanying editorial

> *"The authors are congratulated on providing an updated understanding of NEC-related morbidity and mortality. Overall, contemporary data continue to demonstrate that NEC remains a significant health burden, yet there remains limited literature evaluating NEC-associated morbidity outcomes. Further, it is important to recognize the variability of disease definitions used in the current literature. To better understand this vulnerable population and their long-term outcomes, future research initiatives are crucial."* Flahive *et al.* J Pediatr 2020; 220:7-9.[200]

2.2 Introduction

Necrotising enterocolitis (NEC) remains a great scourge of neonatal intensive care units. Despite decades of research and significant improvements in neonatal care, studies that specifically compare different time periods show that outcomes for NEC are essentially unchanged.[201] Whilst real advances in neonatal care have led to improved outcomes overall for premature babies, NEC still conveys a significant mortality and morbidity.[36,187] Moreover, the increased survival of very premature babies means an increase in the size of the at-risk population, which is reflected in some studies reporting a long term increase in the incidence of NEC.[202]

Understanding the true burden of the disease is vital for assessing any potential future treatments, for clinical decision-making and counselling parents of affected neonates, and for justifying research funding. We aimed to understand the current burden of NEC in terms of mortality, as well as assessing the morbidity for survivors, by including the outcomes of neurodevelopmental disability (NDD) and intestinal failure (IF) as these two encompass the majority of post-NEC morbidity. Hence, there are two key purposes to this review. Firstly, to provide a practical answer to the clinical question of prognosis for an individual baby diagnosed with NEC. Secondly, to establish with greater accuracy the true disease burden of NEC that justifies the search for new treatments and management strategies of which this thesis is a part.

2.3 Methods

2.3.1 Search Strategy

We performed a systematic review in accordance with the PRISMA Statement[203] and registered the protocol in advance with PROSPERO (CRD42018094791). A search strategy was developed to identify studies of infants that reported important longer-term outcomes of NEC, including mortality, neurodevelopmental disability and intestinal failure. The Medline database was interrogated using Pubmed in January 2018 using a search that included the terms “NEC”, “outcomes”, “mortality”, “morbidity”, “neurodevelopmental outcome”, and “intestinal failure”. Details of the search strategy are summarised in Table 2-1. References of included studies were checked for additional eligible studies.

2.3.2 Inclusion / Exclusion criteria

Studies were included in this review if satisfying all of the following criteria:

1. Full text in English;

2. Reported NEC outcomes of interest;
3. Published since 1st January 2010;
4. Reported international, national, regional or multicentre data;
5. Data reported was from high-income countries, defined as members of the Organisation for Economic Co-operation and Development (OECD).

Excluded studies:

1. Review articles rather than primary data;
2. Outcomes of NEC not retrievable from published data. Studies that report NEC outcomes were included for qualitative assessment, even if the data reported could not be included in quantitative analysis. This includes papers that reported odds ratios but not the primary data and studies that examined the causes of neonatal mortality.
3. Studies were also excluded if they combined outcomes for spontaneous intestinal perforation (SIP) and NEC.

Table 2-1 Summary of Search Strategy:
Combining NEC with each of the outcomes of interest and limiting to studies published since 2010

("Necrotising enterocolitis" or "necrotising enterocolitis" or "NEC")[All Fields] OR [MeSH terms])	AND	"Outcomes" [All Fields] OR [MeSH terms]	AND	(English[Language]) AND ("2010/01/01"[PDAT] : "3000"[PDAT]) NOT review[Publication Type])
	AND	"Mortality" [All Fields] OR [MeSH terms]	AND	
	AND	"Morbidity" [All Fields] OR [MeSH terms]	AND	
	AND	"Neurodevelopmental outcomes" [All Fields] OR [MeSH terms]	AND	
	AND	"Disability" [All Fields] OR [MeSH terms]	AND	
	AND	"Intestinal Failure" [All Fields] OR [MeSH terms]	AND	
	AND	("Parenteral nutrition dependence" OR "Parenteral nutrition" OR total parenteral nutrition") [All Fields] OR [MeSH terms]	AND	
	AND	Epidemiology[All Fields] OR [MeSH terms]	AND	

The OECD was used as a simple proxy for high-income countries. This is important because neonatal outcomes are dramatically different between high- and low- income countries. In 2019, Europe had an overall neonatal mortality of 5 per 1000 live births compared to 27 per 1000 live births in Sub-Saharan Africa.[204] This 5-fold disparity means that it would be inappropriate to combine studies from both high- and low- income countries.

After duplicates had been removed, the abstracts were assessed against both inclusion and exclusion criteria. Articles identified as potentially eligible underwent a full text review. Articles that satisfied the inclusion/exclusion criteria were included and the data extracted. All stages of this process were performed independently by two reviewers and any disagreements resolved by discussion. Where papers reported outcomes from the same (or largely overlapping) datasets, only the most recent papers were included for quantitative analysis. If papers were published in the same year, the larger dataset was used. In each paper, the source of the data, the definition of NEC used, a description of the population and the definition(s) of mortality, NDD and IF used were extracted and reported.

2.3.3 Statistical analysis

Statistical analysis was performed using *metaphor*[205] and *meta*[206] in *R*(version 3.5.1)[207]. Meta-analyses of the proportions (using a random effects model) were used to combine the outcome data from the included studies. Double Arcsine transformation was used to normalise the datasets and heterogeneity was estimated by means of tau, Q and I^2 statistics. The *R* script used is reproduced in Appendix A.

2.3.4 Subgroup analysis

Since the relationship between birthweight and NEC outcome, particularly mortality, is well-established (infants with birthweights of less than 750g have a mortality rate that is three times higher than those weighing between 1250g and 1500g at birth[36]), we determined *a priori* to perform subgroup analysis stratified by birthweight. Similarly, some studies only report outcomes for patients who underwent surgery for NEC, a subset known to have worse outcomes. Hence, it would be inappropriate to combine data from studies only examining a surgical population with data from those examining infants who did not have surgery. The following subgroups were therefore used for meta-analyses: all neonates with NEC, all neonates with surgical NEC, neonates with BW <1500g (Bell 2a+), neonates with BW <1000g (Bell 2a+), neonates with BW <1500g and surgical NEC, and neonates with BW<1000g and surgical NEC. These subgroups were defined on the basis of the data available since it was not possible to determine which subgroups would be usable prior to completing the search.

2.4 Results

2.4.1 Included Studies

The initial search returned 1371 articles after duplicates were excluded. After screening of the abstracts, the full text of 89 articles was obtained for assessment. Four further papers that met the inclusion criteria were identified from the reference list of included papers. In total, 38 articles reported at least one of the outcomes of interest (Figure 2-1). Table 2-2 shows the papers included in this analysis and the data sources.

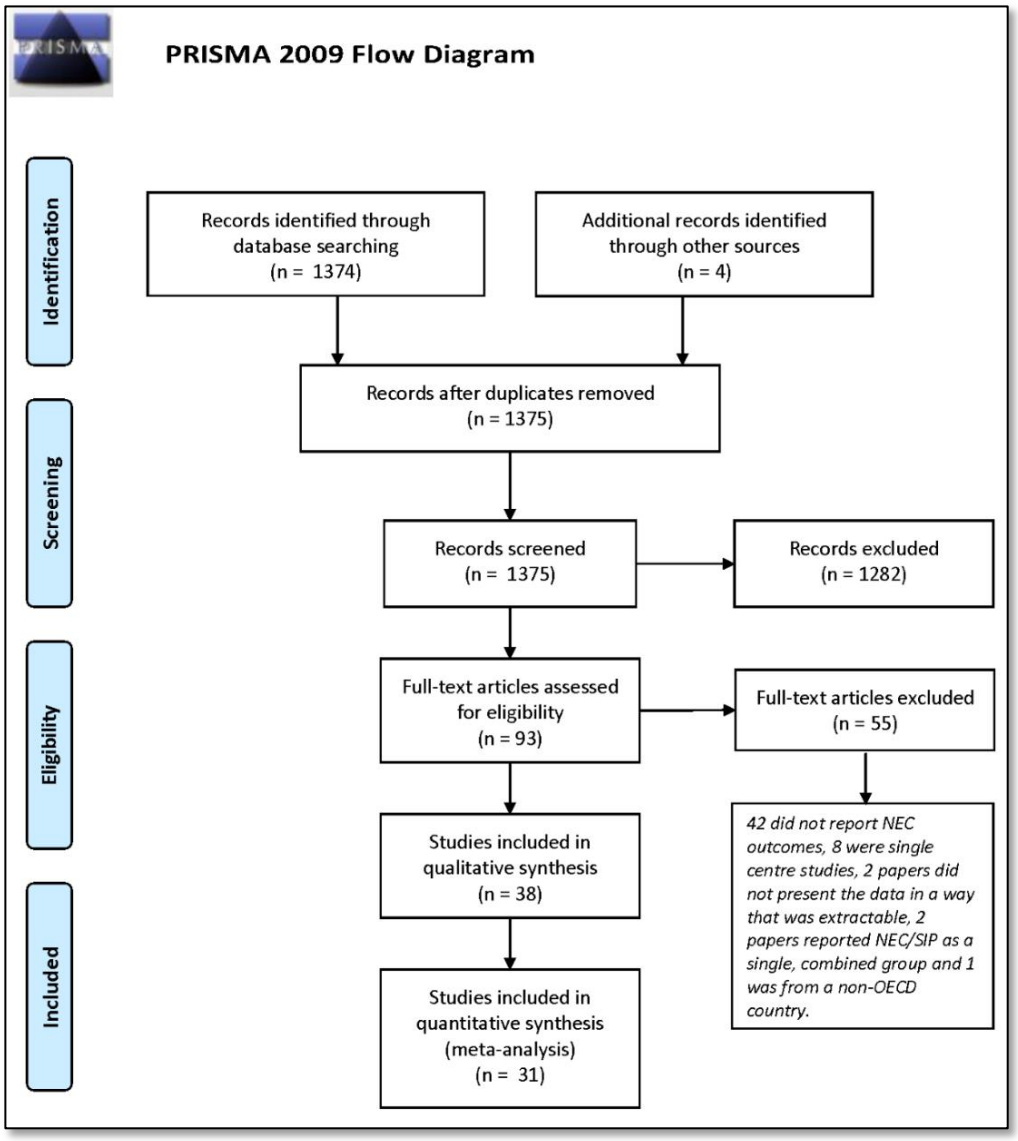


Figure 2-1 PRISMA Flow Diagram
As recommended by Moher *et al.*[203]

Paper	Reports NEC Mortality (n)	Reports NDD associated with NEC (n)	Reports IF associated with NEC (n)	Location: data source*	Data collections (years)
Abdullah 2010[208]	20822			USA: NIS & KID	88, 96, 02, 03
Adams-Chapman 2013[209]	✔			USA	06-08
Allin 2017[210]	189			UK	13-14
Allin 2018[191]	159			UK	13-14
Autmizguine 2014[211]	2780			USA: PMG	97-02
Battersby 2017[212]	531			UK	12-13
Berrington 2012[213]	✔			UK	88-08
Bhatt 2017[214]	223		223	USA: CHCA	09-15
Choo 2011 [215]	4657			USA: NIS & KID	88-05
Clark 2012[216]	7099			USA: PM7	97-09
Duro 2010 [217]			394	USA	04-07
Fisher 2014[218]	4072			USA: VON	06-10
Fullerton 2016[219]	4328			USA:VON	09-13
Fullerton 2017[169]	2881	866		USA:VON	99-12
Ganapathy 2013[220]	316	103		USA: Texas	02-03
Hayakawa 2015[221]	44	18		Japan	03-12
Heida 2017[222]	441			Netherlands	05-13
Hull 2014[223]	17156			USA: VON	06-10
Kastenberg 2015[224]	1879			USA: California	05-11
Kelley-Quon 2012[225]	1272			USA: California	99-07
Martin 2010[226]	✔			USA	02-04
Mukherjee 2010[45]	194			USA: NIS & KID	88-03
Murthy 2014[227]	753			USA	10-13
Patel 2015[228]	✔			USA	00-11
Rees 2010[20]	211			UK	05, 06
Sayari 2016[229]	1542			USA: KID	03, 06, 09
Seeman 2016[230]	✔			USA	10-13
Shah 2012[231]	208			USA	98-09
Shah 2015[232]	784			Canada	10-13
Steurer 2015[233]	✔			Switzerland	02-11
Stey 2015[234]	1375			USA: California	99-07
Synnes 2016[235]		✔		Canada	09-11
Tashiro 2017[236]	886			USA: KID	03-09
Thome 2017[237]	✔			Germany	08-12
Velazco 2017[238]	1629			USA & Canada	09-15
Wadhawan 2014[239]	472	220		USA: NRN	00-05
Youn 2015[240]	149			Korea	13-14
Zhang 2011[241]	5374			USA: NIS & KID	88-03

Table 2-2 All papers included in this review
*Abbreviations: NIS: National (Nationwide) Inpatient Sample[242]/ KID: Kid's Inpatient Database[243]/PMG: Pediatrix Medical Group.[244]/ VON: Vermont Oxford Network[245] / NRN: Neonatal Research Network[246] ✔ indicates reported outcome but data not in a format that allowed extraction.

2.4.2 Mortality

Of these 38 articles, 31 reported mortality from NEC in a format that allowed a series of meta-analyses to be performed. Table 2-3 shows the reported NEC mortality rates in each included study, the timepoint at which mortality was assessed and the definition of NEC used (i.e. Bell 1 to 3 or Bell 2a+). Zhang *et al.* (2011)[241] and Choo *et al.* (2011)[215] are related studies and used the same databases (*Nationwide Inpatient Sample* 1988 – 96, 98, 2001, 02 and *Kids' Inpatient Database* 1997, 2000, 03). For this reason, only Zhang *et al.* (2011) was used in the meta-analysis of neonates with surgical NEC. Similarly, in the subgroup of infants with a birthweight of less than 1500g and requiring surgery, Fisher *et al.* (2014), Fullerton *et al.* (2016) and Hull *et al.* (2014) all drew their datasets from the Vermont-Oxford Network. Hence only the Fullerton *et al.* (2016) data was used for the meta-analysis.

From these datasets, the overall mortality from confirmed NEC (Bell 2a+) is estimated at 23.5% (95% C.I. - 18.5 - 28.8%) with, as-expected, higher rates for infants of lower birth-weights and for those that underwent surgery. Meta-analysis of each of these main sub-groups provides estimates for the mortality rates of each group (Table 2-4, Figure 2-2 to Figure 2-8). The highest mortality was seen in infants with a birthweight of less than 1000g who required surgery for NEC in whom mortality was 50.9% (95% C.I. - 38.1 - 63.5%). Just one study reported mortality for neonates with a birth weight greater than 2500g with overall mortality of 11.0% (8.0% for medical NEC, 22.1% for surgical NEC, n=1629)[238]. Similarly just one study reported outcome of NEC in infants with congenital cardiac disease. Mukerjee *et al.*(2010)[45] reported that NEC mortality in the context of CHD was 19.6%. This is similar to the mortality for CHD alone of 16.1%.[30]

Paper	Population	Definition of NEC	Definition of mortality	NEC deaths	Total number with NEC	Mortality %
Rees 2010[20]	All neonates	Bell 1-3	In-hospital	27	211	12.8%
Abdullah 2010[208]	All neonates	ICD-9	In-hospital	2718	20822	13.1%
Clark 2012 [216]	All neonates	Bell 2+	In-hospital	1505	7099	21.2%
Ganapathy 2013 [220]	All neonates	ICD-9	6 months	66	316	20.9%
Heida 2017 [222]	All neonates	Bell 2+	30 day	117	441	26.5%
Zhang 2011 [241]	All neonates	Surgical NEC	In-hospital	1660	5374	30.9%
Choo 2011[215]	All neonates	Surgical NEC	In-hospital	1115	4657	23.9%
Murthy 2014 [227]	All neonates	Surgical NEC	In-hospital	259	753	34.4%
Stey 2015 [234]	All neonates	Surgical NEC	In-hospital	473	1375	34.4%
Battersby 2017 [212]	All neonates	Surgical NEC	In-hospital	247	531	46.5%
Allin 2017 [210]	All neonates	Surgical NEC	28 days	29	189	15.3%
Allin 2018 [191]	All neonates	Surgical NEC	1 year	41	159	25.8%
Ganapathy 2013 *[220]	All neonates	Surgical NEC	6 months	38	111	34.2%
Hull 2014[223]	<1500g BW	Bell 2+	In-hospital	4804	17156	28.0%
Autmizguine 2014[211]	<1500g BW	All NEC	In-hospital	645	2780	23.2%
Youn 2015[240]	<1500g BW	Bell 2+	In-hospital	63	149	42.3%
Kastenberg 2015[224]	<1500g BW	Bell 2+	unclear	411	1879	21.9%
Hayakawa 2015 [221]	<1500g BW	Bell 2+	In-hospital	17	44	38.6%
Shah 2012[231]	<1000g BW	Bell 2+	In-hospital	105	208	50.5%
Fullerton 2017[169]	<1000g BW	Bell 2+	2 years	952	2881	33.0%
Kelley-Quon 2012[225]	<1500g BW	Surgical NEC	In-hospital	496	1272	39.0%
Fisher 2014[218]	<1500g BW	Surgical NEC	In-hospital	1547	4072	38.0%
Fullerton 2016[219]	<1500g BW	Surgical NEC	In-hospital	1742	4328	40.2%
Hull 2014*[223]	<1500g BW	Surgical NEC	In-hospital	3127	8935	35.0%
Autmizguine 2014*[211]	<1500g BW	Surgical NEC	In-hospital	322	706	45.6%
Youn 2015*[240]	<1500g BW	Surgical NEC	In-hospital	23	77	29.9%
Wadhawan 2014[239]	<1000g BW	Surgical NEC	In-hospital	252	472	53.4%
Tashiro 2017[236]	<1000g BW	Surgical NEC	2 years	522	886	58.9%
Fisher 2014*[218]	<1000g BW	Surgical NEC	In-hospital	1127	2782	40.5%
Fullerton 2017*[169]	<1000g BW	Surgical NEC	2 years	637	1668	38.2%
Mukherjee 2010[45]	CHD	ICD-9	In-hospital	38	194	19.6%
Shah 2015[232]	<32/40	Bell 2+	*2 years*	225	784	28.7%
Sayari 2016[229]	<37/40	ICD-9	In-hospital	528	1542	34.2%
Bhatt 2017[214]	<37/40	Surgical NEC	In-hospital	83	223	37.2%
Velazco 2017[238]	>2500g BW	Bell 2+	In-hospital	179	1629	11.0%
Seeman 2016[230]	All neonates	*NEC as cause of death*	NEC mortality: 12.5/100 000 live births			
Patel 2015[247]	<29/40		10% of infant deaths due to NEC			
Berrington 2012[213]	<32/40		11-21% of infant deaths due to NEC			

*sub-group outcome data reported in paper
BW: Birth weight. <32/40: less than 32 weeks gestation, <29/40: less than 29 weeks gestation. CHD: Congenital heart disease

Table 2-3 Reported NEC Mortality

Three articles (Seeman *et al.* (2016)[230], Patel *et al.* (2015)[247] and Berrington *et al.* (2012)[213]) report NEC as a cause of death. These data sets, based primarily on death certification, do not allow a mortality rate for NEC to be derived as the denominator of the number of cases of NEC is not recorded. However, they do report neonatal mortality and thus the NEC-related infant mortality

rate (IMR). These datasets give an NEC-IMR of 12.5 per 100,000 live births for all neonates,[230] and much higher for premature babies; 2,800 per 100,000 live births between 22 and 29 weeks completed gestation[247] and 1,100 per 100,000 live births between 24 and 31 weeks completed gestation.[213] Overall NEC is responsible for between 10 and 21% of infant mortality in premature babies.[213,247]

Group	N	Mortality % (95% C.I.)
All neonates with NEC (Bell 1-3)	21349	15.3 (10.8 - 20.4)
All neonates with NEC (Bell 2+)	7540	23.5 (18.5 - 28.8)
All neonates with surgical NEC	8303	34.5 (30.1 - 39.2)
Neonates with BW <1500g (Bell 2+)	19228	30.1 (24.3 - 36.2)
Neonates with BW <1000g (Bell 2+)	3089	41.3 (25.0 - 58.7)
Neonates with BW <1500g & Surgical NEC	6383	40.5 (37.2 - 43.8)
Neonates with BW <1000g & Surgical NEC	3668	50.9 (38.1 - 63.5)

Table 2-4 Meta-analyses of NEC mortality by subgroup (see Figures 2-2 to 2-8)

- Figure 2-2 and Figure 2-3 show mortality for all infants with NEC: Figure 2-2 is based on papers that include all infants with a diagnosis of NEC, whilst Figure 2-3 is restricted to only those with Bell Stage 2a+ (confirmed NEC).
- Figure 2-4 shows the mortality for NEC in infants who underwent surgery for their disease.
- Figure 2-5 and Figure 2-6 show the mortality for infants with birth weights less than 1500g and 1000g respectively.
- Figure 2-7 and Figure 2-8 show the mortality for infants who underwent surgery for NEC with birth weights less than 1500g and 1000g respectively,

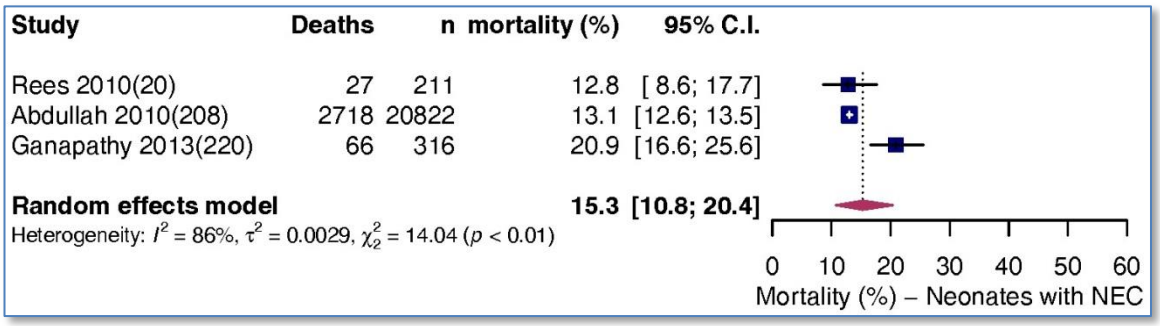


Figure 2-2 Mortality for all infants with NEC (Bell Stage 1-3)

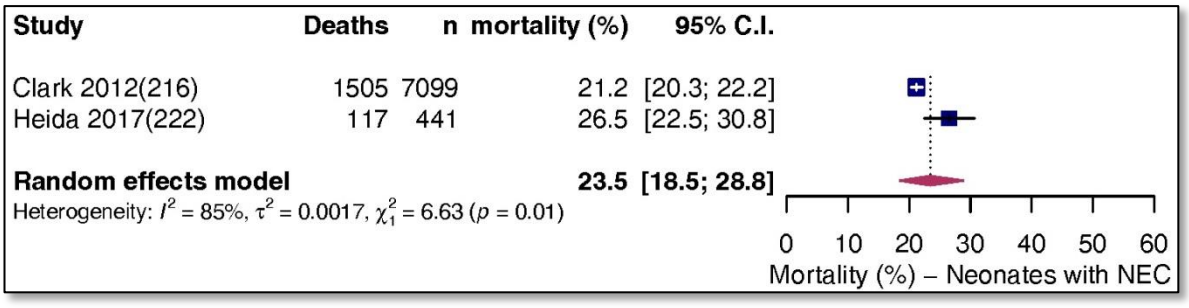


Figure 2-3 Mortality for all infants with NEC (Bell Stage 2a+)

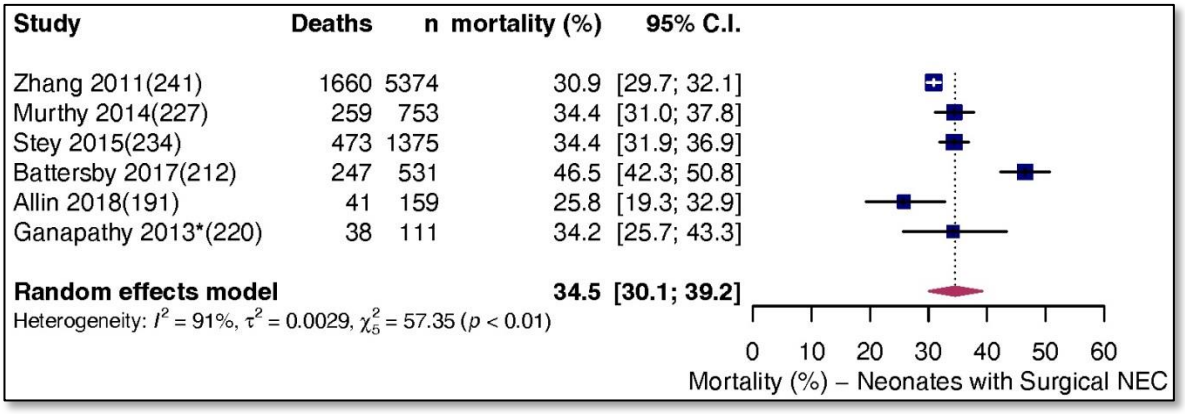


Figure 2-4 Mortality for all infants who underwent surgery for NEC

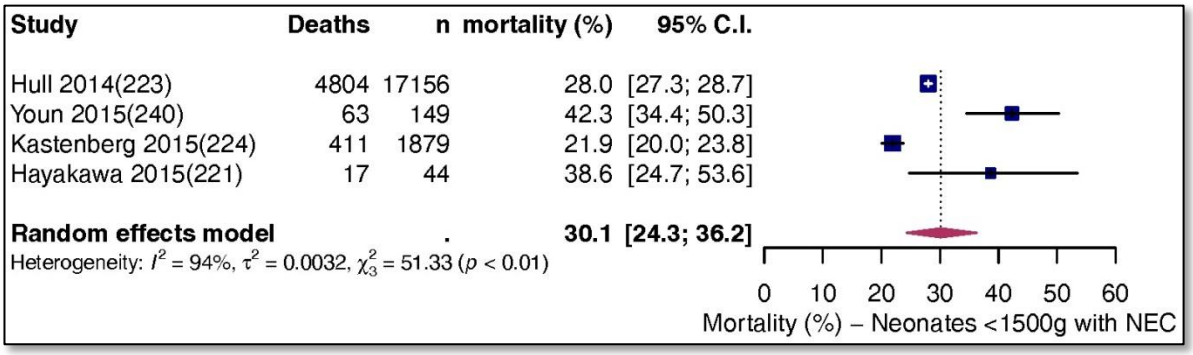


Figure 2-5 Mortality for infants with NEC and a birthweight <1500g

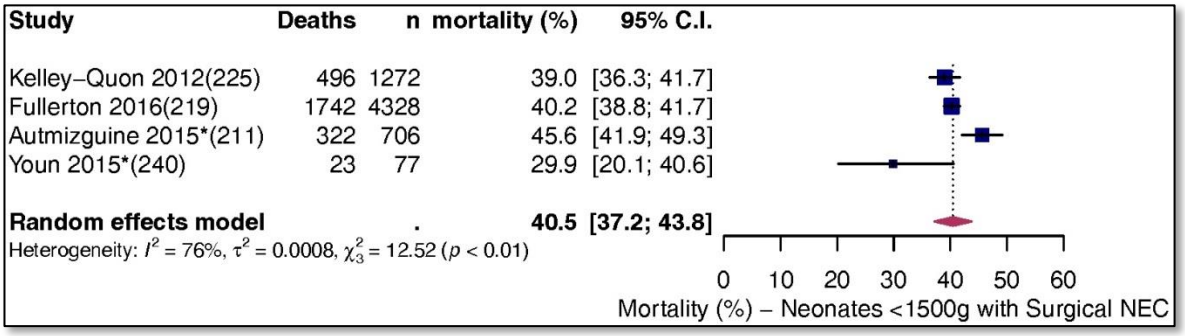


Figure 2-6 Mortality for infants with a birthweight <1500g who underwent surgery for NEC

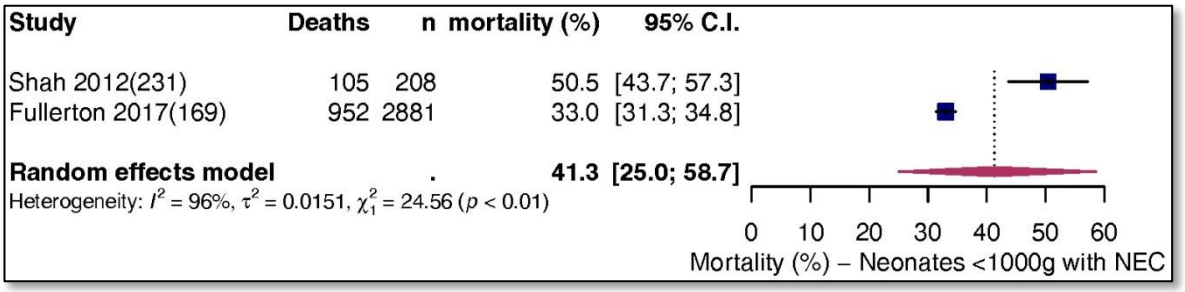


Figure 2-7 Mortality for infants with NEC and a birthweight <1000g

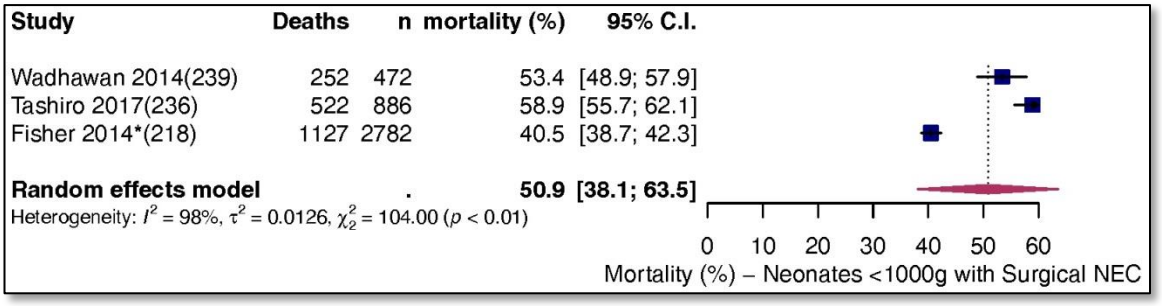


Figure 2-8 Mortality for infants with a birthweight <1000g who underwent surgery for NEC

2.4.3 Neurodevelopmental Disability

Five studies reported data on neurodevelopmental outcomes following NEC. Overall, severe NDD ranges from 24.8%[220] to 59.3%[221] in these series. The exact definition of NDD used varied between studies, as indicated in the table (Table 2-5). The differences in population, definition of NEC and definition of NDD between these studies meant that assimilation with a meta analysis was not possible. Synnes *et al.*[235] reported an increased risk of NDD associated with NEC in infants born before 29 weeks gestation with an odds ratio of 1.88. However, the actual number of patients with NEC and NDD were not reported. These results are summarised in Table 2-5.

Paper	Population	Definition of NEC	Definition of Neurodevlopmental Disability	Number with NDD	n	NDD %
Ganapathy 2013[220]	All neonates	ICD-9	@24-36m - definition unclear	26	105	24.8
Hayakawa 2015[221]	<1500g BW	Bell 2+	18m corrected age: developmental quotient <70, or the presence of neurological sequelae	11	18	61.1
Fullerton 2017[169]	<1000g BW	Bell 2+	Any severe disability (incl BSID: MDI or PDI <70)	267	866	30.8
Fullerton 2017*[169]	<1000g BW + Surgery	Surgical NEC	Any severe disability (incl BSID: MDI or PDI <70) 1 or more of: mod/severe CP, bilateral blindness, bilateral hearing loss needing amplification, MDI or PDI < 70.	169	449	37.6
Wadhawan 2014[239]	<1000g BW + Surgery	Surgical NEC		125	220	56.8
*sub-group outcome data reported in paper						

Table 2-5 Rates of severe neurodevelopmental disability following NEC

2.4.4 Intestinal failure

Three studies report IF rates following NEC (Table 2-6). IF rates vary from 15.2% in all neonates with NEC (n=394) to 35.4% in neonates with NEC requiring surgery (n=147). Whilst the definition of IF used was relatively consistent, the populations and definition of NEC is variable thus making assimilation into a meta-analysis difficult.

Paper	Population	Definition of NEC	Definition of Intestinal Failure	Number with IF	n	IF rates %
Duro 2010[217]	All neonates	Bell 1-3	Failure to achieve full enteral feeds @90d	60	394	15.2
Murthy 2014[227]	Surgical NEC	Surgical NEC	>90d PN	171	753	22.7
Bhatt 2017[214]	Surgical NEC (<37/40)	Surgical NEC	Failure to achieve full enteral feeds @90d	78	223	35.0

Table 2-6 Rates of intestinal failure following NEC

2.5 Discussion

In recent years, several large, multicentre studies have reported outcomes of NEC. The purpose of this review is to collate these studies' results in order to report contemporary outcomes for NEC. Due to limited availability of specialist neonatal care and consequential poorer outcomes outside of high-income countries[248] we excluded data from developing countries. The main outcomes in this review are mortality, neurodevelopmental disability and intestinal failure. The choice of NDD and IF reflects that these two outcomes encapsulate the majority of the longer term morbidity associated with and specifically related to NEC.[27]

In this review, based on large cohorts (at least 3000 infants) we have been able to estimate contemporary mortality in clinically relevant sub-groups of infants with acceptable precision (Table 2-4, Figure 2-2 to Figure 2-8). We have also reported the incidence of NDD and the risk of IF, although the differences in definitions and populations meant it was not possible to combine these in summary statistics. The key findings of our review are that NEC confers a mortality of 23.5% for confirmed cases (Bell stage 2+) rising to over 50% for ELBW infants who undergo surgery for NEC. For survivors, there is significant morbidity with rates of severe NDD of between 25% and 61% and intestinal failure reported at between 15 and 35%. Overall our results confirm that NEC continues to confer a very significant disease burden. In an era when mortality for preterms in high-income countries is actually relatively low (approaching 95% survival for those born between 28 and 32 weeks (90% without impairment)[248]), contracting NEC clearly has a large detrimental effect on the mortality and morbidity of these infants.

2.5.1 Mortality

One of the challenges in providing meaningful estimates of mortality in the context of NEC is variation in the definition of both the population at risk and the timing of death reported. To account for differences in definition or severity of NEC, we have conducted as series of subgroup analyses, aiming to achieve a largely homogenous population within each subgroup and therefore more useful data. Without such stratification the range of mortality is between 12.8 % and

58.9%.[20,236] The time at which mortality is reported also varied between studies. Where clearly stated we have used all-cause mortality for analysis. Of the 21 studies used in the quantitative analysis of mortality, 17 reported only in-hospital mortality rates. Four studies Allin *et al.* (2018)[191], Heida (2017)[222] Fullerton (2017)[169] and Tashiro *et al.* (2017)[236] report both in-hospital and a later mortality. These data show a minority, between 5% and 30.5% of the deaths that occur do so after initial discharge and hence any estimate based on in-hospital mortality is likely to underestimate the true mortality of NEC. This is summarised in Table 2-7.

	In-hospital Mortality			Total Mortality			Proportion of Mortality that is 'late' %
Paper	**Deaths**	**n**	**%**	**deaths**	**n**	**%**	
Allin 2018[191]	29	189	15.3	41	159	25.8	29.3
Heida 2017[222]	117	441	26.5	147	441	33.3	20.4
Tashiro 2017[236]	363	886	41.0	522	886	58.9	30.5
Fullerton 2017[169]	904	2881	31.4	952	2881	33.0	5.0

Table 2-7 Mortality from NEC following discharge (late mortality)

2.5.2 Neurodevelopmental disability

The rates of NDD follow the same pattern as mortality with much higher rates in smaller infants and those who underwent surgery. Whilst the mechanisms remain unclear, NEC has a detrimental effect on the developing brain conferring a worse neurodevelopmental outcome comparable to pre-term infants who do not contract NEC. Foetal brain development is driven by both genetic and environmental factors. Accordingly, the preterm brain is susceptible to damage due to a multitude of environmental factors introduced by premature ex-utero life[249]. This multifactorial encephalopathy is seen in the absence of NEC but rates of severe NDD are not as high. Fullerton *et al.* (2017)[169] followed a cohort of over 10,000 ELBW infants, reporting severe NDD in 17% of these very premature infants in the absence of NEC. Those that contract NEC have much higher rates; 31% overall and 24% and 38% for medical and surgical NEC respectively. A consensus based definition of severe NDD is lacking. The presence of features such as cerebral palsy, sight loss or hearing loss would constitute severe NDD. Similarly, the widely-used Bayley Scales of Infant Development with an appropriate threshold such as a Mental Development Index (MDI) score <70 can be used to define severe NDD. Each of the studies included in this review used similar combinations of these features to define NDD. The variation in the estimates of NDD reported are likely related to differences in definitions used within individual studies but also an overall sparsity of data in this area. However, it is clear that NEC confers a significant risk of severe NDD and all neonates who have had a diagnosis of NEC should therefore undergo long-term neurodevelopmental follow-up.

2.5.3 Intestinal Failure

IF in children is broadly defined as the inability to absorb sufficient nutrition via the gut for normal growth.[196] In the context of NEC, extensive surgical resection may lead to anatomically short-gut but moreover, babies with ostensibly sufficient bowel, can have persistent reduced gut function that precludes adequate nutrition absorption. It is well recognised that NEC is a major cause of intestinal-failure in children.[197] However, the specific risk of IF to an individual baby diagnosed with NEC is much less clear. The commonly used measure of p*arenteral nutrition (PN) dependence at 90 days* is relatively easy to measure but arguably incomplete. Requiring PN at 90 days is a clear indicator of disease burden in the medium term but a proportion of these children will still go on to achieve enteral autonomy.[197] Using this measure we have been able to estimate the overall risk for an infant with surgical NEC of between 22 and 35% but given the limited number of reports identified further characterisation of the burden of IF related to NEC would be useful. Since conducting this search, Bexelius *et al.* (2019)[198] have published a series on IF. These data show IF rates of 6% for infants with NEC. The definition of IF used was based on clinical coding between 14 days of age and two years. This shows a fifteen-fold increase compared to a cohort matched for gestational age (0.4% prevalence). However, these data whilst capturing re-admission for IF will still include patients who are PN-dependent immediately after contracting NEC. A cohort study of IF suggests that over 60% of children whose IF is secondary to NEC will achieve enteral autonomy by 72 months follow-up.[197]

There are several strengths to this review: as far as we are aware, this is the first systematic review of NEC outcomes to be reported. It is based on large, population based datasets with each sub-population estimate of NEC mortality based on large numbers. The main limitations of this review lie in the variety of definitions used for both the exposure and outcomes as already discussed. If agreed definitions of NEC and measures of mortality, severe NDD and IF can be reached and used this would facilitate better assessment of potential treatment interventions as well as enhance comparison between studies. We therefore welcome initiatives such as Core Outcomes In Neonatology that aim to address some of these issues.[250] Due to the level of heterogeneity between studies we used random effects models for meta-analysis. The accuracy of estimates of heterogeneity is known to be limited when the number of studies available for inclusion is small and should be interpreted with caution.[251] Moreover, as the analysis here is of mortality (i.e. a proportion) rather than effect size, the importance of this high level of heterogeneity should not be overstated. In essence, it means that the point estimates for each group are less precise than one might wish but still provide a meaningful answer within the limitations of the published data.

Conclusion

In this review, we report contemporary outcomes for NEC in specific groups of neonates. We estimate overall mortality from confirmed NEC at 23.5%, rising to 50.9% in ELBW infants who require surgery. For survivors, the risk of significant NDD is high (25-61% of survivors). Intestinal failure is common after NEC with 15-35% requiring prolonged intravenous nutrition. Standardised outcome reporting would facilitate comparison between studies.

These data confirm that NEC remains a cause of both mortality and morbidity in the neonatal population. The high levels of both indicate that there is indeed a great need for novel treatment options to better prevent and combat this disease. Undoubtedly, the need justifies both basic science and clinical research efforts. This includes the need for scientific research with animals.

Chapter 3 Remote Ischaemic Conditioning

3.1 Introduction

Remote ischaemic conditioning (RIC) is a putative therapeutic intervention for ischaemia-reperfusion injury (IRI).[252] In Chapter 2, I demonstrated that necrotising enterocolitis (NEC) remains a major cause of mortality and life-long morbidity. In Section 1.5, I discussed the pathophysiology of NEC. Whilst the pathogenesis of NEC is clearly multifactorial, IRI is a key component and arguably a common end-pathway for these multiple factors. In this section, I will discuss the discovery and development of ischaemic conditioning and its potential application in NEC.

3.2 Ischaemia-Reperfusion Injury

Ischaemia-reperfusion injury (IRI) is seen in a group of pathologies and occurs following the loss of an adequate blood supply for a period of time (ischaemia), followed by the restoration of the blood supply (reperfusion) which results in significant tissue or organ damage.[253] This is the central pathological mechanism to multiple common disease processes (such as coronary heart disease and stroke). Transplanted organs also suffer an ischaemic-reperfusion injury.

Whilst the loss of an adequate blood supply is implicitly a non-sustainable state for a prolonged period of time, the paradox is that with short periods of ischaemia (such that the tissue survives), the majority of the damage is done during the reperfusion phase.[254] Figure 3-1 shows this phenomenon schematically. IRI is characterised by a series of cellular changes that result in cell death. This leads to inflammation and further tissue damage. In the event of ischaemia, cells are dependent on anaerobic metabolism which produces a drop in cellular pH. This is buffered by exchanging H^+ ions for Na^+ ions, resulting in a rise in intracellular sodium.[164] Ischaemia also results in a depletion of cellular adenosine triphosphate (ATP) and due to the consequential loss of ATPase function, active Ca^{++} efflux is reduced and calcium overload of the cell is the result.

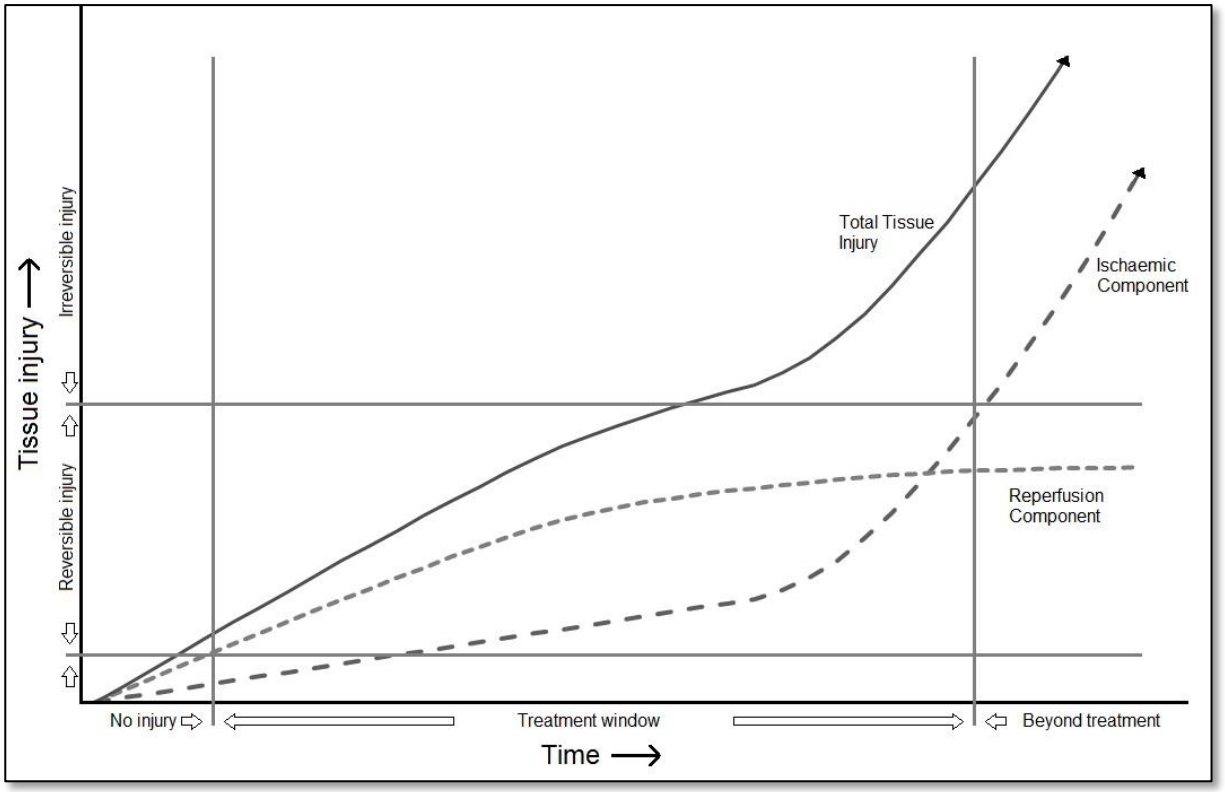


Figure 3-1 Ischaemia-reperfusion injury and tissue damage.
The tissue injury caused by ischaemia and reperfusion are effectively separate processes with their own time courses. Within short time frames (which differs between different tissue types) the majority of the injury occurs in the reperfusion phase. Modified from Buckley *et al.* (1987)[255]

The restoration of sufficient blood flow to the tissues is vital to arrest these processes, but biochemical events triggered by ischaemia when combined with reperfusion, trigger a cascade that leads to further cellular damage (Figure 3-2). Hence the concept of 'ischaemia-reperfusion injury' rather than simply describing it as 'ischaemic injury.' The mechanisms of cell-damage during the reperfusion stage are complex. They include: the generation of reactive oxygen species (ROS), calcium overload, opening of the mitochondria permeability transition pore (MPTP), endothelial dysfunction and a pronounced inflammatory response.[162]

Different organs vary in their susceptibility to IRI and the degree of injury is, unsurprisingly, also dependent on the length and severity of the ischaemia.[255] Endothelial cells are particularly vulnerable to IRI and as a consequence microvascular dysfunction is an early factor in the pathogenesis of IRI.[256] The production of reactive oxygen metabolites increases with a reduction in nitric oxide (NO).[257] In normal circumstances, NO flux scavenges the low-levels of intracellular superoxides. Reduced NO results in the formation and/or accumulation of reactive oxygen metabolites (O_2^- and H_20_2)[258] which initiate or exacerbate acute inflammation. The depletion of NO also alters smooth-muscle tone, increases platelet aggregation and increases adhesive interaction between endothelial cells and leukocytes.[253] Reactive oxygen metabolites activate

genes that encode adhesive molecules such as E-selectin and ICAM-1 (Intercellular adhesion molecule) resulting in on-going inflammation for a prolonged period after IRI.

Ultimately, reperfusion is mandatory to salvage ischaemic tissues but reperfusion also contributes to injury.[259]

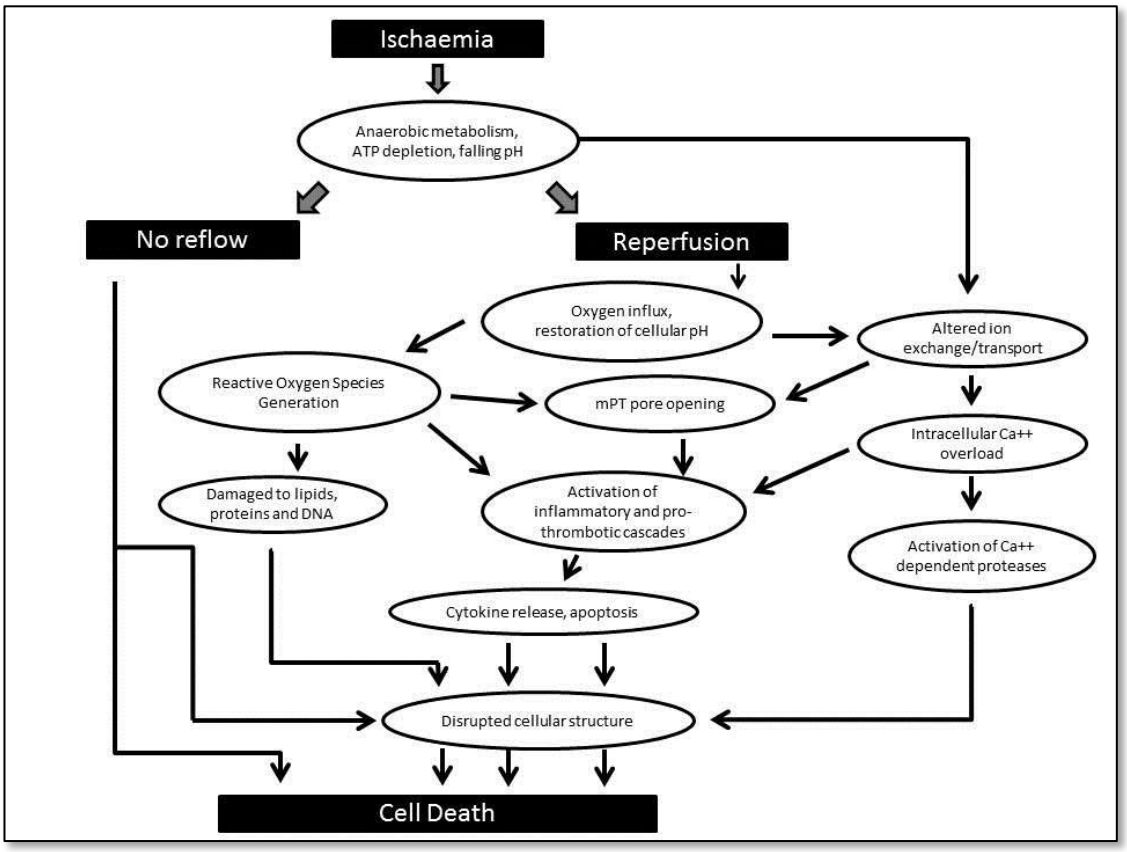


Figure 3-2 Simplified Pathway of Ischaemia-Reperfusion Injury.
Modified from Kalogeris *et al.* (2012)[253]
The paradox that the majority of tissue injury occurs during the reperfusion phase offers the theoretical potential for therapeutic interventions to ameliorate the effect of ischaemia.

3.3 Discovery of Ischaemic Conditioning

Ischaemic Conditioning is an endogenous phenomenon first discovered by Murray *et al.* in 1986.[260] Experiments on canine myocardium demonstrated that brief episodes of non-lethal ischaemia and reperfusion of the left circumflex coronary artery reduced the size of the subsequent infarct by 75% compared to controls when the artery was subsequently occluded to induce a simulated myocardial infarction. The term ischaemic preconditioning was coined. Murray *et al.'s* original experiments are summarised in Figure 3-3 and involved 4 cycles of 5 minutes ischaemia, followed by 5 minutes of reperfusion to the left circumflex artery. When an infarct was subsequently induced by occluding the same artery for 40 minutes, the resulting infarct was

significantly attenuated compared to controls. The same benefit was not seen when 3 hours of occlusion was used, emphasising the importance of timely reperfusion for tissue salvage.

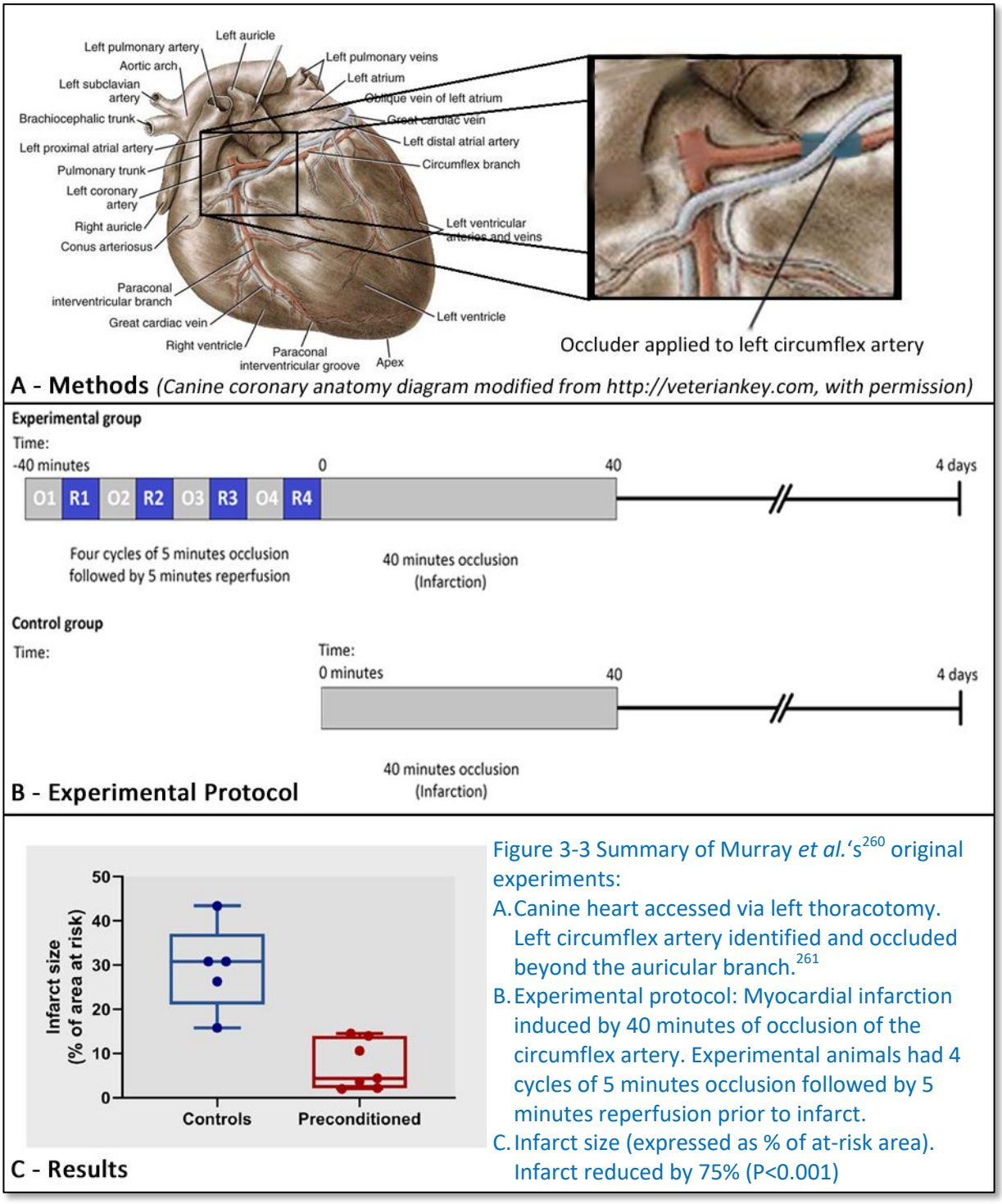


Figure 3-3 Summary of Murray *et al.*'s[260] original experiments:

A. Canine heart accessed via left thoracotomy. Left circumflex artery identified and occluded beyond the auricular branch.[261]
B. Experimental protocol: Myocardial infarction induced by 40 minutes of occlusion of the circumflex artery. Experimental animals had 4 cycles of 5 minutes occlusion followed by 5 minutes reperfusion prior to infarct.
C. Infarct size (expressed as % of at-risk area). Infarct reduced by 75% (P<0.001)

Whilst the mechanisms of this protective effect were not understood, the same phenomenon was demonstrated as a post-hoc effect by Zhao *et al.* [262] Using a similar dog model, Zhao *et al.* applied brief periods of ischaemia during the reperfusion phase and demonstrated similar reductions in infarct size and preservation of endothelial function. The same phenomenon has since been

demonstrated (in the heart) in multiple species, including pigs[263], rabbits[264], rats[265], sheep[266] and mice.[267,268]

These findings, whilst dramatic, are clearly of limited direct clinical use. There is no obvious way in which to replicate coronary artery occlusion in clinical practice. Similarly, the unpredictable nature of acute myocardial events makes the timing of such a procedure impossible. However, the undeniably impressive protective effect of *ischaemic conditioning* offered the tantalising prospect of a therapeutic intervention that could be protective against the detrimental effect of IRI which is central to the pathology of coronary heart disease. This level of protection was greater than anything that could be achieved even by combined pharmaceutical therapy.[259]

Whilst the application of ischaemic conditioning directly to the coronary vessels is not practical, a study of patients who suffered acute myocardial infarction (MI) showed that the presence of angina in the 48 hours prior to the MI was protective with a 4-fold reduction in the in-hospital mortality rate.[259] Although not conclusive, this implies that these patients have a form of endogenous conditioning that is cardioprotective.

3.4 Remote Ischaemic Conditioning

The discovery that the protective effect of ischaemic conditioning could be achieved remotely has important implications in terms of understanding the mechanisms involved but moreover opened up the potential for clinically useful interventions to be developed. Przyklenk *at al.* in 1993[269] first demonstrated that the protective effect of conditioning was not limited to the conditioned tissue. This was done by applying the ischaemic conditioning to the circumflex artery territory of canine hearts before occluding the left anterior descending artery (thus causing IRI to a different area of cardiac muscle). Subsequently it was discovered that the application of the non-lethal ischaemia-reperfusion could be applied remotely from the heart and still derive cardio-protective effects. McClanahan *et al.*[270] did this by applying the ischaemic stimulus to the kidney. Similarly, application of ischaemic stimulus to the small intestine derived cardio-protective effects.[271]

From a practical perspective, arguably the most significant breakthrough, was the observation by Birnbaum *et al.* (1997) that skeletal muscle could be used to apply ischaemic conditioning.[272] The technique used by Birnbaum's team was relatively invasive; reduced flow in the femoral artery being achieved by partial occlusion with an occluder balloon and stimulation of the gastrocnemius muscle. Subsequently RIC was shown to be effective using a simple tourniquet to apply the ischaemic stimulus to the hind limb of rats.[273] Hence, for the first time a method of delivering remote ischaemic conditioning that is non-invasive and clinically applicable was available. Subsequent human trials have used modifications of this method.

3.5 Proposed Mechanisms of Action

Whilst, remote ischaemic conditioning has been shown to confer a significant protective effect in many different contexts, the exact mechanisms by which this benefit is conferred are not well understood. There are literally thousands of studies reporting over 100 different signalling molecules and mechanisms.[259]

3.5.1 Transmission of remote conditioning to the target organ

There are essentially three – interlocking – putative pathways: *Neuronal, Humoral and Systemic*.

3.5.1.1 Neuronal pathway

The role of the autonomic nervous system in RIC is supported by studies using ganglion blockers whereby, the administration of hexamethonium or trimetaphan inhibit the protective effect of RIC.[271,274-277] Similarly, nerve resection prior to administration of RIC reduces the effect of RIC. Mouse studies of remote lower limb preconditioning demonstrated that resection of the sciatic or femoral nerves partially blocked the protective effect of RIC.[278] The neuronal pathway appears to be mediated by the release of endogenous autocoids in the conditioned organ which then stimulate efferent nerves that terminate in the target organ. (Autocoids are endogenous organic molecules with potent pharmacologic effects, that are not part of traditional immune or autonomic groups[279] and are often described as 'local hormones' with an effect in the immediate vicinity of their site of production.[280] Examples include histamine, bradykinin and leukotrienes). Neuropeptides such as CGRP (calcitonin gene-related peptide),[281] adenosine[282] and bradykinin[276] are released by the afferent nerve fibres exposed to conditioning. This protective effect can be mimicked by exogenous administration of neurotransmitters which induces cardioprotection, supporting the theory that RIC is transmitted (in part) by these molecules.[259] Catecholamines act through α-adrenoceptors and this effect is blocked by naloxone. In remote ischaemic conditioning, activation of the α-adrenoceptor leads to the formation of adenosine and activation of Protein Kinase C (PKC) as well as up-regulation of inducible nitric oxide synthase (NOS).[259] The pathways in the target organ are discussed in section 3.6.2.

3.5.1.2 Humeral pathway

Dickson *et al.* (1999)[283] elegantly demonstrated that, to some extent at least, the protective effect of RIC is mediated via a blood-born component by taking effluent from a conditioning heart and administering it to a naïve heart which conferred protection against IRI. Similarly, blood taken from an RIC-exposed animal provided protection from IRI to cardiac muscle.[284] The exact nature of

this putative 'humoral factor' is unclear but Shimizu *et al* in 2009[285] using remote limb ischaemia as the stimulus provided some clues. Plasma taken from exposed animals was used in four experimental groups: i) plasma; ii) dialysate of plasma (dialysed with a 15kDa cut-off dialysis membrane); iii) dialysate passed through a C18 hydrophobic column; and iv) Eludate from the C18 column. In each case the plasma was administered to Langendorff-perfused hearts that were then subjected to IRI. (A Langendorff perfused heart is removed from the donor animal and then perfused in a retrograde fashion via the aorta.[286] This means that the perfusion fluid fills the coronary vessels and provides a stable cardiac model that can have various pharmaceutical agents added to assess response) Plasma(i) and dialysate of the plasma(ii) conferred protection, whilst the flow from the C18 hydrophobic column(iii) did not. However the Eludate washed from the column(iv) had the same protective effect as plasma. Hence humoral factor(s) responsible for RIC must be less than 15kDa in size, and hydrophobic. Similarly, Serejo *et al*. [287] used effluent from ischaemic-conditioned rat hearts to provide a protective effect to hearts not exposed to pre-conditioning. They also used a protein kinase C inhibitor which abolished the protective effect; demonstrating that the protective mechanism of RIC is, at least in part, dependent on the protein kinase C pathway.

Nikkola et al. (2015)[288] examined the effect of RIC on the gene expression in the blood of patients with a diagnosis of sub-arachnoid haemorrhage (SAH). This study took blood samples both before and after conditioning cycles. They found that within the blood transcriptome, 164 differentially expressed genes as well as multiple changes in the methylation of DNA following RIC. Overall, these changes suggested that key genes involved in the mitotic cell cycle as well as inflammatory responses. This supports the concept that RIC induces a global anti-inflammatory response that is protective to the end organ.

3.5.1.3 Systemic pathway

Remote ischaemic conditioning induces systemic changes which appear to act on the end organ in a protective manner which is therefore referred to as the systemic pathway. The evidence for this pathway, is again, cross-species and includes some interesting human studies. The application of forearm ischaemia modifies the gene-expression in human leukocytes.[289] There are significant changes in gene expression in response to RIC. The exact details of the molecular pathways remain frustratingly elusive but pro-inflammatory cytokines are reduced including CD11b and P-selectin. Conversely anti-inflammatory factors like heat shock protein (HSP) 70 and calpastain are upregulated. Tumour necrosis factor alpha (TNF-α) is thought to be especially significant as Peralta *et al.* demonstrated that remote ischaemia applied to the liver attenuates P-selectin expression and neutrophil infiltration of multiple remote organs through inhibition of TNF-α

production.[290] The use of antibodies against TNF-α or P-selectin had the same protective effect on lung tissue as RIC. Conversely application of supplemental TNF-α abolished the benefit of RIC.

Konstantinov *et al.* [289] and Huda *et al.* [291] in mice studies have shown that there is an upregulation in genes associated with cytoprotection, growth and metabolism and DNA repair. Changes in the levels of several other cytokines have been shown with RIC, including IL-6[292] and IL-10[293] suggesting that the systemic pathway is mediated by multiple cytokines.

The evidence thus far points to a significant complexity in the pathways by which RIC confers a protective effect, summarised in Figure 3-4. This complexity necessitates animal-based experiments when looking at the potential of RIC in a different disease process.

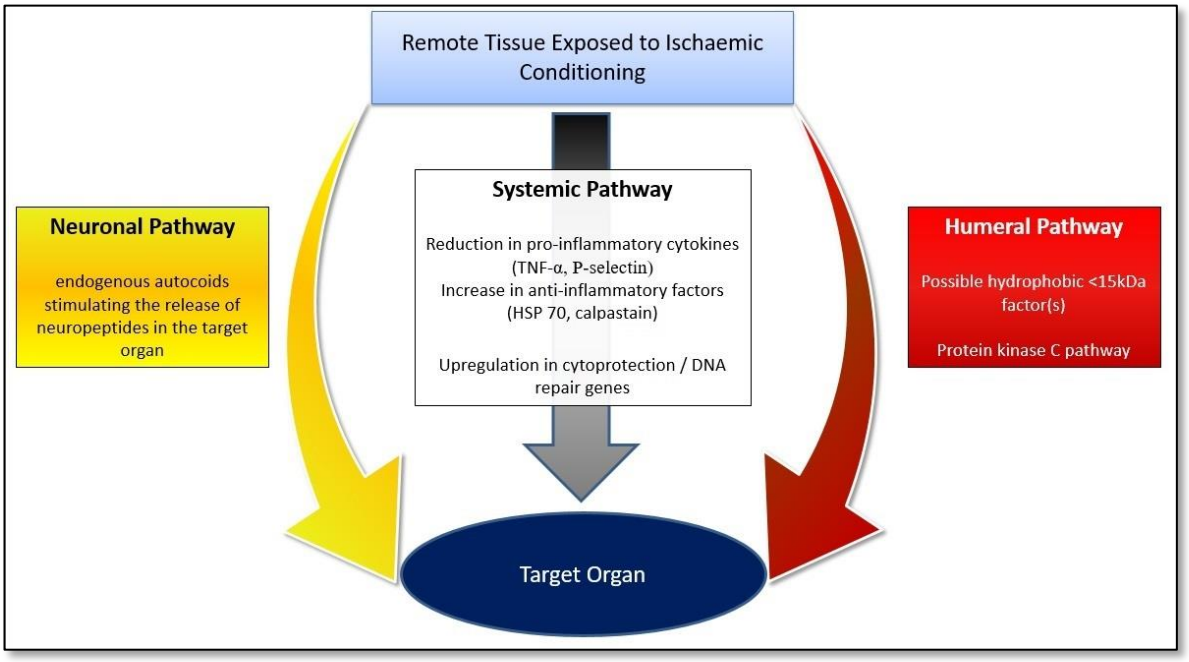


Figure 3-4 Summary of Proposed Mechanisms of Action of Remote Ischaemic Conditioning. Evidence suggests that neuronal autocoid, a change in systemic cytokine levels and one (or more) humeral factor(s) less than 15 kDa all act on the target organ (all organs) to reduce ischaemia-reperfusion injury.

3.5.2 End-organ effectors of remote ischaemic conditioning

At the level of the end-organ, cytological changes and biochemical pathways are activated by RIC that are thus implicated in making the tissue resistant to ischaemia-reperfusion injury. Microarray analysis of renal tissue exposed to IRI and pre- or post- ischaemic conditioning revealed changes in the expression of multiple genes.[294] Unsurprisingly, the majority of these genes were related to oxidation reduction, apoptosis and the inflammatory response. This also points to multiple mechanisms at the end-organ/cellular level, by which the total reduction in

ischaemic injury is achieved. However, there are significant issues with this data as the model of RIC used didn't show a protective effect on the kidneys (in terms of the main outcome measure of creatinine levels)[295] and the tissue samples were taken more than 48 hours after the exposure. Thus the changes in gene expression seen may be entirely different to the immediate effect of RIC.[294] Lukovic *et al.* performed whole-transcriptome analysis on cardiac tissue in a porcine model of myocardical infarction. They concluded that ischaemic conditioning down regulates ECM-proteinases, ribosomal subunits and platelet and leukocyte adhesion molecules. Additionally, post-conditioning inhibited the activation of inflammatory leukocytes.

As with most things related to ischaemic conditioning, the bulk of the evidence is derived from studies of cardiac tissue. Whether the mechanisms are similar or distinctly different in other tissues is an open question.

3.5.2.1 Protein Kinase C

The role of protein kinase C (PKC) in the protective effect of ischaemic conditioning has been nicely demonstrated as the use of three different PKC inhibitors abrogate this protection.[296,297] PKC has multiple isoforms and the increasing evidence is that they act in different ways in both injury and protection.[259] The pathway is complex but in part seems to be that PKC acts on the mitochondrial K_{ATP} channels.[298]

3.5.2.2 Mitogen-Activated Protein Kinases

p38 is a mitogen-activated protein kinase that plays a major role in RIC (other mitogen activated protein kinases may be important such as jun-activated kinase[299]). In cardiac studies, p38 is increased by (direct) ischaemic conditioning[300] and inhibition reduces the demonstrable protective effect – specifically resulting in less reduction of the infarct size.[301,302] Intriguingly the expression of p38 does not change with remote conditioning[303] but inhibition of p38 still abrogates the protective effect of RIC.[304]

3.5.2.3 Nitric Oxide and Protein Kinase G

Endothelial nitric oxide synthase (eNOS) is activated after G protein-coupled receptor activation. The sequence of signals include PI3K, phosphoionositide-dependent kinase and protein kinase B (Akt).[305,306] The resulting increase in NO oxide has multiple effects, including activation of protein kinase G (PKG). PKG acts on the K_{ATP} channel and the sodium-proton exchanger and delays the normalisation of acidosis. The PKG-independent action of NO occurs at the time of reperfusion.[305]

3.5.2.4 RISK Pathway

In the reperfusion phase a group of kinases, when activated, promote cell survival. These have been collectively termed the reperfusion injury salvage kinase pathway (RISK pathway).[307] The RISK pathway is an anti-apoptotic cascade and works by inhibiting the opening of the mitochondrial permeability transition pore (mPTP). This pathway is probably the most extensively studied mechanism of RIC/IRI. In essence, whilst necrosis rather than apoptosis is more deleterious to tissue, because apoptosis is a controlled process of programmed cell death, it is also reversible in the reperfusion phase.[308] The RISK pathway (Figure 3-5) is actually two parallel pathways starting with Phosphoinositide 3-kinase (PI3K) and MEK 1/2 (Mitogen-activated protein kinase/ Extracellular signal-regulated kinase) that result in inhibiting the opening of the mitochondria permeability transition pore (MPTP). Each step of this pathway has been elucidated by the use of pharmacological antagonists.[309] In summary, PI3K phosphorylates Protein-kinase B

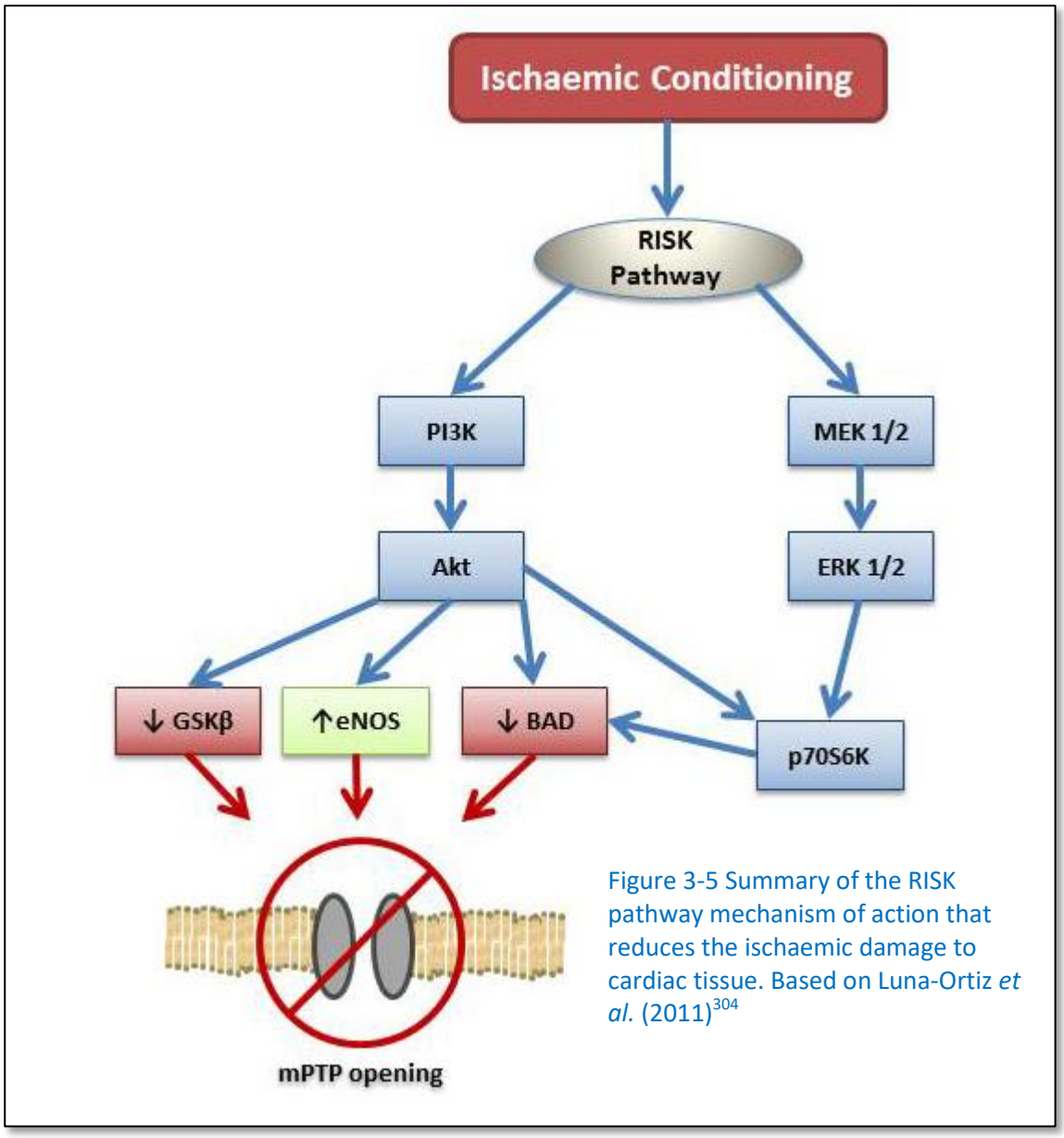


Figure 3-5 Summary of the RISK pathway mechanism of action that reduces the ischaemic damage to cardiac tissue. Based on Luna-Ortiz *et al.* (2011)[304]

(Akt) which inhibits glycogen synthase kinase-3β (GSKβ) and Bcl-2-associated death promoter

(BAD), but activates eNOS and p70S6K. MEK 1/2 phosphorylate ERK 1/2 which also activates p70S6K. p70S6K also reduces BAD. As noted above, the role of eNOS is important in other ways as well during the reperfusion phase.[310]

Acute activation of these kinases is protective, whilst it has long been known that chronic activation is associated with promoting cell-growth and oncogenesis.

3.5.2.5 SAFE Pathway

The survivor-activating factor enhancement (SAFE) pathway is triggered by exogenous TNF-α. Coronary effluent from a preconditioned heart activates Signal transducer and activator of transcription 3 (STAT3) in the acceptor heart and induces functional protection. The mechanism of TNF-α binding the TNF-α receptor subtype 2 activates STAT3 constitutes the SAFE pathway. STAT3 has multiple effects, including upregulating protective proteins[311] and acutely improving mitochondrial respiration.[312]

3.5.2.6 Hypoxia-Inducible Factor-1α

The importance of Hypoxia-Inducible Factor-1α (HIF-1α) in ischaemia was briefly discussed in chapter 1 (section 1.5.7). Evidence from HIF-1α deficient mice shows that it plays an important role in ischaemic conditioning.[313] HIF-1α is a master regulator of cellular oxygen homeostasis and a requisite for mitochondrial ROS formation. HIF-1α's role in ischaemic conditioning seems to be via the mitochondrial MPTP[314] (Section 3.2).

3.5.2.7 End-organ effectors of remote ischaemic conditioning and NEC

Extensive research has elucidated multiple, interrelated biochemical pathways for remote ischaemic conditioning. There is still much that is not known. For example, the understandable focus on cardiac tissue means that little is known about the effector mechanisms in other organs systems (including the bowel). As discussed in Section 1.5.7, NEC is an ischaemia-reperfusion disease. However, it is not only an IRI disease, with IRI potentially being the common end-point of multiple factors (Figure 1-2 NEC Pathophysiology). Importantly the protective pathways discussed in Section 3.5.2 show considerable overlap with NEC pathophysiology. Theoretically, this suggests that RIC could be protective against NEC at other points in the pathogenesis as well as IRI. Furthermore, recent work has shown RIC is indeed a powerful anti-inflammatory process and this may be at-least as important for overall outcomes as the 'direct' anti-IRI mechanisms.[315]

3.6 Remote Ischaemic Conditioning in the clinical context

As already intimated, the predominance of ischaemic conditioning research has been in the context of cardiac disease or stroke. A systematic review of RIC for patients undergoing primary PCI (percutaneous cardiac intervention) following a ST-elevation myocardial infarction (STEMI) found 14 studies including a total of 3165 subjects. This showed that RIC improved some indirect-measures of cardiac outcome but the infarct size was not statistically-significantly different from PCI alone.[316] Trials of RIC in the context of post-stroke management are on-going.[317]

These results notwithstanding, the acute nature of cardiac events does indeed make human clinical trials challenging, Inevitably, RIC is only given once the IRI has been diagnosed. it is therefore unsurprising that many trials have focused on cardiac surgery (including in children) and elective PCI. Table 3-1 summarises the results of clinical trials of RIC during elective cardiac surgery. Sixteen of 27 studies report some positive effect of RIC. However, the endpoints used were often biochemical markers and hence the clinical significance of such a result is debatable. Similar mixed results have been seen in clinical trials with PCI and those looking at renal impairment post cardiac surgery.[318] Among the many clinical studies, Wu *et al.*[319] showed a clear dose effect (or threshold for RIC). With one protocol of three 5 minute cycles of inflation of the blood pressure cuff on the upper limb there was no difference between the experimental group and the controls. When a further two 10 minute cycles of inflation on the lower limb, they found a significant reduction in Troponin I (standard marker of cardiac damage) compared to controls undergoing mitral valve surgery. A Cochrane review of published studies concluded there was no evidence of a clinical benefit of RIC in the context of coronary artery by-pass surgery.[320] Animal studies, regardless of the target organ, have found a profound protective effect from RIC. Unfortunately, as illustrated with the trials in cardiac surgery, the translation to clinical practice has not been as impressive. Whilst several studies have shown a benefit in multiple situations, including: liver and kidney transplantation,[321] major abdominal surgery,[322] acute kidney injury,[323] and stroke,[324] the endogenous effect seems to be less profound. Indeed Abdelnoor *et al.*[325] and Brevoord *et al.*[326] showed no benefit from RIC. Given how effective RIC has been shown to be in animal models, this inevitably begs the question of why the translation to clinical practice has been so disappointing and moreover whether this is a surmountable problem.

Study	Number of patients (RIC/control)	Type of Surgery	RIC regimen (Limb: No. of cycles: I/R time)	Endpoint	Outcome
Günaydin et al., 2000 (i)	4/4	CABG	UL: 2 cycles: 3/2 min	Creatine Phosphokinase / Lactate dehydrogenase	No effect
Cheung et al., 2006 (ii)	17/20	Pediatric cardiac surgery	LL: 4 cycles: 5/5 min	Troponin I (TnI) Post operatively	**Reduced TnI; reduced inotropic support; reduced airway**
Hausenloy et al., 2007 (iii)	27/30	CABG	UL: 3 cycles: 5/5 min	Troponin T	**43% reduction of TnT**
Venugopal et al 2009 (iv)	23/22	CABG (cold blood cardioplegia)	UL: 3 cycles: 5/5 min	Troponin T	**42% reduction of TnT**
Hong et al., 2010 (v)	65/65	CABG (off-pump)	UL: 4 cycles: 5/5 min	Troponin I	26% reduction of TnI, NS
Rahman et al., 2010 (vi)	80/82	CABG	UL: 4 cycles: 5/5 min	Troponin T	No effect
Thielmann et al., 2010 (vii)	27/26	CABG (crystalloid cardioplegic arrest)	UL: 3 cycles: 5/5 min	Troponin I	**45% reduction of TnI**
Li (2010) (viii)	26/27	Valve replacement	LL: 3 cycles: 4/4 min	Troponin I	**40% reduction of TnI**
Zhou (2010) (ix)	30/30	Pediatric cardiac surgery	UL: 2 cycles: 5/5min (*24 h and 1 h prior to operation*)	CK-MB Inflammatory biomarkers (Plasma levels 2, 4, 12, and 24 h after surgery) Lung function	**Reduced CK-MB & inflammatory markers, Improved postoperative lung function**
Wagner et al., 2010 (x)	32/34	CABG (cold crystalloid cardioplegia)	UL: 3 cycles: 5/5 min (*18 h prior to operation*)	Troponin I	**Reduced TnI**
Ali et al., 2010 (xi)	50/50	CABG	UL: 3 cycles: 5/5 min	CK-MB (Plasma levels 8, 16, 24, and 48 h after surgery)	**Reduced CK-MB**
Karuppasam y et al., 2011 (xii)	27/27	CABG	UL: 3 cycles: 5/5 min	Troponin I	No effect
Wu et al., 2011 (xiii)	25,25/25	Mitral valve replacement	a) UL: 3 cycles: 5/5 min *or* b) UL: 3 cycles: 5/5 min + LL: 2 cycles: 10/10 min	Troponin I (Plasma levels 4, 8, 12, 24, 48, and 72 h after surgery)	**Reduced TnI with *b)* but not *a)***
Kottenberg et al., 2012 (xiv)	20/19	CABG Propofol vs. isoflurane anesthesia	UL: 3 cycles: 5/5 min	Troponin I	**Reduced TnI with isoflurane, but not propofol anesthesia**
Young et al., 2012 (xv)	48/48	Cardiac surgery (high-risk CABG and valve	UL: 3 cycles: 5/5 min	TnT (Plasma levels 6 and 12 h surgery)	No effect
Heusch et al., 2012 (xvi)	12/12	CABG	UL: 3 cycles: 5/5 min	Signal transducer and activator of transcription 5 (STAT5)	**Reduced STAT5 activation**
Lee et al., 2012 (xvii)	27/28	Pulmonary hypertensive infants	LL: 4 cycles: 5/5 min	Troponin I 24 hours post surgery	No effect
Pavione et al., 2012 (xviii)	12/10	Pediatric cardiac surgery	LL: 4 cycles: 5/5 min	Troponin I (Plasma levels 4, 12, 24, and 48 h after surgery)	No effect
Lucchinetti et al., 2012 (xix)	27/28	CABG Opioids and propofol for induction	LL: 4 cycles: 5/5 min	Troponin T (Plasma levels 24, 48, and 72 h after surgery	No effect
Xie et al., 2012 (xx)	38/35	Valve surgery	UL: 3 cycles: 5/5 min	Troponin I (Plasma levels 6, 12, 24, 48, and 72 h after surgery	**Reduced TnI**
Thielmann et al 2013 (xxi)	162/167	CABG	UL: 3 cycles: 5/5 min	Troponin I	**27% reduction of TnI and Reduced all-cause mortality**
Ahmad et al., 2014 (xxii)	35/32	CABG (On-pump)	UL: 3 cycles: 5/5 min	CK-MB (Plasma levels 1, 12, 24, and 48 h after surgery)	No effect
Hong et al., 2014 (xxiii)	644/636	Cardiac surgery (cardiac valve surgery; CABG; arch surgery; and congenital heart defect repair)	UL: 4 cycles: 5/5 min (2 cycles before, 2 after cardiopulmonary by-pass)	Clinical outcome (composite of: death; myocardial infarction; arrhythmia; stroke; coma; renal damage; respiratory failure; gastrointestinal complications; and multiorgan failure)	No effect
Slagsvold et al., 2014 (xxiv)	30/30	CABG	UL: 3 cycles: 5/5 min	Mitochondrial oxidation	**Improved mitochondrial respiration**
McCrindle et al., 2014 (xxv)	148/151	Pediatric cardiac surgery	LL: 4 cycles: 5/5 min	Duration of post-operative hospital stay	No effect
Holmberg et al., 2014 (xxvi)	23/23	Cardiac surgery (CABG; valve surgery;	UL: 3 cycles: 5/5 min	Troponin T	25% reduction of TnT, NS
Candilio et al., 2014 (xxvii)	90/90	CABG	UL & LL: 2 cycles: 5/5min	Troponin T	**26% in TnT**

AF = atrial fibrillation; CABG = coronary artery bypass grafting; CK = creatine kinase; CM-MB = creatine kinase-myocardial band; LL = lower limb; NS = not (statistically) significant; TnI = troponin I; TnT = troponin T; UL = upper limb

Table 3-1 Clinical trials of RIC in cardiac surgery
Modified and updated from Heusch, G. et al. (2015)[327]

It would be wrong to conclude however that RIC does not translate to measurable benefits in human patients. Veighey *et al.*[328] showed that long-term renal function is improved by the application of RIC to renal transplant recipients prior to induction of anaesthetic. This benefit of renal function (measured by estimated Glomerular Filtration Rate (eGFR)) is preserved to at least 60 months post-transplant (Figure 3-6). The importance of this work is that it shows that RIC potential confers an eGFR that is 4.7 ml min^{-1} higher. Post-transplant, the GFR deteriorates at approximately 0.7 ml min^{-1} per year and thus this benefit implies several more years of graft survival post-transplant.

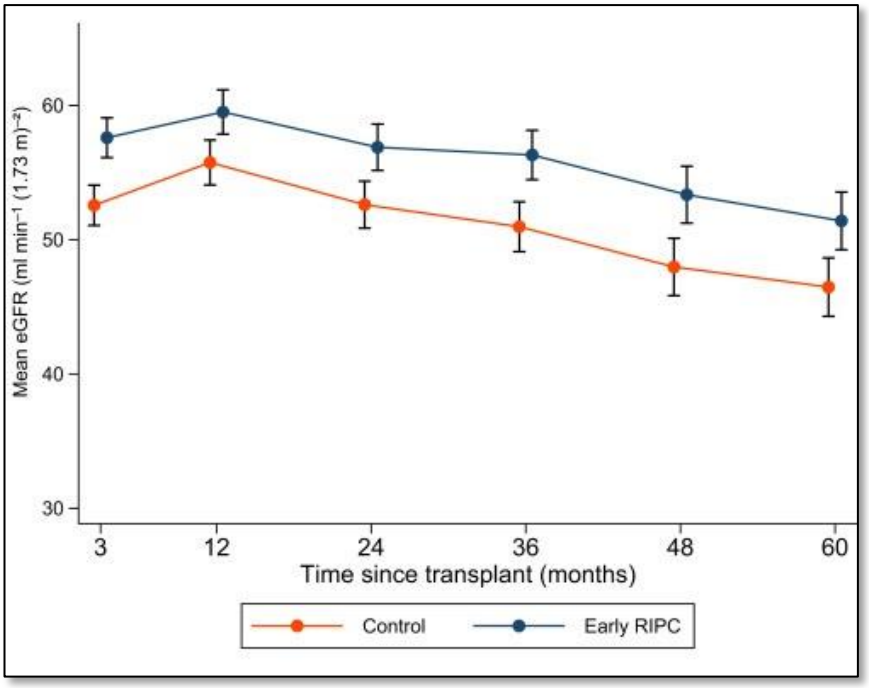


Figure 3-6 The effect of RIC on Allograft function post renal transplant.
eGFR = estimated Glomerular Filtration Rate. Graph shows a persistently higher eGFR 60 months post transplant in transplants where RIC was used compared to controls. *From* Veighey *et al.* (2019)[328]

3.7 Remote ischaemic conditioning and Necrotising enterocolitis

McCafferty *et al.*[329] explored how comorbidities attenuate the effects of RIC. As they note, in animal studies, the participants are often juvenile, of the same age with no comorbidities, as well as inbred, kept in the same environment and with the same diet. As such, there are significant biological differences between the animals used and human patients with coronary artery disease, stroke or any other of the myriad of IRI conditions where RIC has shown promise. Implicitly, necrotising enterocolitis (NEC) is a different paradigm whereby the patients are juvenile, of the same age, kept in the same environment and lacking the comorbidities seen in adults.

Hyperlipidaemia was the first such comorbidity to be reported to attenuate the protective effect of ischaemic conditioning.[330] Rats fed a high-fat diet had hearts that did not benefit from preconditioning. Importantly this effect seems to be independent of atherosclerosis as the experimental animals used were prepared in such a way as to have hyperlipidaemia but not have atherosclerotic plaques. Tang *et al.* examined a rabbit model of hypercholesterolemia and found that the upregulation of tetrahydrobiopterin was inhibited.[331] Tetrahydrobiopterin is essential for inducible nitric oxide synthase. Other potential mechanisms for this effect may be due to downregulation of HSP-70 by hypercholesterolemia,[332] which is implicated in the protective mechanism of ischaemic conditioning and increased activation of Capsase-3 which is part of the apoptosis pathway.[333] Two studies in humans using balloon angioplasty and ischaemic changes on

electrocardiogram as the endpoint showed that the beneficial effect of ischaemic conditioning was lost in those patients with the highest cholesterol levels.[334,335]

In a similar way, animal studies suggest that diabetes mellitus is a barrier to effective ischaemic conditioning. Several pathways are implicated, including decreased generation of NO, impaired phosphorylation of PI3K-Akt, abnormal ERK1/2 activity and K_{ATP} dysfunction.[336] In humans, prodromal angina before an anterior myocardial infarction (MI) represents a 'natural ischaemic preconditioning' on the heart which attenuates the severity of acute MI by several measures including in-hospital mortality which is reduced by more than half. In patients with diabetes mellitus this benefit disappears.[337] The proposed mechanisms for this amelioration includes the reduction in the activation of K_{ATP} channels.[338]

The effect of obesity on ischaemic conditioning has been studied. Implicitly it is challenging to elucidate the effect of obesity alone when it most often coexists with other conditions such as diabetes mellitus and hypercholesterolemia. However, studies with obese rats and lean insulin-resistant rats support the idea that obesity independently reduces the protective effect of ischaemic conditioning.[339]

Senescence confers significant changes in the myocardium and is an independent prognostic factor for morbidity and mortality following MI.[340] As such, it is perhaps not surprising that effects seen in experimental infarcts in juvenile animals do not immediately transfer to the human clinical context.

In summary, the body of evidence does support the concept that RIC can be effective in human disease and that multiple common co-morbidities, both individually and in combination attenuate the endogenous protective mechanisms of RIC resulting in a less protective effect being seen in some human trials. They also provide some further information on the biochemical pathways involved in ischaemic conditioning. These factors are not relevant to human neonates and thus if RIC is effective in an animal model of NEC, it would be expected that it would translate to human practice much more readily. One could almost say that NEC is the disease most likely to respond to RIC. The one vital caveat though is that NEC is a more complex process than simply IRI. However, with IRI being a central part of the pathogenesis, there is a clear potential for an intervention that moderates IRI to significantly affect the clinical course of this disease.

3.8 Conclusion: Remote Ischaemic Conditioning as a Novel Therapeutic Strategy for Necrotising Enterocolitis.

Previously, I described the various features of NEC, including the current understanding of its pathophysiology. The role of IRI is central to the pathogenesis of NEC. As such, RIC is a promising avenue for novel therapies. The dramatic benefits of RIC across multiple organ systems is very encouraging and offers the hope of an effective therapy that could be used as both a preventative measure and a treatment once NEC is diagnosed. These concepts will be investigated in the work I present in this thesis.

Main hypothesis: Remote ischaemic conditioning reduces the extent of intestinal injury and inflammation in an experimental rat model of necrotising enterocolitis.

Sub-hypotheses:

1) Reduction in bowel injury will correlate with a reduced systemic inflammatory response.
2) The same or similar effector mechanisms by which RIC protects cardiac tissue from injury will also be found in the intestine. Transcriptome analysis will provide some important insights to the mechanisms.
3) RIC given to mothers in late pregnancy will confer protection from bowel injury on the offspring.

Chapter 4 Methods

4.1 Introduction

In this section, I will outline the various methods used in the following chapters (5-8 and 10). The specific application of each method and exact protocol used for each set of experiments is indicated in the relevant chapter. Chapter 9 describes transcriptome analysis I performed using *RNAseq* and the methods for that work are not included in this chapter.

4.2 Animal Experimentation – the choice of model

Necrotising enterocolitis (NEC) is a complex, systemic disease (section 1.5). Remote ischaemic conditioning (RIC) has shown immense promise in multiple contexts and the mechanisms involved are multiple, complex and overlapping. For these reasons, there is no practical way to study the potential of RIC for NEC other than with an animal model. Furthermore, the nature of *remote* conditioning necessitates a whole organism in order to be able to apply the stimulus to one part of the body and measure its effect on another. There are studies investigating ischaemic conditioning that have been done with isolated organ systems (such as Langendorff perfused heart for example) but this is not possible when studying the effect of remote conditioning on the intestine. The use of a novel therapeutic intervention on infants, especially preterm infants requires a solid empirical basis. This is true even though the likely risks are very low. Moreover the use of an animal model provides the opportunity to gain more understanding of the putative therapeutic approach and develop appropriate protocols that will inform future clinic studies.

There are several potential animal models for NEC that could be used for this work. The diversity of the models reflects the difficulty in producing a good representation of a multifactorial disease.

4.2.1 Rodent models

Various models of NEC exist in both mouse and rats. One of the first such model was developed in 1975 by Barlow and Santulli which involves a combination of gavage feeding of rat pups with formula and intermittent episodes of cold or hypoxic stresses to the animals.[341] Importantly, as well as showing that formula feed on its own was not sufficient to induce disease in these animals, they found a clear dose effect – the incidence of disease was directly related to the number of stress episodes applied to each pup. This model has been refined by several laboratories.[342] The addition of bacterial colonisation by Caplan *et al.* confirmed the necessity of

bacteria in the disease process but importantly in the absence of a hypoxic insult, formula feed and bacterial alone did not induce disease.[343] More recently, the addition of bacterial endotoxin (lipopolysaccharide) to the gavage feeds has supplanted the use of bacteria.[342]

Mouse models of NEC to a large extent mirror the rat models. The smaller size of the mouse is a technical challenge; gavage feeding of neonatal mice is difficult but has become well established.[344] The advantage of a mouse model is primarily due to the potential for genetic manipulation in mice.

4.2.2 Piglet models

Piglets potentially provide a good basis for a model being of similar size and weight to human neonates. The gastrointestinal tract of piglets also more closely resembles the human tract than the rodent does.[345] Sangild *et al.* showed that piglets born pre-term (caesarean section at 92% gestation) and exposed to formula feeding develop a disease which is strikingly similar to human NEC with similar clinical signs such as abdominal distension, bloody diarrhoea and similar histological changes.[345] Other piglet models have used similar methodology to rodent models with gavage feeding, the introduction of endotoxin and hypoxia,[346] or a combination of feeding and ischaemia/reperfusion to the gut.[347]

Of all the animal models available, the model described by Sangild *et al.* is arguably the best analogue of human disease. However, within their model the disease develops in only some of the animals and within the first 24 to 48 hours after birth. This means that it would be impossible to deliver RIC at a fixed time point prior to the development of intestinal injury thus making it unsuitable for this study.

All of the pig models are limited by the inherent difficulties in using a larger animal and consequentially higher cost. For practical purposes, these limitations make any of the piglet models unsuitable for this current work; in order to evaluate the effect of RIC in this context, a larger number of animals with reliable, reproducible disease is required.

4.2.3 The choice of a rat pup intestinal ischaemia-reperfusion-injury model for this work

Ischaemia-reperfusion injury is a process that is important in the pathogenesis of several diseases. In NEC, IRI is probably the common end-point of several interacting pathways, resulting in ischaemic necrosis (Section 1.5.7). Models based on intestinal IRI are well established[348] and produce an injury which is histologically very similar to that seen in human NEC.[342] The mouse is

simply too small for this model (in infant animals) and the piglet is unsuitable for the reasons outlined above. Thus, for this work, the rat is the best choice for an IRI model in infant animals.

There are several advantages to such a model for the present study. Firstly it has been previously used for experimental work looking at the potential therapeutic benefit of hypothermia in NEC[349] and this lead to direct translation into a clinical study.[350] Secondly, an IRI model produces an injury which is similar to NEC in terms of areas of bowel showing severe necrosis and others being less damaged and still others completely spared.[351-353] Thirdly, it produces a reproducible injury and thus is a good basis for analysing a potential therapy. Fourthly, it produces disease at a fixed time point. This is vital for analysing how long any potential therapeutic benefit might last – which is critical empirical information for any putative clinical use. Figure 4-1 shows the histological similarity between ischaemic models of NEC and the human disease.

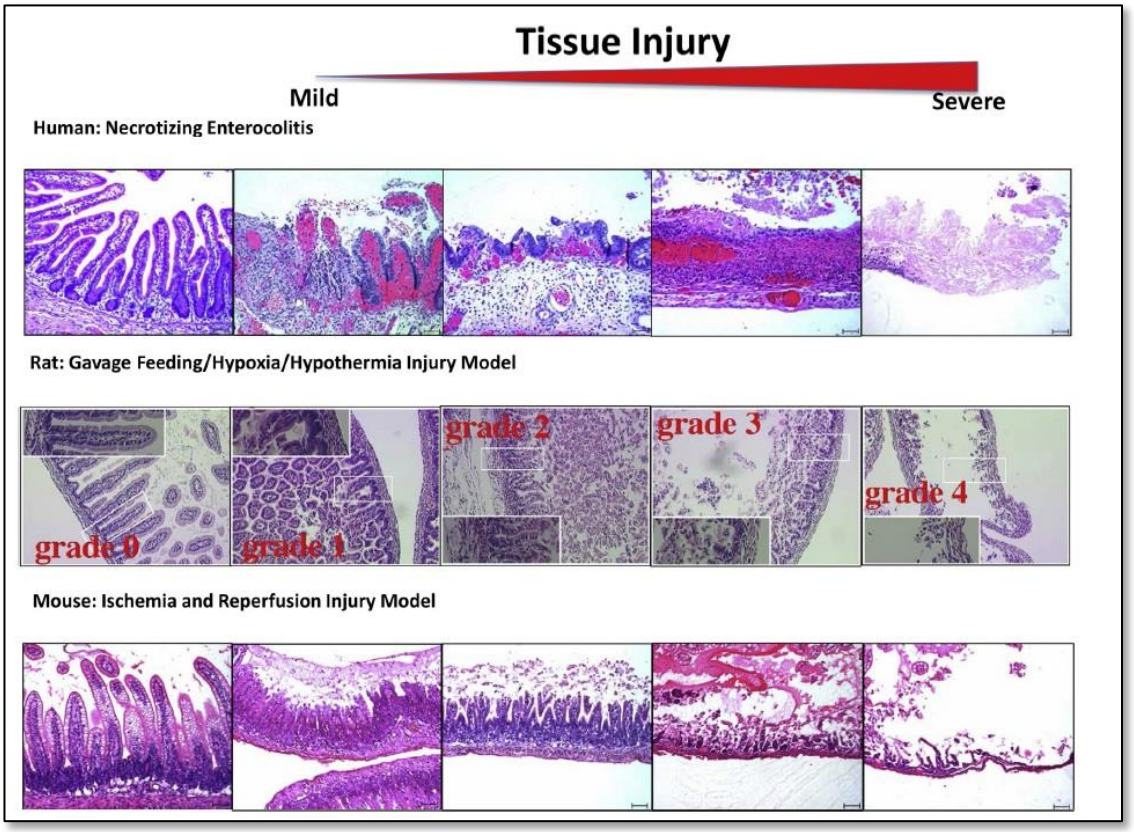


Figure 4-1 Examples of various grades of morphological damage in human NEC vs gavage-based model of NEC and ischaemia-reperfusion injury model (in this case in mice).
Bowel injury pattern in rat is very similar

Ultimately, the end-point of NEC pathogenesis is a common pathway of ischaemic-necrosis regardless of the separate and overlapping-factors that led to that point.[7,78,136] Thus the IRI-model provides a good starting point for investigating the potential effect of RIC on NEC. It has been shown that arterial in-flow obstruction produces a different injury pattern to an arteriovenous or

venous occlusion in rat intestine,[354] which is why this model is more akin to the endpoint of NEC than to a small bowel volvulus.

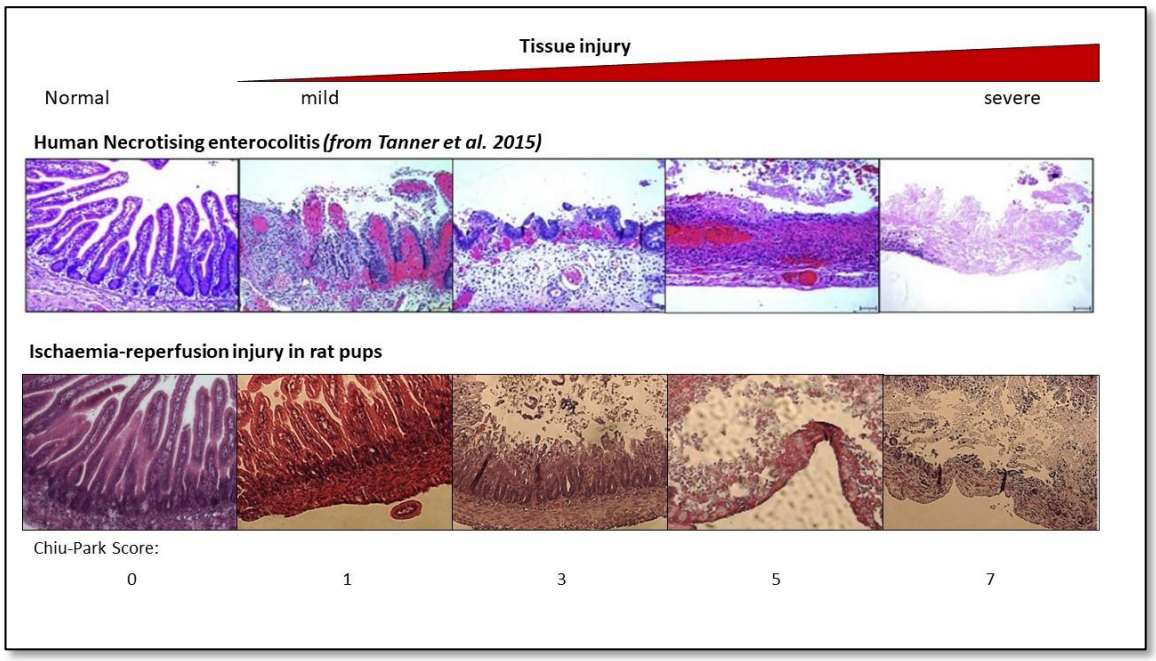


Figure 4-2 Examples of this rat model with their Chiu-Park scores[355] (see Section 4.8.2). Human NEC for comparison from Tanner *et al.* (2015)[353]

4.3 Legal and Ethical Protocols

All animal experiments were carried out according to the Animals in Scientific Procedures Act (APSA) 1986 and revisions. Project licence: PA813F125; personal licence: I0F77E94D. Ethical approval was obtained from the university ethics committee prior to application for the project licence as per standard ASPA procedures. At each stage every effort was made to follow the principals of Replacement, Reduction and Refinement ("the 3 R's") in the use of the animals for scientific experimentation.[356]

My supervisor, Nigel Hall held the project licence but I prepared (under supervision) the licence application. In order to obtain approval for animal work under ASPA, the scientific background, the necessity of animal work as well as the potential benefits of the research must all be established. The non-technical summary of the application (section G) is reproduced in Section 4.3.1

4.3.1 Non-technical summary of Project licence: PA813F125

Describe the aims and objectives of the project (e.g. the scientific unknowns or scientific/clinical needs being addressed):

Necrotising enterocolitis (NEC) is a devastating disease of premature babies. Up to a third sadly die and many of the survivors are left with life-long disability. There are around 3000 cases of NEC in the UK each year.

The causes are complicated but one key aspect of NEC is what is known as 'ischaemic-reperfusion injury.' Whenever any organ of the body does not have enough blood flow this is called 'ischaemia' which results in tissue damage. When the blood supply is restored, further damage occurs and this is called 'reperfusion injury' Ischaemic-reperfusion injuries are part of several common diseases such as heart attacks and stroke.

Experimental work has shown that the use of 'Remote Ischaemic Conditioning' (RIC) can reduce significantly the damage done by a heart attack or other ischaemic-reperfusion injury. Remote Ischaemic Conditioning means giving a small ischaemic 'hit' to some other part of the body. This does not cause any lasting injury or damage. The easiest way to do this is to use a blood-pressure cuff inflated for a period of a few minutes. This conditions the body so that the damage done from the major ischaemic-reperfusion injury is massively reduced.

Our aim is to establish if RIC can be used as a treatment for NEC in premature babies.

What are the potential benefits likely to derive from this project (how science could be advanced or humans or animals could benefit from the project)?:

Our hope is to be able to use a blood pressure-cuff based treatment to treat human babies with NEC. We will also use samples from the animals to study the mechanisms by which RIC works in order to look for other treatments as well. We have previous experience of translating potential treatments from experimental animal research into clinical trials.

What types and approximate numbers of animals do you expect to use and over what period of time?:

Rat pups: around 150 over the course of the project

In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected levels of severity? What will happen to the animals at the end?:

The expected adverse effects are mild. Inflating the blood pressure cuff is well tolerated and has no long lasting effects. Prior to the surgery, animals will be housed in suitable cages, with species appropriate environmental enrichment, within a well-resourced, well equipped, modern facility. These are maintained at the ideal temperature and humidity for the rats. Appropriate food and water are provided at all times. If, at any time, there are concerns about any animals, advice will be sought from the NVS/NACWO and appropriate steps taken to ensure animal welfare including humane killing if necessary. The model of necrotising enterocolitis involves surgery on the abdomen and a temporary interruption to the blood supply to the bowel. At the end of the experiment, multiple samples are taken from each animal to assess the effect of the model and the possible benefit of the intervention. This whole process will be performed under terminal anaesthesia. The animals will not be recovered from the anaesthetic. All animals will be closely monitored under anaesthetic to ensure sufficient depth of anaesthesia and a humane end-point.

Application of the 3Rs

Replacement:

Necrotising enterocolitis is a complex disease affecting not just the intestine but the entire baby. We have developed a model of this disease which closely reflects the human disease. The complex interactions between various parts of the whole animal are necessary for the model to closely replicate the human disease. Furthermore remote ischaemic conditioning must be applied at a site away from the intestine. Such mechanisms can only work in whole animal systems.

Reduction:

Through close attention to experimental design and statistical advice we will use the minimum numbers of animals necessary to gain statistically and clinically meaningful data. We will gain as much information as we can from each experimental animal (i.e. both physiological monitoring and analysis of several organs/fluids) in order to reduce experimental numbers. As

we progress we will continuously review the results to ensure that we minimise confounding factors and do not use more animals that are absolutely necessary whilst maintain experimental rigour.

Refinement:

We will use the rat since the physiology of the rat intestine closely resembles that of the newborn human and it is an appropriate size for the program of work (smaller animals are too small).

To minimise harms, all invasive procedures will be performed under terminal anaesthesia (i.e. without recovery).

4.4 Intestinal ischaemia and reperfusion in rat pups

Mixed sex suckling Sprague-Dawley rat pups were housed with their mother and allowed to suckle freely until the beginning of the experiment. Experimental procedures were performed on pups aged 10-13 days (19.5g – 39.4g).

General anaesthesia was induced by placing the animal in a small anaesthetic chamber (VetTech Solutions Ltd, AN010) and administration of 5% isoflurane with 100% oxygen as the carrier gas. General anaesthesia was confirmed by the absence of webfoot reflex and then maintained with 1-4% isoflurane administered via nose cone (Harvard Apparatus, USA).

Normothermia (36.0^{o}C - 37.0^{o}C) was maintained by means of a heating blanket (Harvard Homeothermic Blanket system) which provides closed-loop temperature control and monitoring by means of a rectal temperature probe placed immediately after induction of anaesthesia. A heating lamp (Crompton Lamps) provided auxiliary heating as necessary to maintain temperature. All animals then received 0.2ml of 0.9% saline injected into the sub-cuticular space using standard technique. Heart rate and oxygen saturations were monitored in anaesthetised animals by means of a pulse oximeter on the left forelimb (Nellcore Oximax). This is a vital confounder to exclude as it has been previously shown that hypothermia protects the intestine from IRI.[349]

Operative procedures were carried out using 2.5x Loupes magnification (Phillips Safety Instruments). The peritoneal cavity was accessed by means of a transverse laparotomy. Traction on the small bowel mesentery allowed identification of the superior mesenteric artery which was dissected out (Figure 4-3A). Experimental animals undergoing intestinal ischaemia and

reperfusion then had the superior mesenteric artery occluded with a microvascular clip (S&T, AG, Switzerland) Complete occlusion was confirmed by the loss of visible pulsation in the arterial arcades, and secondarily by colour-change in the bowel (Figure 4-3B). Sham animals had the artery exposed without the artery clip being applied. The peritoneal cavity was then temporarily closed by means of a single layer continuous suture. After 40 minutes of ischaemic time, the abdomen was reopened; bowel exposed to ischaemia appears oedematous at this stage (Figure 4-3C and Figure 4-3D). In Sham animals, the perfusion of the mesentery was confirmed before temporary closure as previously described. In experimental animals receiving ischaemia and reperfusion, the microvascular clip was removed and the return of mesenteric pulsation confirmed under the magnified view. Reperfusion results in a hyperaemic appearance of the bowel (Figure 4-3E) The abdominal wall was then closed as before with a single layer continuous suture. After 90 minutes of reperfusion the animals were sacrificed by exsanguination by means of cardiac puncture.

Once established, I was able to produce a consistent and reproducible bowel injury, as well as systemic effects analogous to human NEC with this model.

This model had never previously been used in this institution, although my supervisor, Nigel Hall had used it at University College London. The first part of my work therefore was to establish this model and to develop the skills to achieve a reproducible injury.

Particular care must be taken during the dissection, as all of the blood vessels in the mesentery are fragile and the proximity of the underlying aorta when dissecting the SMA is also vulnerable to injury. Injury to any of these vessels results in uncontrolled haemorrhage and death of the animal. The results in Chapters 5-8 and 10 detail the injury produced by this model and the consistency with which it was reproduced.

Additional material: Video (2mins) shows IRI model ("IRI model.MP4")

- On attached CDROM
- ***/IRI_model_video.mp4***
- http://doctor-jones.co.uk/download/IRI%20model%20video.mp4

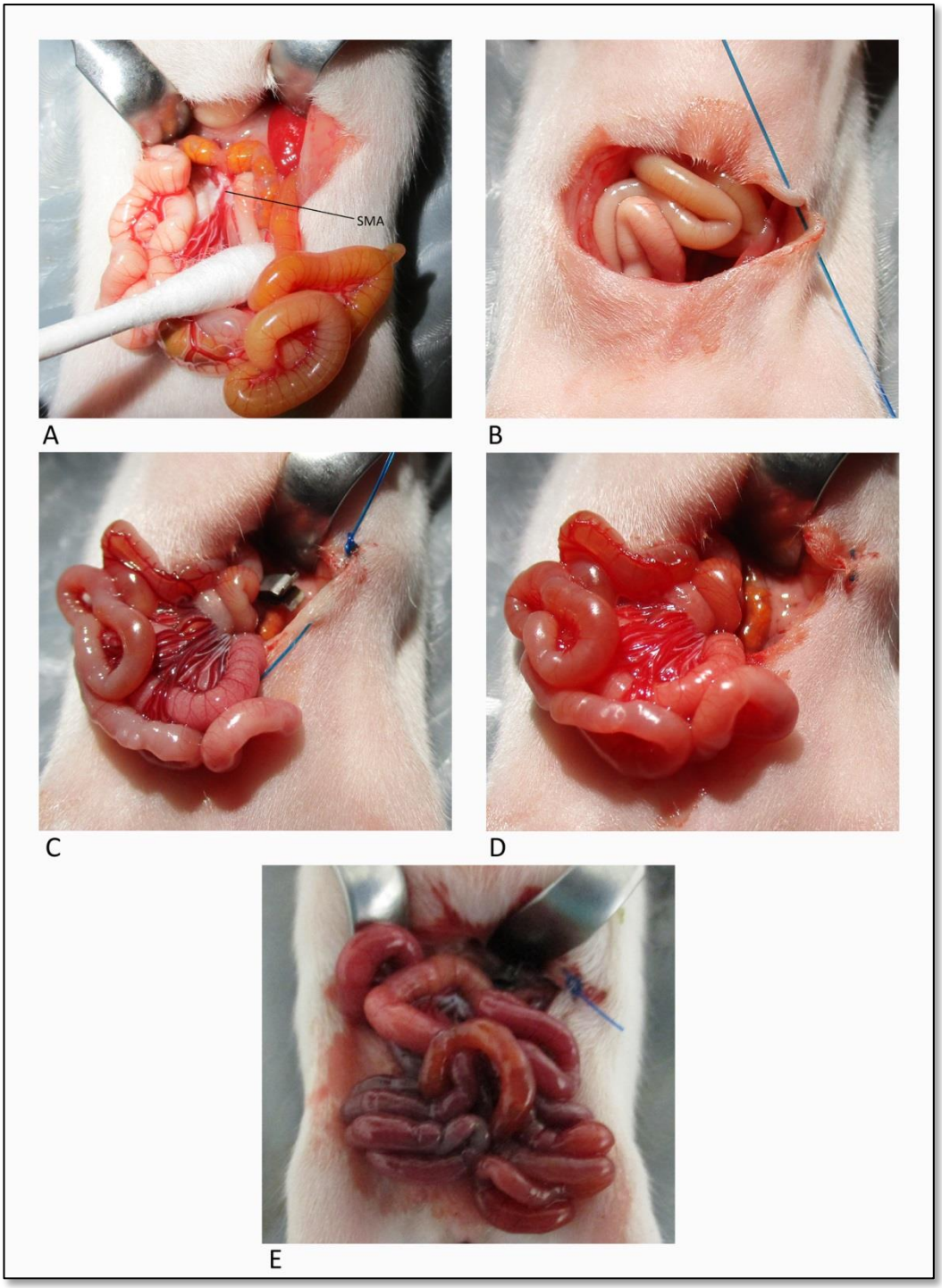


Figure 4-3 IRI model of NEC
4-3A Exposure of superior mesenteric artery (at root of mesentery); **4-3B** Immediate colour change seen is bowel (very pale appearance) due to occlusion of SMA; **4-3C** Oedematous appearance of bowel after 40 minutes of ischaemia (clamp on SMA also visible); **4-3D** Clamp removed after 40 minutes of ischaemia: hyperaemic appearance of bowel due to reperfusion; **4-3E** Typical pattern of bowel injury with necrotic sections and less severely injured portions seen.

4.5 Remote Ischaemic Conditioning

4.5.1 Remote ischaemic conditioning with ligature

Based on previously reported methods used to study remote conditioning in other organs,[339] A vessel loop (Medline Medi-Loop mini) was used as a ligature and was applied to the right hind limb as close to the hip joint as practicable and tightened to achieve arterial obstruction. Loss of arterial in-flow was confirmed by the cyanotic appearance of the hind limb (Figure 4-4). In each case, remote conditioning was performed with cycles of five minutes of occlusion followed by five minutes of reperfusion.

Figure 4-4 RIC by means of hind limb ligature

4.5.2 Remote ischaemic conditioning with blood pressure cuff (Chapter 6)

The occlusion cuff of the appropriate size from Columbus instruments NIBP-8 (Columbus Instruments, USA) was applied to the hind limb (Figure 4-5). This occlusion cuff is designed to measure rodent blood pressure by inflating above systolic pressure and then deflating to assess the distal flow in by means of a second (sensor) cuff. In order to achieve RIC, the cuff was inflated by means of the control and then the pressure tubing clamped when the pressure reached 150mmHg to maintain that pressure until release. As with the ligature method, the loss of arterial inflow was confirmed by the cyanotic appearance of the hind limb. Remote conditioning was performed by three cycles of 5 minutes of occlusion, followed by 5 minutes of reperfusion.

Figure 4-5 RIC by means of rodent blood pressure cuff.
(Internal diameter 6mm or 10mm).

4.6 Tissue Harvesting

Each experimental animal was assigned a random 3-digit numerical code to allow blind analysis of the samples. The effect of the model was assessed in several different ways with the following outcome measures used:

- **Intestine:**
  - **macroscopic assessment of injury (extent of injury)**

- **microscopic injury (primary outcome measure, using a validated, blinded scoring system)**
- **Myeloperoxidase as a measure of neutrophil activity (inflammation marker)**
- **Hypoxia-inducible factor 1 alpha (known to be important in NEC)**
- **Nitric oxide (important for regulation of capillary blood flow)**
- **Malondialedhyde (responds to reactive oxygen and nitrogen species)**

- **Serum**
  - **Pro-inflammatory cytokines to assess the effect of both the model and NEC on the systemic level and to assess possible mediators of RIC**

4.6.1 Bowel

After euthanasia, the entire small bowel was removed and laid out. The extent of macroscopic bowel injury was measured and recorded. Sections of bowel were rated as either 'normal' 'mild injury' or 'severe injury.' The number of centimetres of 'mild' and 'severe' was then recorded for each animal. Samples were removed for study from fixed points in the bowel: 2 cm of ileum (3-5 cm) from the ileo-caecal valve was removed for quantitative RNA analysis. This was immediately flushed with Phosphate Buffered Saline (with calcium chloride and magnesium chloride.) (Gibco / Thermo Fisher Scientific) and then placed in RNAlater™ Stabilization Solution (Qiagen) and stored as per the manufacturer's insturctions. The next 2 cm of ileum (5-7 cm from ileo-caecal valve) was fixed in 10% formaldehyde for 12 hours and embedded in paraffin. 5 µm sections were cut and stained with haematoxylin and eosin for assessment using the validated Park/Chiu scoring system.[355] The next proximal section of ileum (7-9 cm from ileo-caecal valve) was flash-frozen in liquid nitrogen and then stored at -196^{o}C for future analysis. A 2 cm section of small bowel (ileum or jejunum) with the most severe macroscopic injury was also fixed in 10% formaldehyde, embedded in paraffin and 5 µm sections were cut and stained with haematoxylin and eosin for assessment with the Park/Chiu scoring system. A further 2 cm of maximally-injured bowel was flash-frozen in liquid nitrogen and then stored for future analysis at -196^{o}C.

4.6.2 Blood Serum

Whole blood extracted by cardiac puncture was collected in tubes containing Ethylenediaminetetraacetic (EDTA).

4.7 Macroscopic analysis of bowel injury

As Petrat *et al.* (2010)[351] described, this model produces a variable pattern of macroscopic injury, with areas of frankly necrotic bowel, oedematous bowel with haemorrhage and normal bowel seen side by side in the same animal. This variable pattern of injury is esoteric to each animal but there is a simple correlation with the amount of bowel affected and the length of ischaemic time. I.e. animals exposed to more severe ischaemia show more injury than those exposed to less ischaemia by means of shorter ischaemia and reperfusion times. Moreover, Petrat demonstrated that the outward macroscopic appearance of the bowel correlates with the validated Chiu-Park score.[355] Therefore is would be possible to use the non-linear scale that they developed to assess severity of injury. However, the purpose of measuring the macroscopic injury is to assess the extent as the Chiu-Park score can be assessed in a blinded fashion and is validated as a measure of severity.

Bowel tissue rapidly deteriorates once removed from the body. Standardised practice for many analyses is fixation in formaldehyde. Fixation is necessary for the subsequent microscopic analysis also. Significant shrinkage of the bowel tissue (up to 50%) occurs with formaldehyde fixation.[357] Therefore, it is necessary to measure the bowel promptly upon euthanasia and tissue harvesting with subsequent processing of the samples for microscopic analysis. Theoretically it would be possible to photograph specimens with a ruler in place to allow blinded assessment of the macroscopic injury. However, the length of the bowel (approximately 40cm) relative to its narrow diameter 1-2 mm along with variable lighting conditions meant that I was unable to obtain consistent photographs with any reliability or reproducibility. Each small bowel excised required at least three different photographs to encompass the entire bowel with sufficient magnification to visualise the very narrow bowel. As a consequence, even bowel from the same animal often had very different appearances on a series of pictures. Consequently, the assessment of macroscopic injury was done by me and not blinded to the experimental group of the animal.

The extent of bowel injury is important for two reasons. As described, it is a reasonable measure of microscopic appearance and thus does delineate ischaemic injury to the bowel. Moreover, the amount of bowel affected is of massive importance in the clinical context. However, because of the technical limitation described with achieving blinding and because the Chiu-Park score is a validated scoring system, the microscopic assessment was used as the primary outcome measure in the animal experiments.

4.8 Microscopic analysis of bowel Injury

The samples were prepared as described in 4.6.1. and then stained with Mayers Haematoxylin (Mayer P 1904 Z.Wiss.Mikrosk. 20,409) and Eosin.

4.8.1 Haematoxylin and Eosin staining

Sections of the bowel specimens (5 µm in thickness) were cut and dewaxed with Tissue Clear then placed in Mayer's haemotoxylin for 5 minutes. The slides were then rinsed with running water for 5 minutes and stained with eosin for 2.5 minutes.

4.8.2 Chiu-Park Scoring of bowel injury

4.8.2.1 Using the Chiu-Park Scoring system

Three observers conducted independent, blinded analysis of the samples, scoring the injury against the Chiu-Park scoring system[355] recommended by Quaedeckers *et al.* (2000)[358] and Oltean and Olausson (2010).[359] The scoring was performed used light microscopy at various magnifications (100x - 400x). Specimen images are shown in 0. These images are reproduced for reference only – all scoring was done with direct microscopy. Table 4-1 summarises the progressive scoring of ischaemic injury of the small bowel described by Chiu *et al.*[360] and updated by Park *et al.*[355] Example images are shown in Figure 4-6. The three observers were the author, one of the supervisors, Nigel Hall, and a pathology registrar with support from a consultant pathologist. Nigel Hall has previous experience with using this scoring system. Several steps were taken to ensure the robustness of the observations. Each score was obtained independently from the others with no knowledge of the score. As the amount of injury within one section inevitably varies, each score was determined on the basis of the highest seen (i.e worst injury) within the field (with the exclusion of artefact seen due to the slicing process). Once the first tranche of samples were scored (approximately 50 slides), the scores were compared between the three observers. This showed a high-degree of agreement between the observers with no bias towards higher or lower scores of any of the observers. I then discussed a representative sample of these

Table 4-1 Microscopic Criteria for scoring system of bowel injury

Grade	*Description*
0	Normal mucosa
1	Subepithelial space at villus tip
2	More extended subepithelial space
3	Epithelial lifting along villus sides
4	Denuded villi
5	Loss of villus tissue
6	Crypt layer infarction
7	Transmucosal infarction
8	Transmural infarction

Park *et al.* (1990)[355]

slides with the pathologists. The purpose here, with reference to Park *et al.* (1990) was to confirm the key features that identify a sample belonging to one score or another and thus ensure consistency in the scoring process. For the purpose of analysis, the median score of the three scorers was used. Use of more than one value for each animal would distort the dataset by giving the data higher statistical power than it truly justifies.

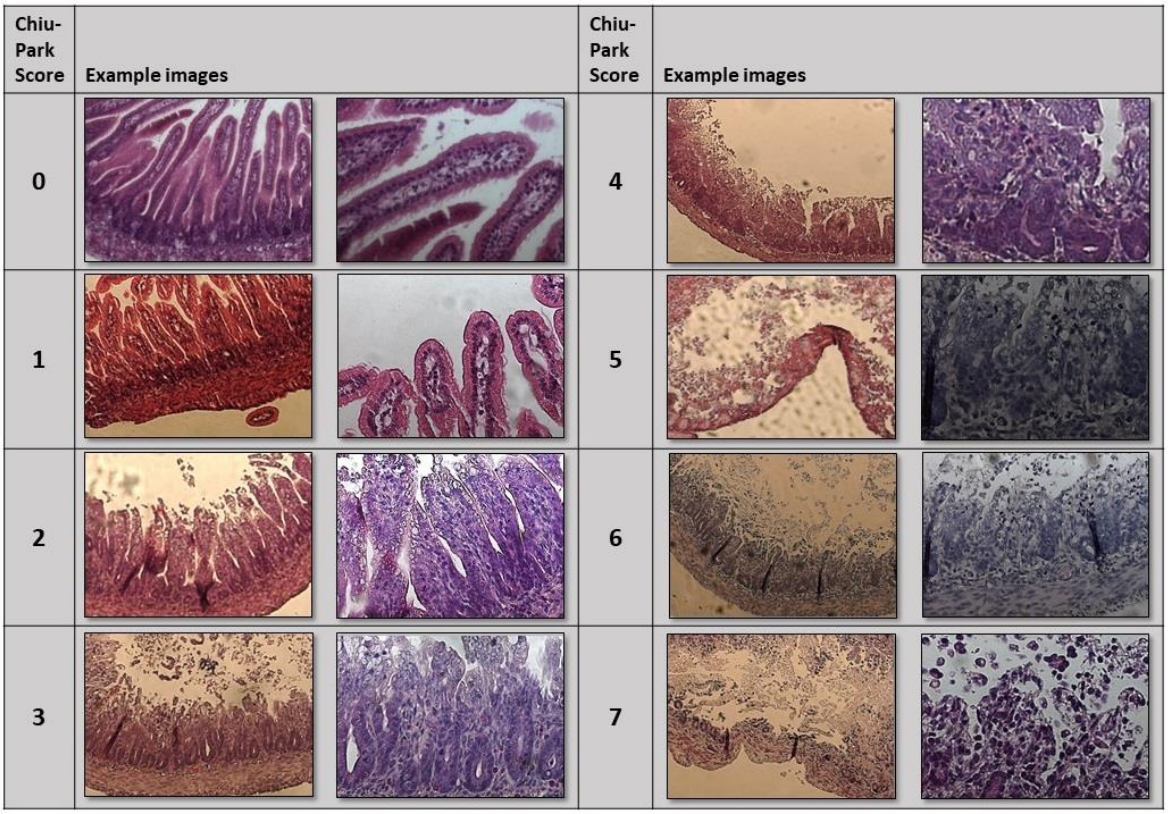


Figure 4-6 Example Images of Chiu-Park scores
Images show samples of bowel from rat pups exemplifying each of the Chiu-Park scores from 0-7. [Park *et al.* 1990][355] Chiu-Park score of 8 is not shown as none of my specimens were graded with a Chiu-Park score of 8. Each score is represented at both low-power (100x) and high-power (400x). Images captured with Zeiss Axioskop 2 microscope and Zeiss Axiocam (Carl Zeiss AG, Germany).

4.8.2.2 Comparison between scorers

The full details of the variations in scores between the three blinded, independent scorers are shown in Appendix B. Figure 4-7 shows the deviation of each scorer from the median score in each individual sample that was scored. This shows a high degree of agreement between the scorers, supporting the robustness and objectivity of the scoring system.

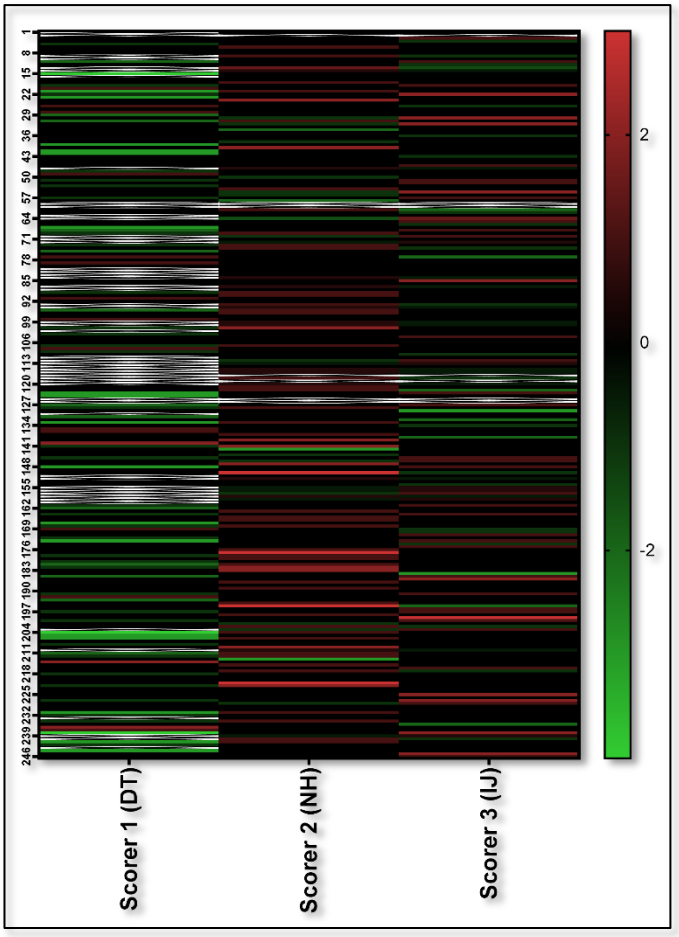


Figure 4-7 Variation in Chiu-Park scores between the three scorers
Heatmap shows the difference in the individual score compared to the median of all three scorers. (*Complete set of scores not yet available from Scorer 1).*[*]

4.9 Blood Serum Cytokine Assay

As described in Section 3.5.1, one of the mechanisms of RIC is via immune modulation. Blood cytokine levels would be expected to reflect systemic sepsis (which this model produces, reflecting the human disease) and also may provide important clues to the effector mechanism of RIC.

Blood Samples were centrifuged at 3000 rpm for 5 minutes to allow extraction of plasma which was then stored at -80^{O}C.

[*] In order to ensure the primary outcome measure (the Chiu-Park scoring) was as robust as possible, three scorers were used. Unfortunately due to the Covid pandemic, at time of writing, the complete set of scores is not available from the third scorer. Personal illness and increased clinical commitments created this delay. All the results presented in chapter 5 are based on three scorers. The subsequent chapters contain some data based on three, some based on 2. The *greyed-out* bars indicate incomplete scores.
The high agreement between the scorers overall means that this will very likely not affect the overall results. All scoring was independent and blinded.

I analysed the plasma extracted from experimental animals using the MSD® MULTI-SPOT Assay System (Proinflammatory Panel 2 (rat)) kit (Meso Scale Discovery®, USA). This assay measures IFN-γ, IL-1β, IL-4, IL-5, IL-6, KC/GRO (equivalent to human IL-8), IL-10, IL-13 and TNF-α in a single sample. Samples were thawed on the day of analysis and processed according to the manufacturer's instructions. Initial analysis with multiple dilutions showed that a single 1 in 4 dilution was sufficient to provide concentrations within the calibration curve and thus provide accurate quantification of each cytokine.

Interferon-gamma (IFN-γ) is produced by lymphocytes and is a potent activator of macrophages. Increased IFN-γ is seen in the intestine in NEC.[99] Serum IFN- γ has been shown to decrease in response to RIC. [361]

Interleukin-1 beta (IL-1β) is a pro-inflamatory cytokine released by activated macrophages. In experimental models of NEC, it has been shown to be raised in gut tissue and to promote Toll-like receptor 4 (TLR4) which is known to be part of the inflammation pathway of NEC.[362] Serum IL-1β rises in systemic sepsis and this rise is reduced by remote ischaemic condition – both pre- and post- conditioning.[363]

Interleukin-4 (IL-4) is produced by TH_2 cells. It activates B-cells and inhibits macrophages.[364] It also supresses IFN-γ production. There is some evidence that IL-4 is protective against NEC.[365] Moreover IL-4 expression may be increased by RIC.[366]

Interleukin-5 (IL-5) has no known role in NEC or RIC.

Interleukin-6 (IL-6) is secreted mainly by T cells and macrophages. It is involved in numerous biological processes, including inflammation, and apoptosis. IL-6 rises are seen after ischaemia-reperfusion injury, peaking at 6 hours post injury.[367] Increased levels of IL-6 are seen in NEC at the tissue level[99] and in the serum in response to sepsis.[363] Higher levels of IL-6 have been seen in response to ischaemia and these levels are increased further by the application of remote pre-conditioning. [292]

KC/GRO (keratinocyte chemoattractant / growth-related oncogene) is the rodent equivalent of IL-8 and is a neutrophil chemoattractant, its release is triggered by both IL-1β and TNF-α. [368] IL-8 is readily produced in the premature gut of humans and is raised both locally and systemically in NEC.[99]

Interleukin-10 (IL-10) is produced by TH_2 cells, promotes B-cell function and inhibits cytokine release by macrophages.[364] IL-10 increases are seen in human NEC.[99] RIC has been shown to reduce the systemic rise in IL-10 in response to ischaemia.[366]

Interleukin-13 (IL-13) is secreted by a range of immune cells and is involved in multiple biological processes. IL-13 increases are seen in human NEC[99] but no role in RIC has been found.

Tumour necrosis factor alpha (TNF-α) is produced by many cell types including macrophages and can induce apoptosis in some tumour cell lines. Tissue and systemic TNF-α increases in NEC as well as other IRI diseases.[99] This ischaemia-related rise of TNF-α is supressed by RIC.[369]

4.10 Hypoxia-inducible Factor 1 alpha Staining

Hypoxia-inducible factor 1 alpha (HIF-1α) accumulates in tissue subject to hypoxia. Chen *et al.* (2016)[136] demonstrated that HIF-1α accumulation can be seen in human NEC. In order to assess the accumulation of HIF-1α, samples processed as described in 4.5.1 and stored in wax underwent immunohistochemical staining with a HIF-1α (C-terminus) polyclonal antibody raised in rabbit. (Cayman Chemical Company, USA).

5 μm sections were cut and dewaxed with Tissue clear. Endogenous peroxidase was inhibited with 0.5% hydrogen peroxide in methanol and then washed with tris buffered saline (TBS). Antigen retrieval was done with microwave heating in a 1mM EDTA buffer (pH 8). (Optimisation work showed the EDTA buffer provided more reproducible staining than the recommended 0.01M citrate buffer (pH 6.0)). Avidin and biotin blocking was then applied (Vector Laboratories, USA) before applying blocking medium (made from Dubecoo's modified Eagle medium, foetal calf serum, bovine serum albumin and goat serum albumin).

The primary antibody (HIF-1α) was then applied at a concentration of 1:25 and incubated overnight at 4^{o}C. This was determined by trial runs with serial dilutions of 1:50, 1:100, 1:200, 1:400 and 1:800. There was significant variability in HIf-1α reactivity between batches. All of the experimental staining was done with a single batch at 1 in 25 as a consequence.

Biotinyated secondary antibody (Goat-anti-rabbit, Vector Laboratories, USA) was then applied for 30 minutes at a concentration of 1 in 1000 (in TBS). At the same time, avidin biotin peroxidase solution (Vector Laboratories, USA) was made at a concentration of 1 in 75 in TBS for each agent and allowed to form complexes. This was then applied for 30 minutes. A 3,3'Diaminobenzidine (DAB) substrate was then applied for chromagen staining for 5 minutes before counterstaining with haematoxylin for 1 minute. Due to the high concentration of HIF1-α antibody needed to get a signal, an isotype control was not performed. However, given the clear variable level of signal

between the samples, there was not particular concern about background staining interfering with the interpretation of the results.

The slides were then scanned with an Olympus VS110 high throughput Virtual Microscopy System (Olympus, Germany). The images were then prepared with Olympus VS desktop software. Based on the methodology described by Prasad *et al.*[370] the immunohistochemistry was quantified by counting the number of cells showing stain in randomly selected fields. For each sample, five randomly selected fields containing apical surface epithelium were examined and the left-most twenty cells examined. The number staining positive was counted. Summation of the numbers from all five fields thus yielded a percentage of cells staining positive.

4.11 Myeloperoxidase assay

Measurement of myeloperoxidase (MPO) activity provides quantitative assessment of neutrophil activity in tissue. This is necessary because whilst it is possible to measure MPO protein level, the actual activity can vary enormously between individuals even if the amount of protein is similar.[371] MPO is the most abundant proinflammatory enzyme stored in azyrophilic granules in neutrophils. It catalyses the formation of hydrochlorus acid from hydrogen peroxide and generates other higher reactive molecules including tyrosyl radicals.

Intestinal samples were homogenised with an Ultra-Turrax homogeniser in 2ml 50mmol/l potassium phosphate buffer, pH 6.0, containing 0.5% (w/v) hexadecyltrimethylammonium bromide. 100µl of homogenate was removed for protein estimation by the method of Peterson[372] and the remainder centrifuged at 18,500rpm for 30 minutes at 4^{O}C. A total of 100µl of supernatant was added to 2.9ml of potassium phosphate buffer containing 0.53mmol/l *O*-dianisidine hydrochloride and 0.0005% hydrogen peroxide. MPO activity was followed with spectrophotometric analysis at 25^{O}C and a wavelength of 460nm. The results are expressed in milliunits (mU) per mg of sample (i.e wet weight) and mU per mg of protein (dry weight). These two measures indicate the activity of the MPO by the tissue sample or by the quantity of protein present, correcting for potential oedema.

4.12 Nitric oxide measurement (nitrate/nitrite assays)

Nitric oxide (NO) is an endogenous mediator of multiple physiological processes. In the context of NEC, the normal function of NO is interrupted resulting in endothelial dysfunction. Measuring NO directly is difficult to measure directly due to its short half-life and low levels in vitro. Nitrite is a stable end product of autoxidation of NO in aqueous solutions. Nitrite is converted to nitrate by oxyhaemoglobin. Therefore NO levels can be reliably assessed indirectly by quantification of nitrite and nitrate levels.[373]

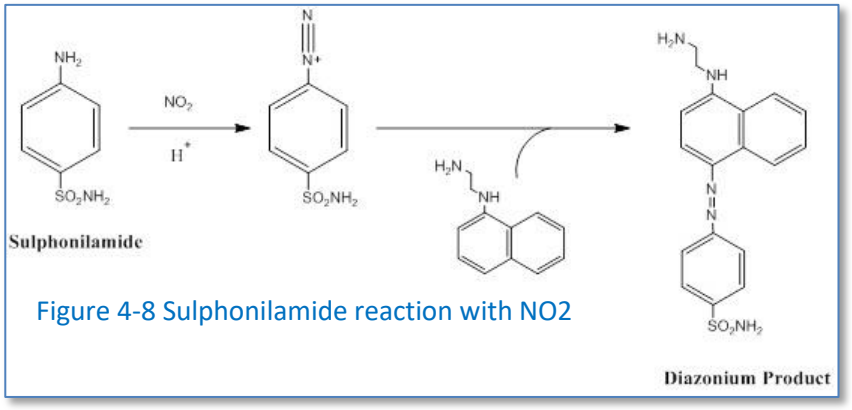


Figure 4-8 Sulphonilamide reaction with NO2

Nitrite ions react with sulfanilamide (in Griess reagent) under acidic conditions followed by coupling to the bicyclic amine N-1-(naphthyl)ethylenediamine (NEDD) to produce diazonium chromophore which can be quantified spectophotometrically at 540nm.

Nitrate is measured by the same method after its reduction to nitrite using Vanadium (III) Chloride.

Intestinal samples processed as described in Section 4.6.1 were used for this analysis. Griess reagent was made with equal parts of the following, 0.1% N-1-(naphthyl)ethylenediamine (NEDD) and 2% Sulphonilamide in 5%(v/v) HCl. Vanadium (III) chloride was produced by dissolving 400mg VCl_3 in 50mls of 1M HCl. Stock solutions of Nitrate (silver nitrate) and Nitrite (sodium nitrite) were used to create standard curves for each as shown in Table 4-2. As the assay measures nitrate by converting in to nitrite, true nitrate levels are determined by subtracting the nitrite assay from the nitrate assay.

$AgNO_3^-$ or $NaNO_2^-$ solution produced (µM)	Vol of 200µM Stock (µl) ($AgNO_3^-$ / $NaNO_2^-$)	Vol of Water (µl)
200	1000	0
100	500	500
75	375	625
50	250	750
20	100	900
10	50	950
2	10	990
0	0	1000

Table 4-2 Nitrate / Nitrite solutions for standard curve

Nitrite assay

100µl of sample (or standard) was added to 100 µl 1M HCl and 100 µl of freshly prepared Griess Reagent, incubated at room temperature for 30-45 minutes and the absorbance read at 540nm and 25^{O}C. Results are reported as nanomole of NO_3 per mg of protein.

Nitrate assay

100µl of sample (or standard) was added to 100 µl VCl_3 solution and 100 µl of freshly prepared Griess Reagent, incubated at room temperature for 30-45 minutes and the absorbance read at 540nm and 25^{O}C. Results are reported as picomole of NO_2 per mg of protein.

4.13 Malondialdehyde assay

Lipid peroxidation is a chain reaction caused by the reaction of reactive oxygen species and reactive nitrogen species with the polyunsaturated fatty acid residues of phospholipids. This is one of the mechanisms of membrane damage and cell death in ischaemia-reperfusion injury. Malondialdehyde (MDA) is a stable product of lipid peroxidation and thus is a usable biomarker of this process.[374]

Intestinal samples processed as described in Section 4.9 were used for this analysis. Standard solutions of MDA were prepared as shown in Table 4-3. 25 µl of sample (or standard) was added to 2 µl of freshly prepared 0.2% 2,6-Di-tert-butyl-4-methylphenol (BHT) in ethanol, 375 µl 1% phosphoric acid (H_3PO_4) and 345 µl of freshly prepared 30mM thiobarbituric acid (TBA).

MDA solution produced (µM)	Volume of 20µM MDA (µl)	Volume of water (µl)
0.0	0	1000
2.5	125	875
5.0	250	750
10	500	500
15	750	250
20	1000	0

Table 4-3 Malondiaehyde solutions for standard curve

Samples were then heated at 100^{o}C for one hour and then allowed to cool to room temperature.

200 µl of each sample was then placed in a fluorescence plate for reading with excitation filter 535 and emission filter 590. Results are reported as picomole of MDA per mg of protein.

The myeloperoxidase, nitric oxide and malondialdehyde assays were performed at University College, London, in the laboratory of Dr Simon Eaton.

4.14 Summary of Protocols

These methods were used for various animal experimentation protocols which are summerised in Table 4-4.

Protocol	Description	Chapter
1a	Remote Ischaemic *pre*-Conditioning delivered with a ligature	Chapter 5
1b	Remote Ischaemic *pre*-Conditioning delivered with a rodent blood pressure cuff	Chapter 6
2	Remote Ischaemic *post*-Conditioning	Chapter 7
3a	Remote Ischaemic *pre*-Conditioning (Early pre-conditioning)	Chapter 8
3b	Remote Ischaemic *pre*-Conditioning (Early *and* immediate pre-conditioning)	Chapter 8
4	Maternal Remote Ischaemic Conditioning	Chapter 10

Table 4-4 Protocols for animal studies.

4.15 Statistical Analysis

Statistical analyses were performed using Graphpad Prism 9.4.1 (Graphpad Software Inc.). For each of these comparisons non-parametric tests were used due to the small group size.

For the macroscopic injury measurement, Chiu-Park scores, cytokines concentrations, Hif-1α, myeloperoxidase, Nitric-oxide concentration and the malondialdehyde assay, a Mann-Whitney or Kruskal-Wallis test with post-hoc testing was used. Correlation testing was done with Spearman's Rank Coefficient.

For the histological assessment (both macroscopic and microscopic) only the direct comparison between the IRI group and the RIC group was performed as the key question is the difference between injury only and injury plus conditioning – i.e. testing if the conditioning changed the injury to the bowel. In the other assays (such as the blood cytokine assays for example) the negative controls provide an important comparator as the basal level of each cytokine (potentially in response to anaesthetic and laparotomy) and hence statistical testing between more than just the two groups was needed.

Chapter 5 Remote Ischaemic preConditioning

5.1 Abstract

5.1.1 Introduction

The purpose of this protocol was to investigate the potential of remote ischaemic pre-conditioning as a therapy in the intestinal IRI model of NEC.

5.1.2 Methods

Related rat-pups aged 10-13 days were randomly assigned to three experimental groups: *Controls*, *IRI only* and *RIC+IRI*. Control animals underwent Sham surgery only. Experimental animals underwent IRI by means of occlusion of the superior mesenteric artery (SMA) for 40 minutes, followed by 90 minutes of reperfusion. RIC was delivered by means of a ligature applied to the hind limb for three cycles of five minutes ischaemia, followed by five minutes of reperfusion.

Bowel injury was assessed macroscopically (by measuring the amount of bowel affected graded as normal, mild or severe) and microscopically using the Chiu-Park scoring system. Pro-inflammatory cytokine levels were measured in each animal and myeloperoxidase(MPO) activity was measured spectrophotometrically.

5.1.3 Results

Intestine of control animals (n=10) was macroscopically normal. The length of intestine that showed any injury was significantly reduced in animals exposed to RIC prior to IRI (n=16) compared to those who had IRI alone with no RIC (n=14, median 100% [IQR 85-100%] vs 58% [15-84%]; p=0.008). The length of intestine with severe necrosis was also shorter in animals exposed to RIC (RIC+IRI 4%(0-21%) vs IRI 78% (30-90%); p=0.002)

Microscopic assessment of intestine demonstrated a significantly reduced injury score in the RIC+IRI group compared to IRI alone. Median score 3.5 [range 0-6] vs 5.5 [4-7] (p=0.0019).

Blood levels of IL-10 and KC/GRO were higher in animals exposed to IRI compared with controls and showed a non-significant trend towards lower levels in animals that had RIC prior to IRI compared with IRI alone.

The median MPO level was 1.2U/mg (range 0.63-2.06) in controls, 3.36U/mg (1.43-9.77) in the IRI only group and 2.38U/mg (1.33-4.19) in the RIC+IRI group (p=0.046).

5.1.4 Conclusion

These results show that remote ischaemic *pre*-conditioning is protective in this model of NEC, supporting the primary hypothesis.

5.2 Introduction

As outlined in Section 3.4, remote ischaemic conditioning has been applied as both a pre- and a post- hoc intervention. In this chapter I will describe and report my experiments using pre-conditioning (preRIC) delivered just prior to anaesthesia and the results derived from this work.

In the clinical context of NEC, the hope is that RIC could be used as both a treatment for NEC and as a prophylactic intervention in at-risk neonates. These experiments are most analogous to the preventative clinical application. These experiments are referred to as protocol 1a.

5.3 Methods

The methods used are described in detail in chapter 4. In this protocol (1a), the animals underwent RIC immediately prior to anaesthesia. RIC was delivery by means of a ligature (Section 4.5.1). The ligature provides the simplest method (and therefore a reliable method) of delivering reliable limb RIC in very small animals. Development of this method to attempt to deliver RIC with measured pressured above systolic are described in Chapter 6.

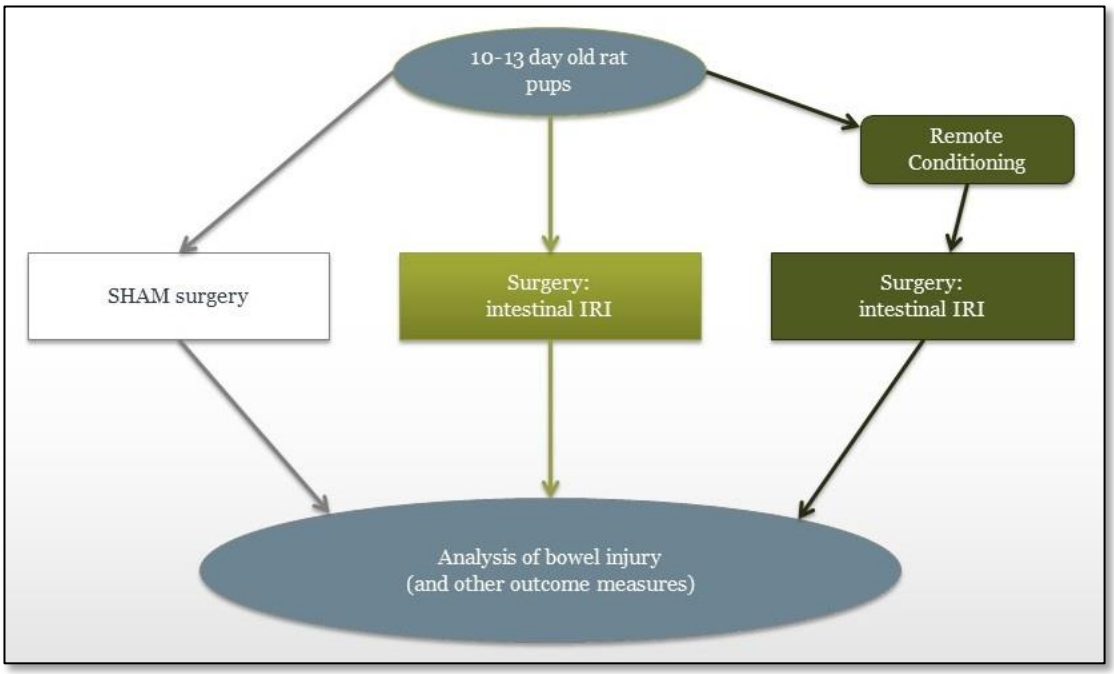


Figure 5-1 Experimental Protocol for Immediate preRIC.
Rat pups from multiple litters were randomly allocated to each experiment group.

Animals from multiple litters were randomly assigned to three groups; IRI only, RIC+IRI and SHAM surgery (Figure 5-1). Experimental animals had three cycles of 5 minutes RIC / 5 minutes reperfusion immediately prior to anaesthetic.

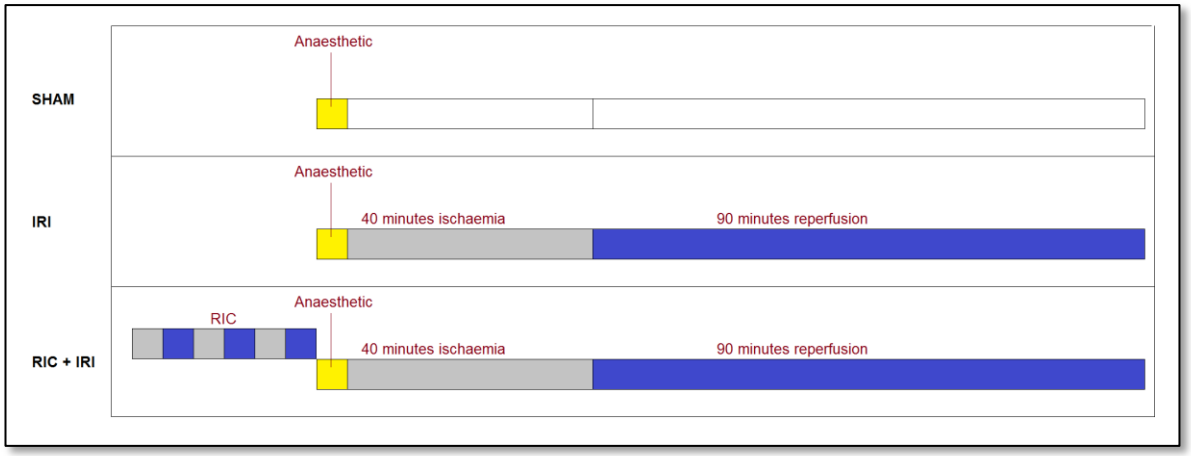


Figure 5-2 Experimental Protocol for preRIC.
Figure shows the timeline of RIC and injury

Macroscopic injury was assessed as described in section 4.7 and microscopic injury was scored with the Chiu-Park scoring system, described in Section 4.8. For each animal, a specimen was taken from the terminal ileum and from the area of maximal macroscopic injury. The median score from three independent scorers, blinded to which experimental group the specimen is derived from was used for analysis. (Comparison between the three scorers is shown in Appendix CAppendix C).

Serum levels of a panel of pro-inflammatory cytokines were measured at the end of the experiment as described in Section 4.9.

Expression of Hypoxia-inducible Factor alpha (HIF-1α) was assessed by immunohistochemistry as described in Section 4.10.

Neutrophil activity in the bowel was measured by means of myeloperoxidase activity (MPO) (Section 4.11). Myeloperoxidase activity is reported by sample weight as units per mg of sample (mU/mg) and by protein content as estimated by the methodology described (units per mg of protein (mU/mg). The samples were from the area of maximal macroscopic injury.

Nitrate and Nitrite levels were measured in bowel taken from the area of maximal macroscopic injury (Section 4.12). The results are reported by weight of protein for each sample. Nitric oxide levels are calculated by adding the nitrate(NO_3) and nitrite (NO_2) concentrations.

MDA was measured in bowel taken from the area of maximal macroscopic injury as described in Section 4.13 and are reported as concentration per mg of protein in each sample.

Spearman rank correlation analysis was used to determine the association between outcome measures; specifically the macroscopic and microscopic intestinal injury measures, the intestinal injury and cytokine serum levels and the intestinal injury and MPO activity.

5.4 Results

5.4.1 Macroscopic bowel injury

Figure 5-3 shows typical examples of the macroscopic injury pattern seen at the end of the reperfusion phase. Figure 5-4 shows an example specimen with normal, healthy bowel, mild ischaemic injury and severe injury present. Control animals showed no macroscopic injury. Animals exposed to IRI had a variable pattern of injury with some areas of normal tissue, some of mild injury and some severe. The median results for each group are recorded in Table 5-1 as a percentage of the total small bowel length. The range of total small bowel length removed from all animals was 27-41cm. (Full results in Appendix B).

Median length of bowel injury			
	Mild	Severe	Total
Controls	0%	0%	0%
IRI only	18%	78%	100%
RIC + IRI	41%	4%	58%

Table 5-1 Protocol 1a: Macroscopic injury. Full data in Table B- 1

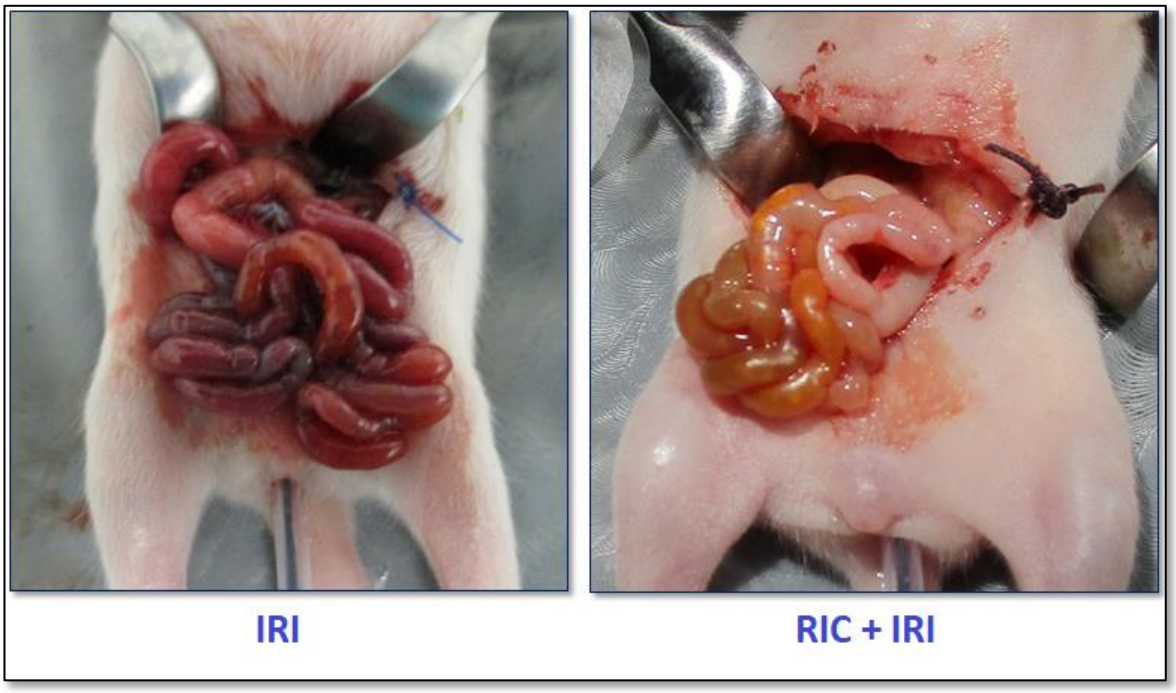


Figure 5-3 Macroscopic injury seen in this model
Images show an example of an animal exposed to IRI alone and an example of one that underwent RIC prior to injury. The animal exposed to IRI alone shows significant ischaemic injury throughout the small intestine, whilst the animal that underwent RIC prior to the same insult to the intestine shows healthy bowel with minimal injury seen.

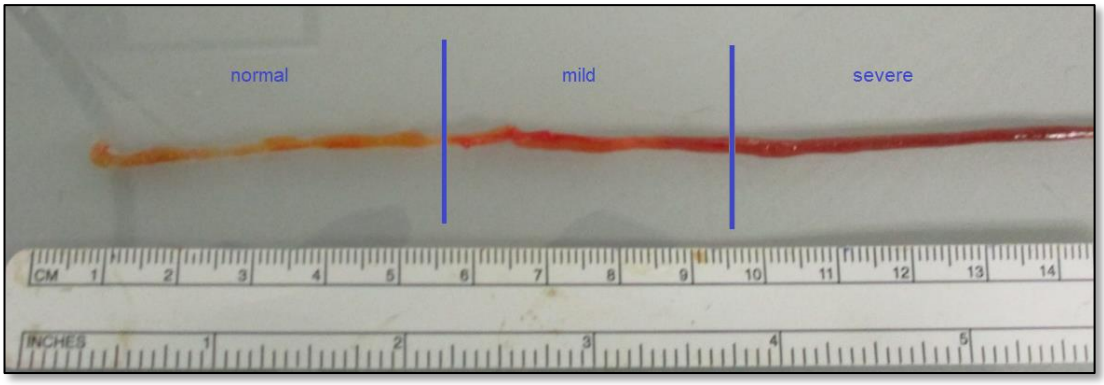


Figure 5-4 Example of macroscopic injury:
This specimen shows (left to right) 5cm normal bowel; 4cm of mild injury and >5cm of severe injury (necrosis).

The median length of injured bowel in the IRI group was 100% (range 0-100%) and 58% (0-100%) in animals that had undergone RIC prior to injury; p = 0.008 (Mann-Whitney test). Similarly animals in the IRI group had a median length of severe injury of 40% (0-100%), whilst in those that had undergone RIC, the median length of severely injured bowel was 0% (0-55%); p = 0.002 (Mann-Whitney Test). Figure 5-5 (total injury) and Figure 5-6 (severe injury) show these data graphically. Each data point is shown, as well as the median and interquartile range (IQR) for each group. Figure 5-7 shows the overall mean injury for each group in the form of pie charts.

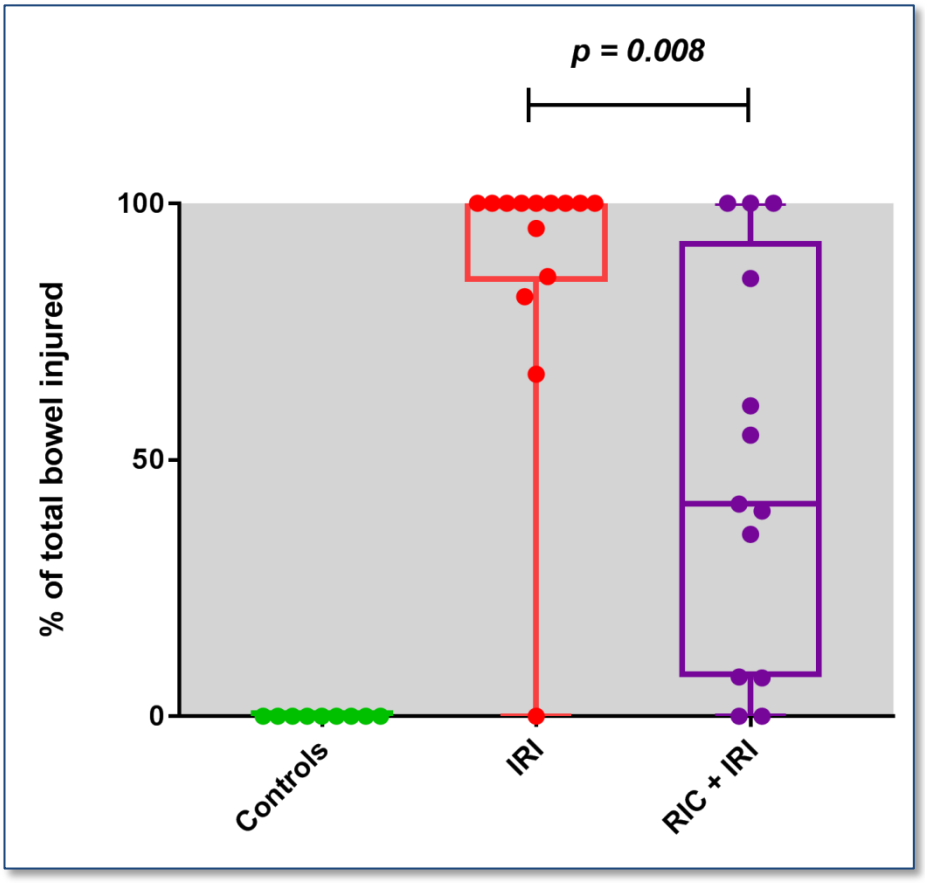


Figure 5-5 Protocol 1a: percentage of total bowel showing macroscopic injury.
Graph shows each data point, inter-quartile range and median

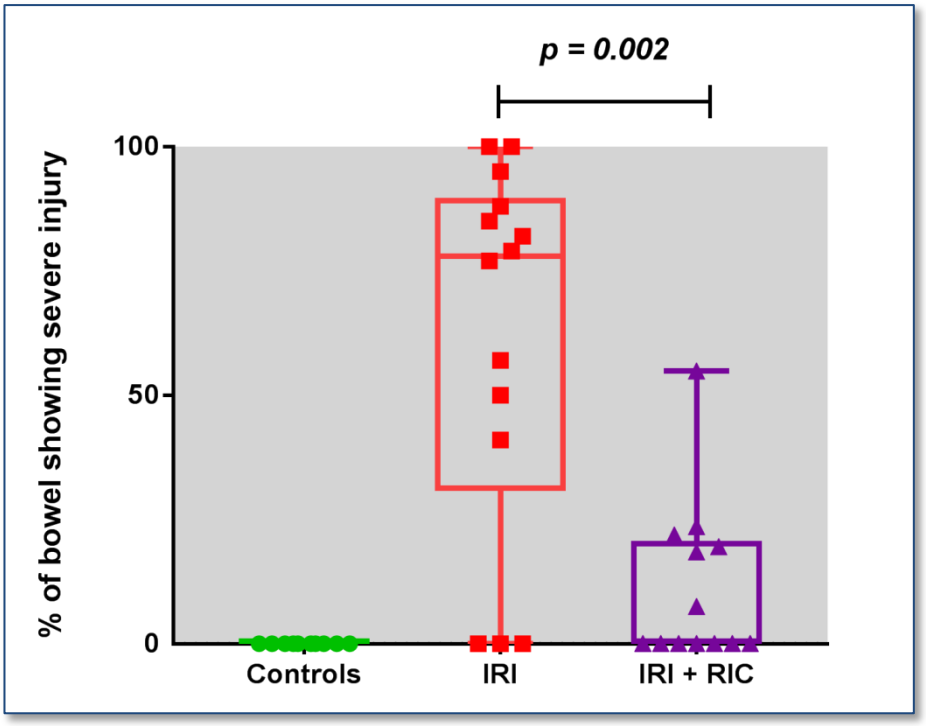


Figure 5-6 Protocol 1a: percentage of total bowel showing severe macroscopic injury. Graph shows each data point, inter-quartile range and median

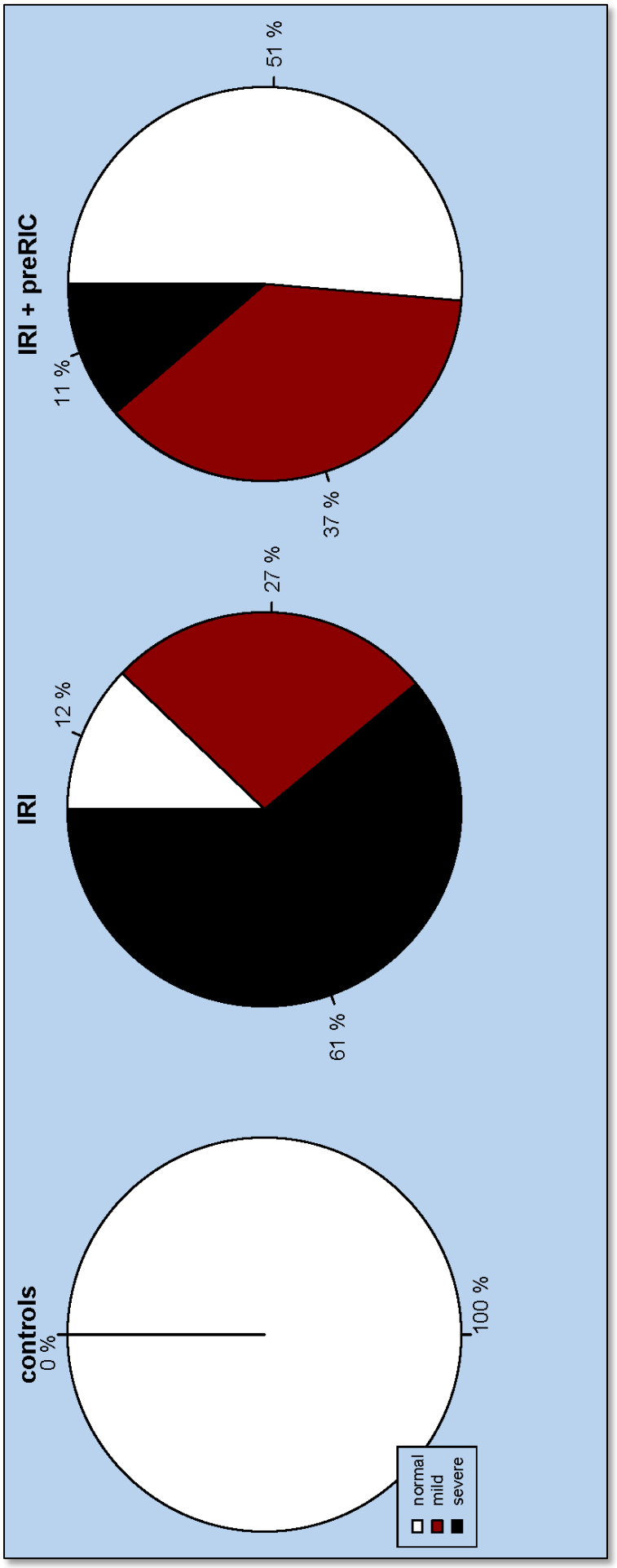


Figure 5-7 Protocoal 1a: Mean macroscopic injury for each group as a percentage of total bowel length. Controls show no macroscopic injury. The IRI group show predominately severe injury and overall the majority of the bowel is affected. In the RIC group, the area of severe injury is reduced as well as the total injury.

5.4.2 Microscopic bowel injury

Table 5-2 shows the Chiu-park scores for this experimental protocol. Control animals had a median Chiu-Park score of 0 with a range from 0-1 for both specimens taken from a fixed location in the terminal ileum and from the area of maximal macroscopic injury.

Animals that had undergone IRI only had a median score of 4 (IQR 3-5) for the terminal ileum sample and 5.5 (4-6) for area of maximal injury. The median scores for the terminal ileum sample in animals that had undergone RIC immediately prior to anaesthesia were 3.5 (1.25-5) and 3.5 (1.25-5). These are shown in Figure 5-8 and Figure 5-9. Statistical analysis with the Mann-Whitney test show no statistically significant difference between the IRI and RIC-IRI groups for the samples from a fixed location in the terminal ileum (p = 0.56, Figure 5-7).

	Controls		IRI only		RIC + IRI	
	TIF	MXF	TIF	MXF	TIF	MXF
Median	0	0	4	5.5	3.5	3.5
Range	0 - 0	0 – 1	0 – 7	4 – 7	0 – 6	0 - 6
IQR	0 - 0	0 – 1	3 – 5	4 - 6	1.25 – 5	1.25 - 5

Table 5-2 Protocol 1a: Median Chiu-Park scores for each group.
TIF = Fixed point in terminal ileum
MXF = Area of maximal macroscopic injury
Full results in Table B- 2

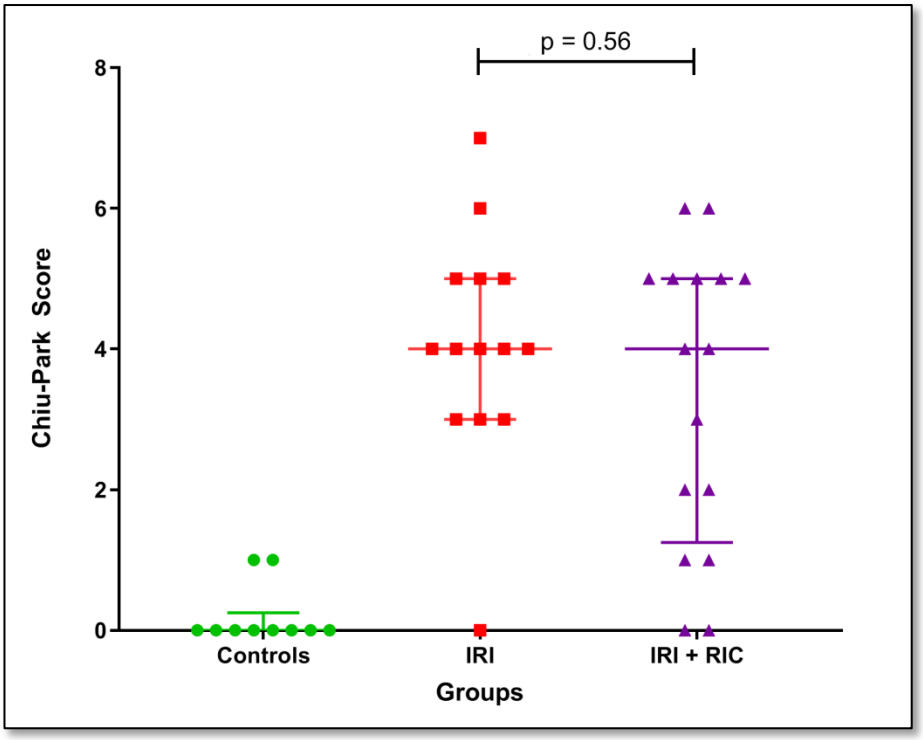


Figure 5-8 Protocol 1a: Chiu-Park Scores – fixed point in the terminal ileum.
There is no statistically significant difference between the IRI group and RIC+IRI group.

Comparison between the areas of maximal macroscopic injury showed that animals who underwent RIC prior to anaesthesia had lower scores than those that had IRI only. (p = 0.002, Mann-Whitney test, Figure 5-9).

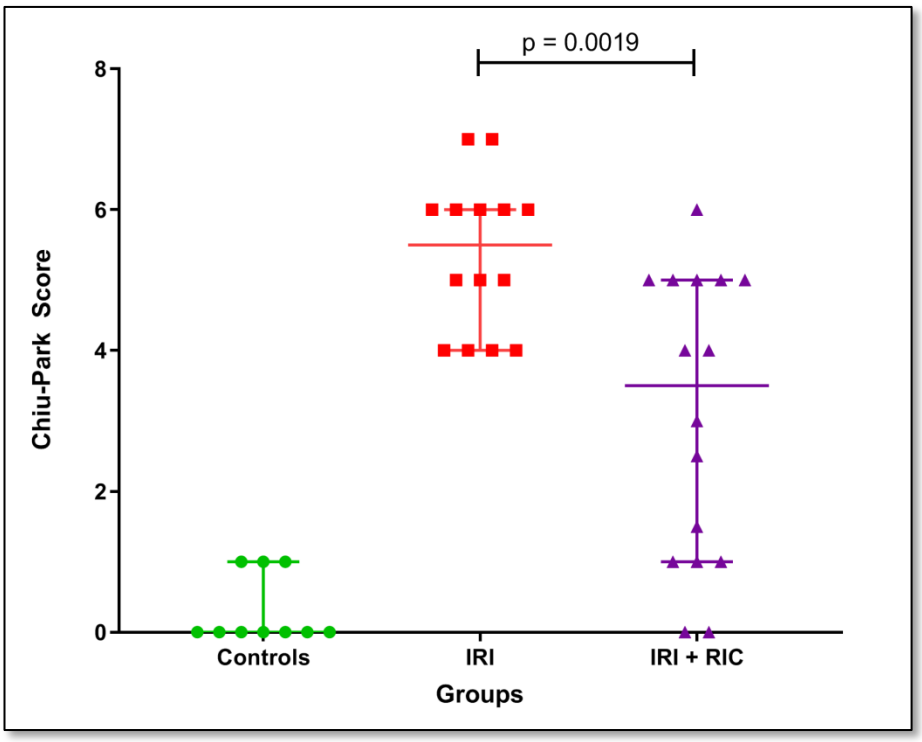


Figure 5-9 Protocol 1a: Chiu-Park Scores – area of maximal macroscopic injury
Animals exposed to RIC prior to IRI had lower scores than animals exposed to IRI alone.

5.4.3 Correlation between macroscopic and microscopic injury

Figure 5-10 and Figure 5-11 show the correlations between the macroscopic (% of bowel injured, unblinded) and microscopic injury (Chiu-Park scores, blinded) at the area of most severe macroscopic injury. This gives Spearman correlation coefficients of 0.57 (p = 0.003) and 0.46 (p = 0.0045) respectively.

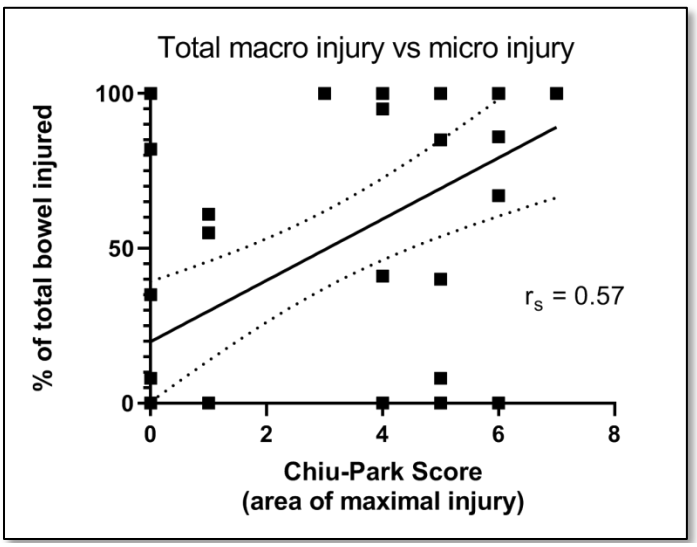


Figure 5-10 Protocol 1a: Total macroscopic injury vs Chiu-Park score (area of maximal injury)

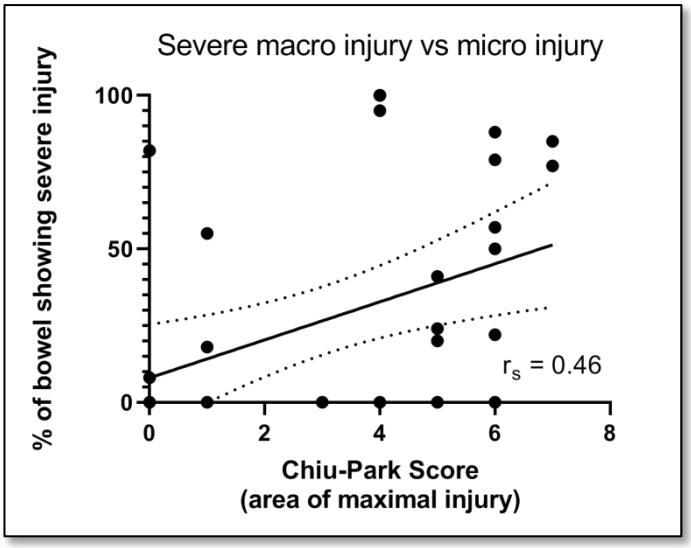


Figure 5-11 Protocol 1a: Severe macroscopic injury vs Chiu-Park score (area of maximal injury)

5.4.4 Hypoxia-inducible Factor Alpha

Staining for HIF-1α was performed as described in section 4.10. Some samples showed no accumulation of HIF-1α (as determined by immunohistochemistry staining). The stain was evident on the apical epithelial cells with no staining seen within the crypts. In many cases, cells that were free within the lumen of the intestine showed strong staining. Examples are shown in Figure 5-13. Quantification of the staining was performed as described, with counts out of 100 of the number of cells showing stain, based on five randomly selected fields. The median score for the SHAM group was 1 (IQR 0-27.5), 34 (19.25-58) for the IRI group and 21 (5-54) for the RIC+IRI group. Kruskal-Wallis analysis (for multiple comparisons of non-parametric data) showed a statistically significant difference between the groups (p =

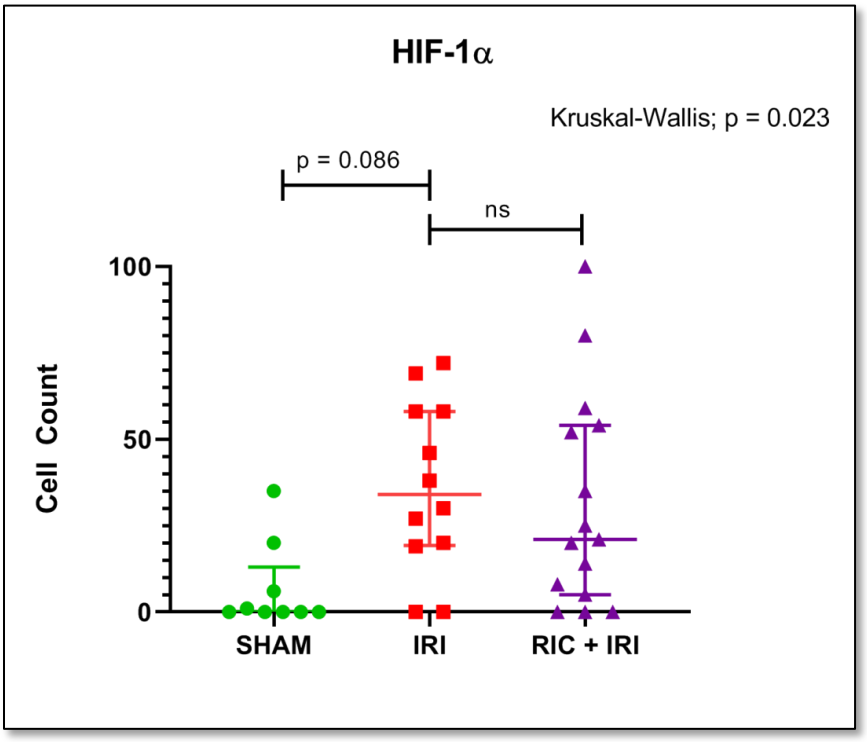


Figure 5-12 Quantification of HIF-1α staining.
Graph shows cell counts for each sample of cells showing positive staining for HIF-1α. Each sample is plotting with median and IQR.

0.023) but post-hoc testing showed no difference (Figure 5-12).

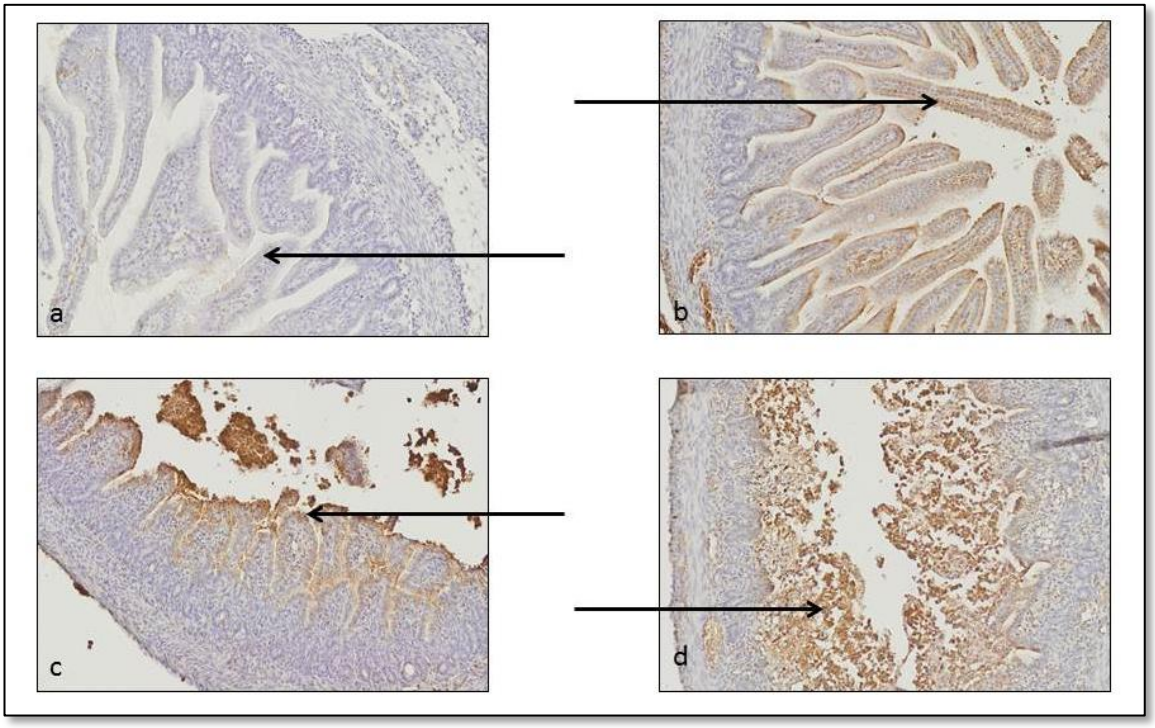


Figure 5-13 HIF-1α Immunohistochemistry: Chromagen staining with haematoxylin counterstain. Images show examples of the staining pattern seen (Brown indicates the presence of HIF-1α. Image a) shows negative staining (no HIF-1α present). Images b) and c) are examples of staining on the epithelial cells. Stain is seen on the apical surface of the villi in b) and on the truncated villi in c) but not in the crypts. Image d) shows cells free within the lumen showing chromagen staining. (These were not counted in the scoring).

5.4.5 Serum Cytokine Analysis

Table 5-3 shows the median concentration, IQR and range for each cytokine for each group.

	Controls			IRI			RIC + IRI		
	Median	IQR	Range	Median	IQR	Range	Median	IQR	Range
IFN-γ *(pg/ml)*	1	0 - 1.6	0 - 3.2	0.6	0 - 1.4	0 - 4.3	0.8	0 - 1.4	0 - 3.1
IL-1β *(pg/ml)*	0	0 - 3.5	0 - 34.1	0	0 - 3.9	0 - 33.1	0	0 – 0	0 - 6.5
IL-10 *(pg/ml)*	16.5	11.0 - 24.2	9.1 - 39.2	45.5	25.7 - 56.5	0 - 74.3	35.3	31.3 - 48.7	0 - 75.8
IL-13 *(pg/ml)*	4	2.4 - 5.2	1.3 - 10.6	2.1	1.1 - 4.5	0 - 8	2.6	1.5 - 4.0	0 - 9.7
IL-4 *(pg/ml)*	0	0 – 0	0 - 0.3	0	0 - 0.2	0 - 0.3	0	0 – 0	0 - 1.8
IL-5 *(pg/ml)*	10.8	0 - 23.1	0 - 57.6	0	0 - 57	0 - 72.3	10.9	0 - 25.2	0 - 70.7
IL-6 *(ng/ml)*	1.1	0.4 - 2.2	0.3 - 4.5	1.8	0.8 - 2.5	0 - 6.6	1.8	1.2 - 2.2	0.8 - 2.5
KC/GRO *(ng/ml)*	13.5	12.0 - 15.4	8.8 - 22.9	21.3	18.4 - 26.4	0 - 39.8	21.3	16.4 - 26.2	13.4 - 29.3
TNF-α *(pg/ml)*	13.4	10.3 - 21.4	8.2 - 27.7	19.15	13.1 - 29.0	0 - 42.2	18.6	13.9 - 27.3	12.6 - 41.8

Table 5-3 Protocol 1a: Blood cytokine levels.
Full results in Table B- 3 Protocol 1a: Blood cytokine levels..

5.4.5.1 IFN-γ, IL-1β, IL-13, Il-4, IL-5 and Il-6

In the assays of these cytokines, there was no statistically significant difference between the groups (Kruskal-Wallis test, Figure 5-14). These box & whisker plots show group median, IQR and range, as well as each data point.

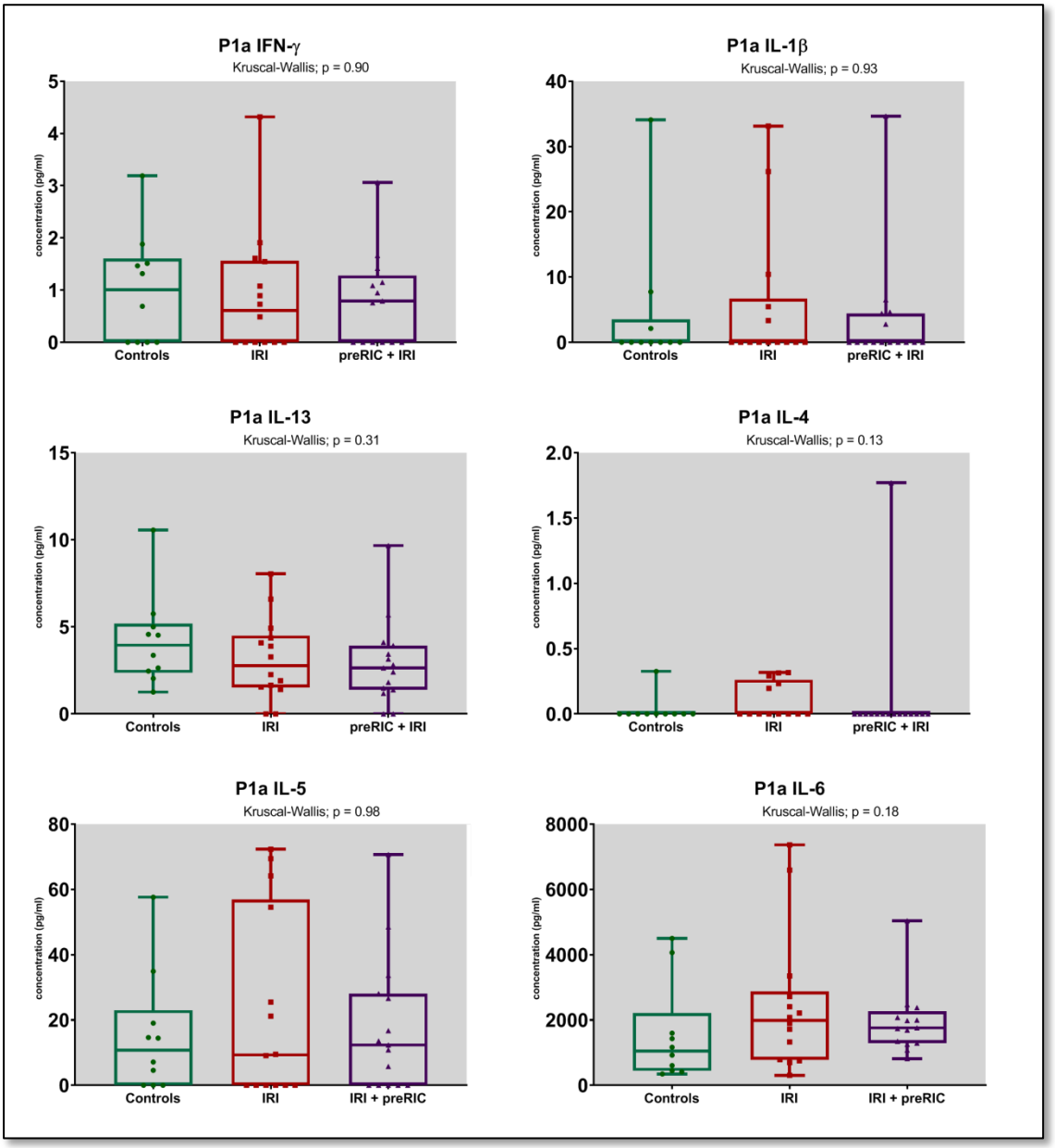


Figure 5-14 Protocol 1a: Box and whisker plots of serum cytokine blood levels
Median, IQR and range as well as each data point shown for IFN-γ, IL-1, IL-13, IL-4, IL-5 and Il-6.

5.4.5.2 IL-10, KC/GRO and TNF-α

Multi-way non-parametric comparison of IL-10 and KC/GRO (which is the rodent equivalent of human IL-8) in each group using Kruskal-Wallis testing, showed that each of these cytokines were significantly raised in animals exposed to IRI. However, in each case, there was a no statistically

significant difference between the animals exposed to RIC prior to IRI compared to IRI only (based on post-hoc testing). These results are shown in Figure 5-15, Figure 5-16 and Figure 5-17.

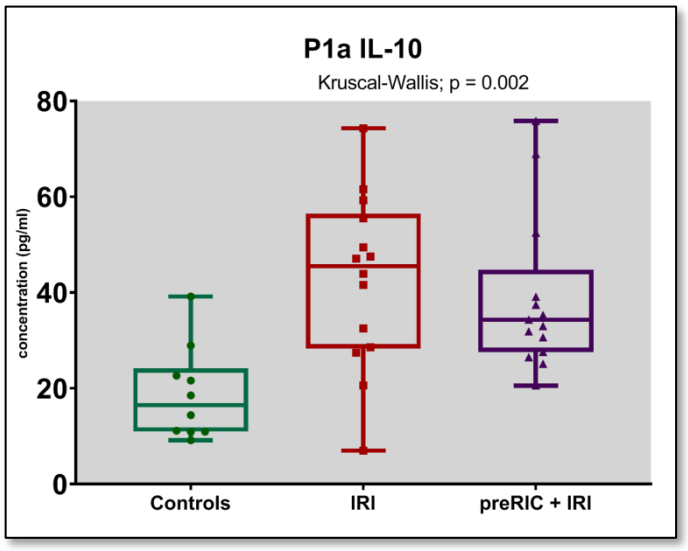


Figure 5-15 Protocol 1a: Box and whisker plots of cytokine serum levels: IL-10
Graph shows each data point, median, IQR and range.

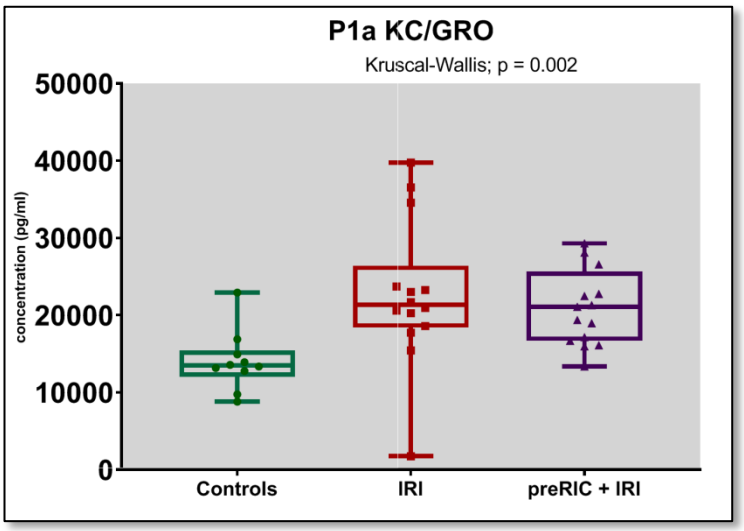


Figure 5-16 Protocol 1a: Box and whisker plots of cytokine serum levels: KC/GRO
Graph shows each data point, median, IQR and range.

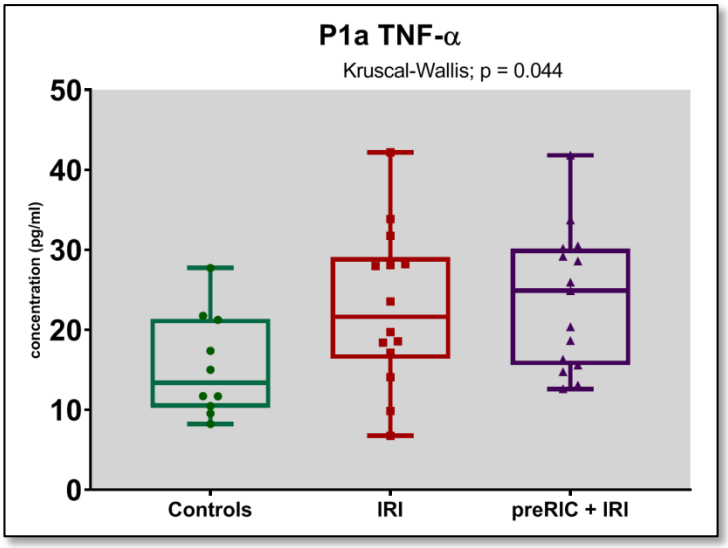


Figure 5-17 Protocol 1a: Box and whisker plots of cytokine serum levels: TNF-α
Graph shows each data point, median, IQR and range.

5.4.5.3 Cytokine and Histological Injury Regression analyses

Regression analyses of the cytokine concentrations with the histological assessment of bowel injury (both macroscopic and microscopic) were performed. Systemic IFN-γ, IL-1β, IL-13, IL-4, IL-5 and Il-6 showed no correlation with the bowel injury. Conversely IL-10, KC/GRO and TNF levels did show a significant positive correlation with the histological assessment of bowel injury (Figure 5-19, Figure 5-18 and Table 5-4).

Cytokine	Macroscopic		Microscopic	
	r_s	P	r_s	P
IL-10	0.59	< 0.0001	0.57	0.0004
KC/GRO	0.57	0.0002	0.60	0.0002
TNF-α	0.47	0.0026	0.47	0.0049

Table 5-4 Protocol 1a: Correlations between bowel injury and cytokine levels (Spearman's rank correlation for no-parametric data).

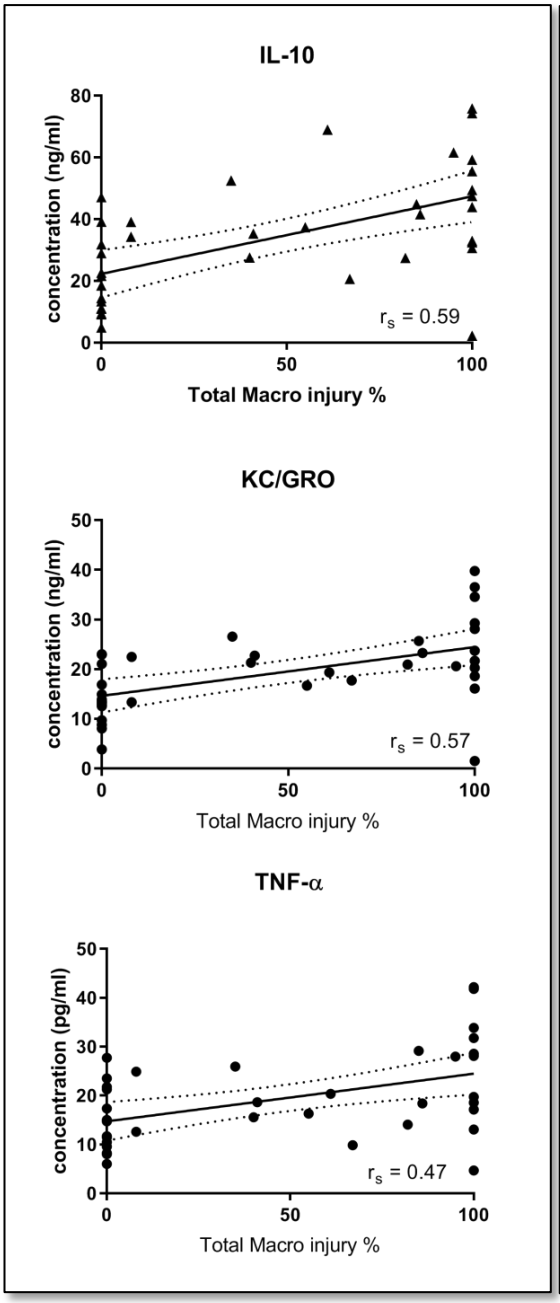


Figure 5-19 Protocol 1a: Macroscopic injury vs serum cytokine levels (IL-10, KC/GRO and TNF-α). Data shown from all three groups (SHAM, IRI and RIC+IRI).

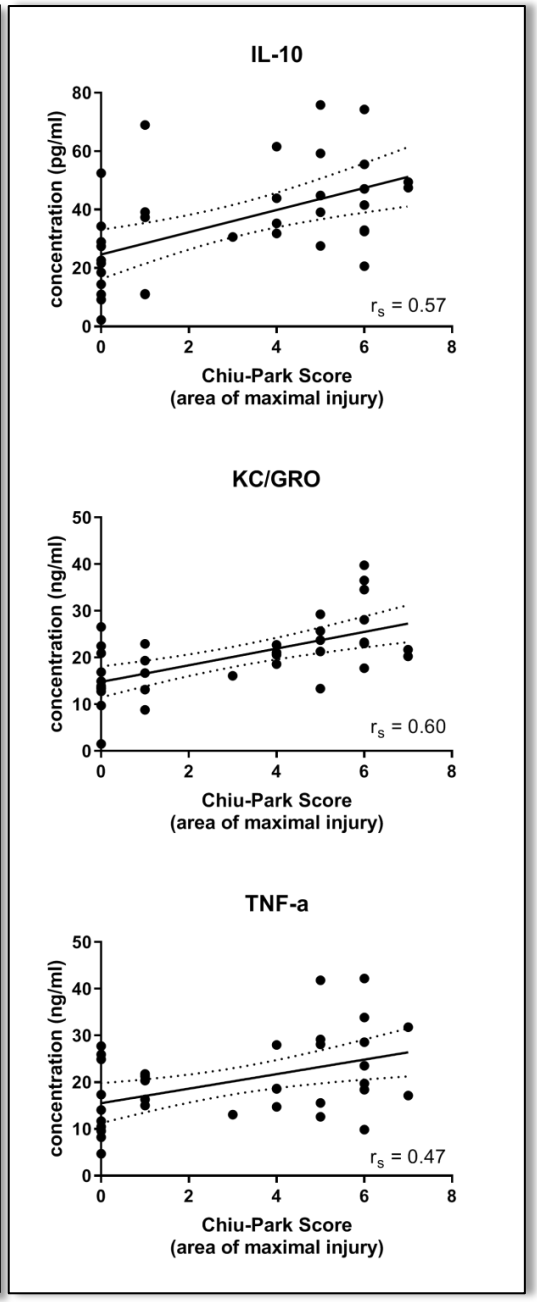


Figure 5-18 Protocol 1a: Microscopic injury vs serum cytokine levels (IL-10, KC/GRO and TNF-α). Data shown from all three groups (SHAM, IRI and RIC+IRI).

5.4.6 Myeloperoxidase

5.4.6.1 Myeloperoxidase activity results

Myeloperoxidase activity was quantified and calibrated by both the weight of the sample and the weight of protein within the sample (corresponding to dry weight). By both measures, myeloperoxidase was higher in animals exposed to injury than the controls. Median activity in the controls was 1.2 mU/mg of tissue and 12.9 mU/mg of protein. In animals exposed to IRI only the median activities were 3.36 mU/mg of tissue and 24.2 mU/mg of protein.

Animals who underwent RIC prior to IRI had lower MPO activity with medians of 2.38 mU/mg (p=0.012, Mann-Whitney) of tissue and 22.2 mU/mg of protein (p=0.59). This is summarised in table 5-5, Figure 5-20 and Figure 5-21.

	Controls		IRI only		RIC + IRI	
	Activity by sample weight	Activity by protein content	Activity by sample weight	Activity by protein content	Activity by sample weight	Activity by protein content
Median	1.2	12.9	3.36	24.2	2.38	22.2
Range	0.63 - 2.06	6.82 - 19.5	1.43 - 9.77	12.2 - 44.0	1.33 - 4.19	14.7 - 37.2
IQR	0.95 - 1.53	9.60 - 15.69	2.50 - 3.71	18.4 - 33.7	1.62 - 2.82	18.2 - 28.8

Table 5-5 Protocol 1a: MPO activity for each group
Full data in Table B- 4

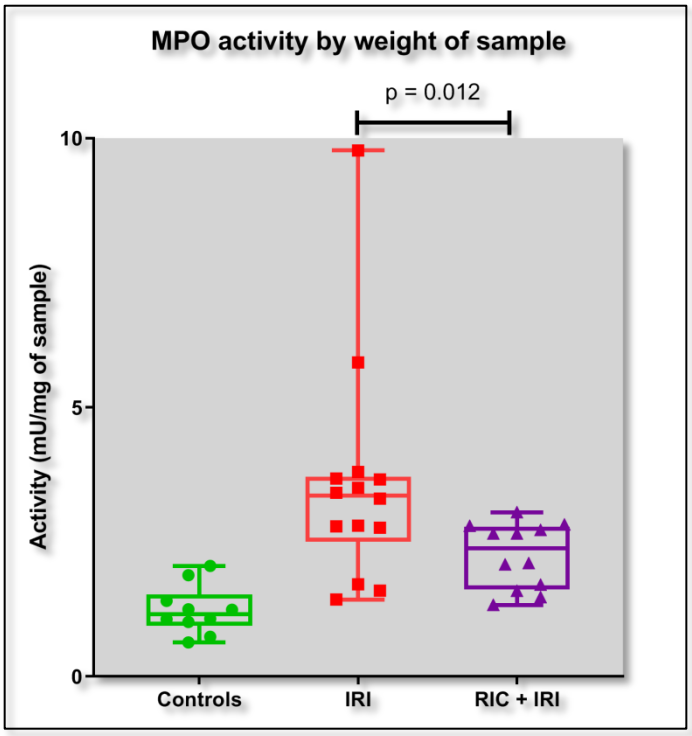


Figure 5-20 Protocol 1a Myeloperoxidase activity by weight of sample.
Graph shows each value, median, IQR and range. Mann-Whitney Test.
Kruskal-Wallis: p < 0.0001

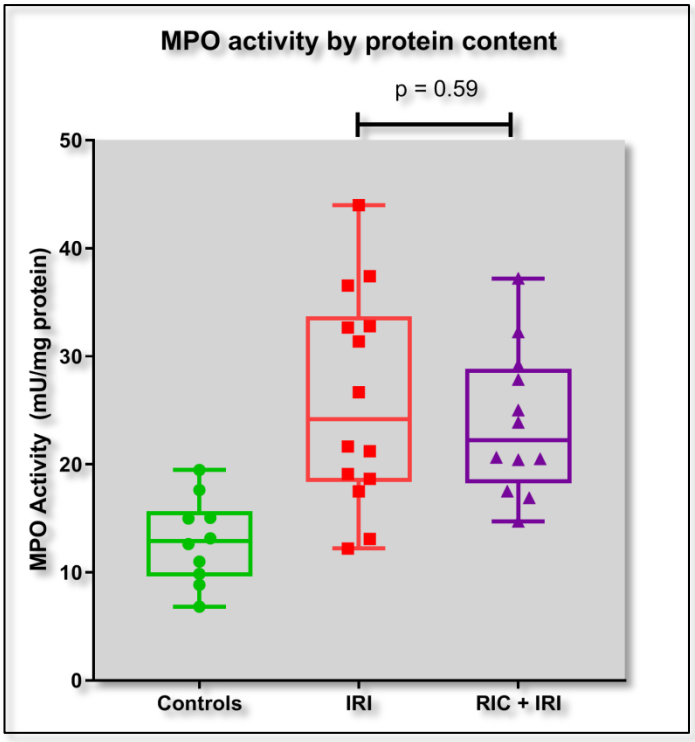


Figure 5-21 Protocol 1a MPO activity by protein content.
Graph shows each value, median, IQR and range. Mann-Whitney Test.
Kruskal-Wallis p = 0.0008

5.4.6.2 Myeloperoxidase activity correlations with histological injury

As described in 5.4, the histological injury was assessed both macroscopically and microscopically with the Chiu-Park scoring system. The MPO activity was compared with both of these and showed moderate, statistically significant correlations between both the microscopic injury scores. The macroscopic injury correlated with the MPO activity by weight of sample (p=0.005) but not by protein content (p = 0.15) (Table 5-6, Figure 5-22).

	MPO activity by weight of sample	**MPO activity by protein content**
Macroscopic injury	r_s = 0.45 (p = 0.0054)	r_s = 0.25 (p = 0.15)
Microscopic injury	r_s = 0.55 (p = 0.018)	r_s = 0.39 (p = 0.0006)

Table 5-6 Correlations between histological injury and MPO activity

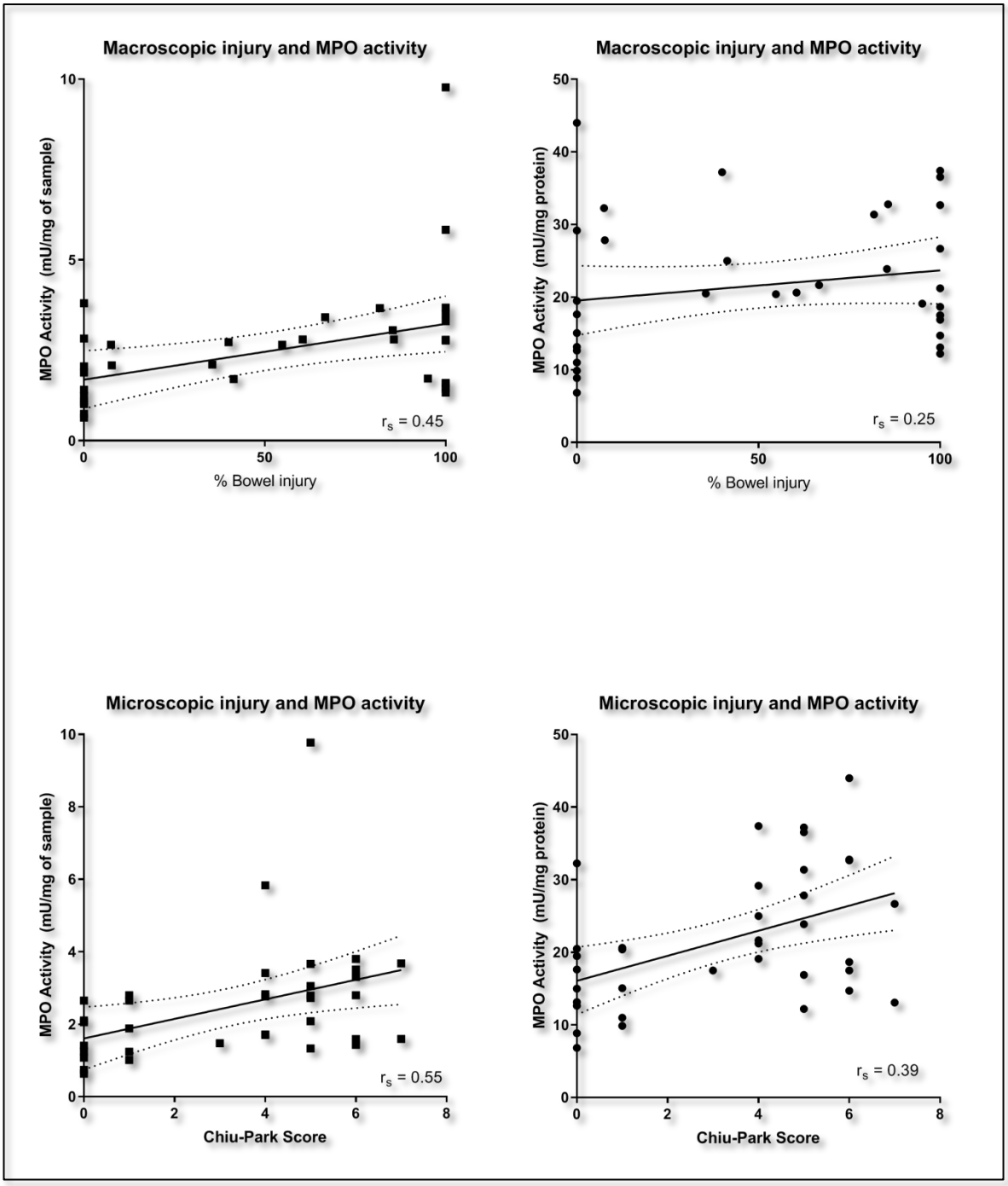


Figure 5-22 Protocol 1a: Correlation between MPO activity and histological injury

5.4.7 Nitric Oxide

Nitric oxide levels are calculated by adding the nitrate (NO_3) and nitrite (NO_2) concentrations. Table 5-7 shows the measured concentrations of nitrite (NO_2) and nitrate (NO_3) in each sample as well as the calculated nitric oxide concentration. The mean concentration of nitric oxide was 3.5µM/mg in controls, 3.42µM/mg in the IRI group and 3.8µM/mg in animals exposed to RIC prior to IRI (p = 0.57, Kruskal-Wallis test).

Figure 5-23 shows the standard concentration curves for nitrite and nitrate. Figure 5-24 shows the nitric oxide results for each group (median, IQR and range shown).

Nitric Oxide (NO_2 + NO_3)	Controls	IRI	RIC+IRI
Median	2.45	3.67	3.6
Range	0.9 – 12.0	1.1 - 7.08	2.2 - 6.7
IQR	1.73 - 3.79	2.13 - 3.85	2.9 - 3.8

Table 5-7 Protocol 1a: Nitric oxide levels in intestinal samples samples.
Full data in Table B- 5.

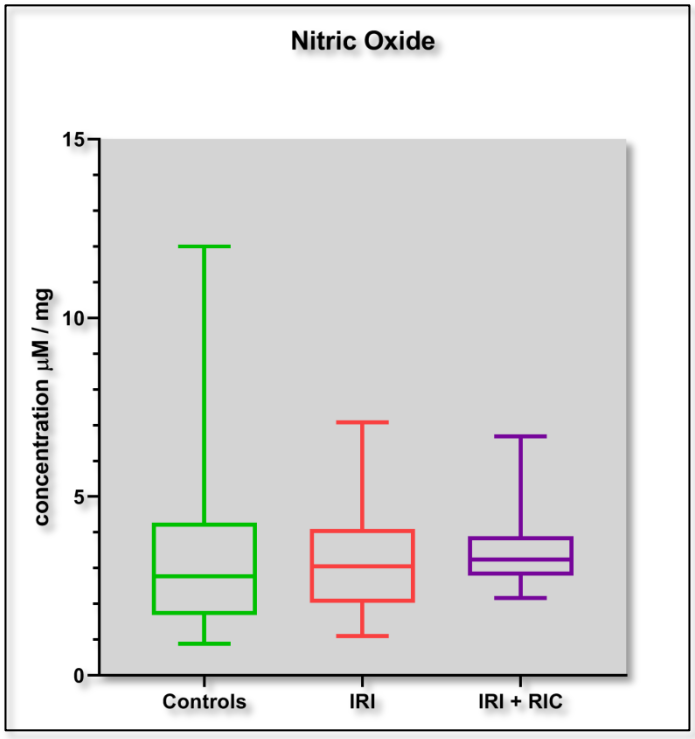


Figure 5-23 Protocol 1a: Nitric Oxide levels in bowel specimens

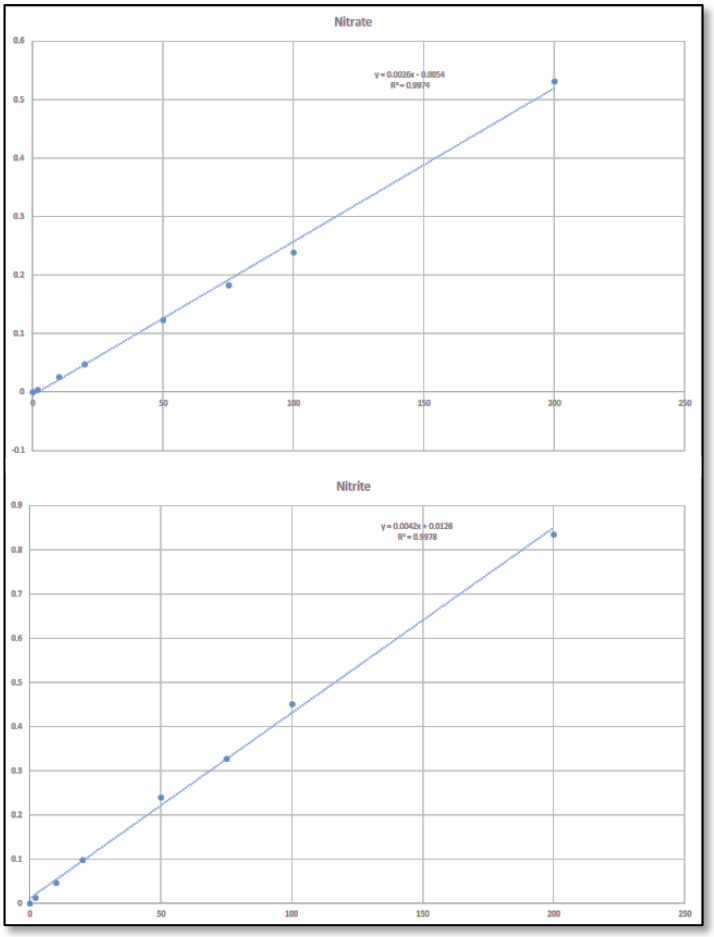


Figure 5-24 Protocol 1a: Standard Nitrate and Nitrite curves

5.4.8 Malondialdehyde assay

The median concentration of MDA was 374pmol/mg in controls, 780pmol/mg in animals exposed to IRI alone and 809pmol/mg (p = 0.39, Kruskal-Wallis test). The results for each sample are shown in Table 5-8 shows the standard curve for MDA and Figure 5-25 shows the results for each group (median, IQR and range shown).

MDA Concentration (pmol/mg)	**Controls**	**IRI only**	**RIC + IRI**
Median	**374**	**524**	**717**
Range	**47 - 1490**	**200 - 1812**	**117 – 2500**
IQR	**99 - 882**	**457 - 1137**	**415 – 920**

Table 5-8 Protocol 1a: MDA levels in bowel samples. Full data in Table B-6

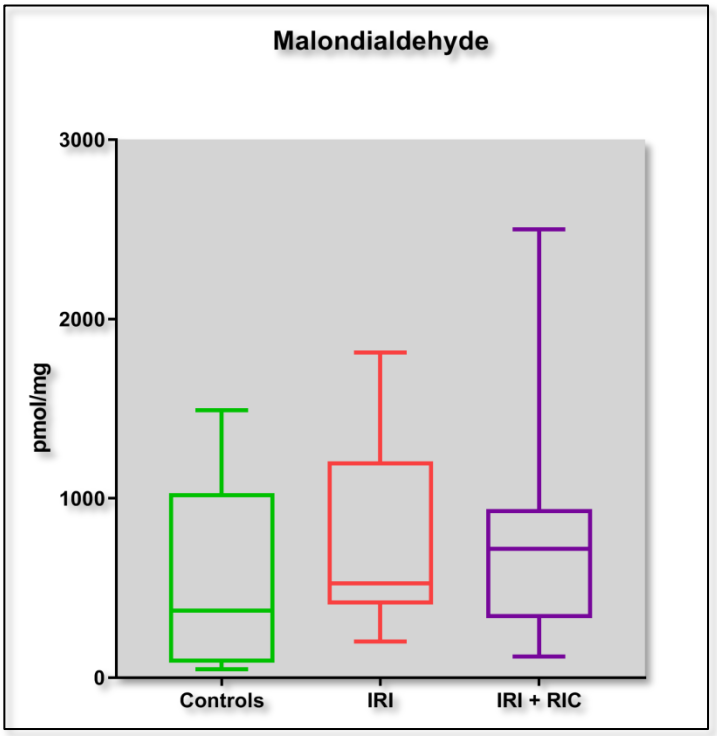


Figure 5-25 Protocol 1a: MDA concentration in bowel samples. Graph shows median, IQR and range.

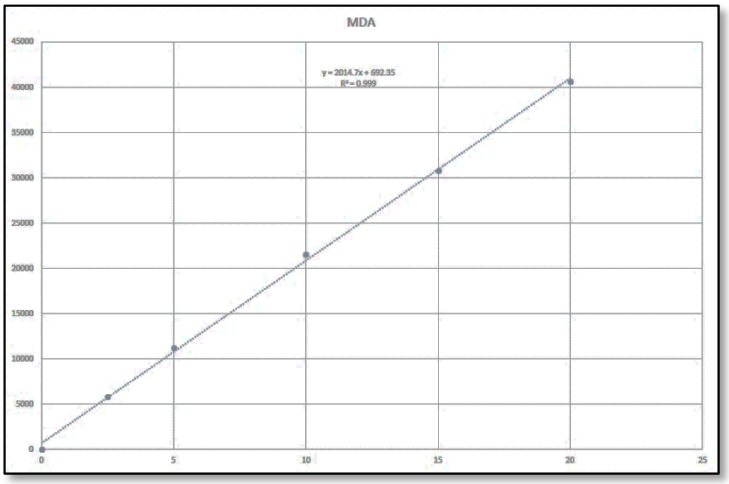


Figure 5-26 Protocol 1a: MDA standard curve

5.5 Discussion

5.5.1 Histological injury

These series of experiments were designed to test the hypothesis that in this model of NEC, RIC would reduce the bowel injury and systemic harmful effects if delivered just prior to injury (remote ischaemic pre-conditioning, RIPC).

Measurement of macroscopic extent of injury is a crude assessment but is directly relevant to human disease. The extent of bowel disease is unsurprisingly correlated with prognosis.[375] This model produces a variable pattern of disease in each individual animal which most likely reflects the variable sensitivity of tissue to ischaemia and the variable nature of collateral circulation in the bowel. This pattern of injury is archetypal of human NEC. These data show a large reduction in the extent of injury when remote conditioning is given prior to injury, although there is variability between individual animals.

The main limitation of this measure is that it is inevitably unblinded. Once the animals are euthanised, there is a rapid change in the quality of the issue and thus only an instant analysis of the extent of injury is possible. Moreover, photographic recording of the whole bowel is difficult to standardise; still pictures do not tend to accurately reflect the state of the tissue. However, the size of the effect seen is likely to exceed any potential observer bias but the validated microscopic scoring was chosen as the primary outcome measure as this is not vulnerable to observer bias. Petrat *et al.* (2010)[351] demonstrated that this macroscopic appearance does correlate with microscopic injury and suggested a non-linear scoring scale that could be used. In these particular experiments this was impractical for a single investigator to perform. However, given that the Chiu-Park score was used as the primary outcome measure, this is not of critical importance. The macroscopic appearance was a vital guide to the fact that the model was working as expected.

Two portions of the bowel were scored with the Chiu-Park system. Tissue taken from a fixed point in the terminal ileum (5-7cm from the ileo-caecal valve) do not show a statistical difference between the IRI and RIC+IRI groups. There is a trend towards RIC+IRI being protective. Where the microscopic analysis and scoring is done on the area of maximal macroscopic injury, there is a clear protective effect of RIC given prior to anaesthetic ($p=0.012$). The IRI group show a clustering around scores 4-7, whereas the RIC+IRI group have a much broader spread, from scores of 0 - indicating no quantifiable injury - up to 6 (essentially the same as the IRI-only group). This pattern suggests that some individuals have a much more profound response to RIC than others. Although this finding could be a reflection of the limitations in the experimental model; specifically the delivery of RIC. The effectiveness of RIC was only demonstrated by the colour change in the limb

as pulse-oximetry proved to be totally unreliable in active (very mobile) rat pups. The fact that samples from the terminal ileum did not show a clear difference most likely reflects the variable vulnerability of the bowel to injury. The RIC+IRI group have a similar pattern of severity to the samples taken from the area of maximal injury (Figure 5-9 and Figure 5-8) but the terminal ileum samples in the IRI-only group did not have consistently high scores in the way that the area of maximal injury did. In human NEC, the terminal ileum is known to be a particularly vulnerable site although the exact reasons for this are unclear. The blood supply is essentially a terminal branch of the superior-mesenteric artery but it is not a true watershed area like the distal transverse colon. There are also anatomical differences between the rat and human bowel, especially in terms of the caecum and the physiology is probably not replicated fully.

Regression analysis of the macroscopic and microscopic injury shows a positive correlation between the two. With total injury the Spearman coefficient (R_s) was 0.57 ($p = 0.003$) and for severe macroscopic injury, $R_s = 0.46$ ($p = 0.0045$). This moderate correlation confirms the relevance of the macroscopic measure. It is obviously, not a perfect correlation but they are measures of different (though related) things. The macroscopic measure reflects the extent of injury across the susceptible bowel. Which in this model is all of the small bowel. The Chiu-Park score is a grading system of severity of injury. The weaker correlation with the proportion of 'severe injury' could be easily over-interpreted but some animals had a relatively small amount of visible severe injury but none of the bowel was normal, hence the 'severe injury' on its own is not a complete measure of the extent of disease. As discussed in Section 4.7, Petrat *et al.* (2010)[351] demonstrated the validity of the macroscopic assessment. This correlation is useful collaboration that this unblinded assessment of bowel injury is valid. Microscopic analysis of every segment of the bowel would be theoretically desirable but impractical. The use of these two measures together provides the same information.

In terms of human disease, both the extent and severity of the intestinal injury are important. Severity of injury correlates with systemic effects, risk of perforation and mortality.[92] Extent of injury also correlates with systemic effects and extensive resection leads to an increase risk of intestinal failure with potentially life-long morbidity.[197]

5.5.2 Hypoxia-inducible Factor Alpha

HIF-1α staining was chosen because of the demonstrated pattern in human disease shown by Chen *et al.* (2016)[136] and because of its ubiquitous role in responding to oxidative stress in all tissues. The down-stream effects of HIF-1 are many and varied and include; angiogenesis, cell proliferation, energy metabolism, wound healing and tissue regeneration.[376]

The regulation of HIF-1 is largely at the post-translational level. Functional HIF-1 is made up of two subunits (α and β). Both of which are constitutionally expressed but in normoxic conditions, the α-subunit is broken down very rapidly.[377-379] The half-life is estimated at only a few minutes.[379] This breakdown is achieved by hydroxylation of two proline residues and thus is an oxygen dependent process. Therefore when oxygen levels fall, this process is inhibited and HIF-1α accumulates.

Reliable antibody staining of HIF-1α can be difficult to achieve. For example, Pampín *et al.* (2006)[380] report that without amplification, they could only achieve staining that was poor and unsatisfactory. Therefore, it is perhaps not surprising that the final dilution needed to achieve signal was 1:25 and thus there is a risk of over-staining. However, many samples and the TBS controls did not show staining suggesting the required specifity. HiF-1α was essentially only seen in the epithelial cells.

The immunohistochemistry staining for HIF-1α showed that it was accumulating on the epilthelial surface of the villi and not within the crypts. In many specimens, the stain was evident in cells that had sloughed off from the epithelial surface. This pattern of expression is consistent with the hypoxic insult to the tissue. The villi tips being most prone to the loss of perfusion. Conservative statistical analysis would rely on Kruskal-Wallis testing for multiple comparisons which does show a statistically significant difference between the groups, but post-hoc testing did not reach statistical significant. The pattern of staining shows that there is more HIF-1α accumulation in the samples from the intestine of animals exposed to IRI than in controls. There is a non-significant trend towards lower scores in animals exposed to RIC prior to IRI.

What is not clear from these data is whether HIF-1 may be simply a marker of ischaemic injury and thus may be expected to be lower in the RIC+IRI group (as they show less injury that IRI alone) or whether the pathways of RIC are downstream from HIF-1 and thus unaffected. I.e. HIF-1α is a marker of hypoxia, whether it plays any role in the protective mechanism of RIC is unknown. There does not appear to be any relevant literature to address this question.

5.5.3 Serum cytokine analysis

Due to the size of the animals and their correspondingly small circulating volumes, a multiplex cytokine analysis was used in order to perform multiple measures on the same sample. The MSD pro-inflammatory panel includes nine cytokines that are potentially informative in this context. Most of these cytokines showed no differences between the groups. Interleukin-10 (IL-10), Keratinocyte chemoattractant / growth-related oncogene (KC/GRO) and TNF-α were higher in animals exposed to IRI than in controls. In each case there was a non-significant trend towards lower levels in animals who underwent RIC prior to IRI.

KC/GRO is the rodent equivalent of IL-8. IL-8 and is released in the premature gut of humans. Analysis has shown that levels are raised both in the gut and systemically in NEC.[99] The raised levels seen in these data show that as well as producing the NEC-like bowel injury, the model is replicating some of the systemic effects. Similarly IL-10 has been shown to increase in human NEC. Moreover, Liu *et al.*(2019) have shown that systemic rises in IL-10 due to IRI are reduced with remote conditioning.[366] The non-significant trend shown in Figure 5-15 Protocol 1a: Box and whisker plots of cytokine serum levels: IL-10is consistent with this finding. IL-10 is released in the presence of IRI but this release is attenuated by RIC. TNF-α is known to rise in NEC and other IRI diseases and this rise is attenuated by the application of RIC.

The major limitation in this analysis is that the cytokine levels are assessed at a single time point (at the end of the reperfusion phase). This is an unavoidable technical limitation of this model in rat pups; it is not possible to obtain sufficient volumes for analysis at any other stage of these experiments as the extraction of even 0.5ml of blood inevitably renders the animals hypovolemic. In addition, these analyses are limited by the small numbers but the experiment was only powered for the primary outcome so this is unsurprising. The single time point also may be the explanation for the lack of observed changes in the other cytokines; systemic responses are not instantaneous and the ideal time point for observing this is not known. It is also important to note that the control animals still underwent a general anaesthetic and a laparotomy and thus have had a significant physiological insult even though it is much less than the experimental groups.

Correlation analysis of each of the cytokines against the macroscopic and microscopic measures of injury showed relationships between IL-10, KC/GRO, TNF-α and the degree of histological injury (Figure 5-19 and **Error! Reference source not found.**). Again, this supports that the model is producing systemic effects that reflect the level of bowel injury.

These cytokine data provide very limited information about the mechanisms of RIC in this context as very few meaningful changes in cytokine levels were detected and the injury seems to be responsible for a far greater change in cytokines that the ischaemic conditioning. Conversely, they do support the effectiveness of the model in mirroring systemic effects and the degree of bowel injury (in both extent and severity) is reflected in systemic changes in cytokine levels for some cytokines at the end point of 90 minutes reperfusion.

5.5.4 Myeloperoxidase Activity

Myeloperoxidase (MPO) activity is a very sensitive marker of tissue inflammation.[381] In these results this is reflected by the fact that the MPO activity levels by both measures are significantly higher in animals that were exposed to IRI compared to controls. When the MPO is quantified by

the protein content, there is a clear trend to lower MPO activity in animals who underwent RIC prior to injury but this was not statistically significant. Conversely, when MPO was quantified by tissue weight, this difference was statistically significant (Figure 5-20 and Figure 5-21). The MPO activity by tissue weight reflects the 'wet weight' of the tissue – i.e. it would be increased by inflammation and hence increase the denominator. Most likely the difference between these two measures (i.e. one reaching statistical significance and the other not) is probably a reflection that the study was underpowered for this measure.

The correlation between MPO activity and histology corroborates and supports the histological assessment of bowel injury. It has previously been reported that MPO is a reliable indicator of inflammation in bowel tissue.[381]

5.5.5 Nitric Oxide

The role of NO in both NEC and IRI is well established.[135,257] As such, one might expect to see some meaningful differences in the NO levels in the bowel whether due to the injury or to the preconditioning. The harvesting of these samples at one time point (i.e. after 90 minutes of reperfusion and over two hours after RIC) may be the explanation for this lack of change being seen. Whilst sampling at other times would be desirable, it is not possible within the limitations of this model. (Figure 5-23).

5.5.6 Malonodialdehyde

Given that MDA is a stable product of lipid damage, it is disappointing that these data do not show either a meaningful difference in animals exposed to injury compared to controls on in those who underwent RIC (Figure 5-25).

5.6 Conclusion

The combination of various measures of bowel injury (macroscopic assessment, microscopic using the Chiu-Park scoring system MPO activity, and HIF-1α immunohistochemistry) collectively show that this model is producing a consistent bowel injury with is to a measurable extent attenuated by the application of RIC prior to injury. Therefore, these data support the main hypothesis that remote ischaemic conditioning will reduce the bowel injury in this model of NEC, a finding which could be applicable to human patients.

Chapter 6 Method Development: Remote Ischaemic Conditioning with a Blood Pressure Cuff

6.1 Abstract

6.1.1 Background

Protocol 1a (Chapter 5) demonstrated that RIC delivered by means of a ligature reduced the extent and severity of bowel injury in this IRI model of NEC. The purpose of this work was to use a blood pressure cuff inflated above systolic pressure to deliver RIC. The aim of this protocol was to establish that the blood pressure cuff could be an effective means of delivering RIC and thus variable pressures could be used to develop empirical data on whether the exact pressures had an impact on the protective effect of RIC.

6.1.2 Methods

Experimental animals underwent IRI by means of occlusion of the superior mesenteric artery (SMA) for 40 minutes, followed by 90 minutes of reperfusion. The comparator groups of Controls (sham surgery) and the IRI-only (injury without RIC) were taken from protocol 1a. Randomly assigned, 11-13 day old related rat pups underwent RIC by means of a rodent blood pressure cuff inflated to 150mmHg on the hind limb for three cycles of five minutes ischaemia, followed by five minutes of reperfusion.

Bowel injury was assessed macroscopically (by measuring the amount of bowel affected; graded as normal, mild or severe) and microscopically using the Chiu-Park scoring system.

6.1.3 Results

The blood pressure cuff used did not reliably maintain the pressure on the limb above systolic.

Animals exposed to RIC prior to IRI had a median of 68% of the bowel showing injury (range 59-86%) compared with 100% for those that had IRI only (p= 0.0009). Similarly, the median length of severe necrosis was 15% of the bowel (0-74%) compared with 61% for those that had IRI alone (p = 0.012).

The median Chiu-Park score was 5.5 (range 4-6) in the area of maximal injury in animals who underwent RIC by means of the blood pressure cuff compared to 5.5 (4-7) in the animals who had IRI alone (p = 0.97).

6.1.4 Discussion

This blood pressure cuff system does not deliver RIC as reliably as the simple ligature (despite various attempted modifications). For this reason, in the subsequent protocols looking at the different timings of RIC, the ligature rather than the blood pressure cuff will be used.

6.2 Introduction

The experimental work, detailed in Chapter 5 demonstrated that hind-limb RIC delivered using a simple ligature reduced both the extent and the severity of the bowel injury in this model of NEC. The purpose of this experimental work was to reproduce these results with a murine blood pressure cuff applied to the hind limb. This is desirable as the ligature only provides an 'all or nothing' approach to inducing the short periods of limb ischaemia necessary for RIC. Little empirical data exists on what pressures are necessary to achieve effective RIC. Logically, it is expected that the cuff merely needs to maintain a pressure slightly above systolic blood pressure for the time periods. However, this has not been formally demonstrated and in the large body of experimental work that has looked at RIC, multiple different protocols have been used. Moreover given the known neuronal pathway of RIC, it is conceivable that the effectiveness of RIC is not purely determined by achieving a pressure above systolic, and there may be additional benefits to higher pressures.

6.3 Methods

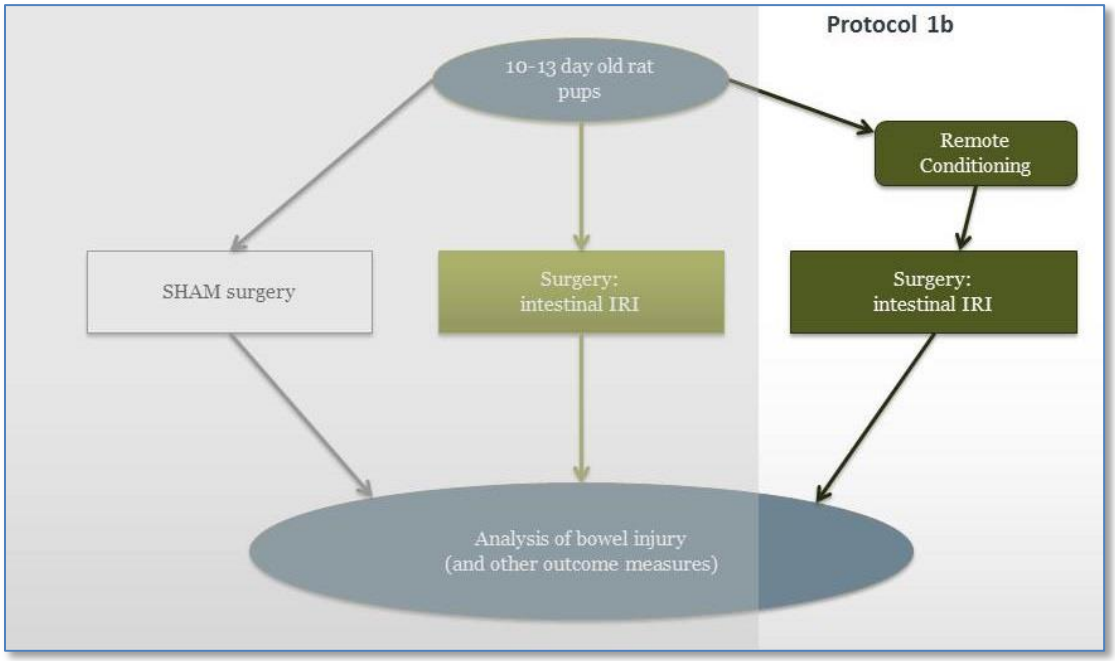


Figure 6-1 Experimental Protocol 1b - preRIC delivered by means of a blood pressure cuff Sham surgery and IRI only animals were from Protocol 1a.

The methods used are described in detail in Chapter 4. In this protocol (1b), the animals underwent RIC immediately prior to anaesthetic (as in protocol 1a). RIC was delivered by means of the Columbus instruments NIBP-8 (Columbus Instruments, USA) blood pressure cuff (Section 4.5.2) inflated to 150mmHg. Statistical analysis was performed using GraphPad Prism 9.1.2. The macroscopic injury measurements and the Chiu-Park scores were analysed with the Mann-

Whitney Test. This experimental group is directly equivalent to the 'RIC+IRI' group in Chapter 5 (protocol 1a). The only difference is the precise means by which the RIC was delivered. As such the comparator groups (Sham surgery and IRI only) are taken from the previous protocol. This was done to minimise the number of animals used (in keeping with the standard '3 R's principle') and because logically these positive and negative controls remain appropriate to these experiments. Interim analysis of protocol 1a showed a positive result for RIC and hence 1b was started before 1a was completed. Similarly 1b was run concurrently with protocol 2 (Chapter 7). This was done so that the animals were randomly chosen from each litter to minimise variability between the experimental groups. Interim analysis was performed with 11 animals in this group.

6.4 Results

The key observation in these experiments was that the blood pressure cuff did not maintain its tension throughout the timed periods and thus the delivery of remote conditioning was not as reliable as with a ligature. Despite clamping the tubing once the desired pressure was achieved, the cuff had a 'slow-leak' which meant the taughtness evident in the cuff initially was lost. There was no reliable way with this system to measure or maintain the pressure.

6.4.1 Macroscopic Bowel Injury

The median length of bowel showing mild injury was 59% of the bowel and severe 15%. The Median total injury was 68% (range 59-86%, Table 6-1). The range of bowel harvested was 31-44cm in total. Figure 6-2 and Figure 6-3 show these data plotted against the Controls and IRI only groups from protocol 1a. Both the total injury and the severe injury were significantly different to the IRI-only animals, p = 0.0009 and p = 0.012 respectively.

Median length of bowel injury			
	Mild	Severe	Total
Controls*	0%	0%	0%
IRI only*	18%	78%	100%
RIC + IRI	59%	15%	68%

Table 6-1 Protocol 1b: Macroscopic injury.
*Controls (Sham surgery) and IRI only taken from protocol 1a.
Full data in Table B-1

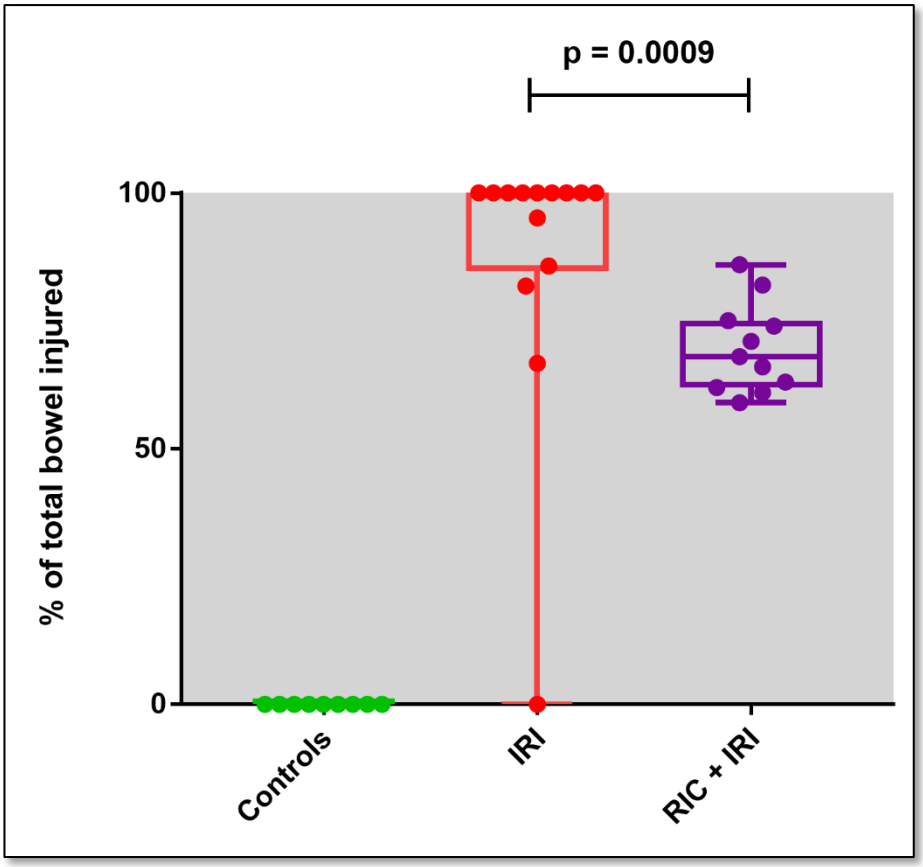


Figure 6-2 Protocol 1b: percentage of total bowel showing macroscopic injury. Graph shows each data point, inter-quartile range and median

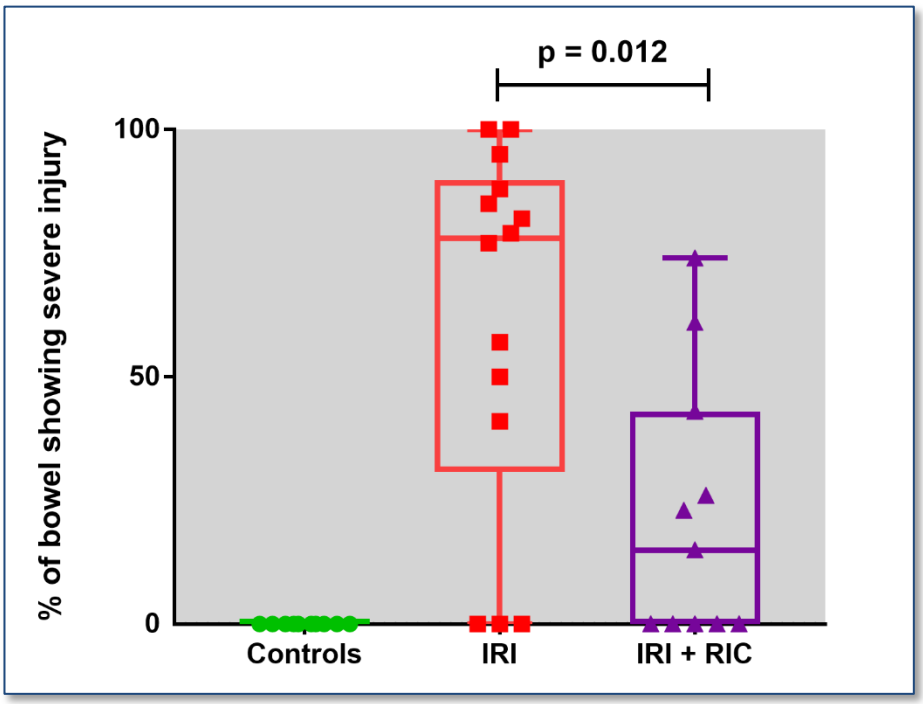


Figure 6-3 Protocol 1b: percentage of total bowel showing severe macroscopic injury. Graph shows each data point, inter-quartile range and median

6.4.2 Microscopic Bowel Injury

Table 6-2 shows the Chiu-park scores for this experimental protocol.

	Controls*		IRI only*		RIC + IRI	
	TIF	MXF	TIF	MXF	TIF	MXF
Median	0	0	4	5.5	4	5.5
Range	0 – 0	0 – 1	0 – 7	4 – 7	3– 6	4- 6
IQR	0 - 0.25	0 – 1	3 – 5	4 – 6	4 - 5	4.625-6

Table 6-2 Protocol 1b: Median Chiu-Park scores for each group.
TIF = Fixed point in terminal ileum
MXF = Area of maximal macroscopic injury
*Controls and IRI only animals from Protocol 1a.
Full data in Table B-1

Animals that had undergone RIC (by means of the blood pressure cuff) prior to IRI had a median score of 4 (IQR 3-6) for the bowel specimen taken from a fixed point in the terminal ileum sample and 5.5 (4.625-6) for area of maximal injury. These scores were essentially the same as for animals who underwent IRI only. Figure 6-4 and Figure 6-5 show the groups and each of the data points.

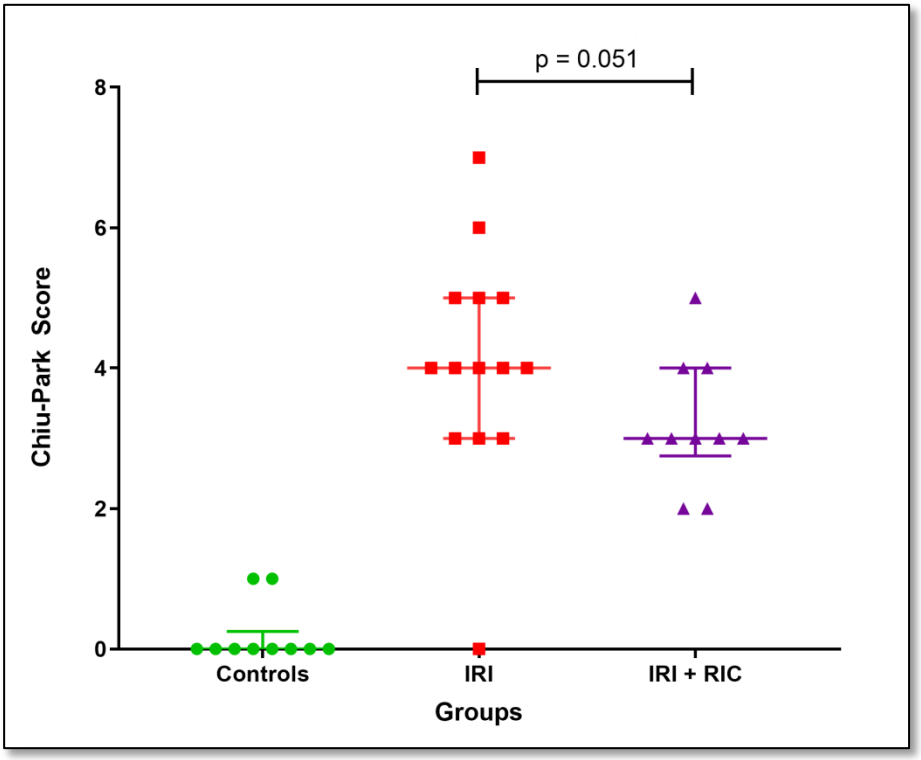


Figure 6-4 Protocol 1b: Chiu-Park Scores – fixed point in the terminal ileum

Unlike in protocol 1a, the area of maximal injury did not have statistically significantly lower scores in the animals who underwent RIC prior to anaesthesia compared to those who underwent IRI alone. (Figure 6-5, p = 0.29).

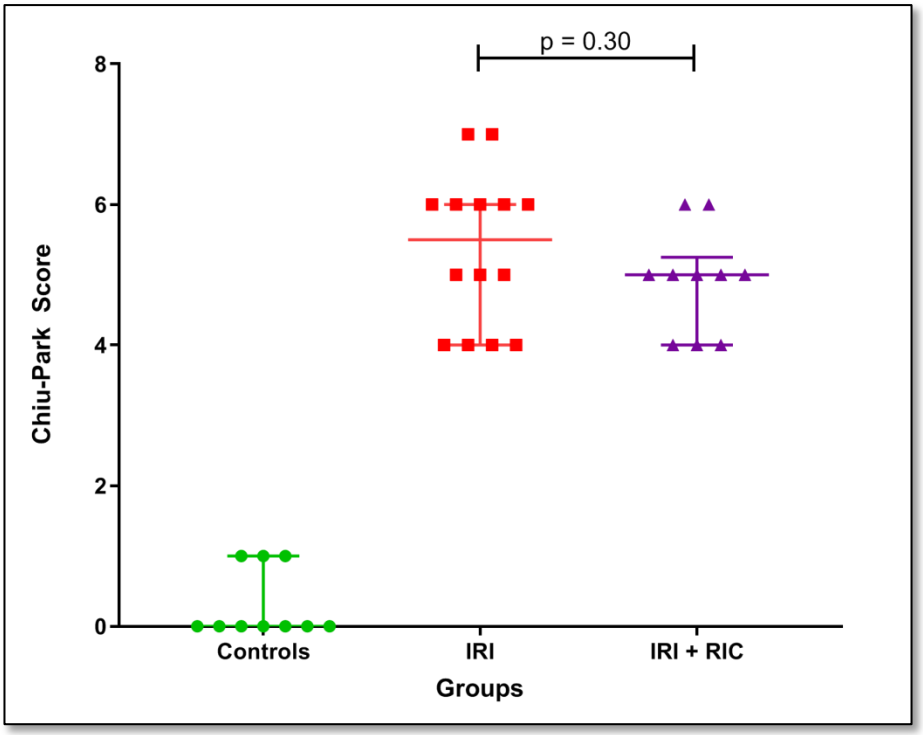


Figure 6-5 Protocol 1b: Chiu-Park Scores – area of maximal macroscopic injury

6.5 Discussion

The Columbus instruments NIBP-8 blood pressure cuff is designed to measure rodent blood pressure in the tail by means of a two cuff system. The 'occluder' cuff inflates to a high pressure and then reduces rapidly whilst the 'sensor' cuff detects the turburlent flow once the pressure falls below systolic and the subsequent loss of signal once the pressure is below diastolic. In adult rats, a modified neonatal cuff has been successfully used on the hind limb to reproducibly deliver RIC.[382] The hope that by arresting the drop of pressure in the occluder cuff on a hind limb, a quantified pressure could be maintained. The cuffs are a combination of a rubber sleave and rubber seals that allow the cuff to be inflated (Figure 4-5). Unfortunately, whilst this combination is clearly sufficiently airtight to allow the rapid inflation and deflation necessary for blood pressure measurement it did not reliably maintain the pressure for five minute periods.

The blood pressure cuff used is a sophisticated instrument designed for non-invasive measurement of blood pressure in rodents using the tail. As such, it is not designed to maintain the pressure for more than a few seconds in order for it to assess the changing waveform as the pressure is reduced. Multiple modifications were attempted, including changes to the software. However, ultimately it was not able to maintain the pressure reliably for a period of time. The exact pressure was not measurable but it was visually obvious after 1-2 minutes in each case that cuff no longer had the tension in it that was evident when it was first inflated.

Despite the concerns about the effectiveness of the RIC delivered by this method, the macroscopic results do clearly show a reduction in the extent of bowel injury. This is not mirrored by the microscopic results with the Chiu-Park scores being the same as animals who underwent IRI only. This potentially poses an important question about the usefulness of these two measures together; in the context where the RIC delivery is not ideal, a clear statistical reduction in macroscopic injury is seen but not in the primary outcome of the microscopic injury. However, comparison of the macroscopic injury between protocol 1a and 1b – i.e. between animals where RIC was delivered by means of a ligature and by the blood pressure cuff shows that the protective effect appears to be greater in animals who received RIC by means of the ligature. The total and severe macroscopic injury extent for both preRIC groups are shown in Table 6-3 and Figure 6-6 Comparison of macroscopic injury between Protocol 1a and 1b. In both cases there is a clear trend although this does not reach statistical significance. Given the range of Chiu-Park scores in this group, it is difficult to justify the use of further animals as this would be unlikely to deliver a statistically significant difference in this, the primary outcome.

Median length of bowel injury			
	Mild	Severe	Total
RIC (ligature) + IRI	40%	0%	41%
RIC (cuff) + IRI	59%	15%	68%

Table 6-3 Protocol 1a and 1b: Macroscopic injury.

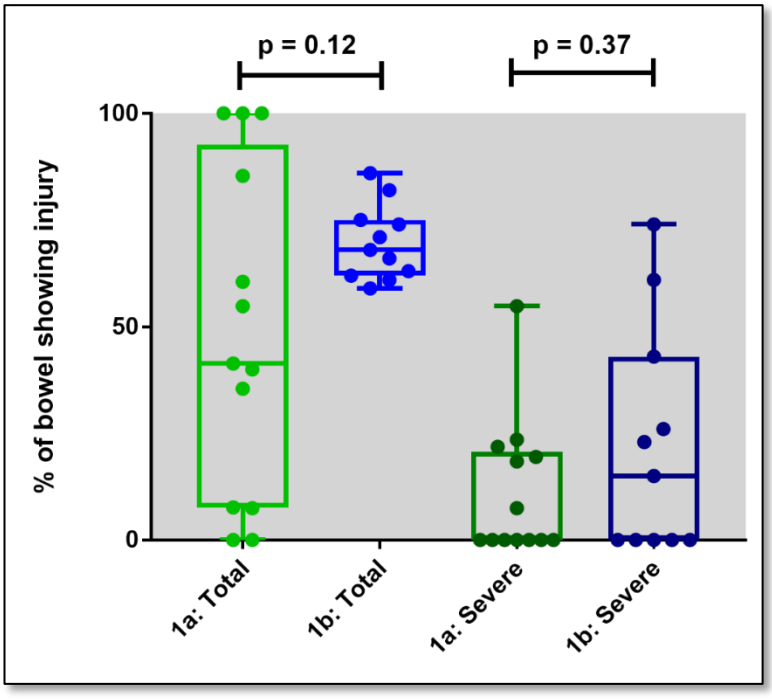


Figure 6-6 Comparison of macroscopic injury between Protocol 1a and 1b.

Protocol 1a – preRIC by means of a ligature; protocol 1b – preRIC by means of a blood pressure cuff. Graph shows both total injury (as a % of total bowel removed) and % severe injury. Box-and-Whisker plots show median, IQR and range and each data point. Mann-Whitney Test used for analysis.

One explanation of these data is that this is a 'dose effect.' The RIC delivered in these experiments is not as effective as that delivered by the ligature. The expectation is that had the pressure been reliably maintained above systolic pressure, the results would have been similar. The dose-effect

explanation is supported by the fact that the macroscopic injury was reduced compared to IRI alone but not as much as when the ligature was used. And, presumably, the effect of this (sub-optimal) RIC was not sufficient to be reflected in the microscopic scoring.

In view of these results showing that the cuff was not equivalent to the ligature in rat pups, the other analyses (HIF-1α, blood cytokines, myeloperoxidase etc.) were not performed on these samples.

6.6 Conclusion

Whilst the macroscopic injury pattern shown in these animals is less than that seen in animals exposed to intestinal IRI without RIC, the effect is less pronounced than when the ligature was used to deliver RIC in protocol 1a. Moreover the microscopic analysis showed no difference in the extent of injury. The technical limitations of the rodent blood pressure cuff may well explain these differences. This does not detract from the potential of using a blood pressure cuff system in human patients, which is well established. However, for the purposes of this work, the unreliability of the cuff system compared with the ligature means that empirical work on the ideal pressure needed to achieve effective RIC is not possible and that for the other protocols looking at the postRIC (Chapter 7) and delivery of preRIC at different times (Chapter 8) only the ligature was used.

Chapter 7 Remote Ischaemic postConditioning

7.1 Abstract

7.1.1 Background

Protocol 1a (Chapter 5) demonstrated that remote ischaemic conditioning (RIC) delivered by means of a ligature reduced the extent and severity of bowel injury in this IRI model of NEC. The aim of this protocol was to investigate the effectiveness of RIC delivered after the ischaemic insult (postRIC). Therefore, RIC was delivered at the same time as the reperfusion phase. The aim here is to assess if postRIC is effective at reducing the injury to the bowel.

7.1.2 Methods

Experimental animals underwent IRI by means of occlusion of the superior mesenteric artery (SMA) for 40 minutes, followed by 90 minutes of reperfusion. The comparator groups of Controls (Sham surgery) and the IRI-only (injury without RIC) were taken from protocol 1a. Randomly assigned, 10-13 day old related rat pups underwent RIC by means of a ligature on the hind limb for three cycles of five minutes ischaemia, followed by five minutes of reperfusion. This was done at the beginning of the reperfusion phase of the bowel injury.

Bowel injury was assessed macroscopically (by measuring the amount of bowel affected graded as normal, mild or severe) and microscopically using the Chiu-Park scoring system. Pro-inflammatory cytokine levels were measured in each animal in blood taken at the end of the procedure.

7.1.3 Results

Animals exposed to IRI only had a median of 100% of the bowel showing injury (IQR 85-100%). In animals that underwent postRIC the median length of bowel showing any injury was 68% (56-80%, $p = 0.0006$). Similarly, the median length of severe necrosis was 4% of the bowel in post-conditioned animals, compared with 78% for those that had IRI alone ($p = 0.012$).

The median Chiu-Park score was 4.25 (range 0-6) in the area of maximal injury in animals who underwent postRIC, compared to 5.5 (4-6) in the animals who had IRI alone ($p = 0.031$).

Serum levels of Interleukin-10 were increased in animals exposed to IRI compared to SHAM controls whilst IL-4 was unaffected by IRI but was seen to increase in animals exposed to postRIC.

7.1.4 Discussion

In this model of NEC, the application of postRIC reduces the intestinal injury due to IRI significantly. The effect, whilst significant is smaller than that seen in the preRIC experimental group. Several cytokines, most notably IL-4 may be important in the mechanism of RIC transmission.

7.2 Introduction

Chapter 5 outlines the results of Remote Ischaemic pre-Conditioning (preRIC), using this model. This chapter describes the experimental procedure and results from post-Conditioning. The controls for this experimental work derive from Chapter 5. This was done to minimise the number of animals used under the '3 'R's' principals. The comparator groups for this work are both the animals who underwent sham surgery ('controls') and those that underwent ischaemia-reperfusion injury only ('IRI'). The purpose of this protocol (protocol 2) is to investigate the potential benefit of RIC as a post-hoc treatment; i.e. after the initial insult has already occurred. This could be analogous to giving RIC to human patients after the putative diagnosis of NEC has been made.

7.3 Methods

7.3.1 Power Calculation

In all of the animal experiments, the blinded analysis of the microscopic histological injury using the Chiu-Park score was the primary outcome measure. I performed a power calculation based on my dataset for pre-conditioning. A sample size of 18 in each group has an 80% power to detect a difference between means of 2 with a significance level (alpha) of 0.05 (two-tailed).

Given that this is a relatively large group size; interim analysis of the outcomes was performed with 12 animals in this group to assess if further numbers were needed. (i.e. If a significant difference was seen with 12 animals, the further 6 would not have been needed).

7.3.2 Experimental protocol

The methods used are described in detail in chapter 4. In this protocol (2), the animals (n=17) underwent RIC immediately at the beginning of the reperfusion phase. Animals underwent 40 minutes of ischaemia and at the commencement of the 90 minutes reperfusion they had three cycles of 5 minutes of limb ischaemia and 5 minutes of limb reperfusion. RIC was delivery by means of a ligature (Section 4.5.1) as chapter 6 showed the blood pressure cuff was not a reliable means of delivering RIC in rat pups. Statistical analysis was performed using GraphPad Prism 9.1.2.

Animals from multiple, related litters were randomly assigned to Protocol 1b (Chapter 6), Protocol 2 (this chapter) and Protocol 3 (Chapter 8).

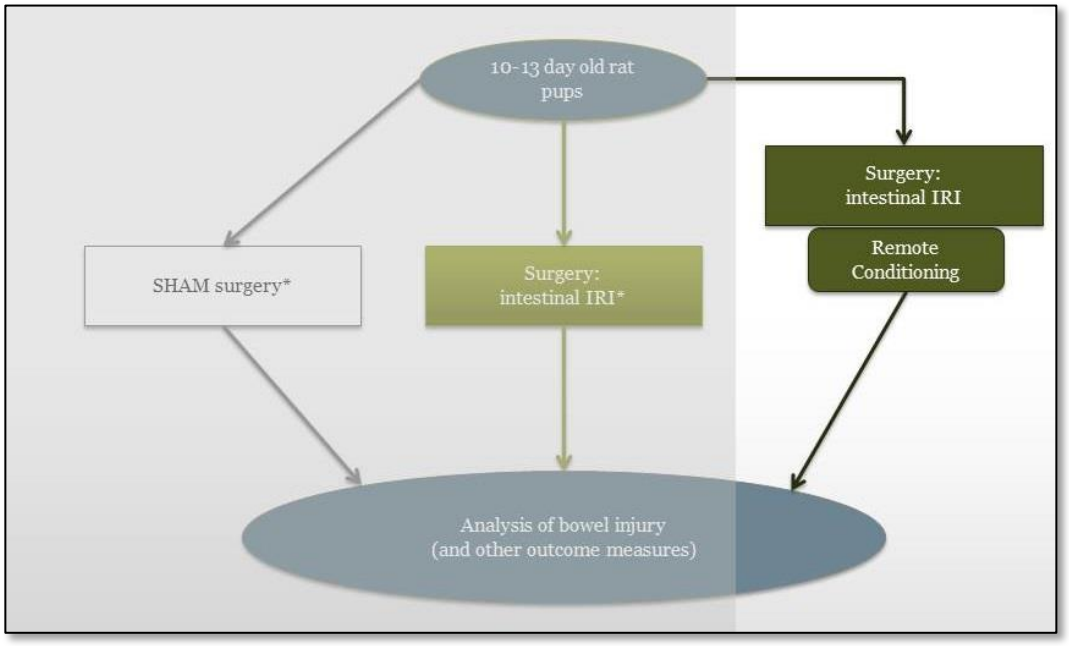


Figure 7-1 Experimental Protocol for postRIC
*Sham Surgery and IRI animals for comparison from previous experiments (Chapter 5).

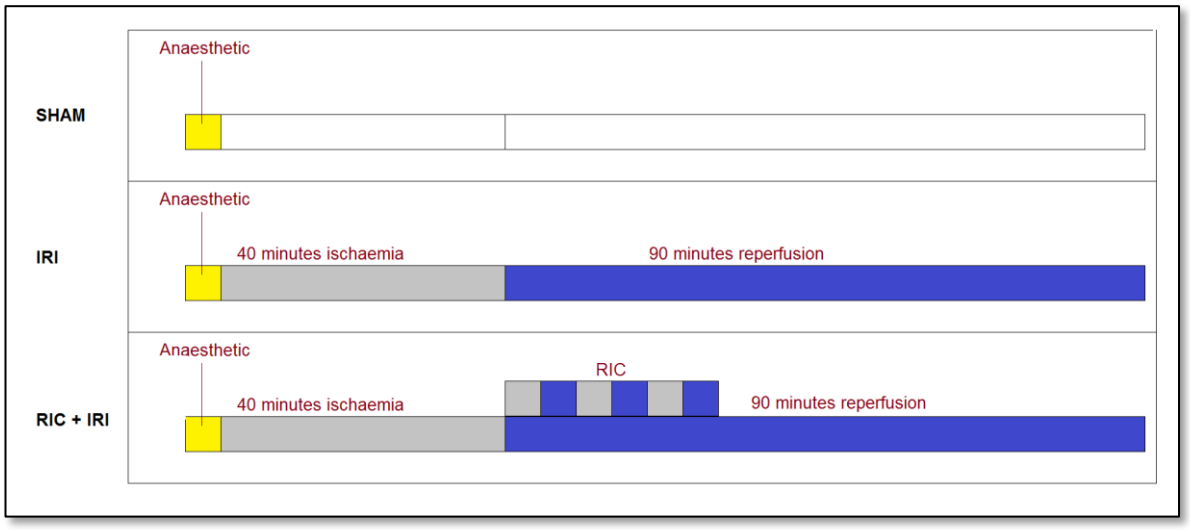


Figure 7-2 Experimental Protocol for postRIC
*Sham Surgery and IRI animals for comparison from previous experiments (Chapter 5).

Macroscopic injury was assessed as described in Section 4.7 and microscopic injury was scored with the Chiu-Park scoring system, described in Section 4.8. For each animal, a specimen was taken from the terminal ileum and from the area of maximal macroscopic injury. The median score from three independent scorers, blinded to which experimental group the specimen is derived was used for analysis. Blood levels of a panel of pro-inflammatory cytokines were measured at the end of the experiment as described in Section 4.9.

7.4 Results

Interim analysis with 12 animals showed a non-significant trend, so further animals were used. With n = 16, a statistically significant difference in the primary outcome measure was seen.

7.4.1 Macroscopic Bowel Injury

Mild injury was seen in a median length of bowel of 44% and the median length of bowel showing severe injury was 4%. The median total injury was 68% (range 37-100%, Table 7-1). The range of total bowel harvested was 29-41cm.

Median length of bowel injury			
	Mild	Severe	Total
Controls*	0%	0%	0%
IRI only*	18%	78%	100%
RIC + IRI	44%	4%	68%

Table 7-1 Protocol 2: Macroscopic injury. *Controls (Sham surgery) and IRI only taken from protocol 1a.
Full results are shown in Appendix B (Table B-7)

Figure 7-3 and Figure 7-4 show these data plotted against the Controls and IRI only groups from protocol 1a. Both the total injury and the severe injury were significantly different to the IRI-only animals, p = 0.0006 and p = 0.012 respectively.

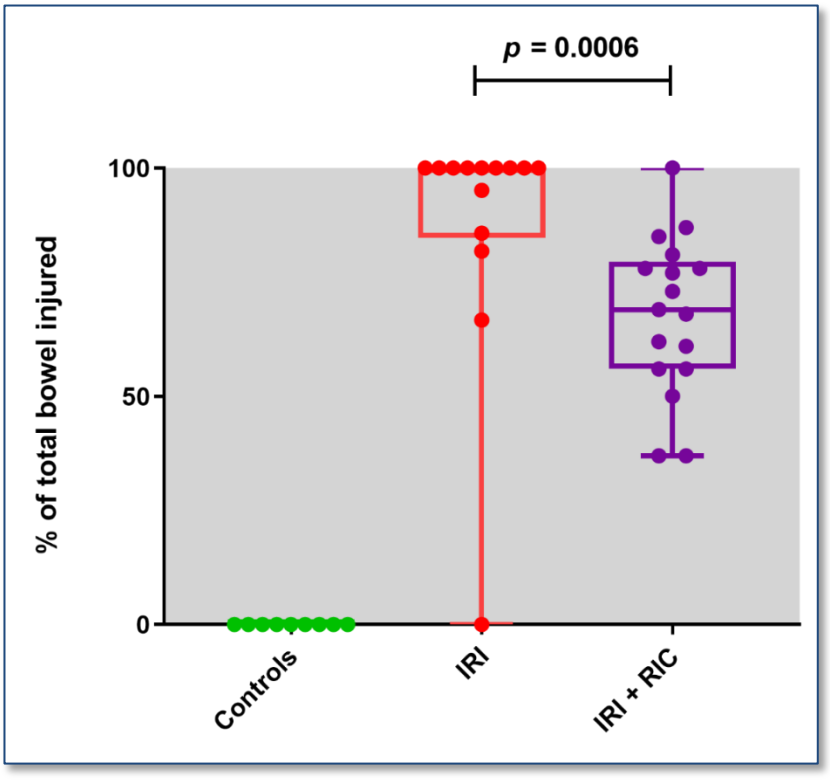


Figure 7-3 Protocol 2: percentage of total bowel showing macroscopic injury. Graph shows each data point, inter-quartile range and median

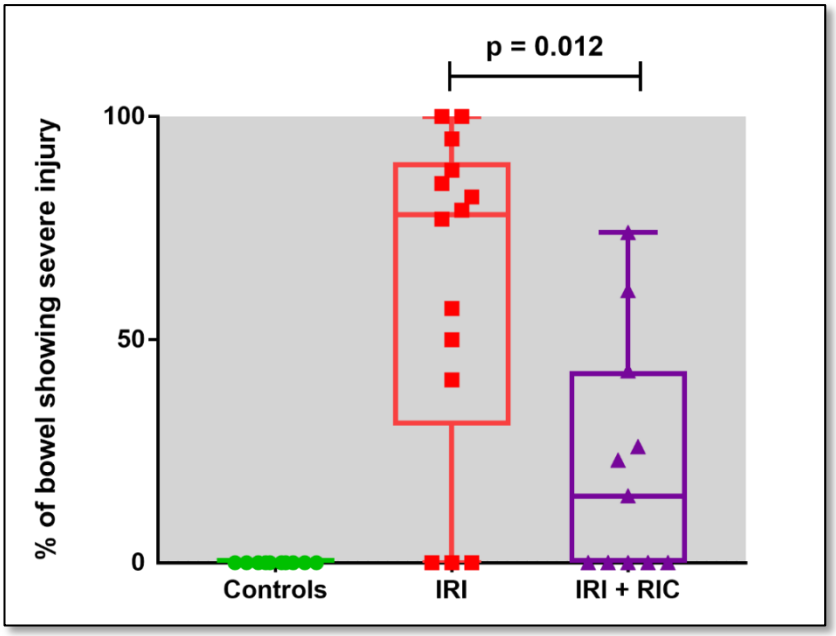


Figure 7-4 Protocol 2: percentage of total bowel showing severe macroscopic injury.
Graph shows each data point, inter-quartile range and median

7.4.2 Microscopic Bowel Injury

Table 7-2 shows the Chiu-park scores for this experimental protocol. Animals that underwent RIC at the start of the reperfusion phase of IRI had a median score of 4 (IQR 3-4.75) for the bowel specimen taken from a fixed point in the terminal ileum sample compared to 4 (3-5) for animals who underwent IRI without conditioning (p = 0.53). The specimens from the area of maximal injury had a median score of 4.25 (4-5) compared to 5.5 (4-6) for IRI only (p = 0.031). Figure 7-5 and Figure 7-6 show the groups and each of the data points.

	Controls*		IRI only*		IRI + RIC	
	TIF	MXF	TIF	MXF	TIF	MXF
Median	0	0	4	5.5	4	4.25
Range	0 – 0	0 – 1	0 – 7	4 – 7	0 – 6	0 – 6
IQR	0 – 0	0 – 1	3 – 5	4 – 6	3 – 4.75	4 – 5

Table 7-2 Protocol 2: Median Chiu-Park scores for each group.
TIF = Fixed point in terminal ileum
MXF = Area of maximal macroscopic injury
*Controls and IRI only animals from Protocol 1a.
Full results are shown in Appendix B (Table B-8)

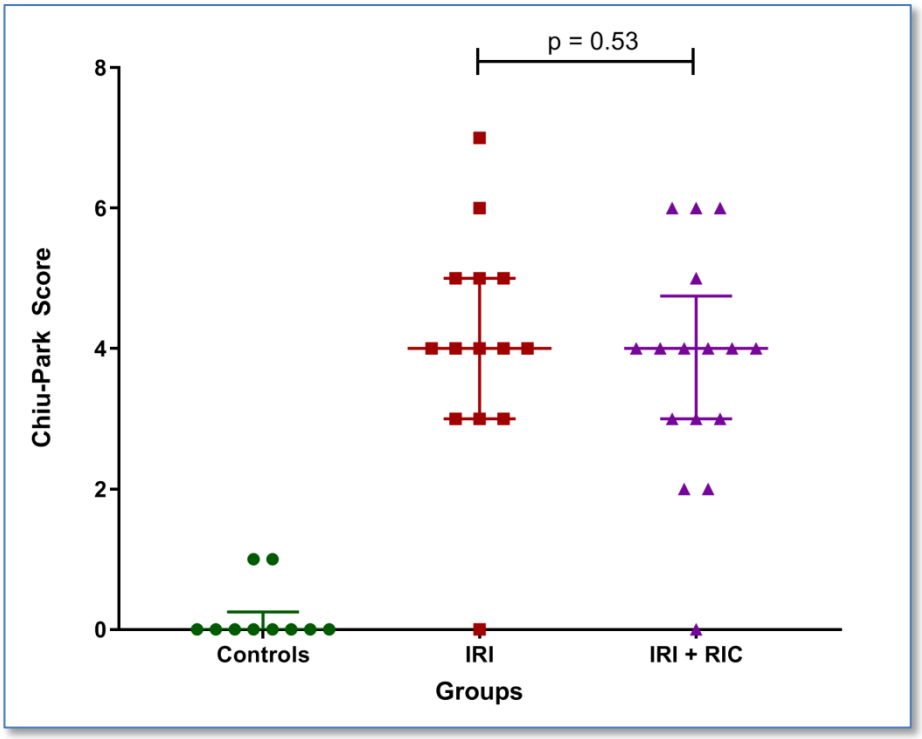


Figure 7-5 Protocol 2: Chiu-Park Scores – fixed point in the terminal ileum

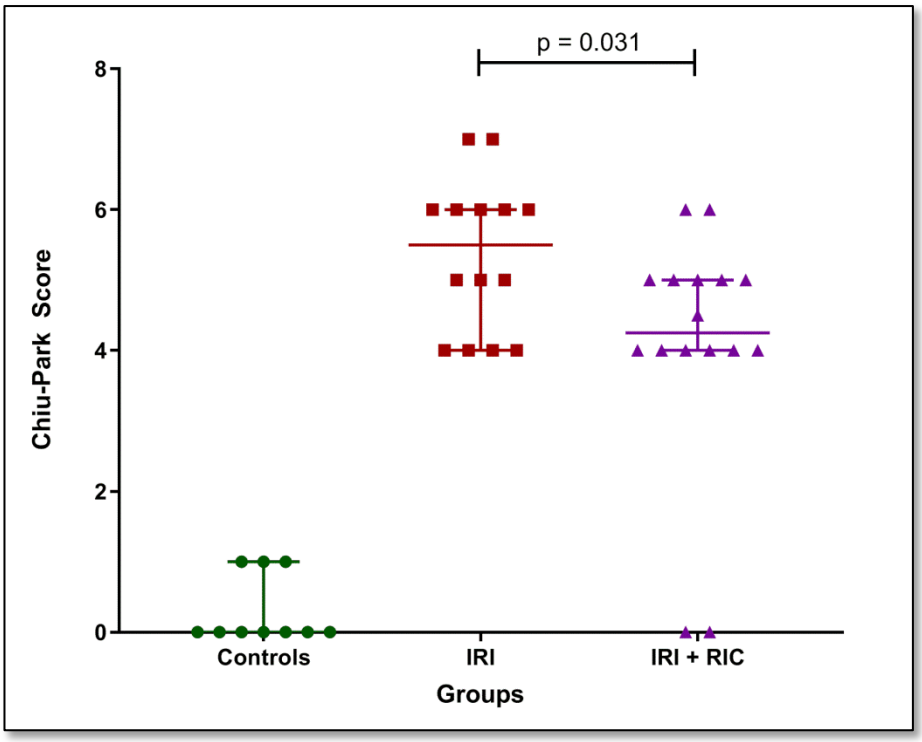


Figure 7-6 Protocol 2: Chiu-Park Scores – area of maximal macroscopic injury

7.4.3 Serum cytokine analysis

Pro-inflammatory cytokines were measured in serum taken from the animals at the end of the procedure. The results are summarised in Table 7-3 Protocol 2: Blood cytokine levels.

	Controls			IRI			RIC + IRI		
	Median	IQR	Range	Median	IQR	Range	Median	IQR	Range
IFN-γ *(pg/ml)*	1	0 - 1.6	0 - 3.2	0.6	0 - 1.4	0 - 4.3	1.9	1.3 – 2.3	1.1 – 2.5
IL-1β *(pg/ml)*	0	0 - 3.5	0 - 34.1	0	0 - 3.9	0 - 33.1	27.8	3.3 – 41.0	0 – 56.7
IL-10 *(pg/ml)*	16.5	11.0 - 24.2	9.1 - 39.2	45.5	25.7 - 56.5	0 - 74.3	26.3	21.0 – 48.1	16.0 – 74.9
IL-13 *(pg/ml)*	4	2.4 - 5.2	1.3 - 10.6	2.1	1.1 - 4.5	0 - 8	5.0	4.4 – 6.0	0 – 8.4
IL-4 *(pg/ml)*	0	0 – 0	0 - 0.3	0	0 - 0.2	0 - 0.3	0.2	0 - 6.0	0 – 8.4
IL-5 *(pg/ml)*	10.8	0 - 23.1	0 - 57.6	0	0 - 57	0 - 72.3	44.7	31.9 – 50.3	26.7-50.3
IL-6 *(ng/ml)*	1.1	0.4 - 2.2	0.3 - 4.5	1.8	0.8 - 2.5	0 - 6.6	3.3	2.2 – 4.9	1.0- 4.9
KC/GRO *(ng/ml)*	13.5	12.0 - 15.4	8.8 - 22.9	21.3	18.4 - 26.4	0 - 39.8	26.8	25.1 – 32.1	17.1 – 55.8
TNF-α *(pg/ml)*	13.4	10.3 - 21.4	8.2 - 27.7	19.15	13.1 - 29.0	0 - 42.2	25.3	20.2 – 31.5	13.9 – 63.9

Table 7-3 Protocol 2: Blood cytokine levels
Full results are shown in Appendix B (Table B-9)

7.4.3.1 Interleukin-10

As described in Section 5.4.5.2, the cytokines IL-10, KC/GRO and TNF-α were increased in animals exposed to IRI alone. There was a trend towards lower levels of each of these in the animals given preRIC suggesting that they correlate with the bowel injury. In this protocol the same pattern was seen for IL-10. (Figure 7-7)

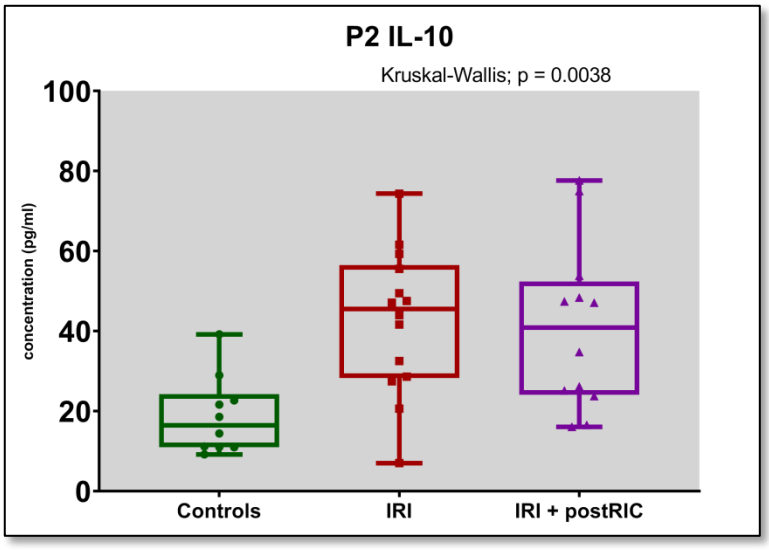


Figure 7-7 Protocol 2: Box and whisker plots of cytokine serum levels: IL-10
Graph shows each data point, median, IQR and range.

7.4.3.2 Tumour necrosis factor alpha

In contrast to IL-10, TNF-α levels were very similar in animals exposed to postRIC as they were in animals that underwent IRI alone. Again the levels of TNF-α appear to be higher in both of these groups compared to controls that did not undergo IRI.

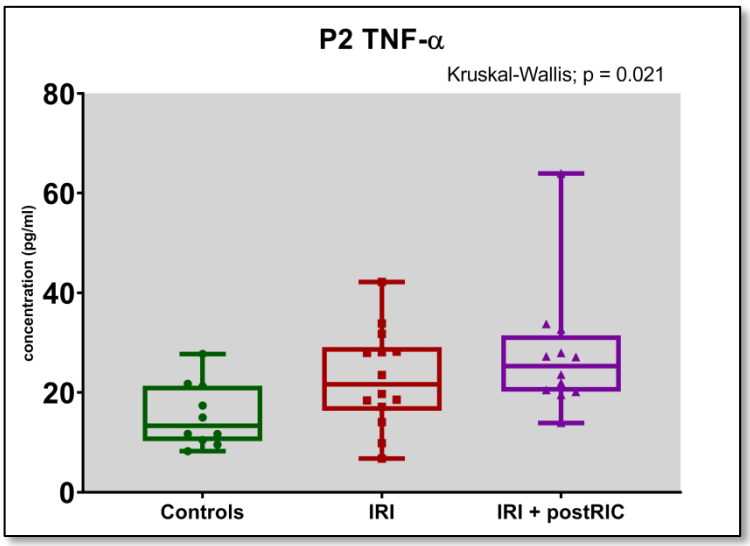


Figure 7-8 Protocol 2: Box and whisker plots of cytokine serum levels: TNF-α.
Graph shows each data point, median, IQR and range

7.4.3.3 Keratinocyte chemoattractant / growth-related oncogene

In animals exposed to the preRIC protocol (Section 5.4.5.2), KC/GRO (along with the IL-10, and TNF-α) were increased in animals exposed to IRI compared to controls but this increase appeared to be mitigated by preRIC. Conversely, when postRIC was applied, the levels of KC/GRO were higher in this group compared to animals exposed to IRI alone. Kruskal-Wallis testing for multiple comparisons of non-parametric data reveals a Kurskal-Wallis statistic of 17.9 with a p-value of 0.0001. Post-hoc comparison of the postRIC group vs IRI alone returned a p-value of 0.27. Mann-Whitney testing of the same comparison gives a p-value of 0.053. Thus there is a demonstrable increase in systemic KC/GRO in animals exposed to IRI and clear trend towards higher levels in those that underwent postRIC as well.

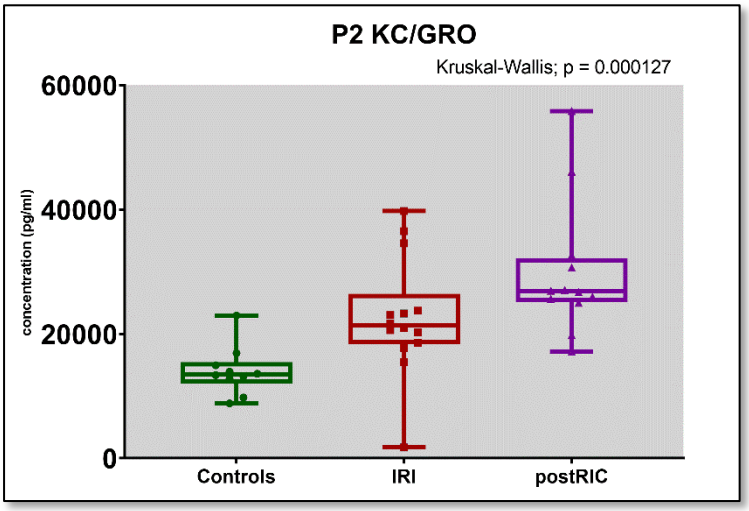


Figure 7-9 Protocol 2: Box and whisker plots of cytokine serum levels: KC/GRO
Graph shows each data point, median, IQR and range.

7.4.3.4 Interferon gamma, Interleukin-1, Interleukin-13, Interluekin-4, Interluekin-5 and Interleukin-6

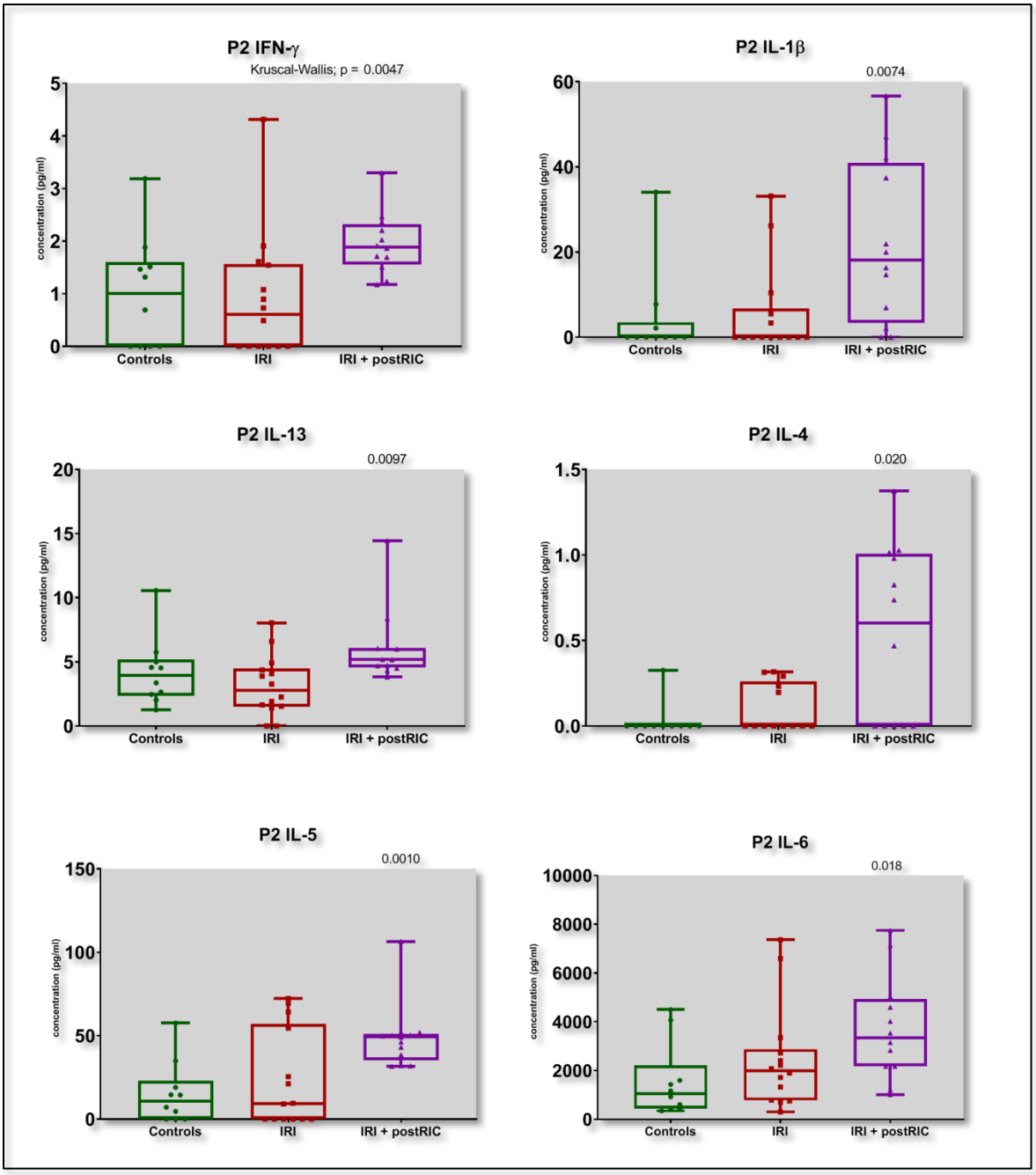


Figure 7-10 Protocol 2: Box and whisker plots of cytokine serum levels: IFN-γ, IL-1β, IL-13, IL-4, IL-5 and IL-6.
Graphs show each data point, median, IQR and range. Kraskal-Wallis multiple comparison p-values shown.

In the preRIC protocol, none of these cytokines showed any difference between the groups. Both IRI and preRIC did not result in a measurable difference in the systemic levels of these cytokines.

With the application of postRIC, in each case there is a change in the serum of level of these cytokines. The post-hoc comparisons between postRIC and controls (no IRI) and postRIC and IRI alone are shown in Figure 7-10 and Table 7-4. Therefore, these cytokines can be said to be raised 1 hour after the application of RIC (and following IRI).

	postRIC vs controls (post-hoc comparison)	**postRIC vs IRI (post-hoc comparison)**
IFN-γ	***p = 0.036***	***p = 0.006***
IL-1β	***p = 0.019***	***p = 0.022***
IL-13	p = 0.20	***p = 0.0075***
IL-4	***p = 0.020***	p = 0.17
IL-5	***p = 0.017***	***p = 0.049***
IL-6	***p = 0.015***	p = 0.24

Table 7-4 Protocol 2; postRIC: Kuskal-Wallis testing, multiple comparisons, serum levels of selected cytokines

7.5 Discussion

7.5.1 Histological injury

The application of post-conditioning significantly reduces the macroscopic bowel injury compared to animals that were exposed to IRI only. The size of the effect was less that that seen in animals who underwent pre-conditioning (Chapter 5). This is summarised in Table 7-1. The areas showing severe injury were reduced significantly as well (from 78% to 4%). As the graphs (Figure 7-3 and Figure 7-4) show there was a lot of variability in the response in individual animals similar to that seen in previous protocols. This suggests that the response to RIC is to some-extent idiosyncratic.

As with the pre-conditioning , the samples taken from a fixed point in the terminal ileum show no statistically significant differences. As Figure 7-5 shows the spread of scores for the postRIC group is very similar to animals exposed to IRI only. The samples taken from the area of maximal injury show less severe injury in the postRIC group (Figure 7-6). The effect is probably smaller than that seen in the preRIC group but postRIC is indeed protective.

7.5.2 Serum cytokine analysis

Analysis of the systemic cytokines shows a very different pattern to the animals exposed to pre-conditioning. An important consideration here is that the pre-conditioned animals have their serum harvested 130 minutes after exposure to RIC, whilst in the post-conditioned animals it is only 60 minutes from when RIC finished. Therefore, the level of cytokines in the serum potentially reflects better the effects of RIC than was seen in the preRIC group.

7.5.2.1 Interleukin-10, Tumour necrosis factor alpha and Keratinocyte chemoattractant / growth-related oncogene

Interleukin-10 (IL-10) followed the same pattern seen in the preRIC group. It is increased by exposure to IRI and this effect is mitigated by RIC therefore most likely the level of IL-10 is simply a reflection of the amount of bowel injury caused by the IRI. Liu *et al.*(2019) reported systemic rises in IL-10 due to IRI that are reduced with remote conditioning.[366] These data suggest that postRIC is having a very similar effect as preRIC.

The patterns seen with TNF-α and KC/GRO (IL-8-equivalent) diverge from that seen in the preRIC protocol. In the preRIC analysis, TNF-α showed a non-significant trend towards reduced levels in animals exposed to RIC prior to IRI, compared to those exposed to IRI alone. In the postRIC group this potential difference in not seen. In the case of KC/GRO there is a non-significant trend towards higher levels in animals who underwent postRIC as well as IRI. Within this model, it is not possible to obtain blood samples at multiple time points nor to identify where the KC/GRO is being produced thus any conclusion from these data is inevitable speculative. However, the most obvious explanation here is that separate to the effect of IRI on KC/GRO levels, RIC is causing a rise in systemic KC/GRO. There is very limited literature on KC/GRO and/or IL-8 in the context of RIC. A PubMed search (March 2021) revealed no results for KC/GRO and remote ischaemic conditioning. Searching for IL-8 in the context of RIC revealed one paper in which the authors investigated RIC in a pig-model of renal transplantation. Their data showed that RIC had no effect on IL-8 levels.[383]

The changes in IFN-γ, IL-1β, IL-13, IL-4, IL-5 and IL-6 levels seen in this group may reflect an effect of RIC as they were not seen at all in the pre-RIC group. The interaction between RIC and IRI is also a potential factor here.

7.5.2.2 Interferon gamma and interleukin-1 beta

Interferon gamma (IFN-γ) is a potent activator of macrophages and is produced by lymphocytes. It has been shown in the human intestine that IFN-γ is increased in NEC.[99] Joseph et al. (2017)

reported that serum IFN-γ was reduced by RIC. In their experiments, an IFN-γ rise was induced by a septic trigger and then seen to decline in response to RIC but there was no positive control group and thus it is unclear what the trend of IFN-γ would be without RIC.[361]

Interleukin-1 beta is a released by activated macrophages and is a pro-inflammatory cytokine. It has been shown to rise in response to sepsis[363] and to be raised in human NEC.[362] It is also part of the pathway that activates Toll-like Receptor-4 which is undoubtedly a key part of the pathogenesis of NEC as detailed in Section 1.5.4.

7.5.2.3 Interleukin-13

No previous role for IL-13 has been established in remote ischaemic conditioning. These data suggest a rise in IL-13 60 minutes after the application of RIC. However, in other contexts it is well established that IL-13 has overlapping function with IL-4.[384] Moreover, IL-13 activates ERK-1 and ERK-2[384] which are known to be important in the protective mechanism of RIC at the cellular level in cardiac tissue, as described in Section 3.5.2.4.

7.5.2.4 Interleukin-4

It has previously been reported that IL-4 may rise in response to RIC; Liu *et al.* 2019 showed in a mice model that IL-4 mRNA level was increased in the target organ (brain).[366] Similarly, there is evidence that IL-4 is protective against NEC.[364]

IL-4 is produced by TH_2 cells. This rise in mRNA levels in the brain seen by Liu *et al.*[366] is intriguing as it suggests that there is a change in the behaviour of T-helper cells in response to RIC in the target organ. Liu *et al.* did not report a change in the serum IL-4 level, whereas in these data, there is a rise that can be observed on the systemic level. It is, of course, unknown where this IL-4 is produced.

7.5.2.5 Interleukin-6

A rise in serum IL-6 is seen in response to sepsis[363] and at the tissue level, IL-6 is higher in NEC.[99] Thus a rise in IL-6 in animals exposed to IRI is expected. Indeed higher levels are seen following ischaemia specifically.[292] The rise over and above that of IL-6 following application of RIC has also been previously reported,[292] suggesting a role for IL-6 in the pathway of RIC.

7.5.2.6 Comparison with animals that underwent RIC only

The differences between the cytokine profile of animals that underwent preRIC compared to postRIC may be due to the interplay of RIC's effect on the cytokines and the effect of IRI. Specifically, there are potentially temporal changes here that are complicating the picture.

Cytokines levels may be affected by either of these stimuli (RIC and IRI) and the effects may not be simple. To elucidate this process further would require carefully designed experiments with blood sampling at multiple time points. However, a comparison with animals that only underwent conditioning without IRI might provide further evidence of cytokine changes due to RIC specifically. In order to provide tissue for transcriptomic analysis (Chapter 9), a further group of animals (n=8) underwent RIC without IRI. (As with the controls, these animals had SHAM surgery with laparotomy but no intestinal IRI). Because these animals underwent RIC just prior to anaesthetic (analogous to the preRIC+IRI experimental group), the exposure to RIC was 130 minutes prior to collection of the samples. However, the same cytokine changes in this group would corroborate the findings in the postRIC and support the inference that these changes are due to RIC.

Figure 7-11 shows the levels of IFN-γ, IL-1β and IL-4. IFN-γ and IL-1β both show increases in the postRIC group (compared to IRI alone) and non-significant increases in the RIC only group compared to the SHAM controls. In the case of IL-4, the pattern is reversed with a non-significant trend in the postRIC group and a statistically significant rise in the RIC only group. The small numbers in total in these comparisons and especially in the RIC only group necessitate caution but there is a clear pattern here that 130 minutes after RIC (with no other stimuli), IFN-γ and IL-1β are raised and a similar rise is potentially seen in animals who did also have IRI but had their RIC much closer in time to the sample collection. It should also be noted that in some of the samples, the cytokines are undetectable. This is part of the technical limitations of these experiments but does corroborate the role of these three cytokines in RIC specifically.

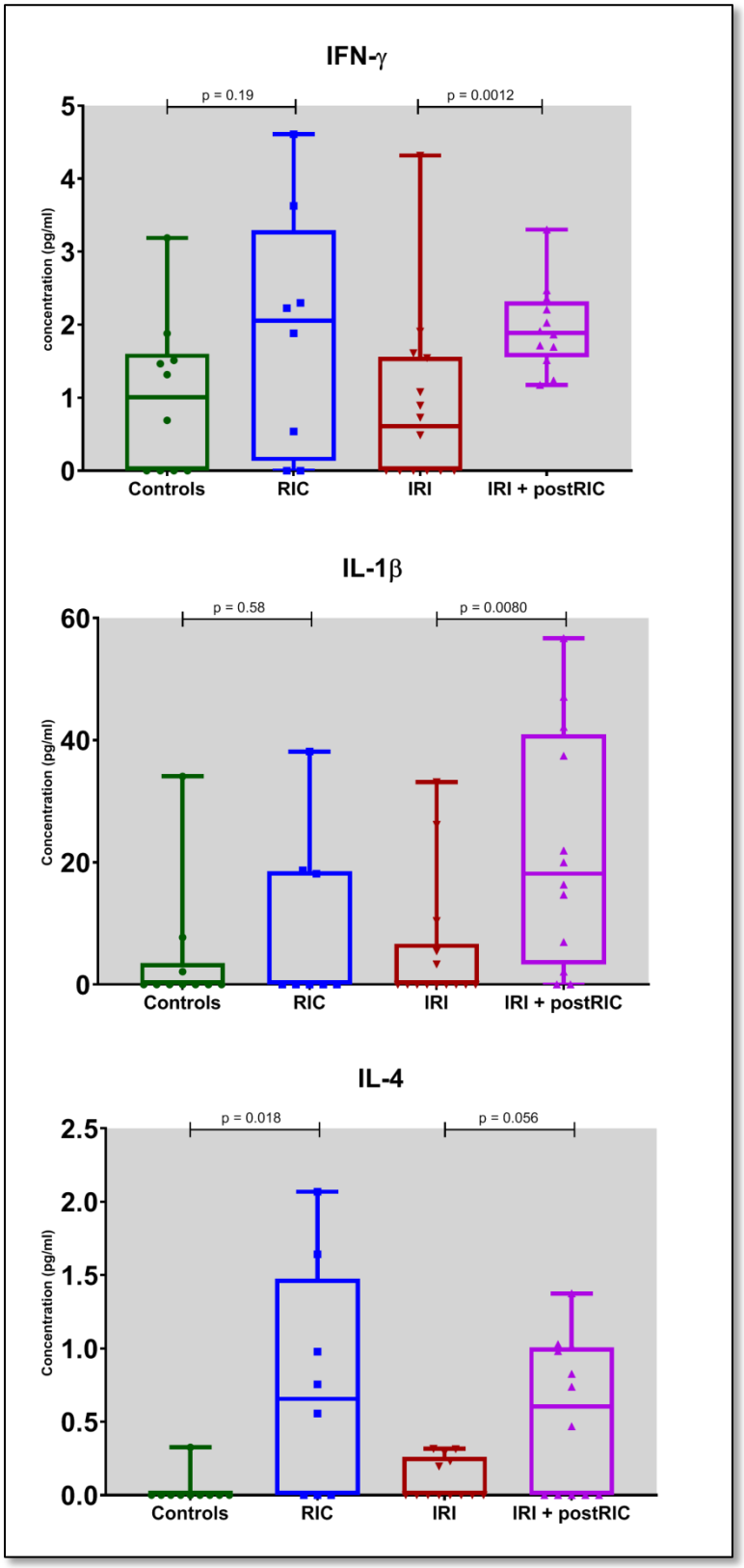


Figure 7-11 Box-plots of serum cytokine levels (IFN-γ, IL-1β and IL-4) in animals that underwent preRIC only and animals that had IRI and postRIC

Graph shows each data point, median, IQR and range. Statistics show Mann-Whitney Tests comparing preRIC and controls and postRIC and IRI alone.

IFN-γ: Non-significant trend towards higher levels in animals exposed to RIC alone (compared to controls). Higher levels in postRIC group compared to IRI alone.

IL-1β: Non-significant trend towards higher levels in animals exposed to RIC alone (compared to controls). Higher levels in postRIC group compared to IRI alone.

IL-4: Higher levels in animals exposed to RIC alone (compared to controls). Non-significant trend towards higher levels in postRIC group compared to IRI alone.

In the preRIC only group, there is no change in serum levels of IL-13 and IL-5 in animals exposed to RIC only (Figure 7-12). This lack of a rise in IL-5 or IL-13 in the preRIC only group in contrast to IFN-γ, IL-1β or IL-4 means that the hypothesis that these changes are an effect of RIC are not so clearly corroborated. Possible explanations include that this is an artifactual finding, that the rise is short lived in response to RIC (i.e. that the time window with postRIC captures this and the preRIC alone window does not) and that there is a two-hit effect here from RIC and IRI. This seems less likely as the same effect is not seen with preRIC and IRI. A different experimental design with multiple time-points of serum cytokine analysis would be necessary to explore this further.

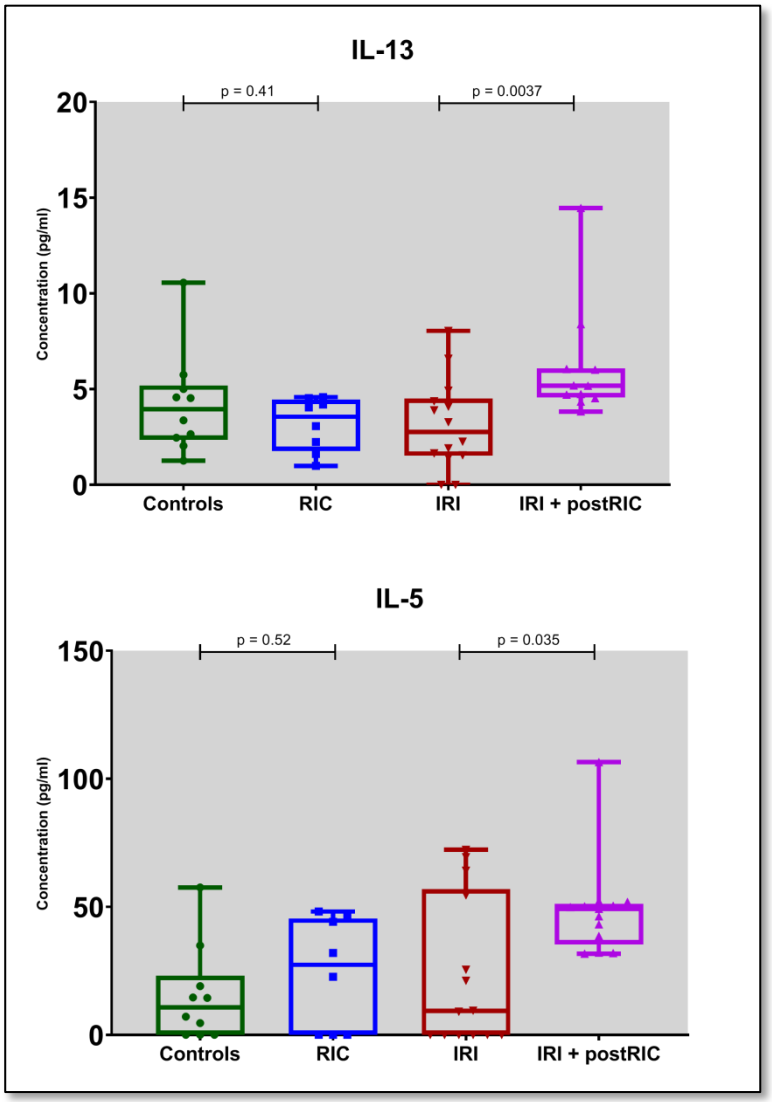


Figure 7-12 Box-plots of serum cytokine levels (IL-13 and IL-5) in animals that underwent preRIC only and animals that had IRI and postRIC.

Graphs show each data point, median, IQR and range.

Statistics show Mann-Whitney Tests comparing preRIC and controls and postRIC and IRI alone.

In both cases, the rise seen in the postRIC group is not seen in animals who underwent RIC alone.

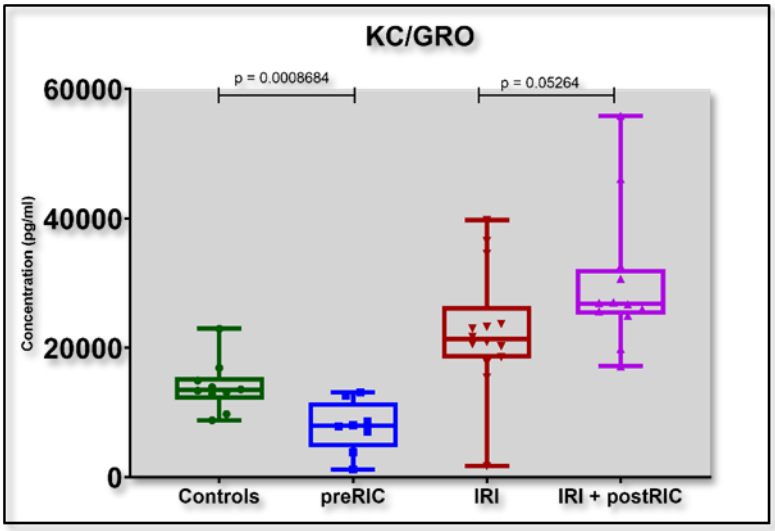


Figure 7-13 Box-plots of serum KC/GRO levels in animals that underwent preRIC only and animals that had IRI and postRIC.

Graph shows each data point, median, IQR and range. Statistics show Mann-Whitney Tests comparing RIC only and controls and postRIC and IRI alone. KC/GRO is seen to rise in the PostRIC group compared to IRI alone and to fall in response to RIC compared to the SHAM controls.

Keratinocyte chemoattractant / growth-related oncogene rises in response to IRI. As described in Section 5.4.5.2, this rise was mitigated in animals exposed to preRIC prior to the injury. This mitigation could be simply explained by the reduced level of intestinal injury seen in the preRIC group; If this is the case, then the serum level of KC/GRO is simply a surrogate for intestinal injury. The postRIC results suggest this is not the case. The rise in KC/GRO after postRIC (i.e. the rise in serum level seen at one hour after the application of RIC) warrants an alternative explanation. However, the RIC only group had lower levels than the SHAM controls (Figure 7-13). The interaction between the two stimuli is a confusing factor here. It could be that postRIC is definitively different to preRIC in terms of the effect on this cytokine. Conversely, the different time-points (relative to the application of RIC) may be the key to understanding these results. Either way, the reduction in KC/GRO in response to RIC (relative to injury alone) seen in protocol 1a (Section 5.4.5.2) is not simply a surrogate of intestinal injury. By contrast, IL-10 seems to have this more simple relationship with the intestinal injury.

These cytokine results suggest a tantalising potential for a role of various cytokines, most notably IL-4, in the transmission of RIC. Further understanding of this would not be possible with this specific model as the only means of harvesting serum is euthanasia due to the very small circulating volume. However, undoubtedly they warrant further investigation as these changes in systemic cytokines imply that IFN-γ, IL-1β and IL-4 are involved in the systemic pathway - discussed in Section 3.5.1.3 - by which RIC transmits its protective effect to the target organ.

7.6 Conclusion

Remote *post*-Ischaemic conditioning does reduce the bowel injury and systemic effects in this model of NEC, whilst the effect demonstrated is smaller than that seen with pre-conditioning. The advantage of post-conditioning in the clinical context is that it can be used once a diagnosis has been made. Thus this smaller effect may indicate that RIC is not as useful with patients who have been diagnosed as it might be as a preventative treatment. However, as discussed in Section 1.5, NEC is a disease process that takes place over hours to days and there is a vicious cycle of necrosis leading to inflammation leading to further injury. Thus, even after diagnosis, the application of RIC to human neonates with NEC is not entirely post-hoc and the benefits of pre-RIC may still be translated.

The collection of serum from a time point that is only 60 minutes after the application of RIC allows the identification of potential systemic factors that are involved in the translation of RIC from the effector organ to the target organ. IFN-γ, IL-1, IL-13, IL-4 and IL-6 are all seen to rise in response to RIC and further study of the role of each of these in RIC would be very interesting.

Chapter 8 Early Remote Ischaemic preConditioning

8.1 Abstract

8.1.1 Background

Protocol 1a (Chapter 5) demonstrated that RIC delivered by means of a ligature reduced the extent and severity of bowel injury in this IRI model of NEC. The aim of this protocol was to investigate the effectiveness of RIC given two days prior to IRI at reducing the injury. There is some evidence that RIC can have a prolonged protective effect and this could be important for clinical translation.

8.1.2 Methods

Experimental animals underwent IRI by means of occlusion of the superior mesenteric artery (SMA) for 40 minutes, followed by 90 minutes of reperfusion. The comparator groups of Controls (Sham surgery) and the IRI-only (injury without RIC) were taken from Protocol 1a. Randomly assigned, 11-13 day old related rat pups underwent RIC by means of a ligature on the hind limb for three cycles of five minutes ischaemia, followed by five minutes of reperfusion. One group underwent RIC two days prior to injury. The other group underwent RIC two days prior to injury and immediately prior to injury.

Bowel injury was assessed macroscopically (by measuring the amount of bowel affected graded as normal, mild or severe) and microscopically using the Chiu-Park scoring system.

8.1.3 Results

Animals exposed to RIC 48 hours prior to injury showed a reduction in the amount of bowel showing severe injury; median 3%, compared to animals who did not undergo RIC prior to injury (median 78%, $p = 0.0054$). Animals who had two applications of RIC (48hrs and immediately prior to injury) showed severe injury in a median of 0% of the bowel harvested, $p = 0.002$.

In terms of the microscopic injury, animals that underwent RIC performed two days prior to injury had median Chiu-Park scores of 5 (Interquartile range 4 – 6) compared to 5.5 (4-6) in those that had injury alone. RIC delivered two days prior and immediately prior to injury resulted in Chiu-Park scores of 3 (1-3), $p = 0.0001$).

8.1.4 Discussion

There is a prolonged protective effect on the intestine from RIC and the application of RIC multiple times has an additive effect, producing more protection than a single application. This prolonged effect could be important for clinical applications of RIC in at-risk neonates.

8.2 Introduction

Chapter 5 outlines the results of Remote Ischaemic pre-Conditioning, using this model and Chapter 7 describes the application of post-Conditioning. This chapter describes the experimental procedure and results from 'early' pre-Conditioning and also animals that underwent both 'early' and immediate pre-conditioning. There is evidence that the protective effect of RIC can last for several days and certainly more than 24 hours.[289]

The purpose here is to establish how long the protective effect lasts and if there is a potential benefit from multiple applications of RIC. This has direct implications for translation to the clinical context. The controls for this experimental work derive from Chapter 5, to avoid replicating the experimental work and thus minimising the number of animals used.

8.3 Methods

8.3.1 Power Calculation

The power calculation is described in Section 7.3.1 – this suggests a group size of 18 in each group has an 80% power to detect a difference between means of 2 with a significance level (alpha) of 0.05 (two-tailed).

Given that this is a relatively large group size; interim analysis of the outcomes was performed with 12 animals in this group to assess if further numbers were needed.

8.3.2 Experimental protocol

The methods used are described in detail in Chapter 4. In this protocol (3), the animals underwent RIC at different time-points. In protocol 3a, animals underwent three cycles of RIC approximately 48 hours prior to injury. In protocol 3b they had RIC both 48 hours prior to injury and immediately prior to injury (the same as in Protocol 1). Following the three cycles of RIC the pups were marked by means of a skin tattoo and returned to their mother.

RIC was delivery by means of a ligature (Section 4.5.1) as Chapter 6 showed the blood pressure cuff was not a reliable means of delivering RIC. Statistical analysis was performed using GraphPad Prism 9.1.2.

Animals from multiple, related litters were randomly assigned to Protocol 1b (Chapter 6), Protocol 2 (Chapter 7) and Protocols 3a and 3b (this chapter).

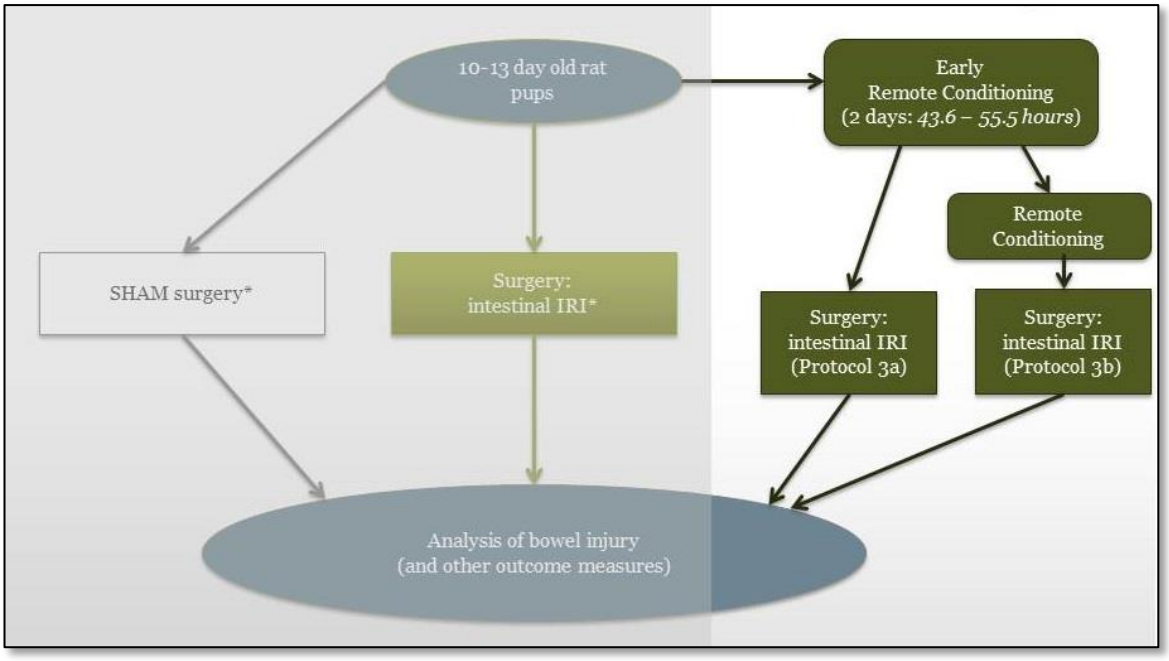


Figure 8-1 Experimental Protocol for early preRIC
*Sham Surgery and IRI animals for comparison from previous experiments (Chapter 5).

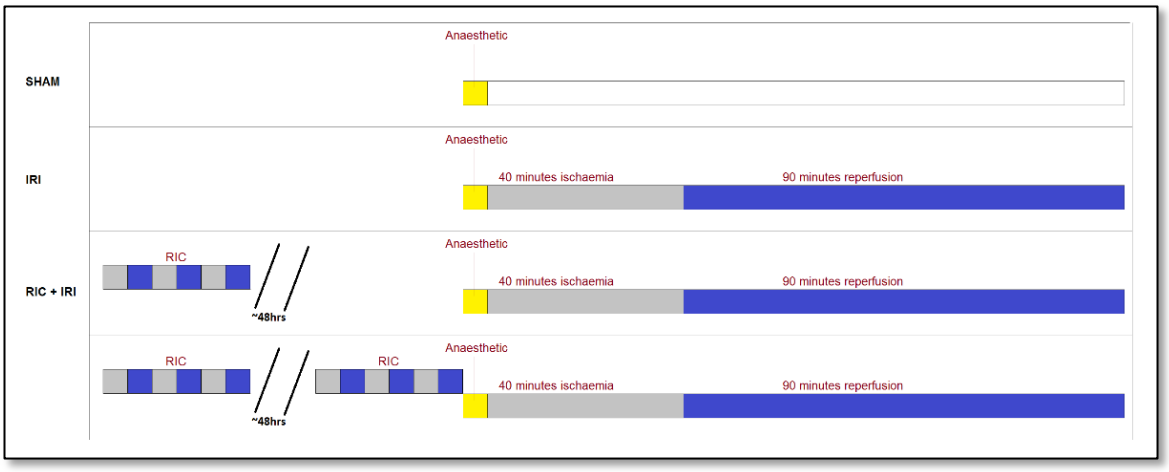


Figure 8-2 Experimental Protocol for early preRIC
*Sham Surgery and IRI animals for comparison from previous experiments (Chapter 5).

Macroscopic injury was assessed as described in Section 4.7 and microscopic injury was scored with the Chiu-Park scoring system, described in Section 4.8. For each animal, a specimen was taken from the terminal ileum and from the area of maximal macroscopic injury. The median score from three independent scores, blinded to which experimental group the specimen is derived from was used for analysis. (Comparison between the three scorers is shown in Appendix C).

8.4 Results – Protocol 3a: Early pre - Remote Ischaemic Conditioning

RIC was applied around 48 hours prior to injury. The range from application of RIC to injury was 43.5-55.5 hours, n = 18 animals.

8.4.1 Macroscopic Bowel Injury

In this protocol the range of total bowel harvested from these animals was 28-43cm. Animals exposed to 'early' preRIC had a median total injury of 68% (IQR 0-77%) and a median severe injury of 3% (0-42%) This compares to a median total injury of 100% (IQR 85-100%) in the IRI comparator group (p=0.0004) and a median of 78% (30-90%) of the bowel showing severe injury (p=0.0054). These are shown in Figures 8-3 and 8-4.

Median length of bowel injury			
	Mild	Severe	Total
Controls*	0%	0%	0%
IRI only*	18%	78%	100%
***Early pre*RIC + IRI**	50%	3%	68%

Table 8-1 Protocol 3a: Macroscopic injury.
*Controls (Sham surgery) and IRI only taken from protocol 1a.
Full data is shown in Table B-10

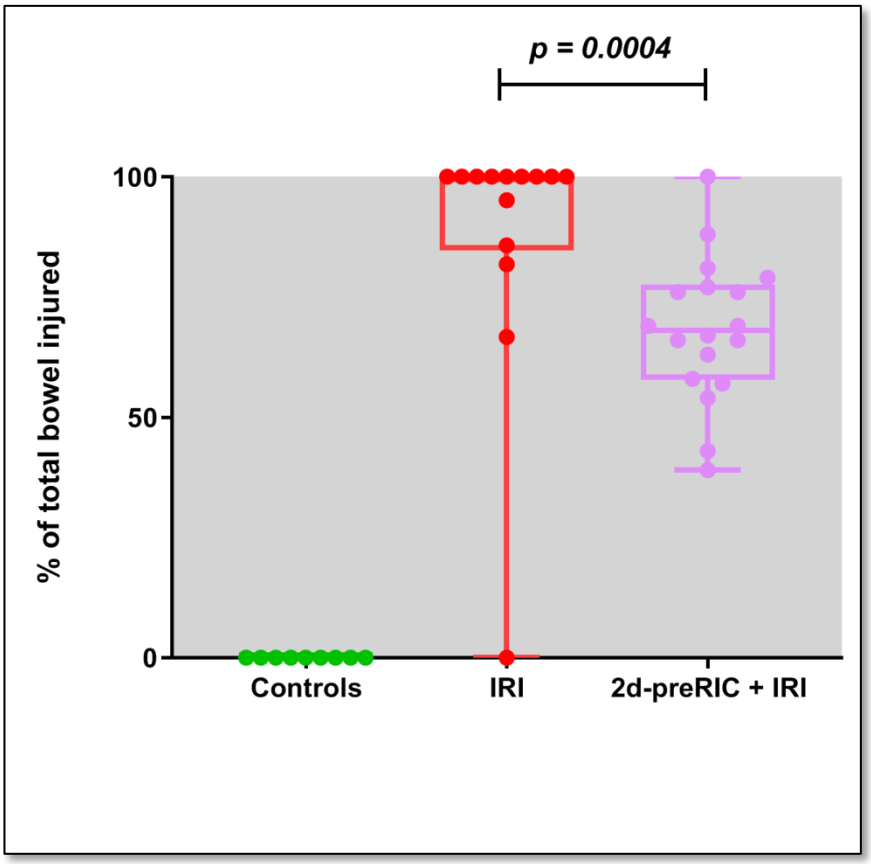


Figure 8-3 Protocol 3a: percentage of total bowel showing macroscopic injury. Graph shows each data point, inter-quartile range and median

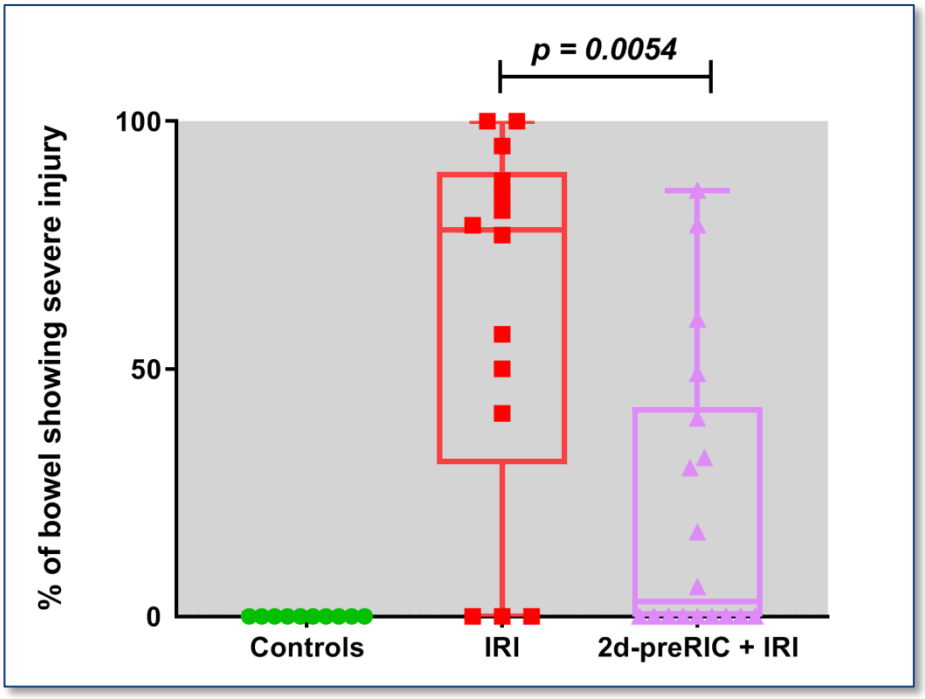


Figure 8-4 Protocol 3a: percentage of total bowel showing severe macroscopic injury. Graph shows each data point, inter-quartile range and median

8.4.1 Microscopic Bowel Injury

Table 8-2 shows the Chiu-park scores for this experimental protocol.

Animals that unwent RIC two days prior to IRI had a median score of 3 (IQR 3-4) for the bowel specimen taken from a fixed point in the terminal ileum sample and 5 (4-6) for area of maximal injury. In both cases, they show a non-significant trend towards lower scores in the animal who underwent conditioning two days prior to injury, (p = 0.07 and 0.17 respectively)

	Controls*		IRI only*		early preRIC + IRI	
	TIF	MXF	TIF	MXF	TIF	MXF
Median	0	0	4	5.5	3	5
Range	0 – 0	0 – 1	0 – 7	4 – 7	1 – 6	1 – 6
IQR	0 – 0	0 – 1	3 – 5	4 – 6	3 – 4	4 – 6

Table 8-2 Protocol 3a: Median Chiu-Park scores for each group.
TIF = Fixed point in terminal ileum
MXF = Area of maximal macroscopic injury
*Controls and IRI only animals from Protocol 1a.
Full data are shown in Appendix B (Table B-10)

Figure 8-5 and Figure 8-6 show the groups and each of the data points.

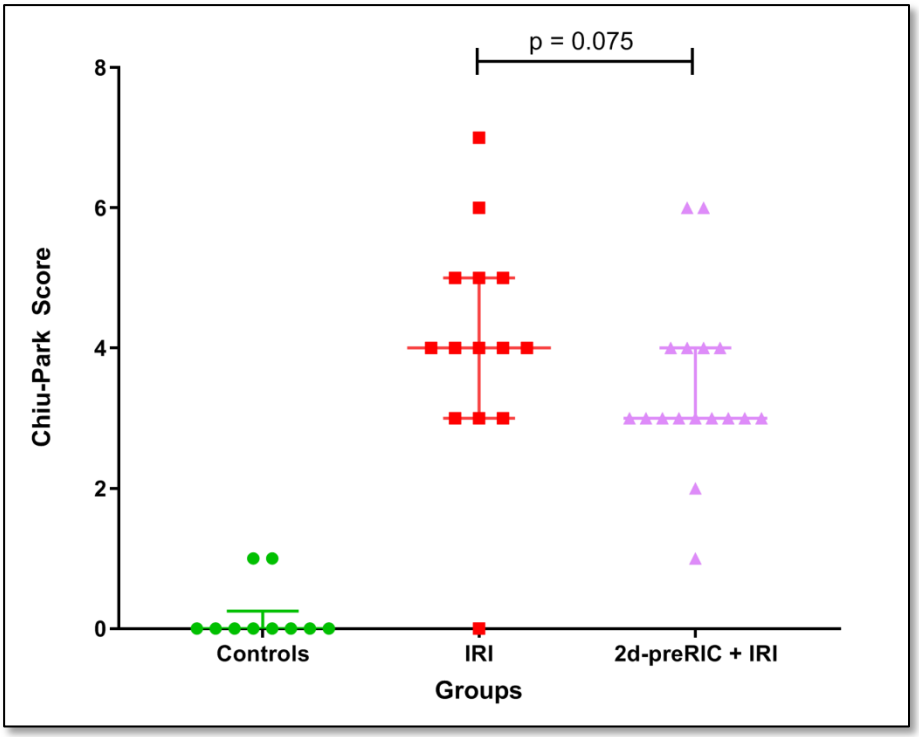


Figure 8-5 Protocol 3a: Chiu-Park Scores – fixed point in the terminal ileum
Graph shows each data point, inter-quartile range and median

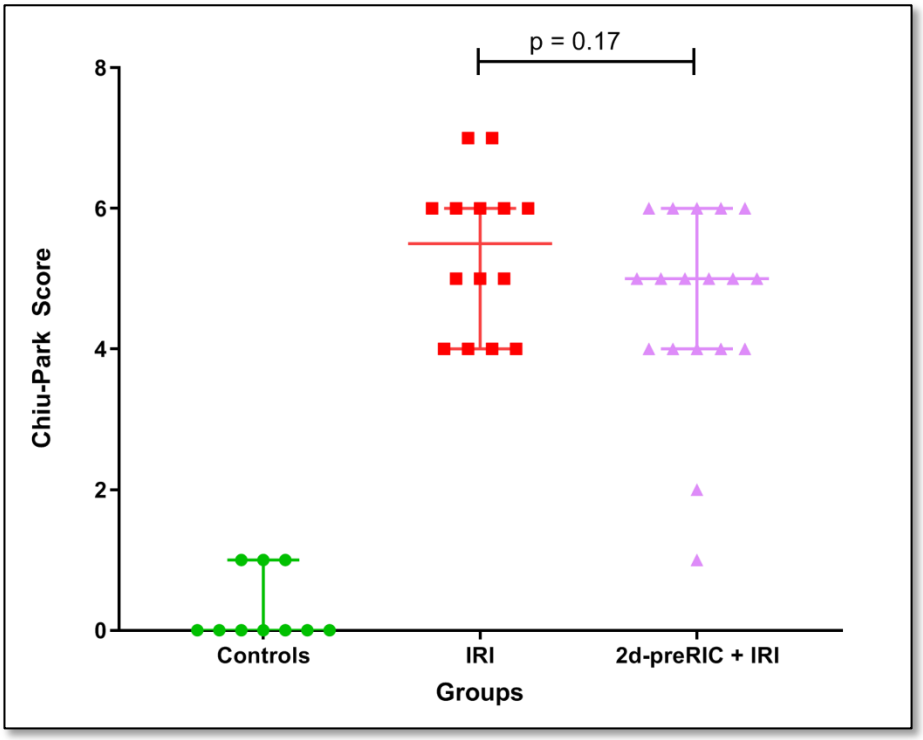


Figure 8-6 Protocol 3a: Chiu-Park Scores – area of maximal macroscopic injury
Graph shows each data point, inter-quartile range and median

8.5 Results – Protocol 3b: Early pre- and immediate pre- Remote Ischaemic Conditioning

RIC was applied approximately 48 hours prior to injury (range of 38.6-55.2 hours) and immediately prior to injury, n = 16 animals.

8.5.1 Macroscopic Bowel Injury

The range of total small bowel harvested in this protocol was 28-46cm. In animals that had both early and immediate preRIC, the total injury was reduced from a median of 100% (IQR 85 - 100%) to 76% (IQR 62 - 91%) p = 0.011 (Figure 8-7). Similarly the two episodes of RIC reduced the median severe injury from 78% (30 - 90%)) to 0% (IQR 0 - 39%) p = 0. 0019 (Figure 8-8).

Median length of bowel injury			
	Mild	Severe	Total
Controls*	0%	0%	0%
IRI only*	18%	78%	100%
***Early* and immediate preRIC + IRI**	64%	0%	76%

Table 8-3 Protocol 3b: Macroscopic injury.
*Controls (Sham surgery) and IRI only taken from protocol 1a. Full data are shown in Appendix B (Table B-11)

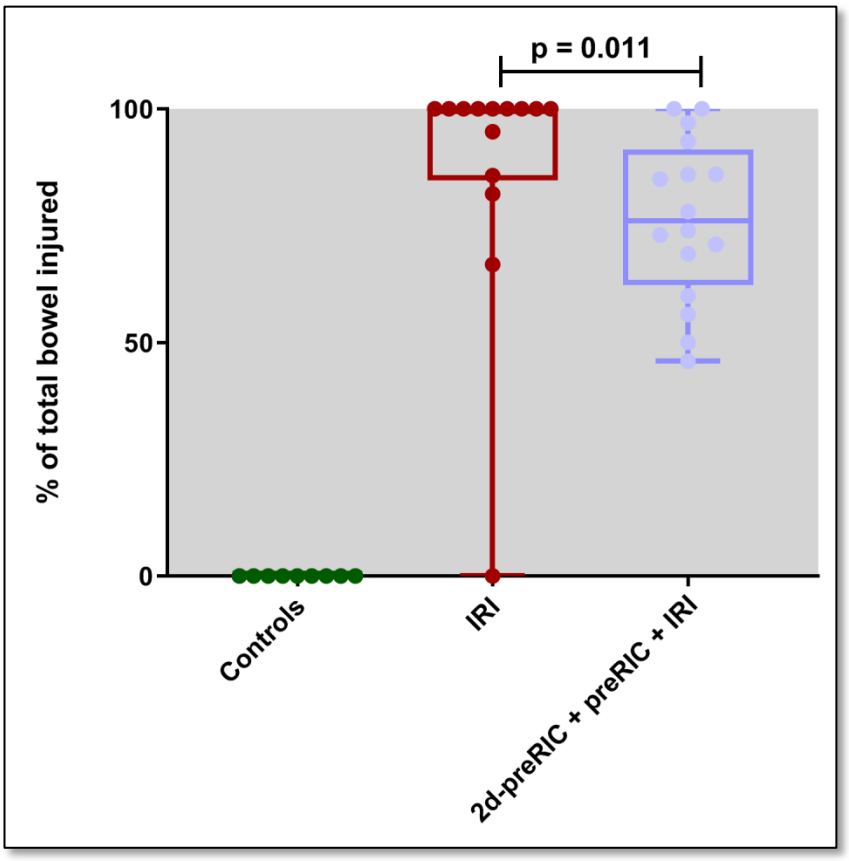


Figure 8-7 Protocol 3b: percentage of total bowel showing macroscopic injury.

Graph shows each data point, inter-quartile range and median

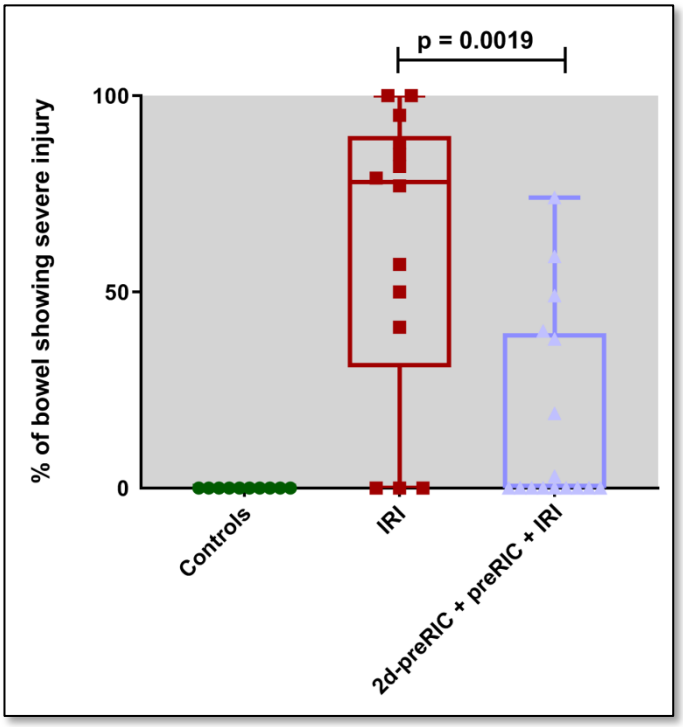


Figure 8-8 Protocol 3b: percentage of total bowel showing severe macroscopic injury.
Graph shows each data point, inter-quartile range and median

8.5.2 Microscopic Bowel Injury

Animals that underwent RIC two days prior to – and immediately prior to injury showed significant reduction in the Chiu-Park scores at both a fixed point in the terminal ileum and in the area of maximal injury. In the fixed point the median score was 2.5 (IQR 1 – 3) compared to 4 (3-5) in the IRI-only group (p = 0.003). In the area of maximal injury the scores were reduced from 5.5 (IQR 5-6) to 3 (1-3), p = 0.0001. These results are summarised in Table 8-4 and shown graphically in Figure 8-9 and Figure 8-10.

	Controls*		IRI only*		Early and immediate preRIC + IRI	
	TIF	MXF	TIF	MXF	TIF	MXF
Median	0	0	4	5.5	2.5	3
Range	0 – 0	0 – 1	0 – 7	4 – 7	0 – 4	0 – 6
IQR	0 – 0	0 – 1	3 – 5	4 – 6	1 – 3	1 -3

Table 8-4 Protocol 3b: Median Chiu-Park scores for each group.
TIF = Fixed point in terminal ileum
MXF = Area of maximal macroscopic injury
*Controls and IRI only animals from Protocol 1a.
Full data are shown in Appendix B (Table B-11)

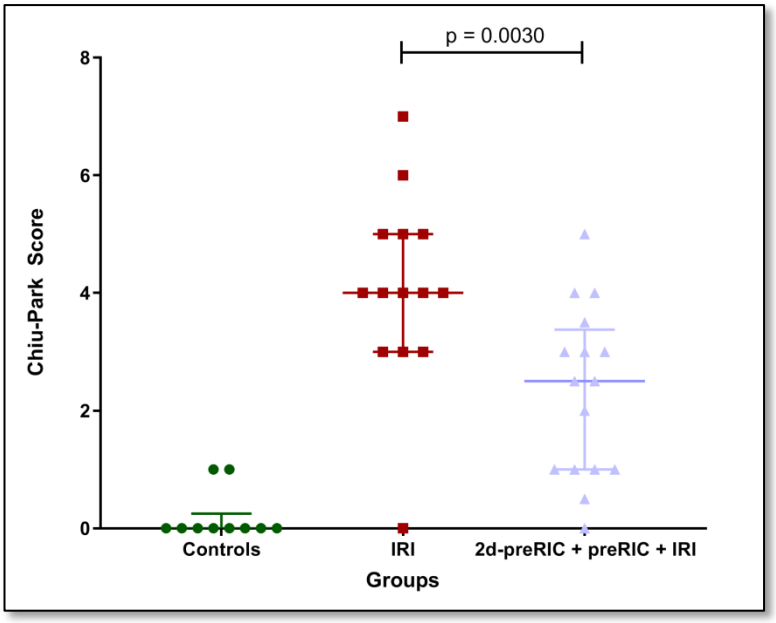


Figure 8-9 Protocol 3b: Chiu-Park Scores – fixed point in the terminal ileum
Graph shows each data point, inter-quartile range and median

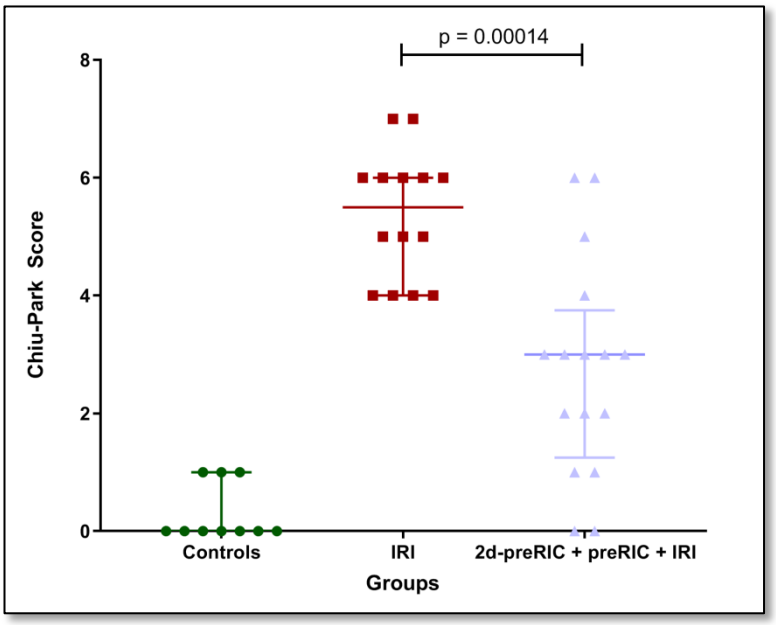


Figure 8-10 Protocol 3b: Chiu-Park Scores – area of maximal intestinal injury
Graph shows each data point, inter-quartile range and median

8.6 Discussion

The purpose of this series of experiments (Protocols 3a and 3b) was two-fold. Firstly to establish if the protective effect of RIC demonstrated when RIC was delivered either immediately before injury (Protocol 1, Chapter 5) or after the onset of injury (postRIC) as seen in Protocol 2 (Chapter 7), can also be found if the RIC was performed an extended time prior to injury. The second purpose was to investigate whether there is an additive benefit from delivering RIC on multiple occasions.

It has previously been reported that the protective effect of RIC can be seen over an extended time period.[277] More recently, Wang *et al.* (2021) demonstrated a clear benefit of RIC on cardiac ischaemia when the ischaemic injury occurs 24 hours after delivery of RIC.[385]

Similarly, 'dose-effects' of RIC whereby more than one application has a benefit over a single application are well described.[319,386]

8.6.1 Macroscopic Analysis

The macroscopic injury pattern in animals that had RIC 48 hours prior to injury was significantly reduced compared to animals that had IRI only; both the total injury and the amount of bowel showing severe necrosis was reduced. Animals that underwent two episodes of RIC had a very similar injury pattern; i.e. significantly reduced compared to injury alone but essentially unchanged compared to the animals that had the 'early' pre-conditioning alone.

These results do support the notion that the extent of bowel injury is reduced by conditioning performed two days prior to injury, hinting at a prolonged protective effect, which could be meaningful in the clinical context. However, in terms of this measure alone, there is no additive benefit from the second application of RIC.

8.6.2 Microscopic Analysis

The microscopic analysis of both a fixed point in the terminal ileum and the area of maximal injury showed no statistically significant differences between animals than had 'early' pre-conditioning and those that were exposed to injury without RIX but in both cases, there is a non-significant trend towards lower Chiu-Park scores in animals who were conditioned.

Animals that had both early- and immediate- pre-conditioning showed lower scores than animals that did not undergo RIC prior to injury. This is of course, expected, given that Protocol 1a has previously shown that immediate-pre-RIC reduces the severity of bowel injury. In previous Potocols, a statistically significant difference in the severity of injury has been seen in the area of maximal injury (median score reduced by 2, $p = 0.002$ for preRIC and by 1.25, $p = 0.031$ for postRIC). In this protocol though a statistically significant lower score was also demonstrated in the fixed point in the terminal ileum samples as well (Figure 8-9).

Figure 8-11 and

Figure 8-12 show the Chiu-Park scores for animals that underwent RIC immediately prior to injury (Protocol 1a), animals that had RIC two days in advance of the injury (Protocol 3a) and animals that had both the early- and immediate pre-RIC (Protocol 3b).

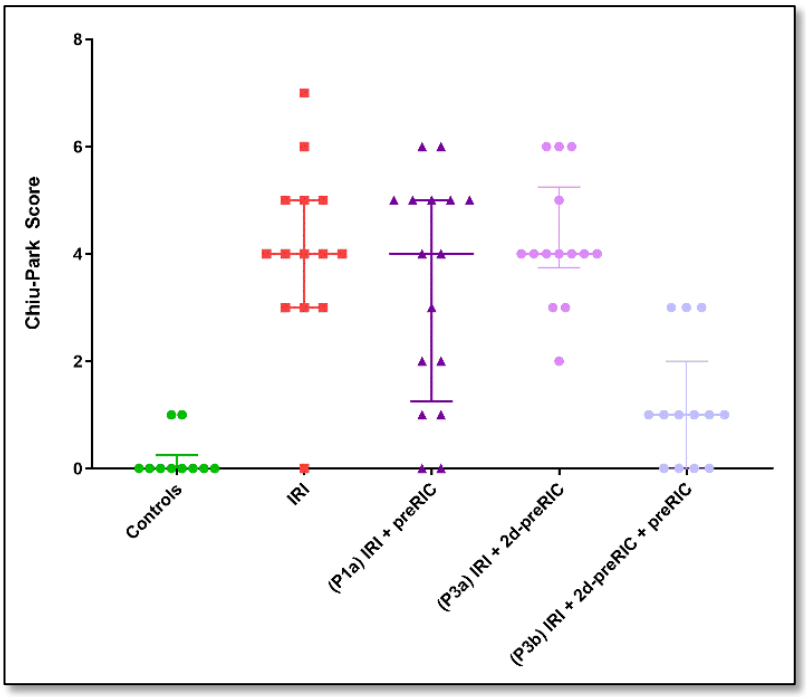


Figure 8-11 Comparison between different time points of RIC application: Chiu-Park Scores – fixed point in the terminal ileum.
Graph shows each data point, inter-quartile range and median. Animals exposed to RIC two days prior to injury had a non-significant trend towards lower scores whilst those that had immediate preRIC as well had the lowest scores.

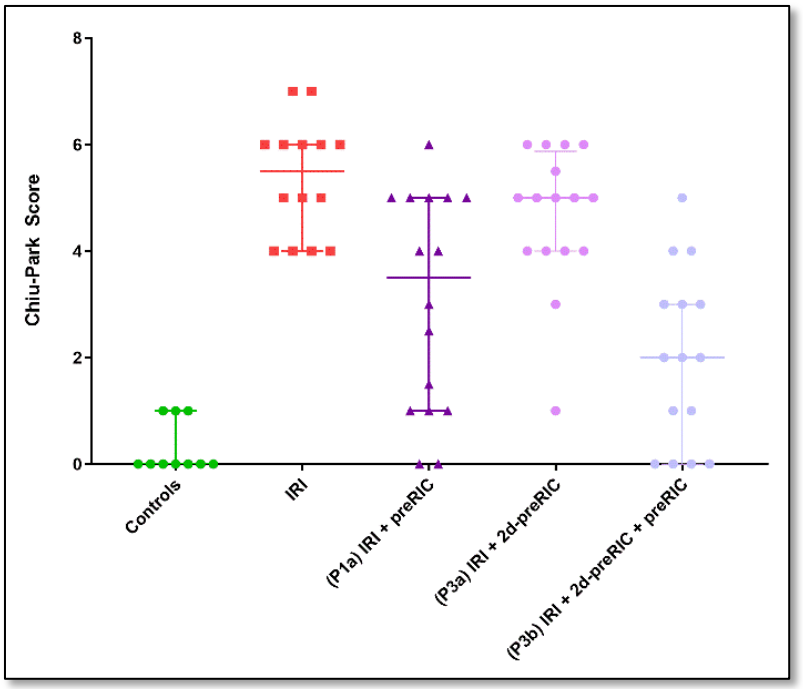


Figure 8-12 Comparison between different time points of RIC application: Chiu-Park Scores – area of maximal injury.
Graph shows each data point, inter-quartile range and median. Animals exposed to RIC two days prior to injury had a non-significant trend towards lower scores whilst those that had immediate preRIC as well had the lowest scores.

In both sets of samples the pattern is the same, the injury scores in animals exposed to RIC twice are lowest. With the samples taken from the fixed point in the terminal ileum, animals exposed to

two 'doses' of RIC are the only ones that are statistically lower than injury alone ($p = 0.003$). In the area of maximal injury, the trends show that whilst early preRIC may reduce the injury, it is a smaller effect than when the RIC is delivered immediately prior to injury but the lowest scores are seen in animals who underwent RIC twice (Median scores of 3 vs 5.5 for IRI alone). Statistical analysis with correction for multiple comparison (Kruskal-Wallis testing with post-hoc analysis) shows that the difference in effect between immediate-pre-RIC and animals that had both early-*and* immediate-pre-RIC does not reach statistical significance however.

8.7 Conclusion

Taken together, these data show that there is a protective effect from RIC up to at least 48 hours from the delivery of RIC. Moreover, there is a dose-like additive effect of giving RIC more than once in advance of injury.

If RIC can be translated to the clinical context, these data would suggest a prolonged effect and an additive effect and thus would inform the design of a protocol that could be used on human neonates to prevent NEC. For example, if RIC was delivered two to three times per week in at risk infants it could result in lower rates and lower severity of NEC.

Chapter 9 Transcriptomic Analysis of the Effects of Remote Ischaemic Conditioning on the Bowel

9.1 Abstract

9.1.1 Introduction

Remote ischaemic conditioning (RIC) provides a powerful protective effect against ischaemia-reperfusion injury (RIC) in multiple organs systems. Hind limb RIC delivered just prior to exposure to intestinal ischaemia-reperfusion (preRIC) reduces both the extent and severity of the resultant intestinal injury. RNAseq is a method of analysing the changes in gene expression by quantitative analysis of the entire transcriptome in tissue exposed to different experimental conditions. In this chapter, I describe the experiments used to investigate the putative mechanisms by which RIC confers such a profoundly protective effect on the intestine.

9.1.2 Methods

Related rat-pups aged between 10 and 13 days old were randomly assigned to four groups; SHAM, Ischaemia-reperfusion injury only (IRI), RIC and RIC+IRI. IRI animals, under terminal anaeasthesia underwent 40 minutes of ischaemic, followed by 90 minutes of reperfusion. Animals that underwent RIC had three cycles of 5 minutes of alternating ischaemia/reperfusion by means of a ligature applied to the hind limb.

Samples from a fixed point in the terminal ileum were collected immediately after the experiment and were stored in RNA-preserving media (RNAlater). The RNA was extracted using the Qiagen RNeasy Plus protocol.

Next generation sequencing was performed using the Illumina NextSeq 550 High Throughput Next Generation Sequencer system and genome alignment was performed with Qiagen's CLC read mapper to produce the gene raw counts.

Transciptome analysis was done using *R* v 3.6.1. Analysis was done with two approaches: differential expression testing and targeted gene analysis.

9.1.3 Results

Quality control assessment confirmed that the resultant samples contained high quality RNA for analysis.

Differential expression testing showed 868 statistically significant differentially expressed genes in animals exposed to RIC alone compared to SHAM and 135 in animals exposed to IRI and RIC compared to IRI alone. Comparison between these two sets of genes showed that 25 genes were differentially expressed in both groups. Of these, several genes involved in pro-inflammatory pathways including NF-κβ2, Cxcl1, SOD2 and Map3k8 all show reduced expression in response to RIC.

Targeted gene analysis revealed increased expression in PI3K which is part of the so-called RISK-pathway which is a key part of the protective mechanisms of RIC in the heart.

9.1.4 Discussion

Expression patterns suggest that within the intestine, RIC suppresses pro-inflammatory pathways, especially the NF-κβ pathway and that an equivalent of the RISK-pathway identified in cardiac tissue may be present in the intestine. There is cross-over between the pro-inflammatory pathways suppressed here and those that are involved in several stages of the pathogenesis of NEC, further supporting the potential for RIC to be effective at treating and preventing the human disease.

9.2 Introduction

Whole transcriptome analysis of mRNA is a very powerful tool for studying complex biochemical pathways. In recent years, a few papers have studied the changes in gene expression triggered by RIC.

As discussed in Section 3.5, there are inherently three stages to the mechanisms of RIC: the effector organ/tissue (skeletal muscle in many cases); the transmission mechanism; and the target organ (bowel in this case).

Yoon *et al.* (2015)[294] used microarray analysis to study the gene expression changes in a porcine model of preRIC and renal Ischaemia reperfusion injury (IRI). They found that RIC had effects on the expression of multiple cytokines (including Interleukin-10 (IL-10) and Transforming growth factor beta (TGF-β)), as well as modulating the complement and coagulation cascades. However, there are significant limitations to these data. Firstly, in their model of renal IRI, RIC did not alter the primary end-point, which was a surrogate for renal function. Secondly the tissues were harvested two days after the IRI and thus do not capture the immediate changes in gene-expression.

The intermediate (or 'transmission') stage of RIC was examined in humans by Nikkola *et al.* (2015).[288] This study involved analysing the blood transciptome in patients undergoing RIC for treatment of aneurysmal subarachnoid haemorrhage. The major strength of this work was the ability to pair the analysis and examine the transcriptome both before and after the application of RIC. As well as demonstrating changes in chromosomal methylation, they showed changes in expression of genes involved in the mitotic cell cycle.

The analysis of gene expression in a porcine model of myocardial infarction with post-ischaemic conditioning was reported by Lukovic *et al.* in 2019. Unsurprisingly many of the gene expression changes seen in the myocardium are the same in animals exposed to post conditioning as those who were not; both groups undergoing a myocardial injury. However, there were distinct genes whose regulation was distinctly different in animals who underwent conditioning. They concluded that ischaemic conditioning downregulates ECM-proteinases, ribosomal subunits and platelet and leukocyte adhesion molecules. Additionally, post-conditioning inhibited the activation of inflammatory leukocytes.

This chapter concerns the changes in expression patterns in the bowel tissue. From a practical perspective, the analysis of the blood seen in Nikkola *et al.* (2015)[288] would be impossible to replicate in this model due to the small circulating blood volume of the rat pups making multiple blood sampling impossible. Given that I was harvesting the bowel, it was very straightforward to

obtain appropriate samples for analysis of the end-organ changes in gene expression. Moreover, it remains the case that the majority of study into RIC is focused on the heart and to a lesser extent the brain. Therefore the understanding of the processes within gut tissue which are important in the context of NEC, are not well understood.

Hypotheses:

1. **Differential Expression Testing will show changes in the expression of genes in animals exposed to Remote Ischaemic Conditioning that are responsible for the protective effect of Remote Ischaemic Conditioning in the intestine.**
2. **Analysis of the gene expression changes in animals exposed to both conditioning and ischaemic insult as well as those exposed to conditioning alone will show a '2-hit.' Exposure to insult will cause further changes in gene expression not evident in animals only exposed to conditioning.**
3. **Targeted analysis of animals exposed to injury alone will better delineate how well the model represents human NEC.**
4. **Targeted analysis will show a significant change in the cell population (i.e. in influx of immune cells).**
5. **Targeted analysis will show that pathways seen in Remote Ischaemic Conditioning in other organ systems are replicated in the intestine.**

9.3 Methods

9.3.1 Animal Experiments

The animal experimentation for this study is described in detail in Chapter 5, with the addition of a fourth group of animals who underwent RIC and sham surgery (Figure 9-1). As detailed in Section 4.6.1, specimens were stored in RNAlater™ Stabilization Solution (Qiagen, Germany) at -20^{o}C. As per the standard protocol; samples were immediately immersed in the RNAlater solution,

stored overnight at 4°C before transfer to -20°C for long term storage.

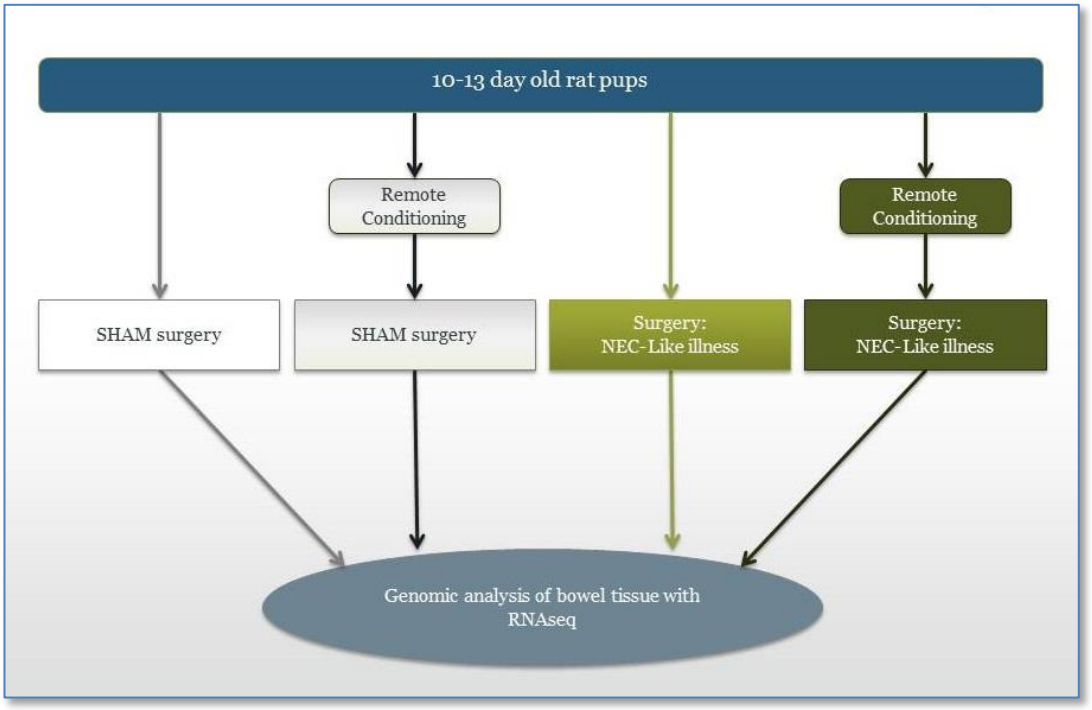


Figure 9-1 Experimental Groups.
Rat pups were randomly allocated to one of four groups: Sham surgery; Remote ischaemic conditioning followed by sham surgery; surgery/ischaemia-reperfusion injury; and remote ischaemic conditioning followed by surgery/ischaemia-reperfusion injury. Animal experiments described in detail in Sections 4.4, 4.5 and 4.6 and Chapter 5.

The RNA extraction, library preparation and next-generation sequencing were performed by Qiagen Genomic Services (Hilden, Germany). Genome alignment was also performed by Qiagen, who provided the raw-count matrix used for analysis of differential expression (Figure 9-2). The formal report, produced by Qiagen, of the laboratory processing is in Appendix E.

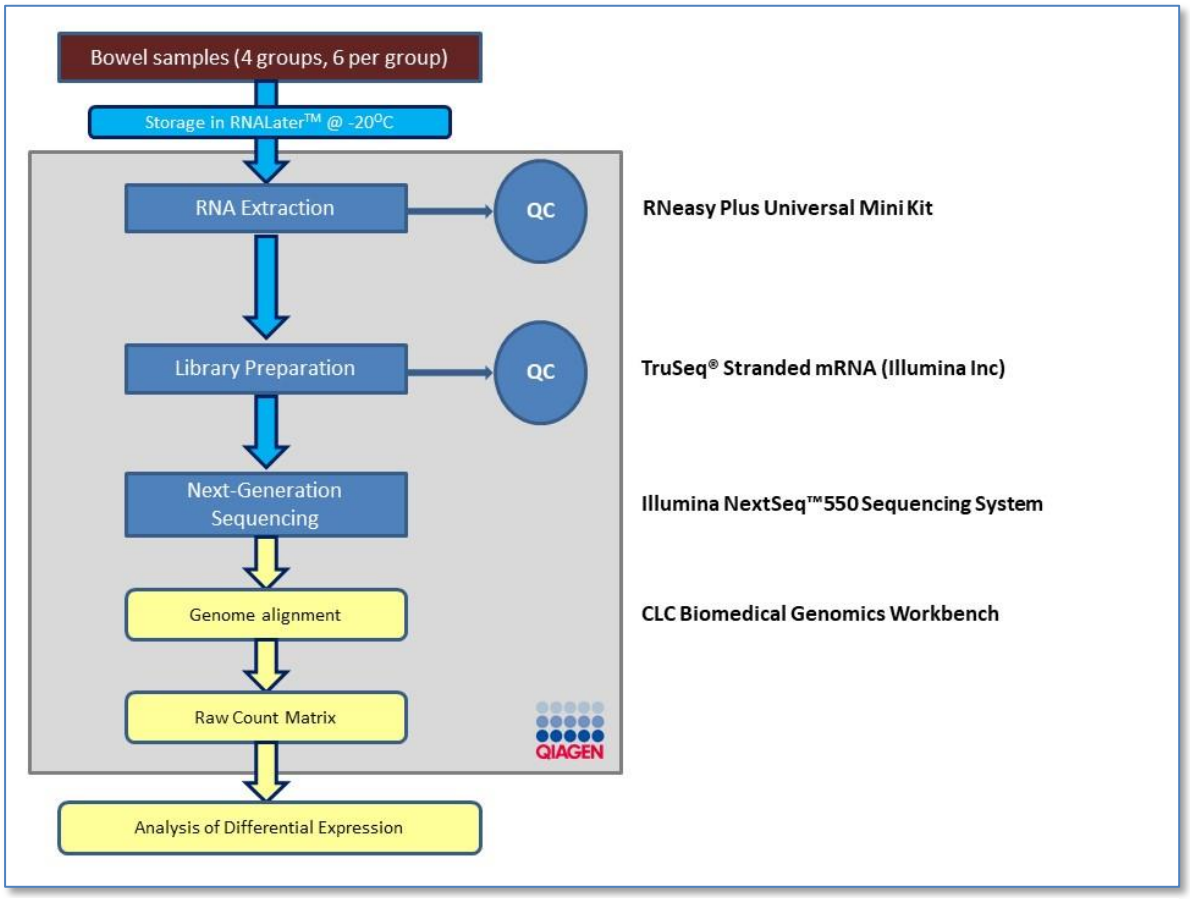


Figure 9-2 RNAseq Workflow
Samples of bowel tissue were sent to Qiagen in RNAlater™. All the steps in the grey box were performed by Qiagen Laboratories. The 'Wet-lab' work prior to RNA extraction is described in detail in Sections 4.4, 4.5 and 4.6 and Chapter 5.

9.3.2 RNA Extraction

RNA Extraction and quality control was performed using the RNeasy Plus protocol which uses phenol/guanidine for lysis and silica-membrane purification of RNA (Figure 9-3).

The samples were removed from RNALater and disrupted and simultaneously homogenised using the TissueLyser LT with 900 µl of QIAzol lysis reagent for each sample.

The lysates were removed by pippette and stored at room temperature for 5 minutes. 100µl gDNA Eliminator Solution was added and the sample shaken for 15 seconds. This reduces the genomic DNA making further DNase treatment unnecessary. 180 µl of chloroform was added to each and manually shaken for 15 seconds before storage at room temperature for 2 - 3 minutes. This was then centrifuged at 12000G for 15 minutes at 4°C. The upper, colourless, aqueous phase was transferred to new tubes and equal volumes of 70% ethanol added. The samples were transferred to RNeasy mini spin columns and centrifuged for 15 seconds at 8000G at room temperature. 700 µl of Buffer RWT was added and then centrifuged for a further 15 seconds at 8000G and room temperature. 500 µl Buffer RPE was added and then centrifuged for a further 15

seconds at 8000H and room temperature to wash the membrane. The flow-through was discarded. This step was repeated. The RNeasy spin column was then placed in a new 1.5ml collection tube. 30-50 µl of RNase-free water was added and then centrifuged for 1 minute at 8000G. This step was then repeated. The second elution gives approximately 30% higher RNA yields.

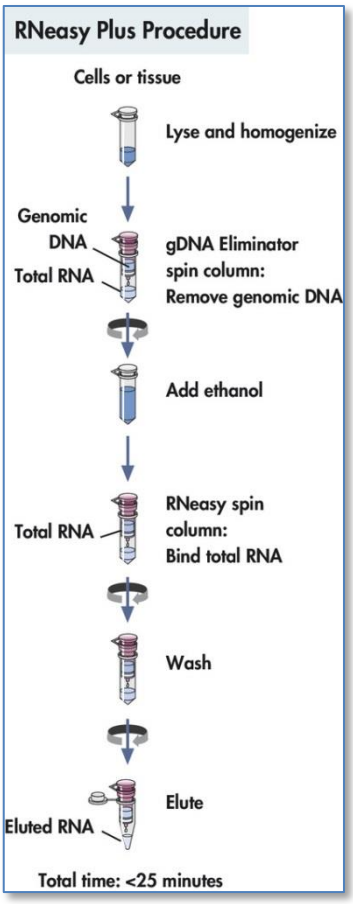


Figure 9-3 RNeasy RNA extraction method. *Figure derived from Qiagen RNeasy instruction manual.*

Sample quality control was then performed on each sample. The quantity of RNA was determined spectrophotometrically by measuring the absorbance at 260 nm. 10 µl of each RNA sample was added to 490 µl of 10 mM Tris·Cl at pH 7.0 in an RNase-free cuvette. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per ml. The integrity of the total RNA purified was then assayed with the Agilent 4200 TapeStation system (Agilent Technologies Ltd, USA). 2 µl of each sample was used to assess RNA purity generating an RNA Integrity Number (RIN). RIN is a validated measure of the level of fragmentation of RNA. RIN scores above 8 are considered 'high quality;' scores of 5 - 7 indicate some degree of fragmentation.[387,388]

9.3.3 Library Preparation

Having confirmed that the quality of the RNA isolated was sufficiently high, I approved proceeding to the next stage – library preparation. Once again, this was done by Qiagen using the TruSeq® Stranded mRNA Sample preparation kit (Illumina Inc). This kit is designed for producing a cDNA library for whole transcriptome analysis. This procedure is summarised in Figure 9-4.

The starting material (500 ng) of total RNA was mRNA enriched using the oligodT bead system. The isolated mRNA was subsequently fragmented using enzymatic fragmentation. Then first strand synthesis and second strand synthesis were performed and the double stranded cDNA was purified (AMPure XP, Beckman Coulter). The cDNA was end repaired, 3' adenylated and Illumina sequencing adaptors ligated onto the fragments ends, and the library was purified (AMPure XP). The mRNA stranded libraries were pre-amplified with PCR and purified (AMPure XP). The libraries size distribution was validated and quality inspected on a Bioanalyzer 2100 or BioAnalyzer 4200 TapeStation (Agilent Technologies). High quality libraries are pooled in equimolar concentrations based on the Bioanalyzer Smear Analysis tool (Agilent Technologies). The library pool(s) were quantified using qPCR and optimal concentration of the library pool used to generate the clusters

on the surface of a flowcell before sequencing on a NextSeq500) instrument (75 cycles, according to the manufacturer instructions (Illumina Inc.).

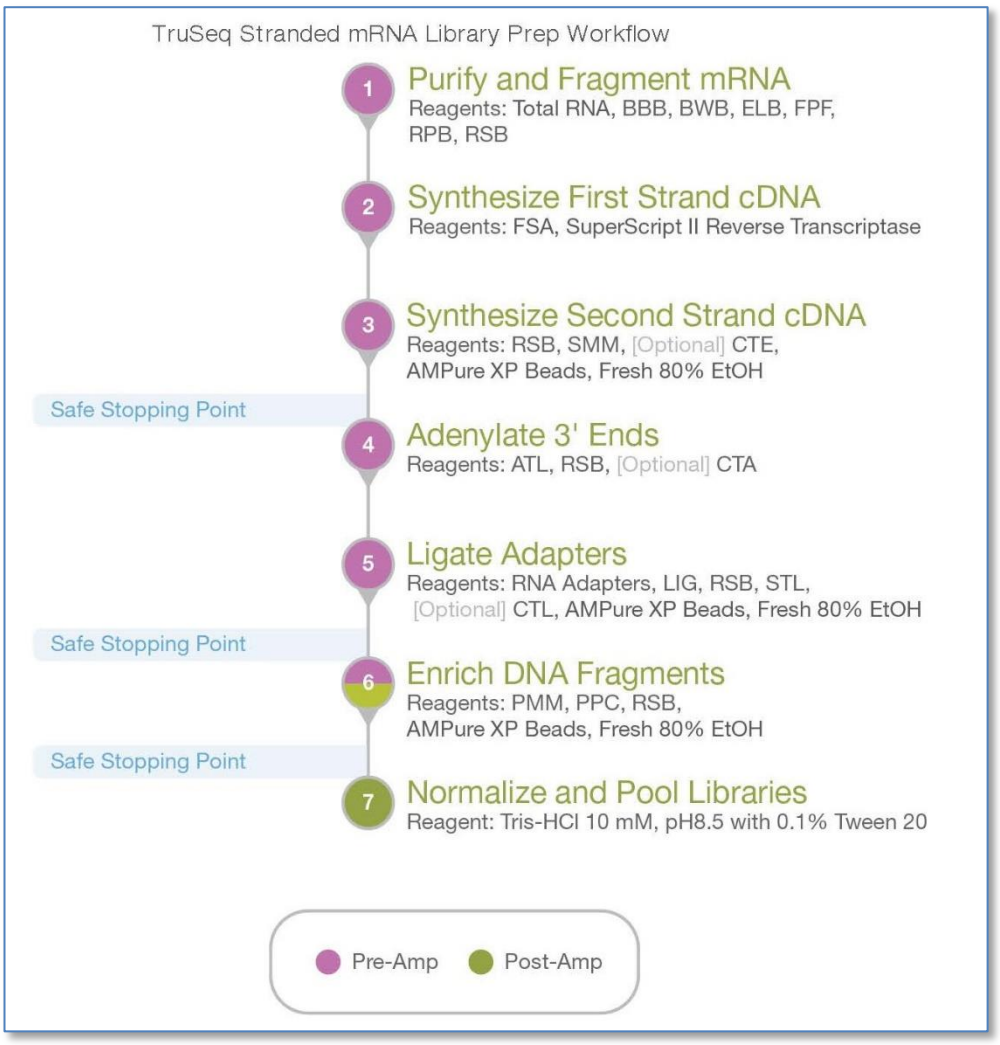


Figure 9-4 Qiagen's Library Preparation Workflow.
Figure derived from Qiagen's published protocol.

9.3.4 Next-generation Sequencing

The sequencing of the RNA was preformed using an Illumina NextSeq 550 High Throughput Next Generation Sequencer. (Qiagen, Germany / Illumina, USA). This standardised process for double-stranded sequencing is illustrated by this proprietary video from Illumina: https://www.youtube.com/watch?v=fCd6B5HRaZ8&feature=youtu.be

The raw counts were produced for genome alignment by using 30M reads and a read depth of 75 base pairs.

9.3.5 Genome Alignment

The genome alignment step of the analysis was performed using CLC read mapper within Qiagen's Biomedical Genomics Workbench software (Qiagen, Germany / CLC bio, Denmark).[389] Baruzzo *et al.* (2017)[390] performed benchmarking using simulated data to compare alignment algorithms. This showed that CLC read mapper out-performs the most-widely used sequences aligning tools (TOPHAT2[391] and STAR[392]).

9.3.6 Analysis

Transciptome analysis was done using *R* v 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria).[393] The *R* Script used is included in Appendix F (Section F.1). To test for potential confounders, known phenotypic data for each animal was inputted. The age in days, gender, weight, from which litter the animal came and the date of the experiment was entered for each animal and combined with the raw read counts. Before formal analysis of the data, the raw data was explored with principal component analysis to ensure quality control and remove any significant outliers from the model if necessary.

9.3.6.1 Data exploration and quality control

Conceptually, in order to be meaningful, the intergroup variability within the datasets, representing differences between experimental conditions in comparison with control conditions, should be greater than the intragroup variability, representing technical or biological variability.[394] For example, these analyses may identify a mislabeled or contaminated gene that would align well and show good read depth. In order to assess this, I used the following steps:

1. A box plot was produced on the raw sample counts of mRNA for each sample to assess the read counts between samples. This was then repeated with lowly expressed mRNA genes excluded. This was done to remove barely expressed genes as they do not add to the analysis. Removing these reads for the samples reduces the need for multiple testing corrections.
2. Using the whole dataset; Hierarchical clustering was performed using the Euclidean distance method; Principal component analysis was performed and plotted; and median vs interquartile range for each raw count was plotted. Finally the PCA was plotted with the samples labelled for the known phenotypical factors.

Any sample that was shown to be an outlier in more than one of these plots would be considered as a true outlier and excluded from further analysis.

9.3.6.2 Differential Expression Testing

The differential gene expression was assessed in four 2-way comparisons, using *edgeR* (version 3.6.1).[395,396] The key biological question is *what is different in the bowel tissue following conditioning in order to provide the protective effect?*

The most obvious analysis to carry out therefore is between animals that have undergone the IRI insult to the bowel (IRI only) and those that had conditioning prior to the injury (RIC+IRI). It is expected that this analysis will be informative but the tissue in question has undergone a significant physiological stress and damage and thus whether the RNA recovered gives meaningful data was not a certainty. Moreover, whilst the control group for the comparison has also undergone the same insult, this will inevitably have diverse and extensive effects on multiple biochemical pathways. The potential for a lot of noise here means that analysis of the effect of RIC without the IRI is also desirable. Conversely, this analysis alone may not be fully informative as it is at least conceivable that the protective processes (in terms of meaningful changes in gene expression) in the end organ are triggered *both* by the conditioning and the stimulus of the ischaemic insult. Hence the decision to focus on both a comparison between animals who underwent RIC vs controls *and* animals who underwent RIC prior to IRI vs IRI alone. This was done using *edgeR*.[395,396] The model was built using the whole dataset and then the direct comparisons between the groups performed.

It is important to note here that SHAM animals have still undergone an anaesthetic and a laparotomy.

gplots v 3.1.1,[397] *edgeR*[395] and *biomaRt*[398] packages were used for these analysis. Volcano plots and heatmaps were produced to display the differentially expressed genes. *biomaRt* allows the gene IDs to be converted to gene and protein name.

9.3.6.3 Differential Expression – Targeted gene analysis

As well as performing differential expression testing – guided by the changes in gene expression that are seen within the samples, I interrogated the data for specific genes of interest. These broke down to various sub-groups. In order to compare these different genes, simple box-plots and t-tests were performed using *R.* The *R* Script and data files are included in Appendix F.

Protein name	Gene name	Marker for
CD45	PTPRC	Immune cells
CD163	CD163	Macrophages
CD164	CD164	Macrophages
CD209	CD209	Macrophages
CD206	MRC1	Macrophages
CD68	CD68	Macrophages
CD11c	ITGAE	Macrophages
CD20	MS4A1	B Cells
CD19	CD19	B Cells
CD3	CD3G	T Cells
CD4	CD4	T Cells
CD8	CD8A	T Cells
CD103	ITGAE	T Cells
CD69	CD69	T Cells
CD56	NCAM1	NK Cells
KIR receptors	KIR3DL1	NK Cells
CD109	CD109	Endothelial Cells
CD31	PECAM1	Monocytes
CD15	FUT4	Neutrophils
Vimentin	VIM	Fibroblasts
N-cadherin	N-cadherin	Fibroblasts
CDH2	CDH2	Fibroblasts
Desmin	DES	alpha smooth muscle
N-cadherin	N-cadherin	alpha smooth muscle
CDH2	CDH2	alpha smooth muscle

Table 9-1 Selected proteins used as markers of the cell populations
The gene names refer to the names used by the Ensembl database.[399]

9.3.6.3.1 Cell types

The tissue samples for this RNAseq was whole bowel, removed and processed on-block. By definition, therefore the samples contained multiple cell types including mucosal, serosal and muscle cells. As well as these structural cell types, the tissue also contains immunological cells. It would not be surprising if these populations of cells changed due to either RIC or IRI or indeed both. For this reasons, a selection of genes that are markers for various cell types were analysed across the four groups. These are summarised in Table 9-1.[400,401]

9.3.6.3.2 Biological Processes

The tissue from experimental animals in this model has been exposed to hypoxic stress. This small number of genes was selected to assess the changes seen in gene expression due to the IRI and whether the application of RIC has an effect on this both in animals exposed to injury but also in those that underwent RIC only.[136,402,403]

Protein name	Gene name	Marker for
HIF1a	HIF1a	Hypoxia
HIF2a	ARNT	Hypoxia
HIF1b	EPAS1	Hypoxia
VEGF	VEGFA	Hypoxia
KRT7	KRT7	Type 1 cytokeratins – cell repair
KRT8	KRT8	Type 1 cytokeratins – cell repair
KRT18	KRT18	Type 2 cytokeratins – cell repair
KRT19	KRT19	Type 2 cytokeratins – cell repair

Table 9-2 Selected proteins used as markers of hypoxia and cellular injury.
The gene names refer to the names used by the *Ensembl* database.[399]

9.3.6.3.3 Necrotising Enterocolitis

Section 1.5 describes the current understanding of the biochemical pathways that are known to be important in the pathophysiology of NEC. The aim of analysing these pathways is two-fold. Firstly if genes known to be significant in NEC pathophysiology show changes in expression due to IRI that could be informative about how well the model is replicating NEC; essentially showing pathways that are similar between an immediate IRI (in the case of the model) and a more complex interplay of factors leading to a IRI (human NEC). The second purpose is to assess what (if any) changes in these genes is seen in response to RIC. [100,101,105,404]

Protein name	Gene name	Function
TLR2	TLR2	Toll-like receptors are part of the innate immune response to bacteria. Activation of TLRs in known to be important in NEC
TLR4	TLR4	
MyD88	MyD88	Downstream signal from TLR4
NF-kB	NFKB1	Cellular response to stress
PAF	PTAFR	Platelet activation / proinflammatory cytokine
CD17	KIT	Marker for regulatory T-cells
iFABP	iFABP	Putative biomarker of NEC.[404]

Table 9-3 Selected proteins shown to be important in the pathophysiology of NEC.
The gene names refer to the names used by the Ensembl database.[399]

9.3.6.3.4 Remote Ischaemic Conditioning

The reperfusion injury salvage kinase pathway (RISK) pathway is described in Section 3.5.2.4 (**Error! Reference source not found.**). This pathway has been shown to be important in the protective effect of RIC in cardiac tissue. For this reason, I interrogated my raw counts for differences in the specific genes known to be part of that pathway. Therefore I looked for differential expression across the four groups of the mRNA for these proteins: [307,311]

Protein name	Gene name	Function
PKC	PRKCA	The Risk Pathway, shown to be important in the mechanisms of RIC in cardiac tissue (Section 3.5.2.4, Figure 3-5)
P38	MAPK14	
PI3K	PIK3C3	
MEK 1	MAP2K1	
MEK 2	MAP2K2	

Table 9-4 Selected proteins shown to be important in the mechanisms of RIC in other target organs.
The gene names refer to the names used by the Ensembl database.[399]

9.3.7 Immunohistochemistry validation of transcriptomic analysis

In order to validate and confirm the transciptomic analysis, immunohistochemistry (IHC) was performed on multiple samples from each of the experimental groups. Whilst practical limitations meant only six samples were used for the RNA analysis, the IHC was performed on all available samples.

9.3.7.1 Hypoxia-inducible Factor 1 alpha (HIF-1α).

HIF-1α was performed on the area of maximal intestinal injury and reported in 5.4.4. The methods are described in detail in 4.10. In brief, dewaxed sections of the intestine were stained with a standard two antibody technique. A HIF-1α primary antibody raised in rabbit was used with a goat anti-rabbit biotinyated secondary antibody and chromagen staining. The slides were then blindly scored.

9.3.7.2 Vimentin, Cytokeratin18 and Desmin

These three proteins were selected for immunohistochemistry analysis as they showed differential expression in this dataset and have reliable, commercially available antibodies for staining.

Vimentin is ubiquitously expressed in normal mesenchymal cells and is known to maintain cellular integrity and provide resistance against stress.[405] It known to be important in maintain the structural integrity of the cell and also has multiple other functions[406]

Cytokeratin 18 (CK18) is a type I intermediate filament protein that is primarily found in epithelial tissues and is localized in the cytoplasm and perinuclear region[407,408] CK18 provides a flexible intracellular scaffolding to structure cytoplasm, resists stresses[409] and maintain normal mitochondrial structures.[410] It also plays a role in apoptosis.[394,]

Desmin is a muscle-specific protein which is a key subunit of the intermediate filament of smooth muscle.[411]

Tissue from a fixed point in the terminal ileum that were fixed in formalin and embedded in wax were used for this analysis (Section 4.6.1). (i.e. tissue immediately adjacent to that used for the transciptomic analysis was used). For each antibody, optimisation was performed to assess the most effective buffer for retrieval and the optimal concentration for the primary antibody.

5µm sections were cut and dewaxed with Tissue clear. Endogenous peroxidase was inhibited with 0.5% hydrogen peroxide in methanol and then washed with tris buffered saline (TBS).

Antigen retrieval was done with microwave heating in a 1mM EDTA buffer (pH 8) for 30 minutes on full power, as per local protocol.

Avidin and biotin blocking was then applied (Vector Laboratories, USA), before applying blocking medium (made from Dubecoo's modified Eagle medium, foetal calf serum, bovine serum albumin and goat serum albumin). The primary antibodies (Vimentin, Abcam plc, UK, ab92547; CK-18 Abcam plc, UK, ab133263; and Desmin Novus biological, USA, SI18-00) were then applied at the optimal concentration (based on the optimisation work) and incubated overnight at 4^{O}C. The optimised concentration for each was as follows; 1:800 for Vimentin, 1:100 for CK18 and 1:200 for Desmin. Positive tissue controls were used for each (Kidney for Vimentin, tonsil and nasal polyp for CK18 and rat colon for Desmin). TBS negative controls were used in each case.

The slides were then scanned with an Olympus VS110 high throughput Virtual Microscopy System (Olympus, Germany). The images were then prepared with Olympus VS desktop software. Based on the methodology described by Prasad *et al.*[370] the immunohistochemistry was quantified by counting the number of cells showing stain in randomly selected fields.

For each sample, five randomly selected fields were used and the left-most twenty cells of interest were examined: For Vimentin the cells within the villi were counted, for CK-18 the apical villi cells were used and in the case of Desmin the smooth muscle layer of the intestine. The

number staining positive was counted. Summation of the numbers from all five fields thus yielded a percentage of cells staining positive.

Each of the antibodies thus provided a further negative control for each of the other. Desmin showed staining within the muscle layer. There was no such staining with CK-18. Similarly, CK-18 staining was confined to the apical surface. (Figure 9-62)

Biotinyated secondary antibody (Goat-anti-rabbit, Vector Laboratories, USA) was then applied for30 minutes at a concentration of 1 in 1000 (in TBS). At the same time, avidin biotin peroxidase solution (Vector Laboratories, USA) was made at a concentration of 1 in 75 in TBS for each agent and allowed to form complexes. This was then applied for 30 minutes. A 3,3'Diaminobenzidine (DAB) substrate was then applied for chromagen staining for 5 minutes before counterstaining with haematoxylin for 1 minute.

9.4 Results

9.4.1 Data Exploration and Quality Control

9.4.1.1 RNA Quality Control

Table 9-5 shows the results of the quality control assessment of all the samples. The RNA integrity number (RIN) was greater than, or equal to 8.0 in all samples except sample 11 (from animal ID 266). This indicates high quality RNA in 23 of the samples and a small amount of fragmentation in this one sample.[387,388]

Sample	Sample ID (Individual animal ID)	Concentration of RNA (ng/µl)	RIN
1	017	1990	8.7
2	518	2460	9.4
3	038	2940	8.3
4	251	2540	9.2
5	535	3520	8.1
6	711	2920	9.1
7	971	2760	9.5
8	297	3780	9.0
9	976	2220	9.4
10	307	2760	9.5
11	266	3360	7.4
12	329	2780	9.3
13	240	2800	8.1
14	993	2980	9.5
15	790	2220	9.5
16	460	5060	8.2
17	701	4500	8.9
18	018	4540	8.2
19	674	3600	9.5
20	244	1620	9.3
21	949	2860	9.7
22	159	3900	9.1
23	205	2820	10.0
24	783	2800	8.9

Table 9-5 Quality of RNA in each sample. Data provided by Qiagen. RIN >8 indicates high quality sample.[387,388]

9.4.1.2 Data Exploration and Quality Control

The following charts summarise the data exploration and quality control I performed before proceeding to analysis of differential gene expression. Figure 9-5 Box plot of Raw Sample Counts of mRNA Hits Figure 9-5 shows the raw sample counts of mRNA hits and Figure 9-6 the

same plot once the lowly expressed mRNAs are removed. These show very similar mRNA counts across all samples.

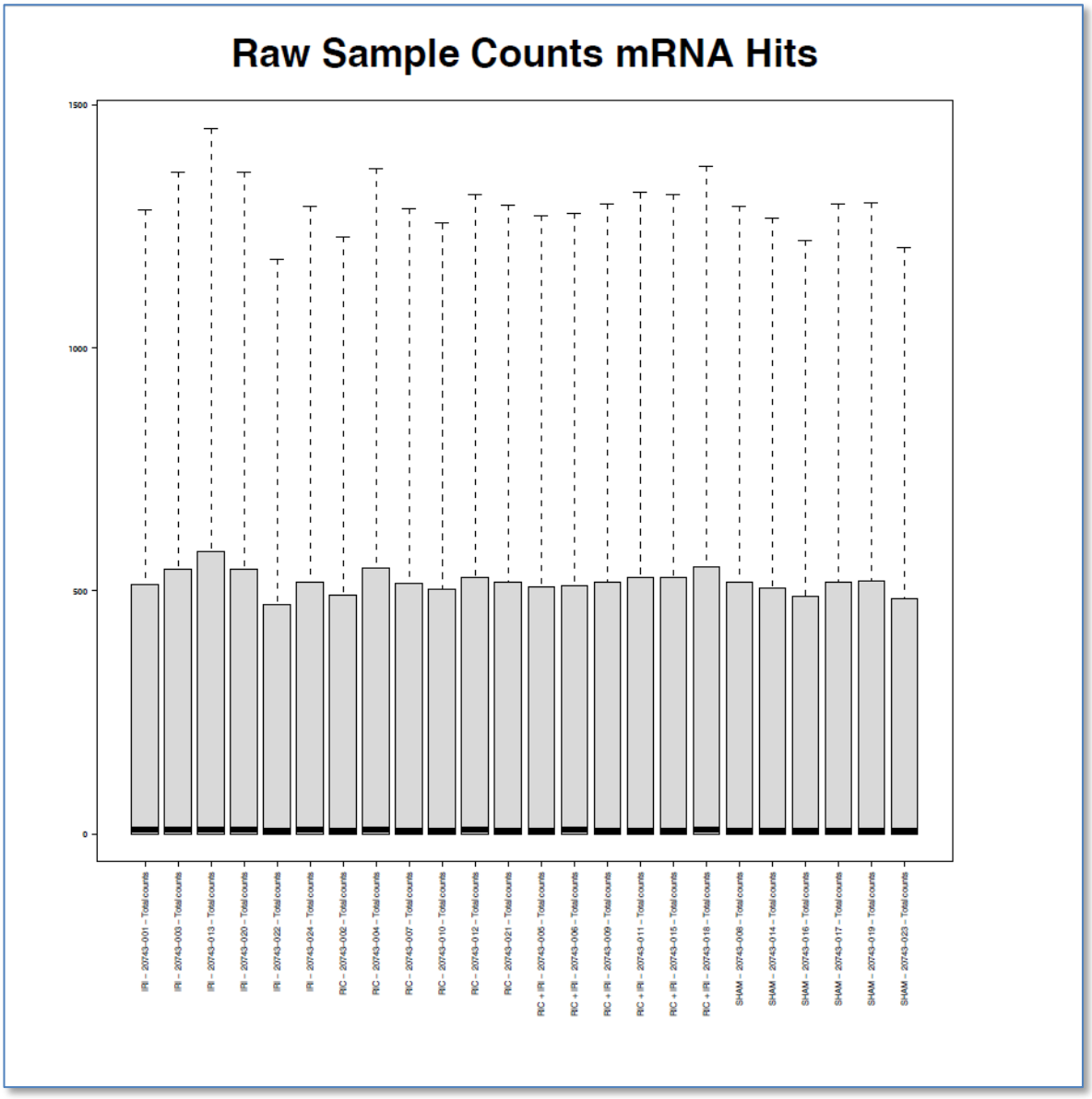


Figure 9-5 Box plot of Raw Sample Counts of mRNA Hits in each sample.
Median shown by black gene. Gene counts range from 0-1500 copies. Box shows 1.5x IQR.

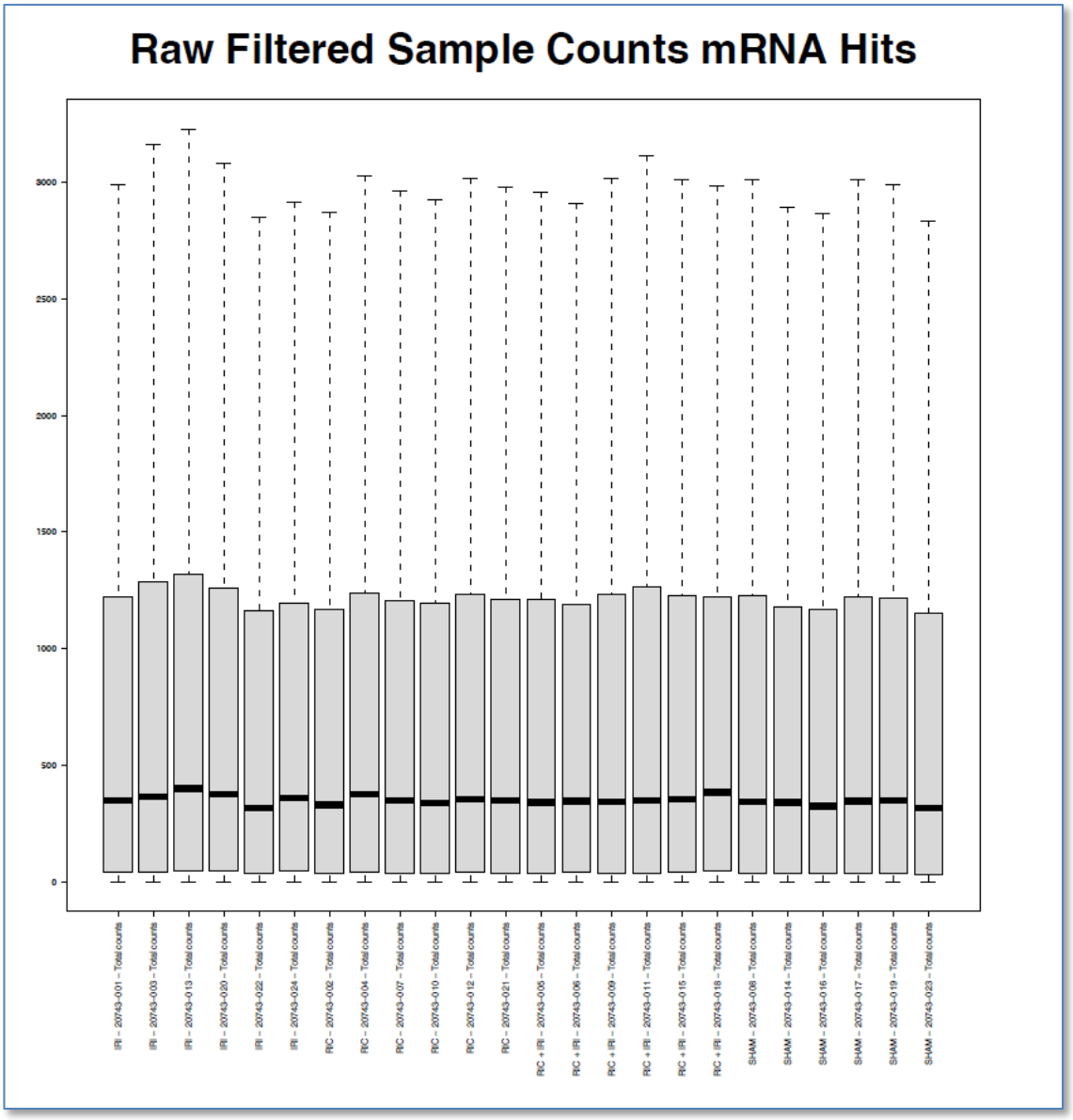


Figure 9-6 Box plot of Filtered Sample Counts of mRNA Hits in each sample; lowly expressed mRNAs removed.
Median shown by black gene. Gene counts range from 0-1500 copies. Box shows 1.5x IQR. This is done to reduce noise and prevent Type II error.

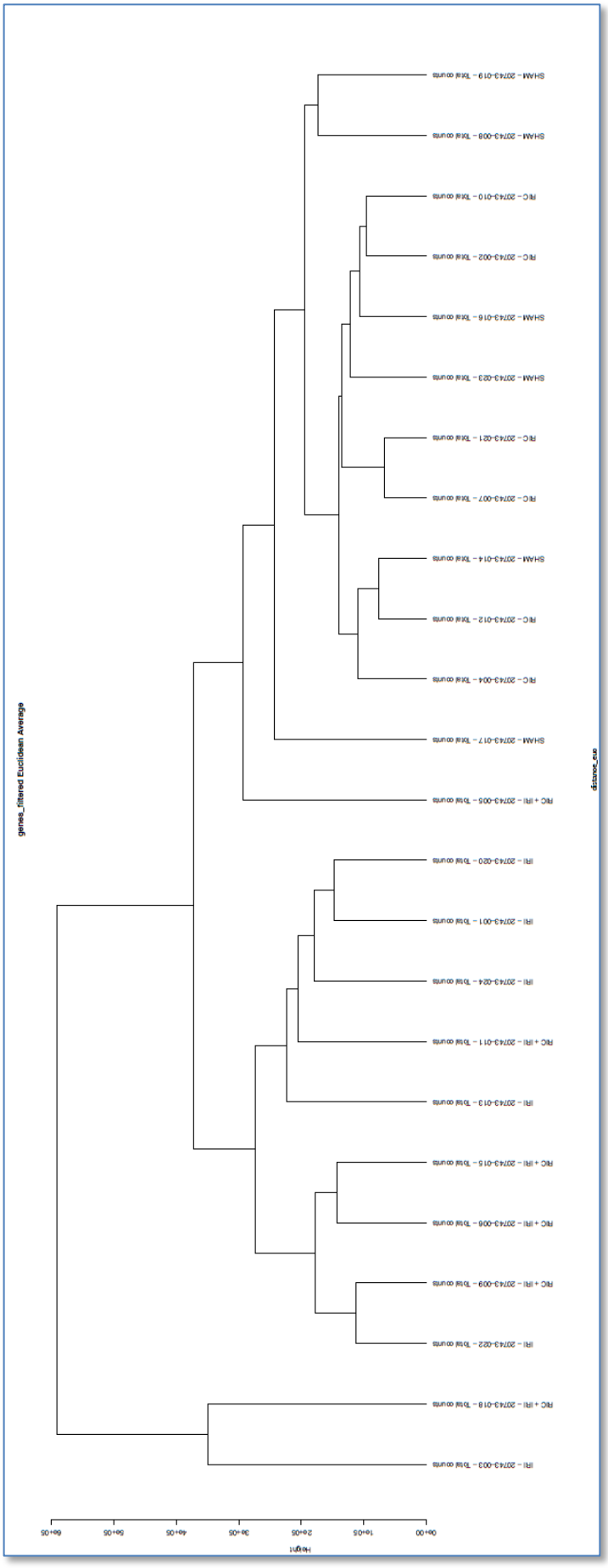


Figure 9-7 Hierarchical clustering of all samples and genes using the Euclidean distance method

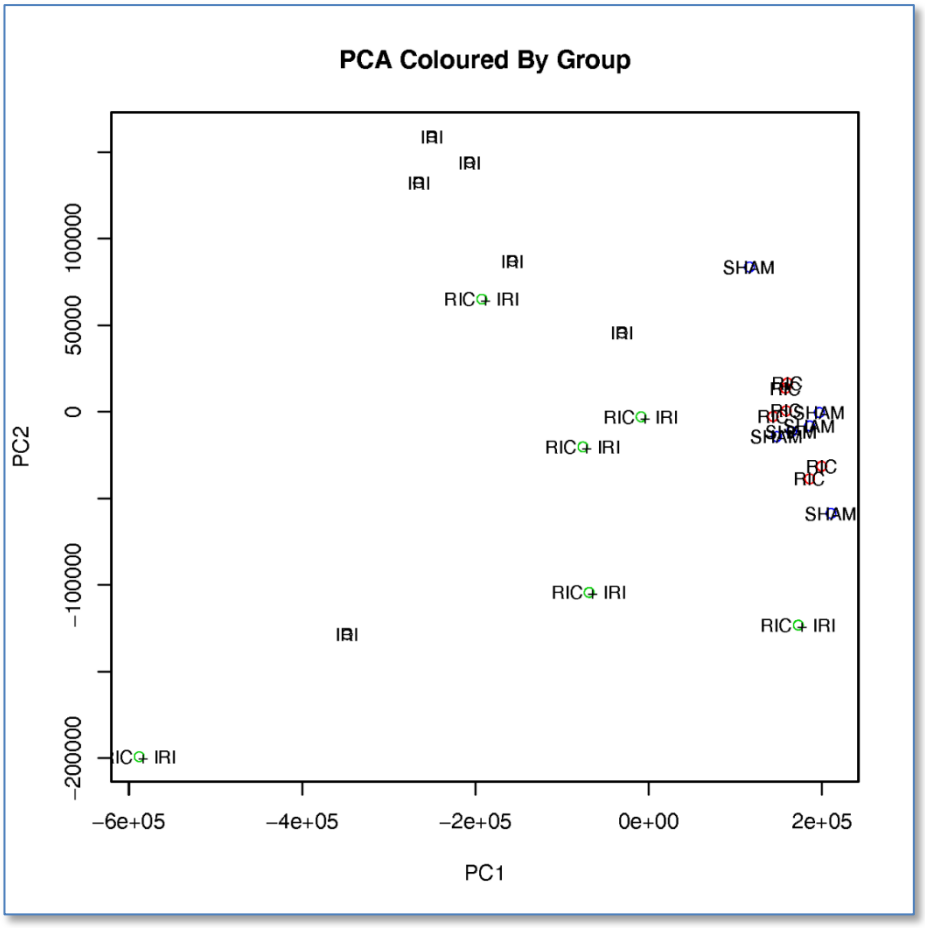


Figure 9-8 Principal Component Analysis of all samples and genes

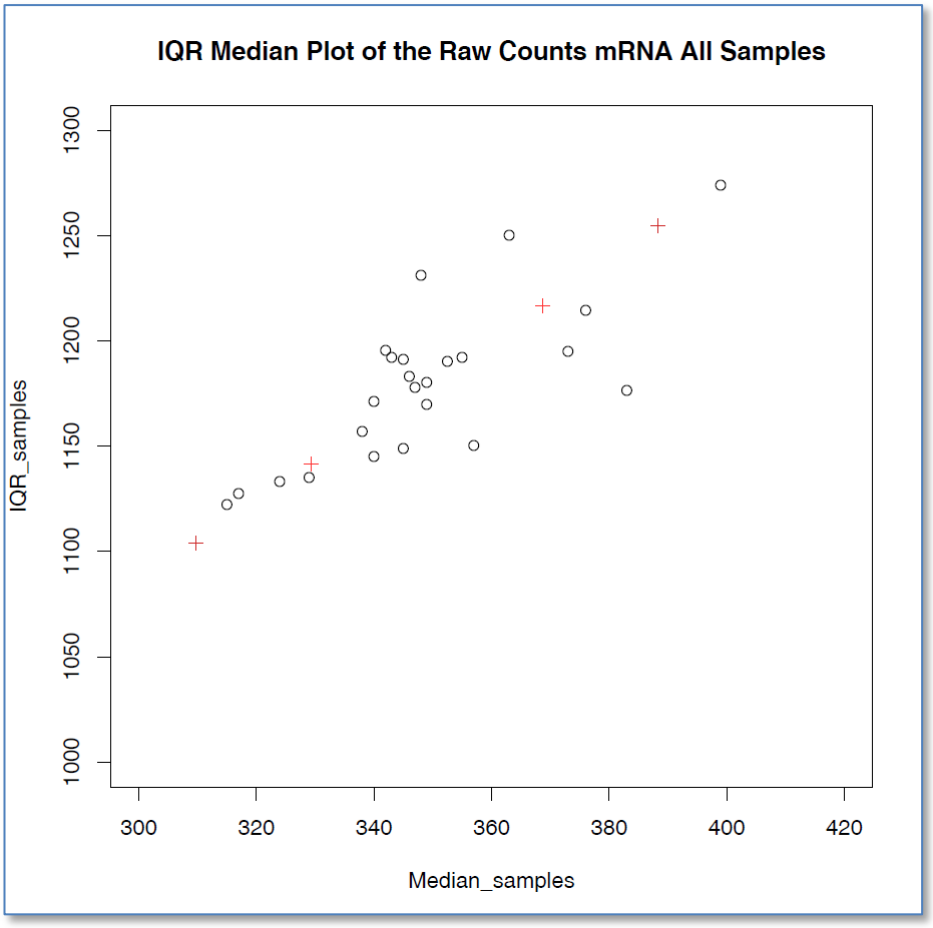


Figure 9-9 Median or Raw Counts plotted against Interquartile range of all samples and genes

The hierarchical clustering (using Euclidean Average) showed that in the whole dataset the IRI vs SHAM dominates over RIC vs SHAM in the patterns of gene expression, with samples from IRI and RIC+IRI clustering together and SHAM and RIC+SHAM clustering together. With the Principal Component analysis labelled by known phenotypical factors (Age in days, gender, weight, litter, and date of procedure) there is no clustering by any of these specific factors.

Sample 003 was a potential outlier in the PCA but not within raw counts/IQR plot.

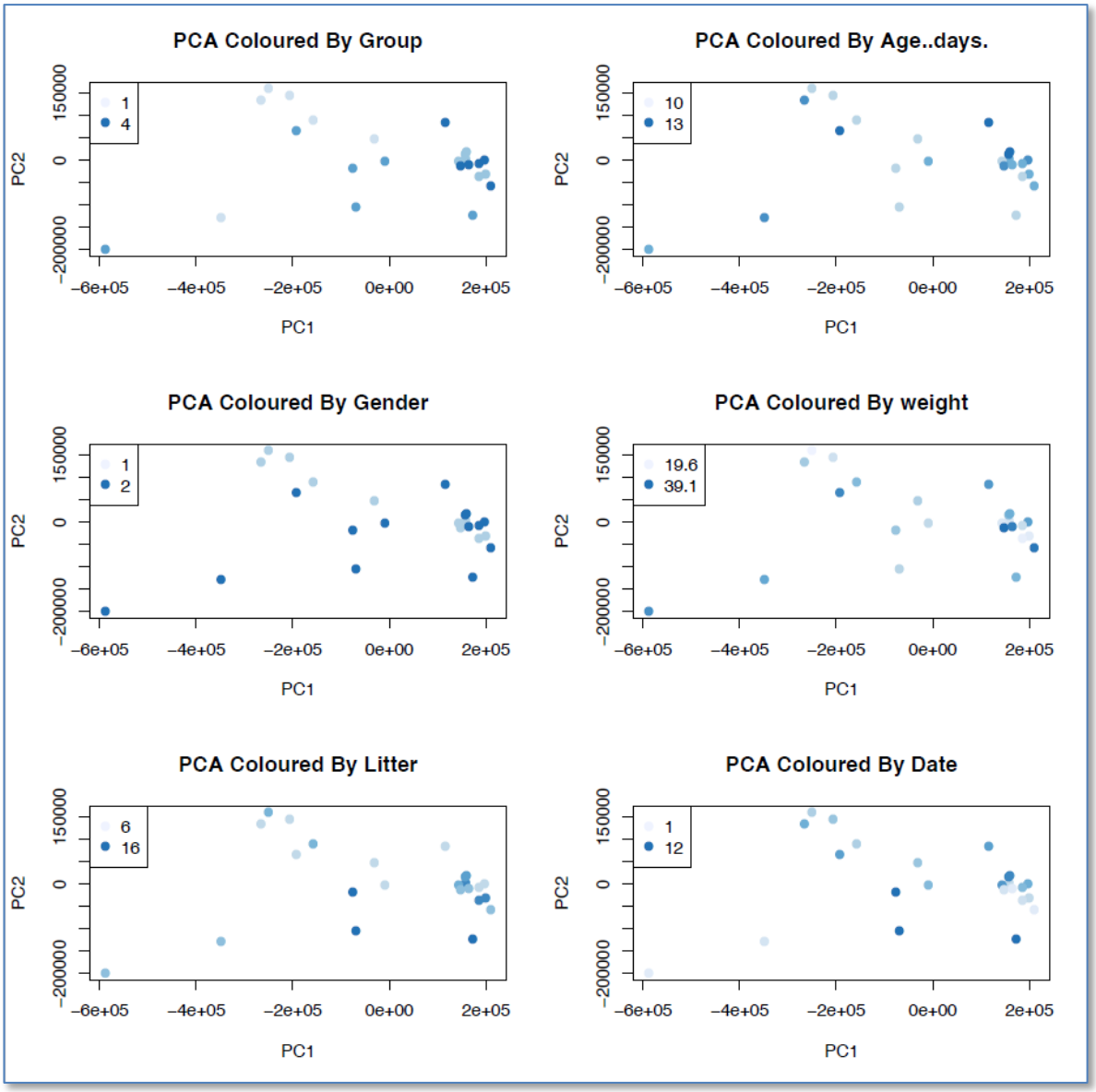


Figure 9-10 Principle-Component Analysis (PCA) and known phenotypic features

On the basis of these analyses, no samples needed to be excluded from further analysis of differential gene expression.

9.4.2 Differential Expression Testing

The lists of differentially expressed genes are in Appendix G. Complete datasets are included in the additional material.

9.4.2.1 Differential Gene Expression – IRI vs SHAM (The effect of the IRI Model on gene expression)

The differential gene expression between animals who underwent IRI compared to SHAM showed statistically significant differential expression is 6772 genes (Appendix G. Section G.1).

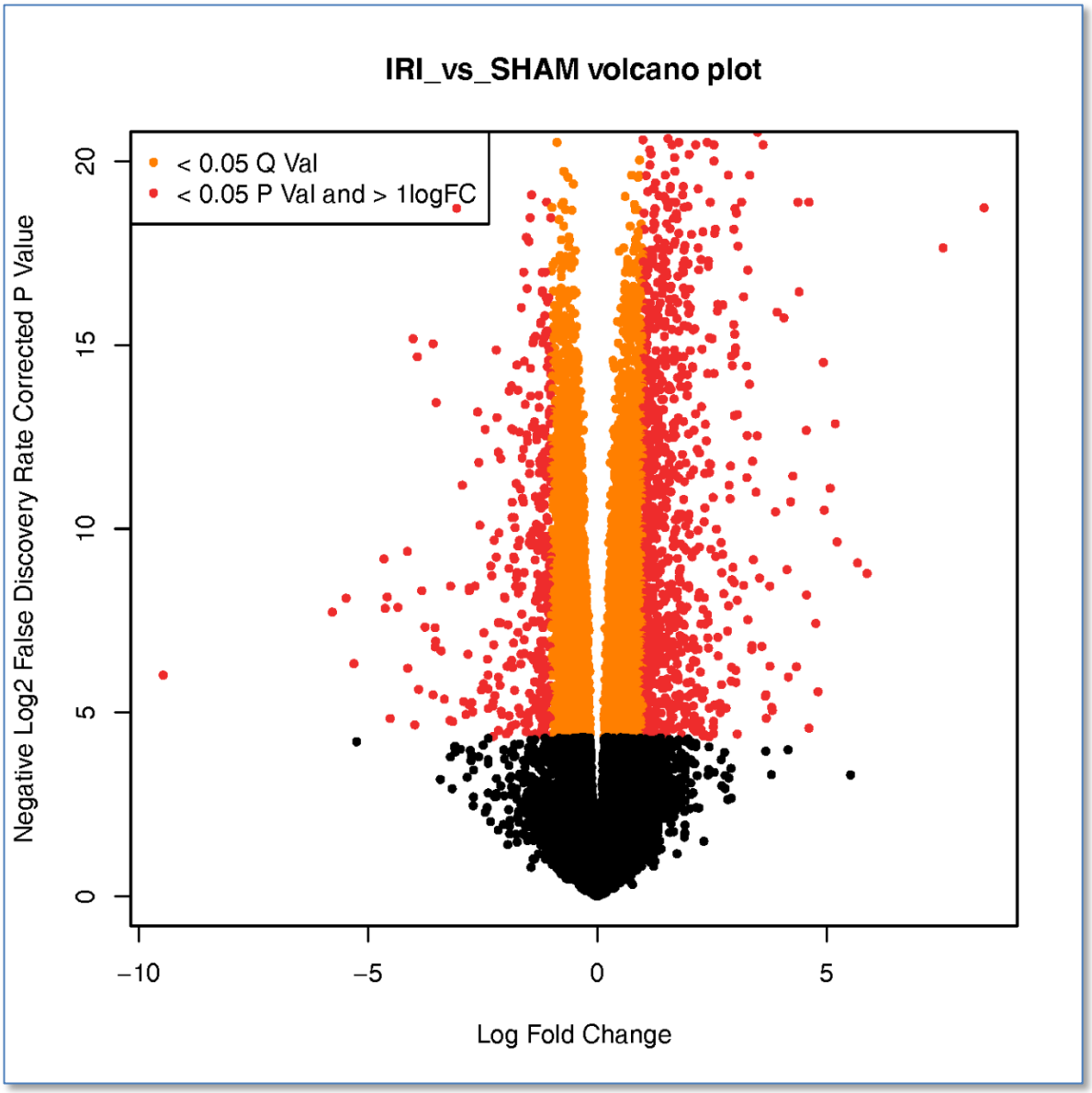


Figure 9-11 Volcano plot of differentially expressed genes IRI vs SHAM
The x-axis shows log Fold Change in gene expression between the groups
The y-axis shows log2 False Discovery Rate corrected p-value. A log2 FDR of less than five indicates a non-significant result. Genes show a log Fold change of +/-1 or more and are statistically significant are shown in red.

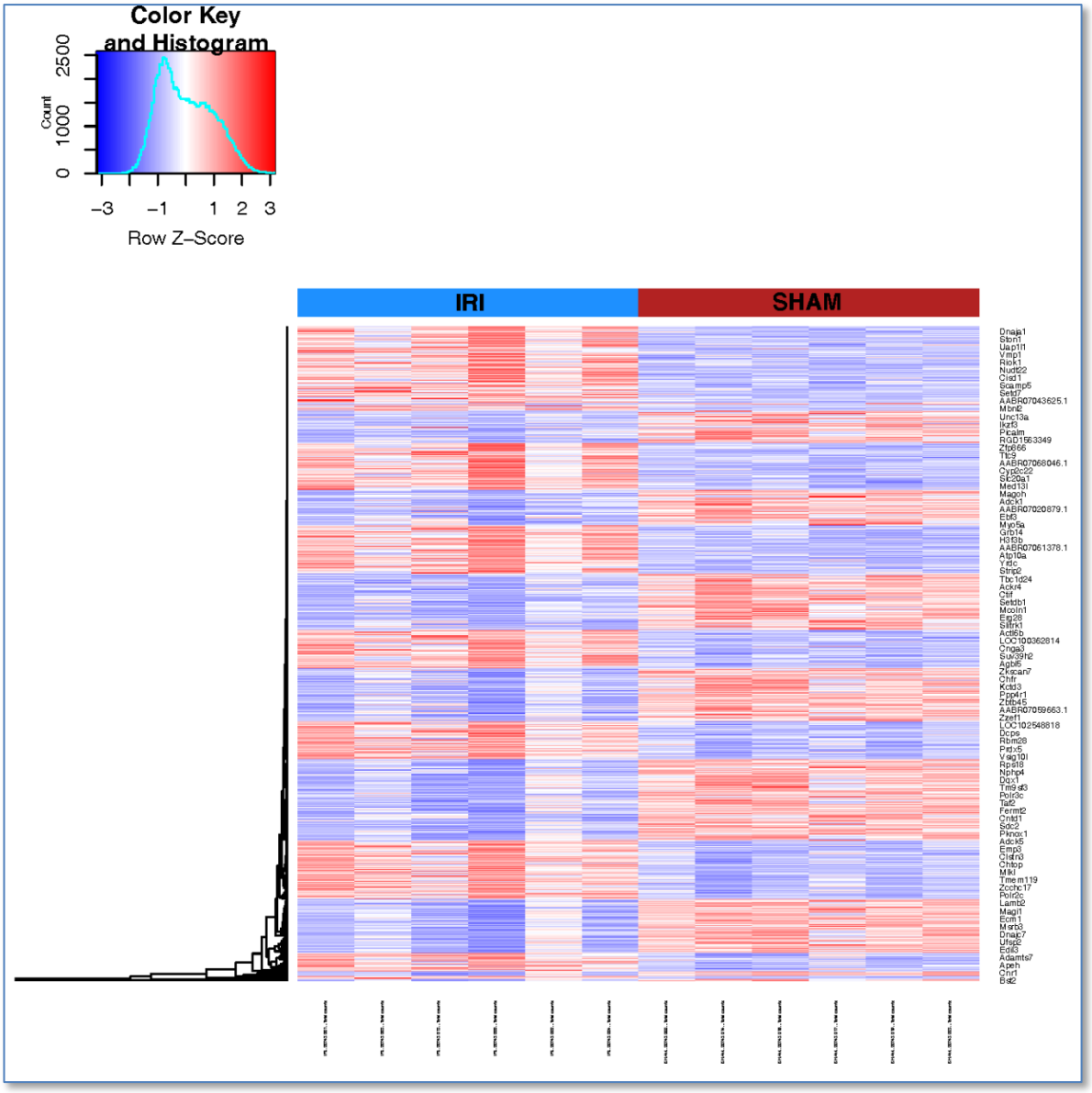


Figure 9-12 Heatmap of differentially Expressed Genes IRI vs SHAM

9.4.2.2 Differential Gene Expression – RIC vs SHAM

In the comparison between animals who underwent RIC and SHAM surgery vs animals who underwent SHAM surgery only, 868 genes showed statistically significant differential expression. (The full list of differentially expressed genes is in Appendix G. Section G.3). Figure 9-13 shows a volcano plot of differentially expressed genes Figure 9-14 a heatmap.

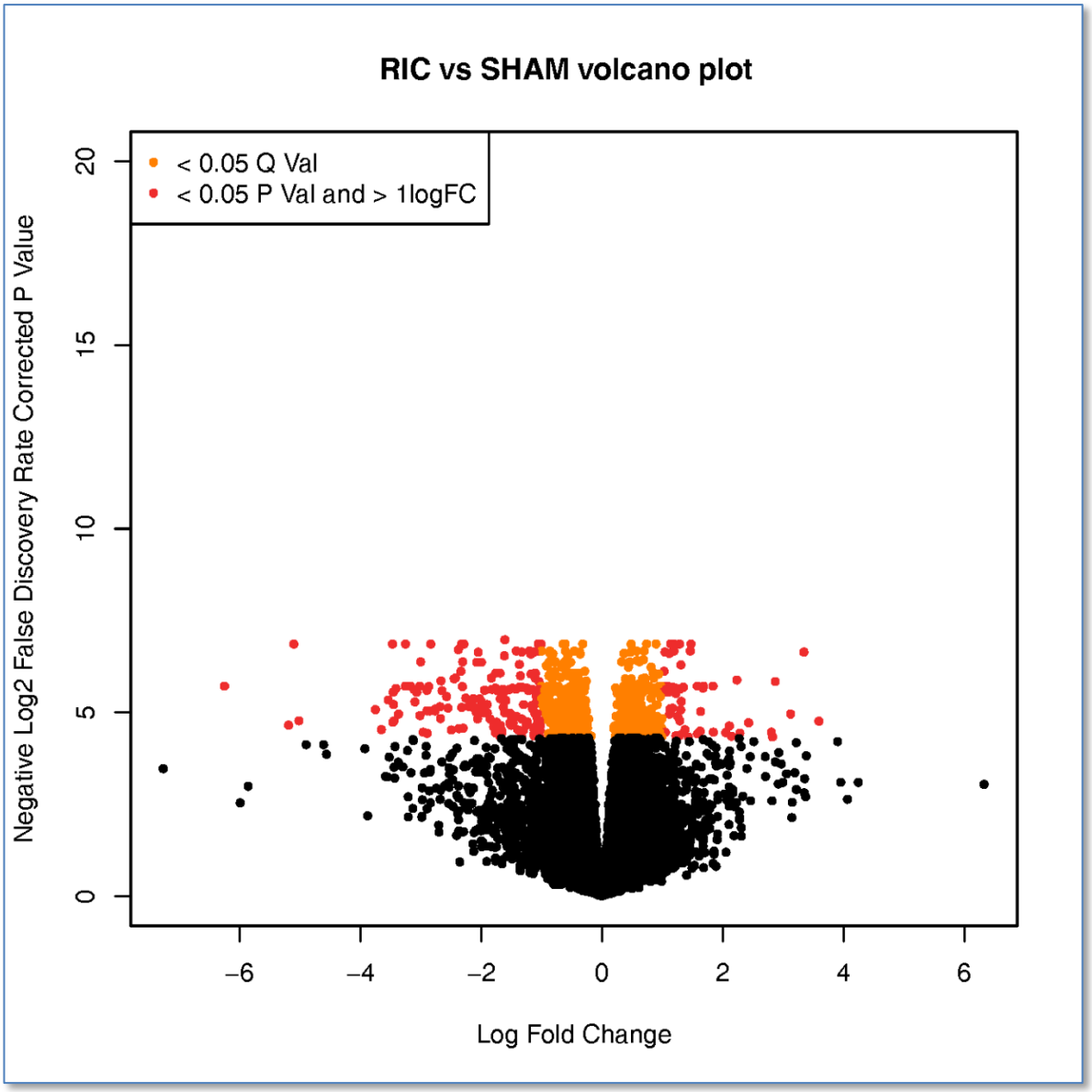


Figure 9-13 Volcano plot of differentially expressed genes RIC vs SHAM
The x-axis shows log Fold Change in gene expression between the groups
The y-axis shows log2 False Discovery Rate corrected p-value. A log2 FDR of less than five indicates a non-significant result. Genes show a log Fold change of +/-1 or more and are statistically significant are shown in red.

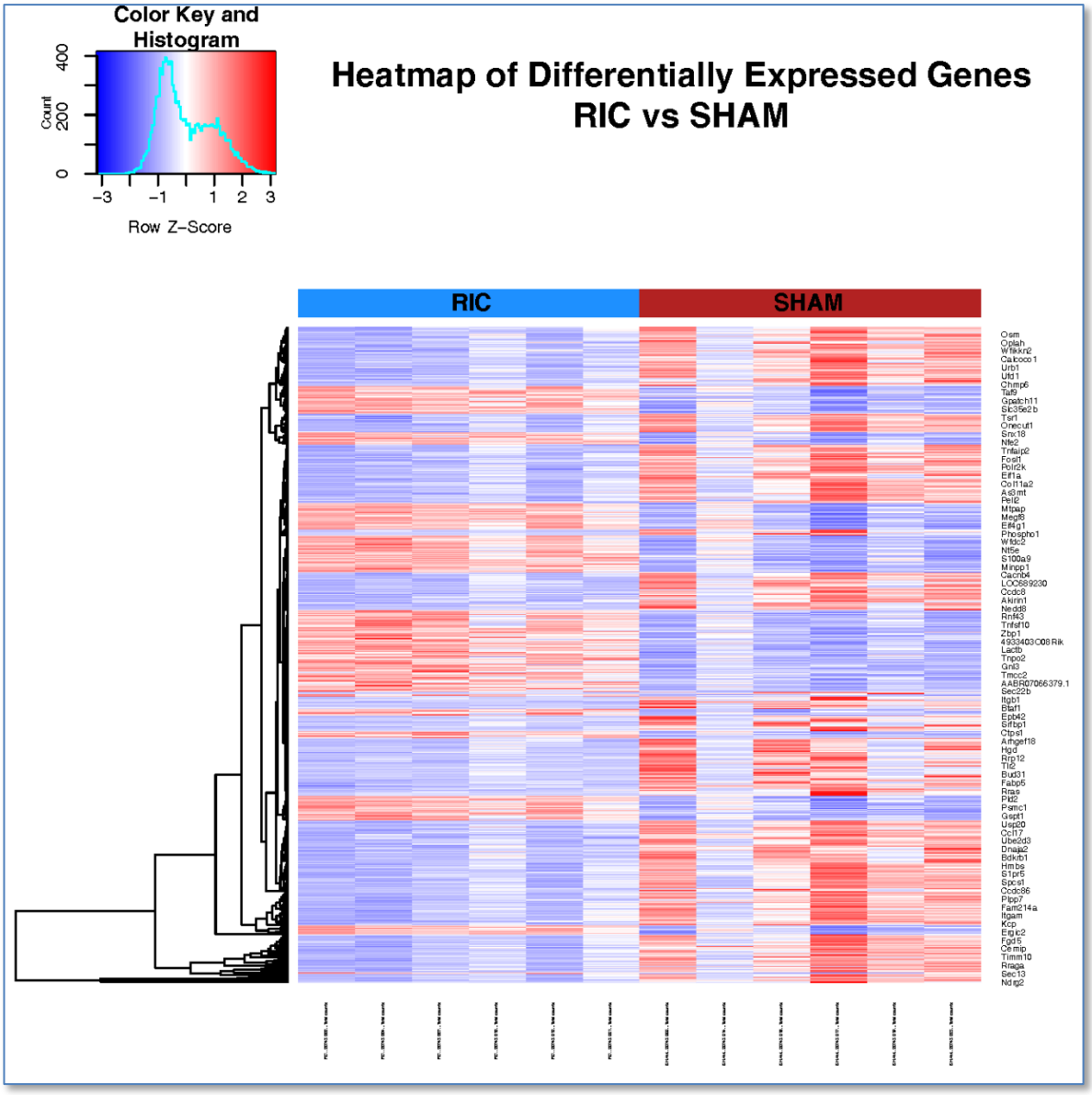


Figure 9-14 Heatmap of differentially Expressed Genes RIC vs SHAM

9.4.2.3 Differential Gene Expression – RIC+IRI vs IRI

In the comparison between animals who underwent RIC+IRI vs animals who underwent IRI alone, 135 genes showed statistically significant differential expression. (The full list of differentially expressed genes is in Appendix G. Section G.3). Figure 9-15 shows a volcano plot of differentially expressed genes Figure 9-16 the heatmap.

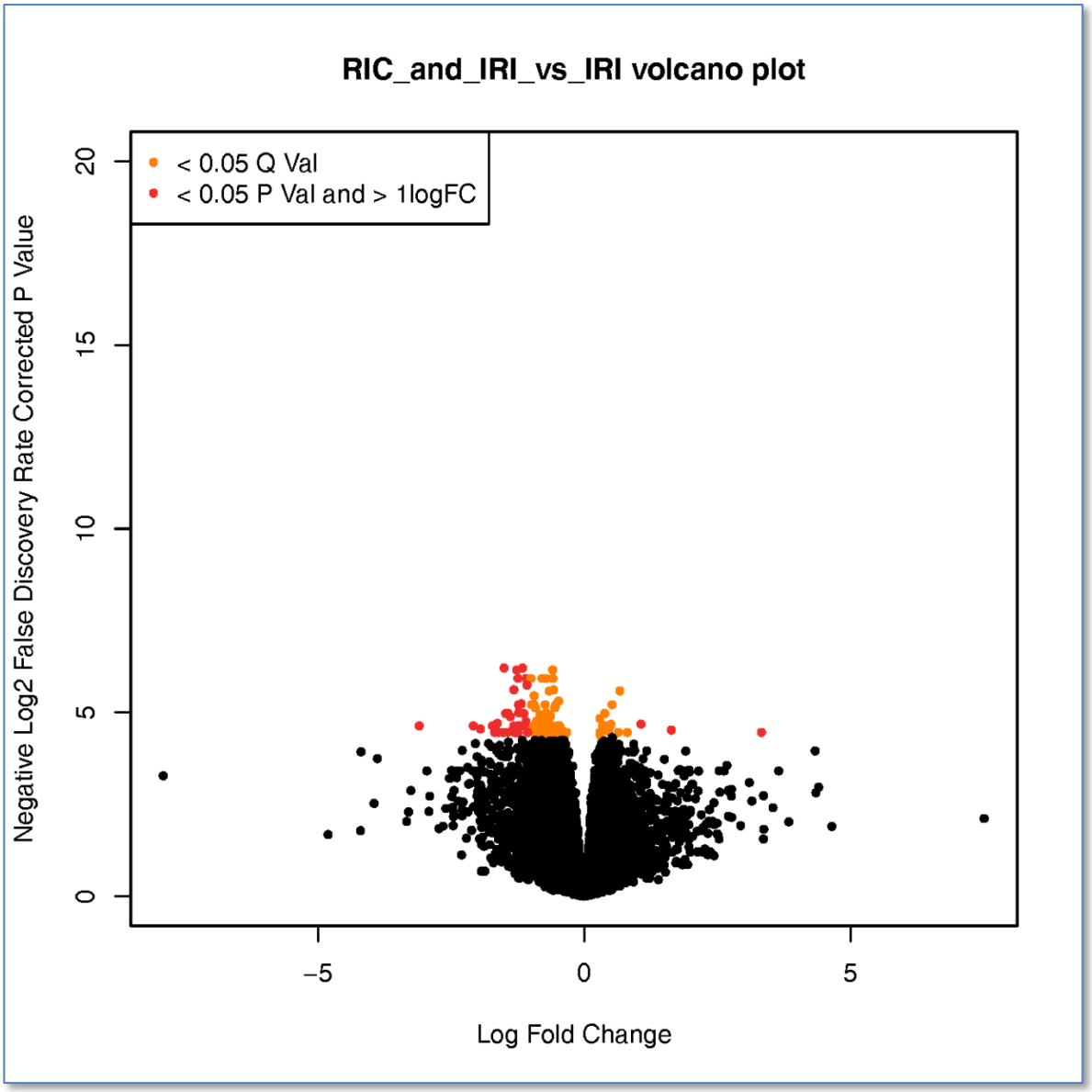


Figure 9-15 Volcano plot of differentially Expressed Genes RIC vs SHAM
The x-axis shows log Fold Change in gene expression between the groups
The y-axis shows log2 False Discovery Rate corrected p-value. A log2 FDR of less than five indicates a non-significant result. Genes show a log Fold change of +/-1 or more and are statistically significant are shown in red.

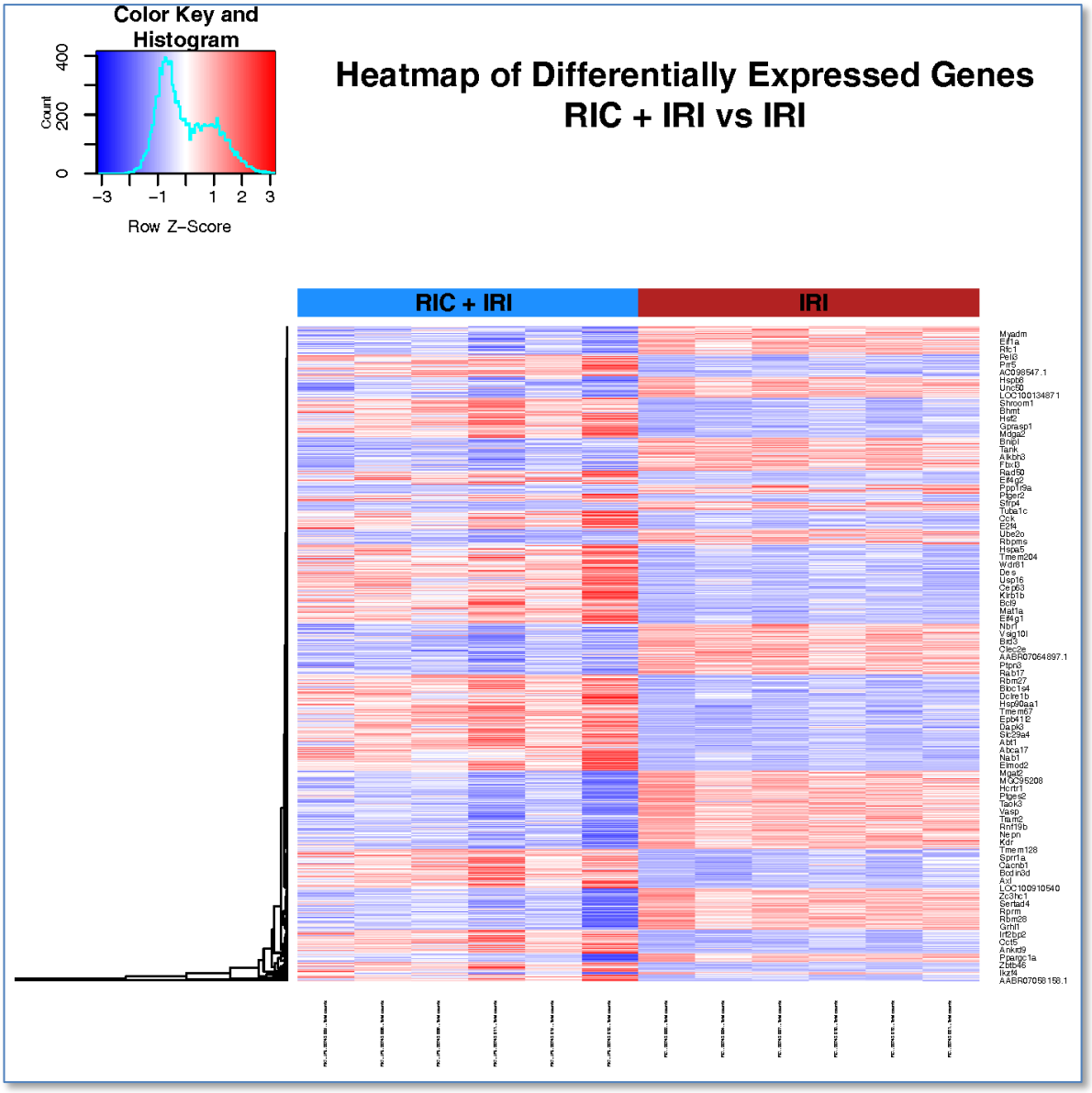


Figure 9-16 Heatmap of differentially Expressed Genes RIC vs SHAM

9.4.2.4 Combined IRI+RIC vs IRI and RIC vs SHAM

The large number of differentially expressed genes in both of these comparisons means there are multiple ways of analysing which ones are likely to be important. One such strategy is to assess which genes are differentially expressed in both groups. Combining these two groups show that 25 genes were differentially expressed in both comparisons. (Figure 9-17, Table 9-6)

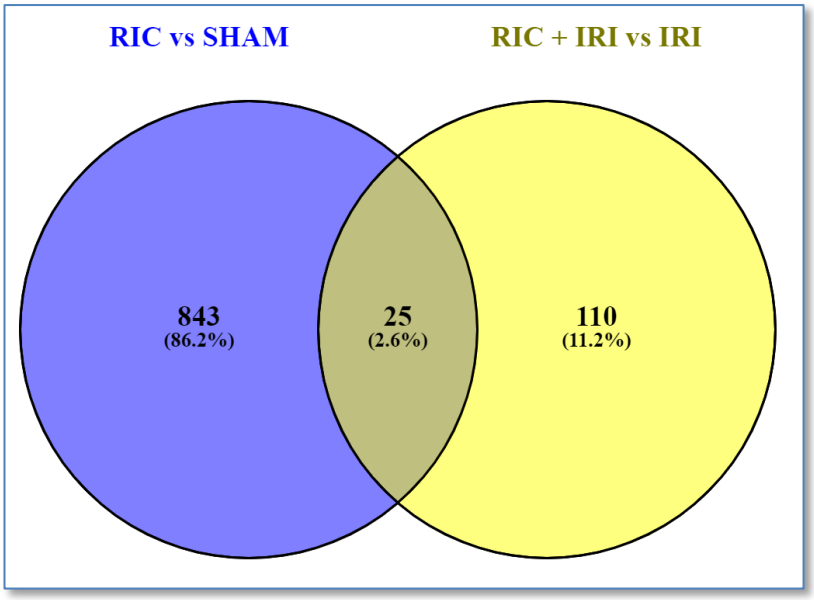


Figure 9-17 Genes that show differential expression in both the RIC vs SHAM comparison and the RIC+IRI vs IRI comparison (Produced using Venny 2.1.0)[412]

Table 9-6 Short names of genes found to be differentially expressed in both RIC vs SHAM and RIC+IRI vs IRI

1	Lrrc8c	10	Malsu1	18	Map3k8
2	Ugdh	11	Spry2	19	Sod2
3	Cxcl1	12	Timp1	20	Nfkb2
4	Adamts4	13	Tfpi2	21	Procr
5	Cd55	14	Plau	22	Usp36
6	Trib1	15	Coq10b	23	Kcne4
7	Adamts8	16	S1pr3	24	Gstm5
8	Pde4b	17	Ifitm3	25	AABR07047256.1
9	RGD1563365				

9.4.2.5 A brief description of each of the genes differentially expressed in both the RIC vs SHAM and RIC+IRI vs IRI comparison

The following information for each gene derived from the GeneCards® database.[413]

1. **Lrrc8c -** Leucine Rich Repeat Containing 8 VRAC Subunit C.
   A Non-essential component of the volume-regulated anion channel (VRAC, also named VSOAC channel), an anion channel required to maintain a constant cell volume in response to extracellular or intracellular osmotic changes.
2. **Ugdh -** UDP-Glucose 6-Dehydrogenase

The protein encoded by this gene converts UDP-glucose to UDP-glucuronate and thereby participates in the biosynthesis of glycosaminoglycans such as hyaluronan, chondroitin sulfate, and heparan sulfate. These glycosylated compounds are common components of the extracellular matrix and likely play roles in signal transduction, cell migration, and cancer growth and metastasis. The expression of this gene is up-regulated by transforming growth factor beta and down-regulated by hypoxia. Alternative splicing results in multiple transcript variants.

3. **Cxcl1 -** C-X-C Motif Chemokine Ligand 1
   This antimicrobial gene encodes a member of the CXC subfamily of chemokines. The encoded protein is a secreted growth factor that signals through the G-protein coupled receptor, CXC receptor 2. This protein plays a role in inflammation and as a chemoattractant for neutrophils. Aberrant expression of this protein is associated with the growth and progression of certain tumors. A naturally occurring processed form of this protein has increased chemotactic activity. Alternate splicing results in coding and non-coding variants of this gene.
4. **Adamts4 -** ADAM Metallopeptidase With Thrombospondin Type 1 Motif 4
   This gene encodes a member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) protein family. Members of this family share several distinct protein modules, including a propeptide region, a metalloproteinase domain, a disintegrin-like domain, and a thrombospondin type 1 (TS) motif. Individual members of this family differ in the number of C-terminal TS motifs, and some have unique C-terminal domains. The enzyme encoded by this gene lacks a C-terminal TS motif. The encoded preproprotein is proteolytically processed to generate the mature protease. This protease is responsible for the degradation of aggrecan, a major proteoglycan of cartilage, and brevican, a brain-specific extracellular matrix protein. The expression of this gene is upregulated in arthritic disease and this may contribute to disease progression through the degradation of aggrecan. Alternative splicing results in multiple transcript variants, at least one of which encodes an isoform that is proteolytically processed.
5. **Cd55 -** CD55 Molecule (Cromer Blood Group)
   This gene encodes a glycoprotein involved in the regulation of the complement cascade**.** Binding of the encoded protein to complement proteins accelerates their decay, thereby disrupting the cascade and preventing damage to host cells. Antigens present on this protein constitute the Cromer blood group system (CROM). Alternative splicing results in multiple transcript variants. The predominant transcript variant encodes a membrane-bound protein, but alternatively spliced transcripts may produce soluble proteins.
6. **Trib1 -** Tribbles Pseudokinase 1

This is an adapter protein involved in protein degradation by interacting with COP1 ubiquitin ligase (PubMed:27041596). The COP1-binding motif is masked by autoinhibitory interactions with the protein kinase domain (PubMed:26455797). Serves to alter COP1 substrate specificity by directing the activity of COP1 toward CEBPA (PubMed:27041596). Binds selectively the recognition sequence of CEBPA (PubMed:26455797). Regulates myeloid cell differentiation by altering the expression of CEBPA in a COP1-dependent manner (By similarity). Controls macrophage, eosinophil and neutrophil differentiation via the COP1-binding domain (By similarity). Interacts with MAPK kinases and regulates activation of MAP kinases, but has no kinase activity (PubMed:15299019, PubMed:26455797). Trib2: This gene encodes one of three members of the Tribbles family. The Tribbles members share a Trb domain, which is homologous to protein serine-threonine kinases, but lacks the active site lysine and probably lacks a catalytic function. The Tribbles proteins interact and modulate the activity of signal transduction pathways in a number of physiological and pathological processes. This Tribbles member induces apoptosis of cells mainly of the hematopoietic origin. It has been identified as a protein up-regulated by inflammatory stimuli in myeloid (THP-1) cells, and also as an oncogene that inactivates the transcription factor C/EBPalpha (CCAAT/enhancer-binding protein alpha) and causes acute myelogenous leukemia. Alternatively spliced transcript variants have been found for this gene.

7. **Adamts8 -** ADAM Metallopeptidase With Thrombospondin Type 1 Motif 8
   This gene encodes a member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) protein family. Members of the family share several distinct protein modules, including a propeptide region, a metalloproteinase domain, a disintegrin-like domain, and a thrombospondin type 1 (TS) motif. Individual members of this family differ in the number of C-terminal TS motifs, and some have unique C-terminal domains. The encoded preproprotein is proteolytically processed to generate the mature enzyme. This enzyme contains two C-terminal TS motifs, and disrupts angiogenesis in vivo. A number of disorders have been mapped in the vicinity of this gene, most notably lung neoplasms. Reduced expression of this gene has been observed in multiple human cancers and this gene has been proposed as a potential tumor suppressor.
8. **Pde4b -** Phosphodiesterase 4B
   This gene is a member of the type IV, cyclic AMP (cAMP)-specific, cyclic nucleotide phosphodiesterase (PDE) family. The encoded protein regulates the cellular concentrations of cyclic nucleotides and thereby play a role in signal transduction. Altered activity of this protein has been associated with schizophrenia and bipolar affective

disorder. Alternative splicing and the use of alternative promoters results in multiple transcript variants encoding different isoforms.

9. **RGD1563365 -** Chromosome 12 Open Reading Frame 45
10. **Malsu1 -** Mitochondrial Assembly Of Ribosomal Large Subunit 1
    Required for normal mitochondrial ribosome function and mitochondrial translation (PubMed:22238375, PubMed:23171548). May play a role in ribosome biogenesis by preventing premature association of the 28S and 39S ribosomal subunits (Probable). Interacts with mitochondrial ribosomal protein L14 (MRPL14), probably blocking formation of intersubunit bridge B8, preventing association of the 28S and 39S ribosomal subunits (Probable). Addition to isolated mitochondrial ribosomal subunits partially inhibits translation, probably by interfering with the association of the 28S and 39S ribosomal subunits and the formation of functional ribosomes (Probable). May also participate in the assembly and/or regulation of the stability of the large subunit of the mitochondrial ribosome (PubMed:22238376, PubMed:23171548). May function as a ribosomal silencing factor (Probable)
11. **Spry2 -** Sprouty RTK Signaling Antagonist 2
    This gene encodes a protein belonging to the sprouty family. The encoded protein contains a carboxyl-terminal cysteine-rich domain essential for the inhibitory activity on receptor tyrosine kinase signaling proteins and is required for growth factor stimulated translocation of the protein to membrane ruffles. In primary dermal endothelial cells this gene is transiently upregulated in response to fibroblast growth factor two. This protein is indirectly involved in the non-cell autonomous inhibitory effect on fibroblast growth factor two signaling. The protein interacts with Cas-Br-M (murine) ectropic retroviral transforming sequence, and can function as a bimodal regulator of epidermal growth factor receptor/mitogen-activated protein kinase signaling. This protein may play a role in alveoli branching during lung development as shown by a similar mouse protein.
12. **Timp1 -** TIMP Metallopeptidase Inhibitor 1
    This gene belongs to the TIMP gene family. The proteins encoded by this gene family are natural inhibitors of the matrix metalloproteinases (MMPs), a group of peptidases involved in degradation of the extracellular matrix. In addition to its inhibitory role against most of the known MMPs, the encoded protein is able to promote cell proliferation in a wide range of cell types, and may also have an anti-apoptotic function. Transcription of this gene is highly inducible in response to many cytokines and hormones. In addition, the expression from some but not all inactive X chromosomes suggests that this gene inactivation is polymorphic in human females. This gene is located within intron 6 of the synapsin I gene and is transcribed in the opposite direction (in humans).

13. **Tfpi2 -** Tissue Factor Pathway Inhibitor 2

    TFPI2 (Tissue Factor Pathway Inhibitor 2) is a Protein Coding gene. Diseases associated with TFPI2 include Fibrosarcoma and Choriocarcinoma. Among its related pathways are Matrix Metalloproteinases and Cell adhesion Plasmin signaling. Gene Ontology (GO) annotations related to this gene include *serine-type endopeptidase inhibitor activity* and *peptidase inhibitor activity*. An important paralog of this gene is TFPI.

14. **Plau -** Plasminogen Activator, Urokinase

    This gene encodes a secreted serine protease that converts plasminogen to plasmin. The encoded preproprotein is proteolytically processed to generate A and B polypeptide chains. These chains associate via a single disulfide bond to form the catalytically inactive high molecular weight urokinase-type plasminogen activator (HMW-uPA). HMW-uPA can be further processed into the catalytically active low molecular weight urokinase-type plasminogen activator (LMW-uPA). This low molecular weight form does not bind to the urokinase-type plasminogen activator receptor. Mutations in this gene may be associated with Quebec platelet disorder and late-onset Alzheimer's disease. Alternative splicing results in multiple transcript variants, at least one of which encodes an isoform that is proteolytically processed.

15. **Coq10b -** Coenzyme Q10B

    COQ10B (Coenzyme Q10B) is a Protein Coding gene. Diseases associated with COQ10B include Autosomal Dominant Non-Syndromic Intellectual Disability 2. Among its related pathways are Metabolism and Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins.. An important paralog of this gene is COQ10A.

16. **S1pr3 -** Sphingosine-1-Phosphate Receptor 3

    This gene encodes a member of the EDG family of receptors, which are G protein-coupled receptors. This protein has been identified as a functional receptor for sphingosine 1-phosphate and likely contributes to the regulation of angiogenesis and vascular endothelial cell function.

17. **Ifitm3 -** Interferon Induced Transmembrane Protein 3

    The protein encoded by this gene is an interferon-induced membrane protein that helps confer immunity to influenza A H1N1 virus, West Nile virus, and dengue virus. Two transcript variants, only one of them protein-coding, have been found for this gene. Another variant encoding an N-terminally truncated isoform has been reported, but the full-length nature of this variant has not been determined.

18. **Map3k8 -** Mitogen-Activated Protein Kinase Kinase Kinase 8

This gene is an oncogene that encodes a member of the serine/threonine protein kinase family. The encoded protein localizes to the cytoplasm and can activate both the MAP kinase and JNK kinase pathways. This protein was shown to activate IkappaB kinases, and thus induce the nuclear production of NF-kappaB. This protein was also found to promote the production of TNF-alpha and IL-2 during T lymphocyte activation. This gene may also utilize a downstream in-frame translation start codon, and thus produce an isoform containing a shorter N-terminus. The shorter isoform has been shown to display weaker transforming activity. Alternate splicing results in multiple transcript variants that encode the same protein.

19. **Sod2 -** Superoxide Dismutase 2

    SOD2 (Superoxide Dismutase 2) is a Protein Coding gene. Diseases associated with SOD2 include Microvascular Complications Of Diabetes 6 and Ischemia. Among its related pathways are FoxO family signaling and Detoxification of Reactive Oxygen Species. Gene Ontology (GO) annotations related to this gene include *identical protein binding* and *oxygen binding*. An important paralog of this gene is ENSG00000285441.

20. **Nfkb2 -** Nuclear Factor Kappa B Subunit 2

    This gene encodes a subunit of the transcription factor complex nuclear factor-kappa-B (NFkB). The NFkB complex is expressed in numerous cell types and functions as a central activator of genes involved in inflammation and immune function. The protein encoded by this gene can function as both a transcriptional activator or repressor depending on its dimerization partner. The p100 full-length protein is co-translationally processed into a p52 active form. Chromosomal rearrangements and translocations of this locus have been observed in B cell lymphomas, some of which may result in the formation of fusion proteins. There is a pseudogene for this gene on chromosome 18. Alternative splicing results in multiple transcript variants.

21. **Procr -** Protein C Receptor

    The protein encoded by this gene is a receptor for activated protein C, a serine protease activated by and involved in the blood coagulation pathway. The encoded protein is an N-glycosylated type I membrane protein that enhances the activation of protein C. Mutations in this gene have been associated with venous thromboembolism and myocardial infarction, as well as with late foetal loss during pregnancy. The encoded protein may also play a role in malarial infection and has been associated with cancer.

22. **Usp36 -** Ubiquitin Specific Peptidase 36

    This gene encodes a member of the peptidase C19 or ubiquitin-specific protease family of cysteine proteases. Members of this family remove ubiquitin molecules from polyubiquitinated proteins. The encoded protein may deubiquitinate and stabilize the

transcription factor c-Myc, also known as MYC, an important oncoprotein known to be upregulated in most human cancers. The encoded protease may also regulate the activation of autophagy. This gene exhibits elevated expression in some breast and lung cancers.

23. **Kcne4 -** Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 4
    Voltage-gated potassium (Kv) channels represent the most complex class of voltage-gated ion channels from both functional and structural standpoints. Their diverse functions include regulating neurotransmitter release, heart rate, insulin secretion, neuronal excitability, epithelial electrolyte transport, smooth muscle contraction, and cell volume. This gene encodes a member of the potassium channel, voltage-gated, isk-related subfamily. This member is a type I membrane protein, and a beta subunit that assembles with a potassium channel alpha-subunit to modulate the gating kinetics and enhance stability of the multimeric complex. This gene is prominently expressed in the embryo and in the adult uterus.
24. **Gstm5 -** Glutathione S-Transferase Mu 5
    GSTM5 (Glutathione S-Transferase Mu 5) is a Protein Coding gene. Diseases associated with GSTM5 include Borderline Glaucoma and Barrett's Adenocarcinoma. Among its related pathways are Drug metabolism - cytochrome P450 and Glutathione metabolism. Gene Ontology (GO) annotations related to this gene include *glutathione transferase activity*. An important paralog of this gene is GSTM1.
25. **AABR07047256.1**

Of these genes, CXCL1, TIMP1, Cd55, Map3k8, Sod2, NfKb2, Procr and USP36 are good candidates as putative pathways for RIC protection in the bowel. In most cases, these genes are down regulated by RIC, relative to the comparable groups. In the cases of TIMP1 and Cd55 these genes are upregulated in RIC alone and down-regulated in animals exposed to RIC and IRI. The details of the differential expression data are shown in Table 9-7.

Genes		logFC	logCPM	PValue	FDR
CXCL1	*RIC+IRI*	-0.73581	8.295629	9.37E-06	0.016462
	RIC	-0.59385	7.581155	6.20E-05	0.014521
TIMP1	*RIC+IRI*	-1.08748	4.695321	9.35E-05	0.036933
	RIC	0.584135	6.188187	0.00031	0.021415
Cd55	*RIC+IRI*	-0.56804	7.283793	1.78E-05	0.020411
	RIC	0.487938	6.174565	9.45E-05	0.016591
Map3k8	*RIC+IRI*	-0.75824	4.211773	0.000186	0.042433
	RIC	-2.7615	2.360486	0.000722	0.030387
Sod2	*RIC+IRI*	-0.98012	7.372327	0.000217	0.043591
	RIC	-1.47528	3.472257	0.000946	0.033794
Nfkb2	*RIC+IRI*	-0.49015	4.366177	0.000222	0.043961
	RIC	-0.3912	4.601592	0.000969	0.034104
Procr	*RIC+IRI*	-1.34504	0.765029	0.000227	0.044383
	RIC	-0.40365	7.534098	0.000969	0.034104
Usp36	*RIC+IRI*	-1.60342	-1.88456	0.000267	0.045566
	RIC	-0.80179	2.778097	0.001428	0.040053

Table 9-7 Genes showing differential expression in both the RIC vs SHAM and RIC+IRI vs IRI groups.
logFC = log fold-change in gene expression; logCPM = log Counts per Million; PValue = raw p-value; FDR = False discovery rate (p-value corrected for multiple comparisons).

9.4.3 Targeted gene analysis

The results of the various targeted analyses are summarised below. In each gene, the purpose is to determine whether IRI affects the expression or whether RIC affects the expression pattern and how the two different processes interact.

9.4.3.1 Cell types

As the RNAseq was performed on whole tissue, the purpose here was to elucidate if there was a measurable change in the cell population – i.e. by the influx of immune cells. Table 9-8 shows these selected genes arrange by the cell types for which they are markers and the expression pattern seen. In the following figures, statistically significant p-values are shown in bold.

Protein name	Expression Pattern	Figure
Immune cells		
CD45	Expression *unchanged* by either RIC or IRI **Expression of patterns not affected by either IRI or RIC**	Figure 9-18
Macrophages		
CD163	Expression is *unchanged* IRI relative to SHAM (p = 0.35) Expression is *increased* in RIC relative to SHAM (p = 0.003) but *unchanged* in IRI+RIC relative to IRI (p = 0.88) **CD163 *increased* by RIC but this effect is *not seen* in IRI+RIC**	Figure 9-19
CD164	Expression *unchanged* by either RIC or IRI **Expression patterns not affected by either IRI or RIC**	Figure 9-20
CD206	Expression *unchanged* in IRI relative to SHAM (p = 0.19) Expression *unchanged* in RIC relative to SHAM (p = 0.87) Expression *unchanged* in IRI+RIC relative to IRI (p = 0.34) (although does show a tread to lower expression in RIC+IRI relative to IRI) Expression *decreased* in IRI+RIC relative to RIC (p = 0.006) **CD206 *reduced* by the *combination* of IRI and RIC but not by either alone**	Figure 9-21
CD68	Expression *unchanged* in RIC relative to SHAM (p = 0.14) and RIC+IRI relative to IRI (p = 0.16) Expression *unchanged* in IRI relative to SHAM (p = 0.50) *And* RIC+IRI relative to RIC (p = 0.19) (although does show a trend to higher expression in IRI relative to SHAM and RIC+IRI relative to RIC) **CD68 *unchanged* by RIC and *potentially unchanged* by IRI**	Figure 9-22
CD11c	Expression *unchanged* in IRI relative to SHAM (p = 0.30) Expression *decreased* in RIC relative to SHAM (p = 0.01) but *unchanged* in RIC+IRI relative to IRI (p = 0.11) (although does show a trend towards *lower* expression in RIC+IRI relative to IRI) **CD11c expression is *decreased* by RIC and this affect is masked by IRI**	Figure 9-23
B-Lymphocytes		
CD20	Expression *unchanged* by either RIC or IRI **Expression patterns not affected by either IRI or RIC**	Figure 9-24
CD19	Expression *unchanged* by either RIC or IRI **Expression patterns not affected by either IRI or RIC**	Figure 9-25

T-Lymphocytes		
CD3	Expression *unchanged* in IRI relative to SHAM (p = 0.06) (although does show a trend toward *higher* expression in IRI relative to SHAM) Expression *increased* in IRI+RIC relative to RIC (p = 0.05) Expression *decreased* in RIC relative SHAM (p = 0.02) *and* RIC+IRI relative to IRI (p = 0.05) **CD3 expression *increased* by IRI and *decreased* by RIC**	Figure 9-26
CD4	Expression *unchanged* by either RIC or IRI **Expression of patterns not affected by either IRI or RIC**	Figure 9-27
CD8a	Expression *unchanged* by either RIC or IRI **Expression of patterns not affected by either IRI or RIC**	Figure 9-28
CD69	Expression *increased* in IRI relative to SHAM (p = 0.002) *and* RIC+IRI relative to RIC (p = 0.003) Expression unchanged in RIC relative to SHAM (p = 0.36) *and RIC+IRI* relative to IRI (p = 0.07) (although does show a trend towards *lower* expression in RIC+IRI relative to RIC) **CD69 expression *increased* by IRI and this is possibly *mitigated* by RIC**	Figure 9-29
CD103	Expression *unchanged* by either RIC or IRI **Expression of patterns not affected by either IRI or RIC**	Figure 9-29
Natural Killer Cells		
CD56	Expression *increased* in IRI relative to SHAM (p = 0.03) *but unchanged* in RIC+IRI relative to RIC (p = 0.74) Expression *increased* in RIC relative to SHAM (p=0.0003) *but unchanged* in RIC+IRI relative to IRI (p = 0.53) **CD59 expression is *increased* by both IRI and RIC. There does not appear to be any interaction between the two processes**	Figure 9-31
KIR receptors	Expression *unchanged* by either RIC or IRI **Expression of patterns not affected by either IRI or RIC**	Figure 9-32
Endothelial Cells		
CD109	Expression *unchanged* by either RIC or IRI **Expression of patterns not affected by either IRI or RIC**	Figure 9-33
Neutrophils		
CD15	Expression *decreased* in IRI relative to SHAM (p = 0 .0001) *and* IRI+RIC relative to RIC (0.003) Expression *increased* in RIC+IRI relative to IRI (p = 0.043) Expression *unchanged* in RIC relative to SHAM (p = 0.34) **CD15 expression *decreased* by IRI and this effect is *mitigated* by RIC**	Figure 9-34

Fibroblasts		
Vimentin	Expression *increased* in IRI relative to SHAM (p = 0.0002) *and* IRI+RIC relative to RIC (p = 0.02) Expression *unchanged* in RIC relative to SHAM (p = 0.41) Expression *decreased* in RIC+IRI relative to IRI (p = 0.02) **Vimentin expression *increased* by IRI and this effect is mitigated by RIC**	Figure 9-35
CDH2	Expression *unchanged* by either RIC or IRI **Expression of patterns not affected by either IRI or RIC**	Figure 9-36
Acta2	Expression *increased* in IRI relative to SHAM (p = 0.02) *and* IRI+RIC relative to RIC (p = 0.04) Expression *decreased* in RIC relative to SHAM (p = 0.0005) *but unchanged* in IRI+RIC relative to IRI (p = 0.57) **Acta2 expression *increased* by IRI** **Acta2 expression *decreased* by RIC but this effect is not seen in animals exposed to IRI as well as RIC**	Figure 9-37
PDGFA	Expression is *decreased* in IRI relative to SHAM (p = 0.049) *but unchanged* in IRI+RIC relative to RIC (p = 0.70) Expression is *decreased* in RIC relative to SHAM (p = 0.10) *but unchanged* in IRI+RIC relative to IRI (p = 0.65) **PDGFA expression decreased by IRI** **PDGFA expression decreased by RIC** **There appears to be no interaction between these two processes**	Figure 9-36
Alpha smooth muscle		
CD31		
Desmin	Expression *increased* in IRI relative to SHAM (p = 0.0004) *and* RIC+IRI relative to RIC (p = 0.001) Expression *decreased* in RIC relative to SHAM (p = 0.01) *and* IRI+RIC relative to IRI (p = 0.01) **Desmin expression *increased* by IRI and *decreased* by RIC**	Figure 9-39
CDH2	Expression *unchanged* by either RIC or IRI **Expression of patterns not affected by either IRI or RIC**	

Table 9-8 Expression patterns of selected genes to examine the likely cell population in the tissue

9.4.3.1.1 Immune cells

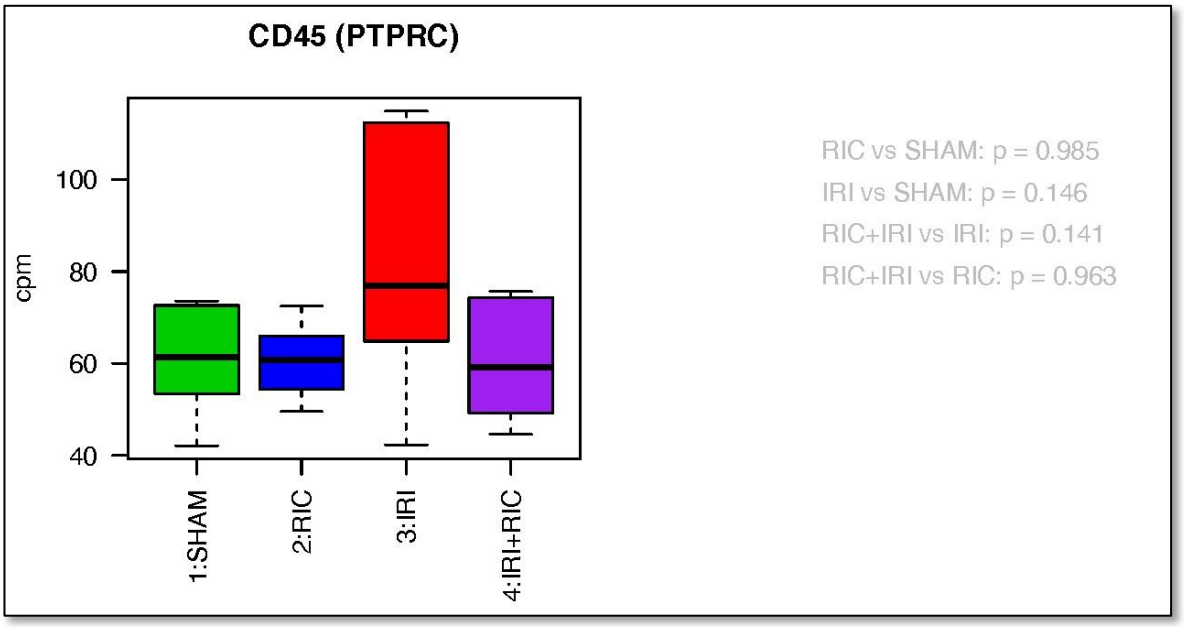


Figure 9-18 Expression pattern across the four groups for CD45 (PTPRC).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

Overall there is no change in the measured expression of CD45 and hence there does not appear to be a significant change in the immune cell population overall within this tissue.

9.4.3.1.2 Macrophages

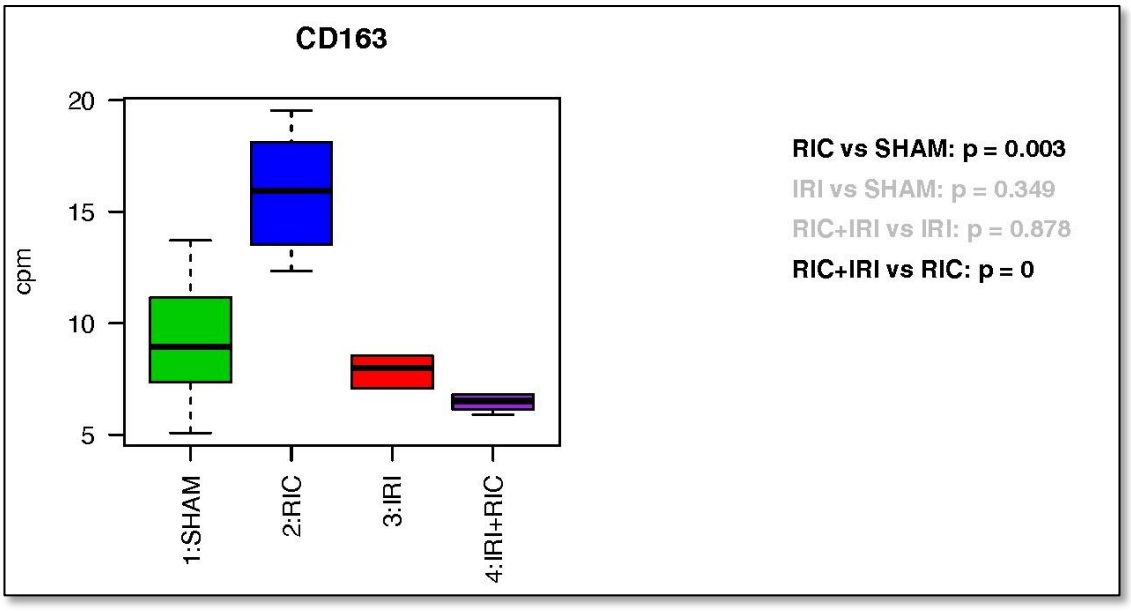


Figure 9-19 Expression pattern across the four groups for CD163.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

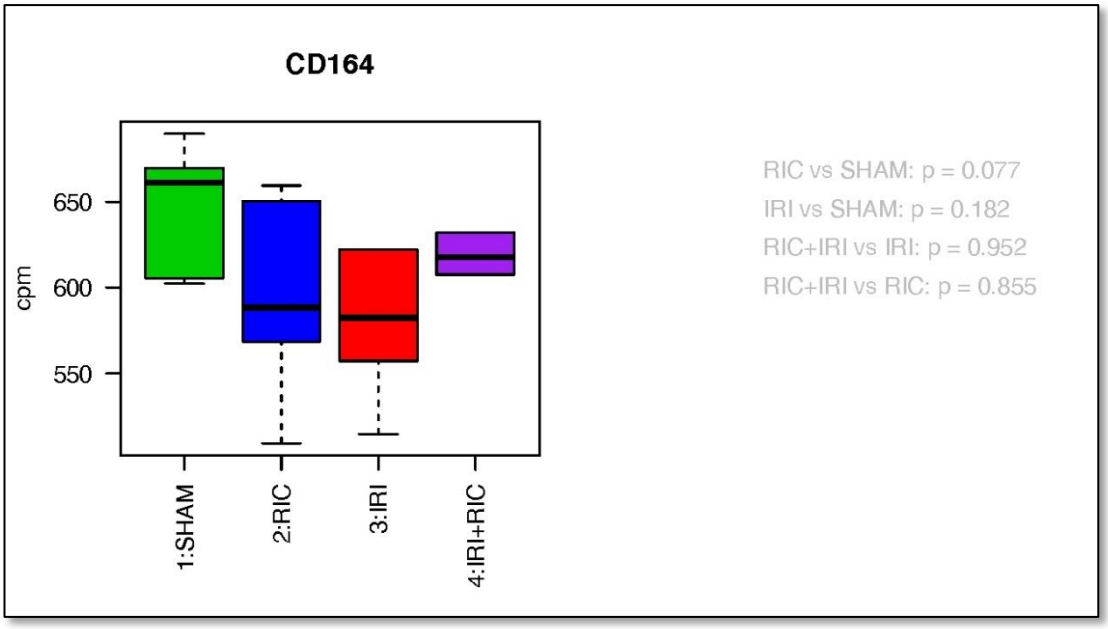


Figure 9-20 Expression pattern across the four groups for CD164.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

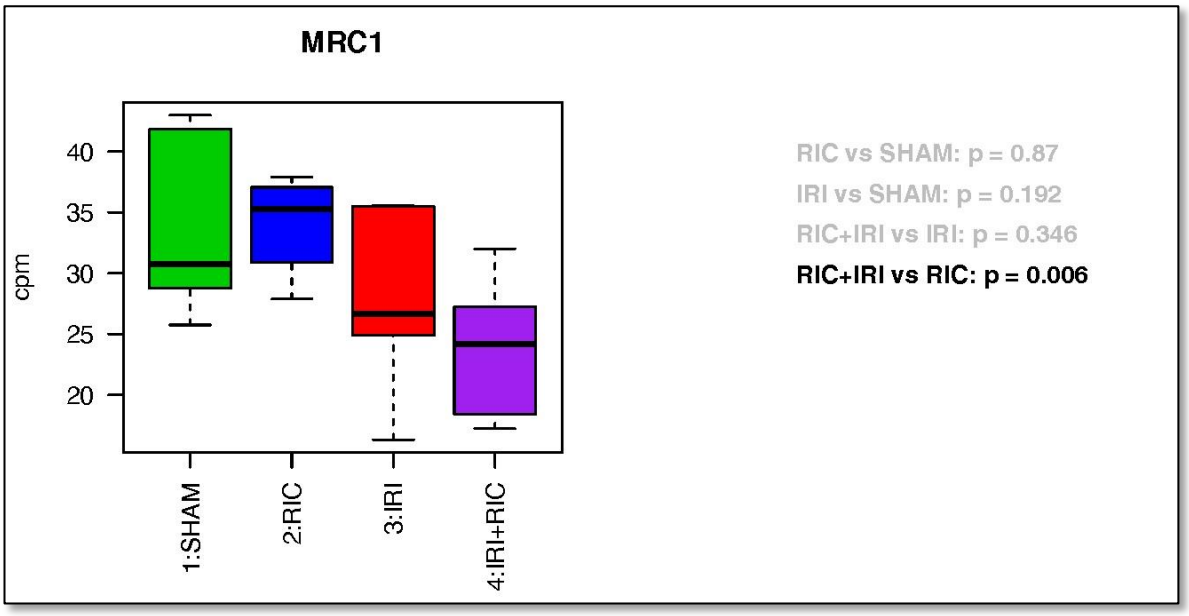


Figure 9-21 Expression pattern across the four groups for CD206 (MRC1).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

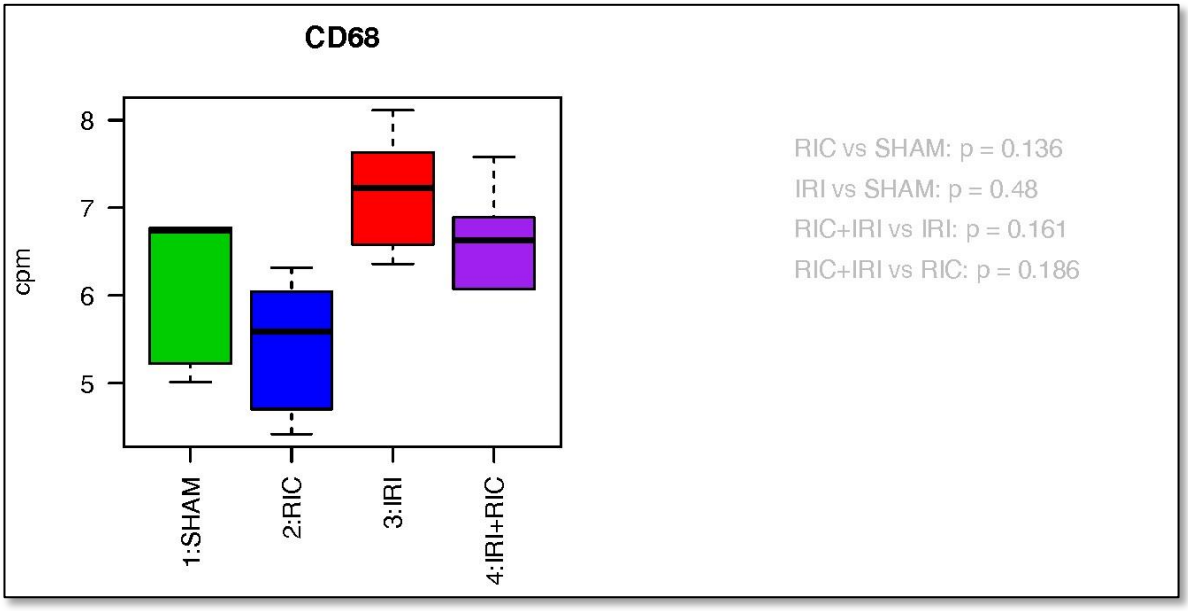


Figure 9-22 Expression pattern across the four groups for CD68.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

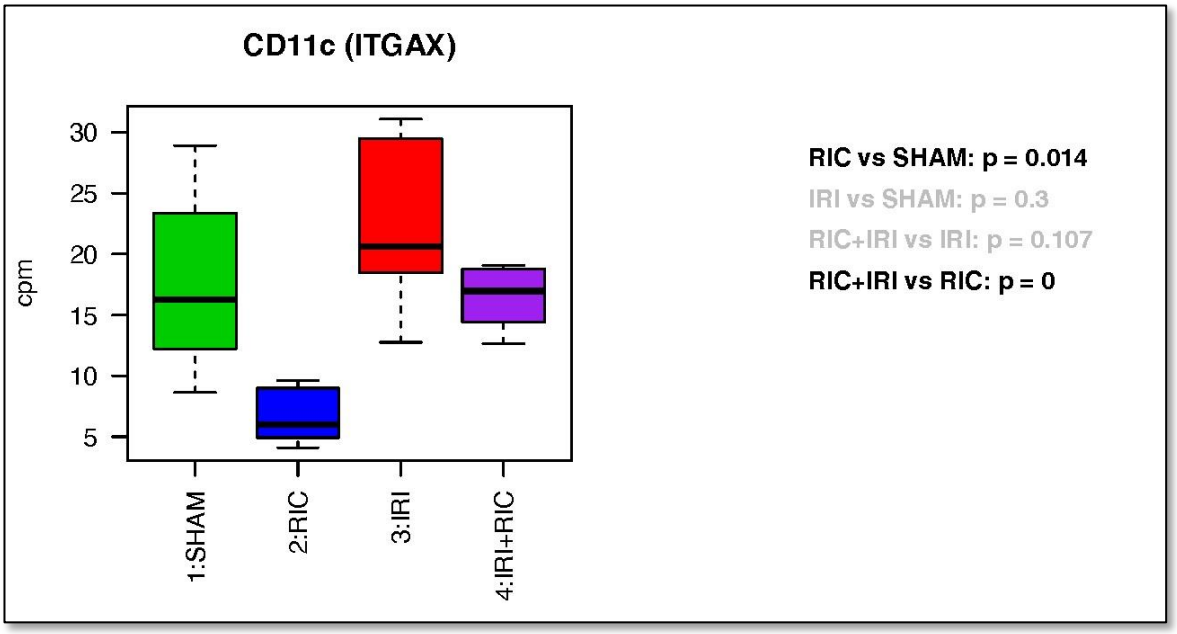


Figure 9-23 Expression pattern across the four groups for CD11c (ITGAX).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

The variable patterns of expression of these markers of macrophages would fit with an alteration in macrophage function rather than a change overall macrophage numbers.

9.4.3.1.3 B-Lymphocytes

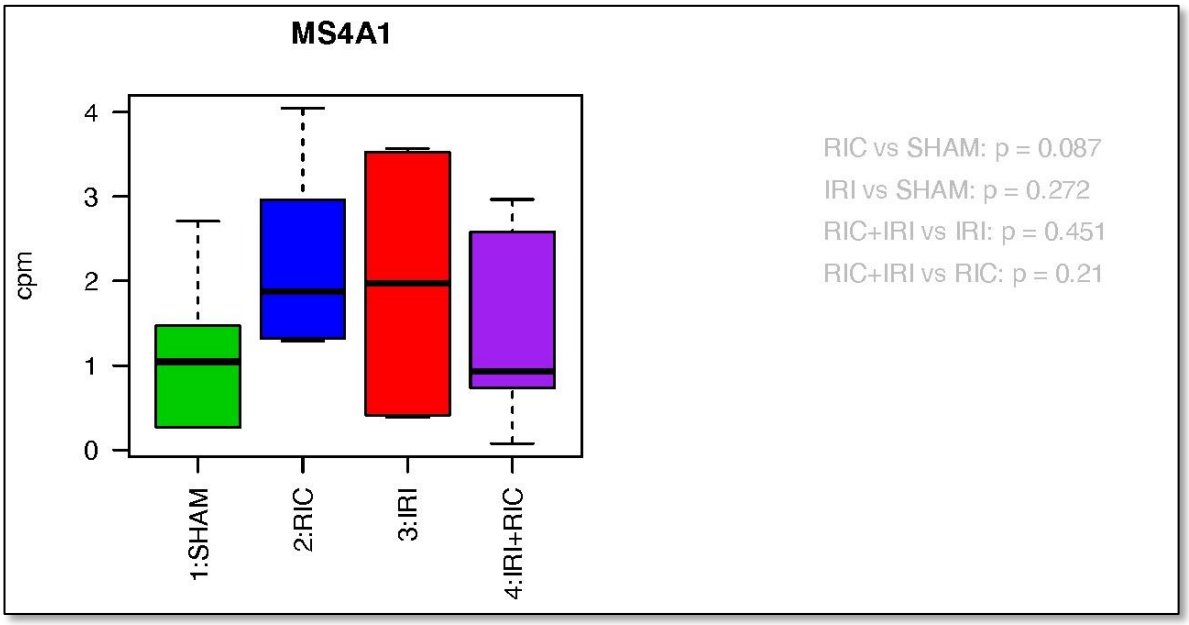


Figure 9-24 Expression pattern across the four groups for CD20 (MS4A1).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

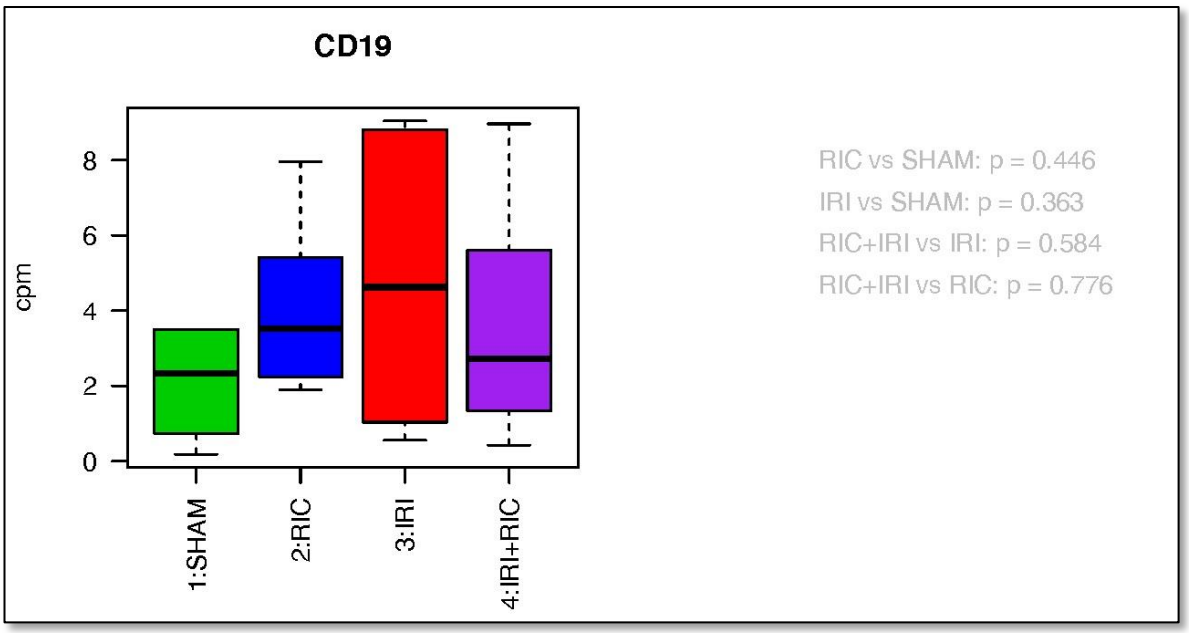


Figure 9-25 Expression pattern across the four groups for CD19.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

Based on these data, the population of B-lymphoctyes within the intestine appears to be unchanged by both IRI and RIC.

9.4.3.1.4 T-Lymphocytes

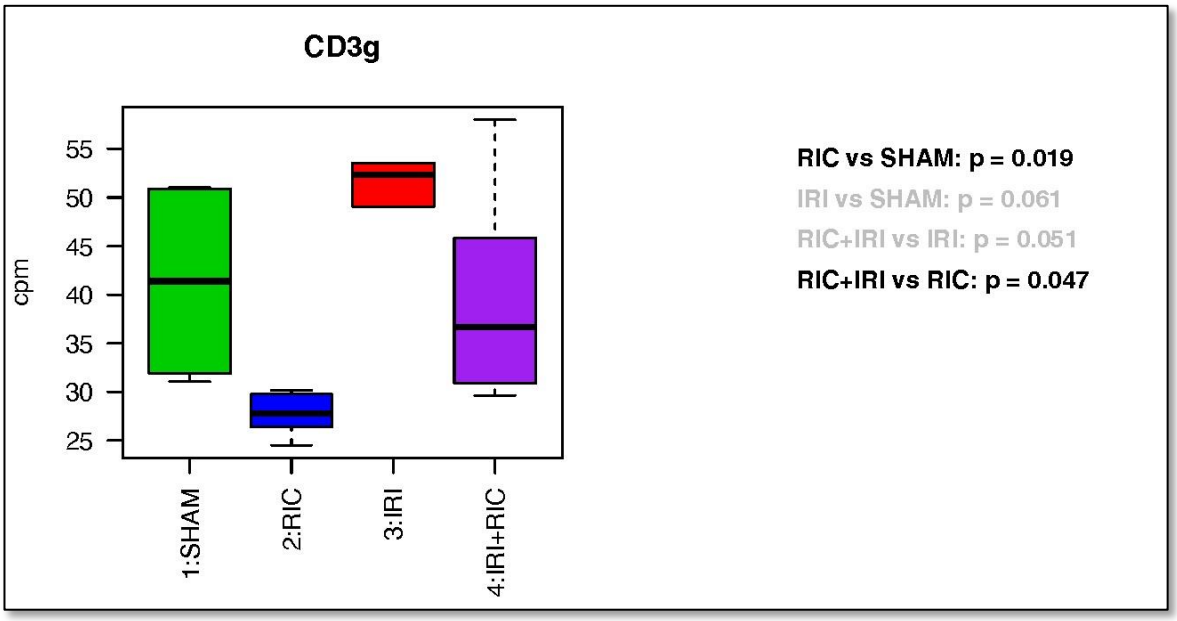


Figure 9-26 Expression pattern across the four groups for CD3γ.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

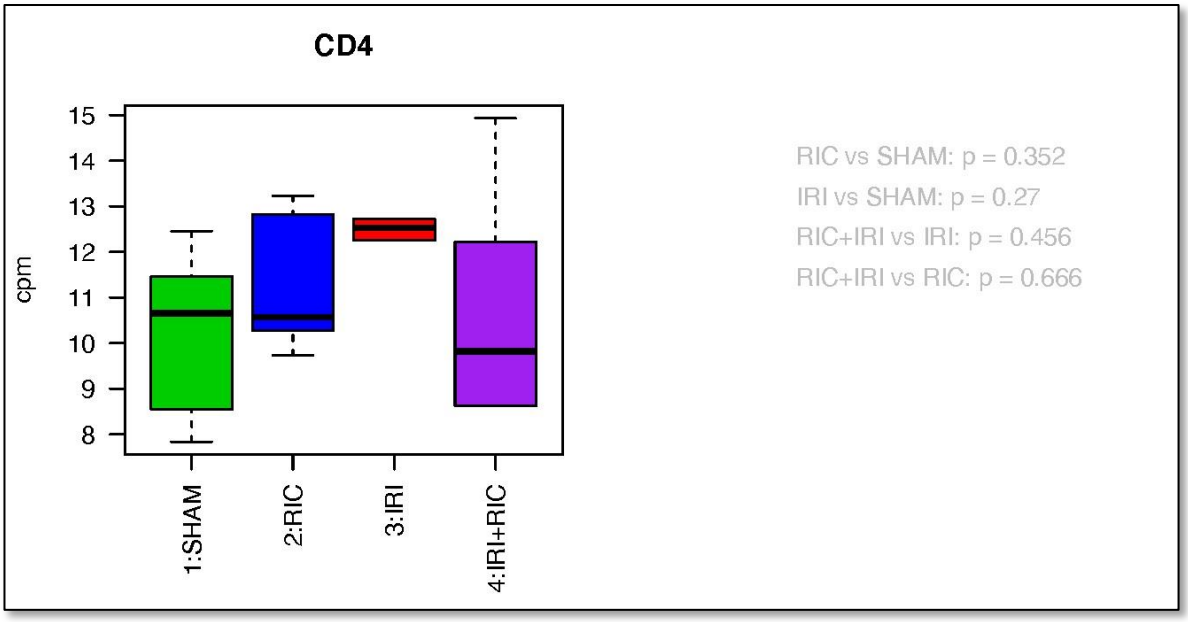


Figure 9-27 Expression pattern across the four groups for CD4.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

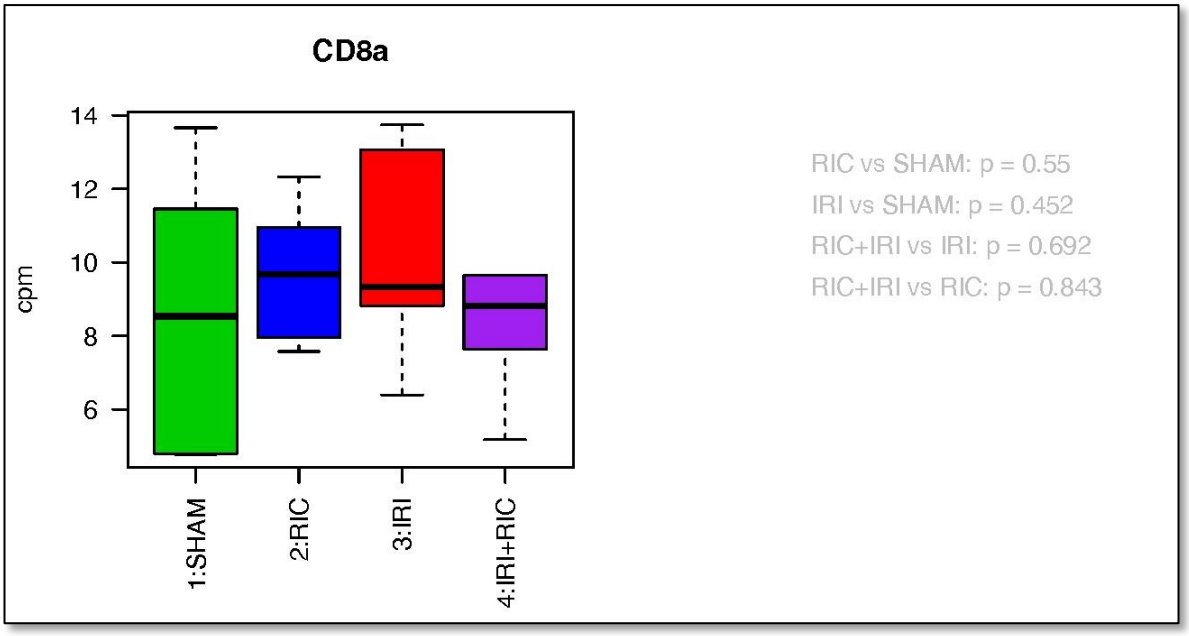


Figure 9-28 Expression pattern across the four groups for CD8a.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

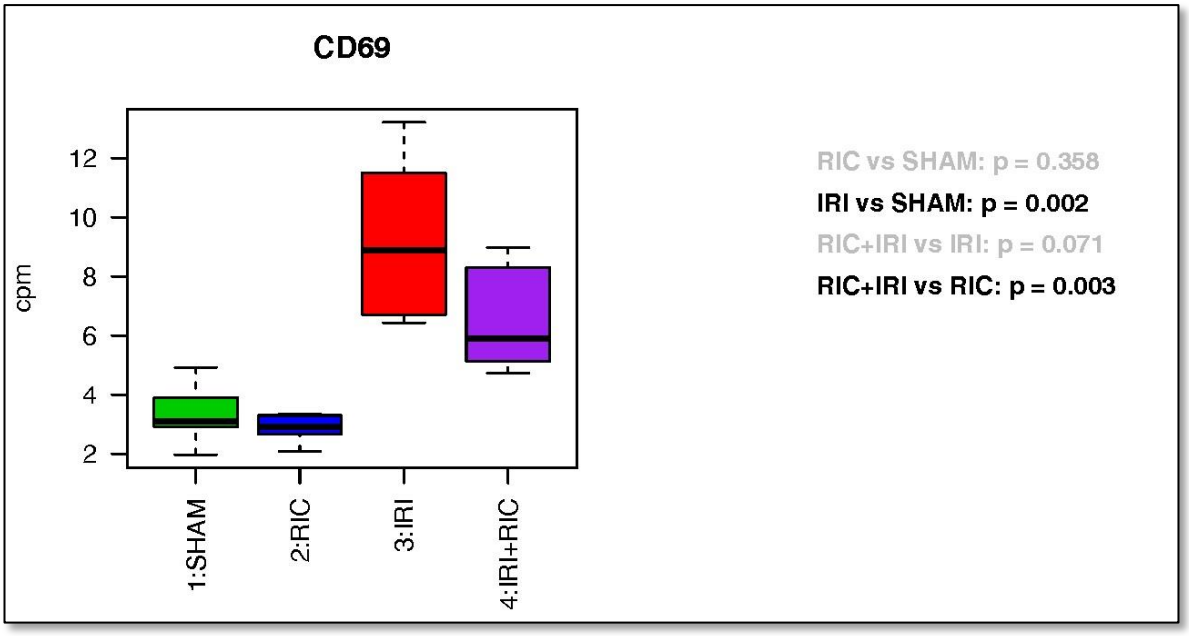


Figure 9-29 Expression pattern across the four groups for CD69.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

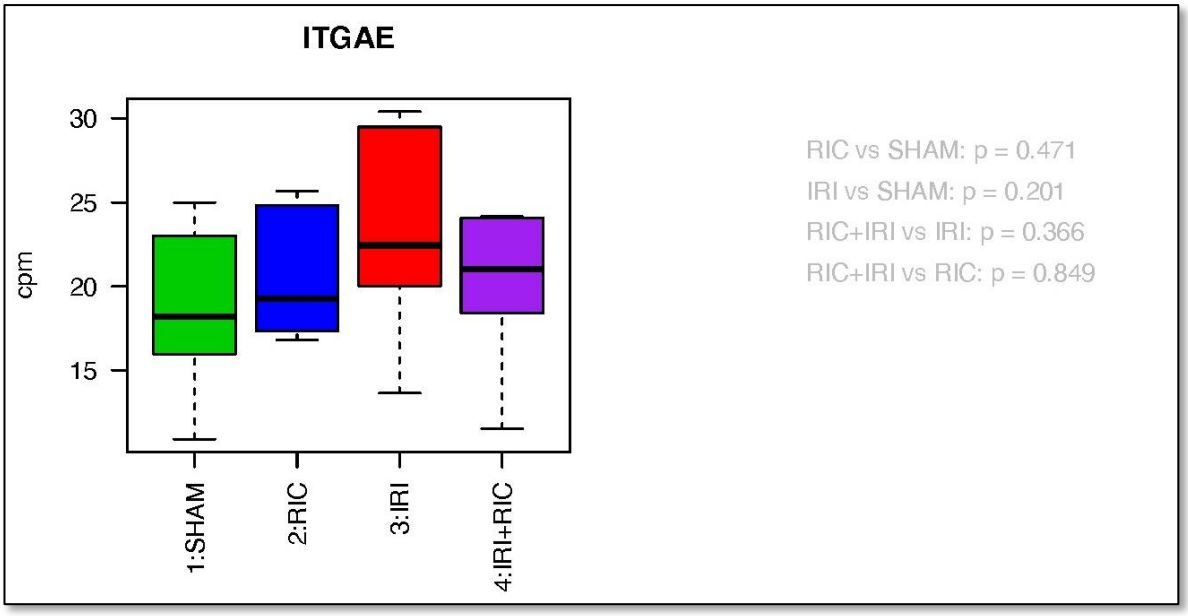


Figure 9-30 Expression pattern across the four groups for CD103 (ITGAE).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

These data suggest that there is an influx of T-cells into the bowel in response to IRI as indicated by the rise in CD3. This influx is less marked in the tissue of animals exposed to RIC prior to IRI. These T-cells do not appear to be either cytotoxic T-cells or T-helper cells as the expression of CD8 and CD4 is unchanged. The expression pattern of CD69 is similar to CD3 suggesting that there is an increase in regulatory T-cells in the IRI groups.

9.4.3.1.5 Natural Killer Cells

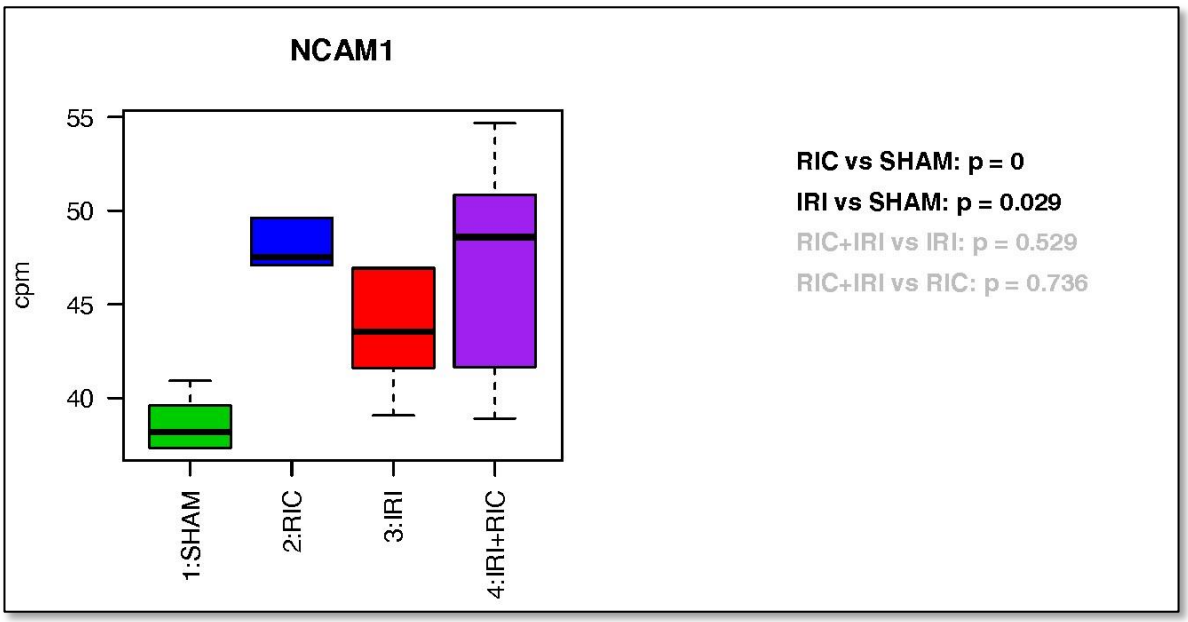


Figure 9-31 Expression pattern across the four groups for CD56 (NCam1).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

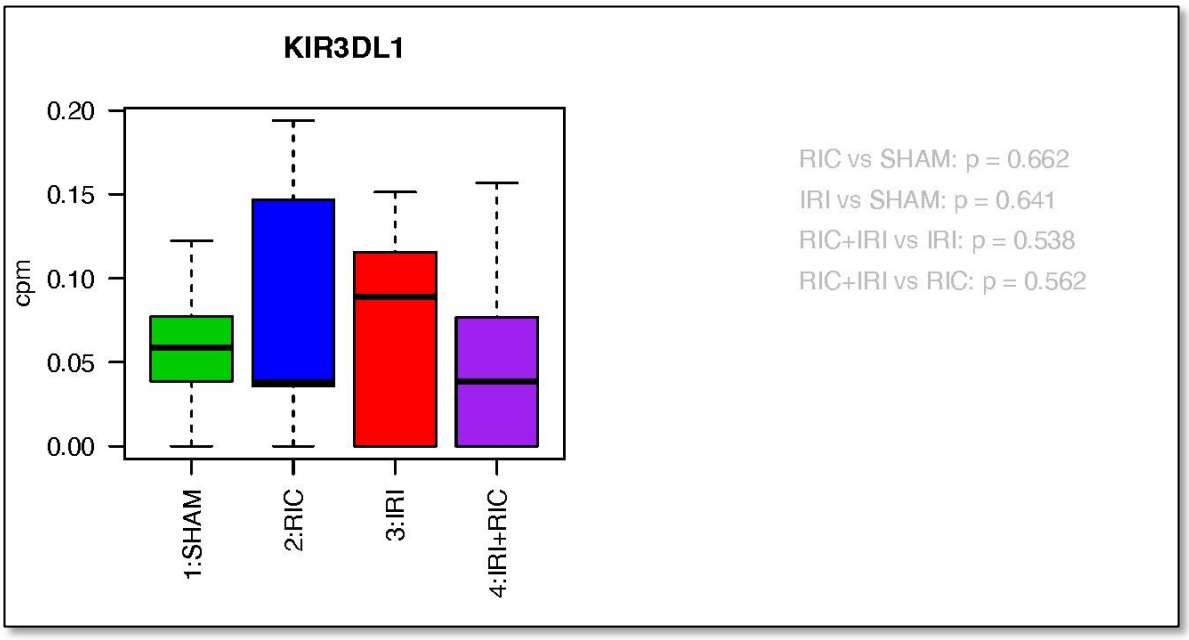


Figure 9-32 Expression pattern across the four groups for KIR receptor.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

Remote ischaemic conditioning increases the expression of NCAM-1. There is no clear difference in the cell population.

9.4.3.1.6 Endothelial Cells

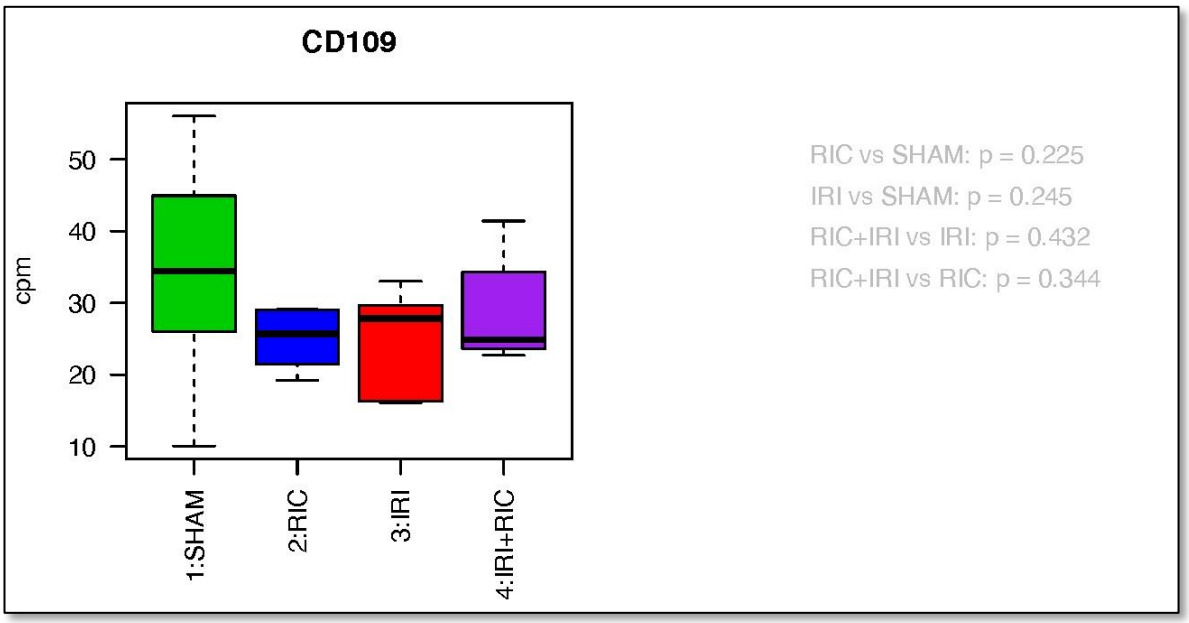


Figure 9-33 Expression pattern across the four groups for CD109.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

There appears to be no change in the pattern of expression of CD109 which would indicate no change in the population of endothelial cells within this intestinal tissue.

9.4.3.1.7 Neutrophils

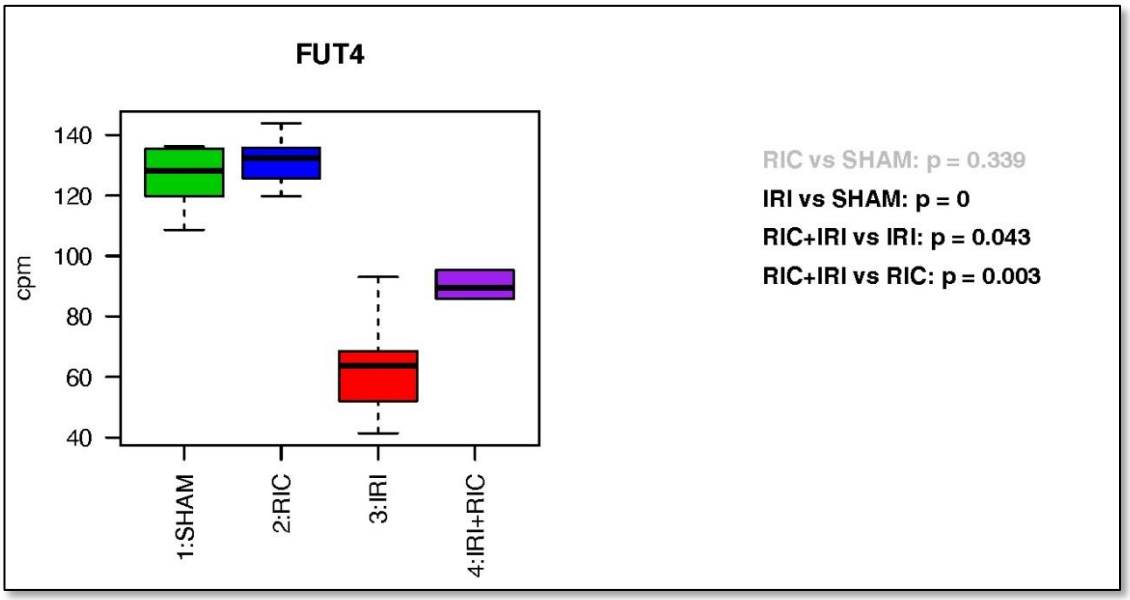


Figure 9-34 Expression pattern across the four groups for CD15 (FUT4).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

CD-15/Fut4 expression tracks with IRI. It is notably reduced by IRI and this effect is mitigated with RIC. The lack of effect of RIC alone here suggest that CD-15 expression is tracking with level of bowel injury.

9.4.3.1.8 Fibroblasts

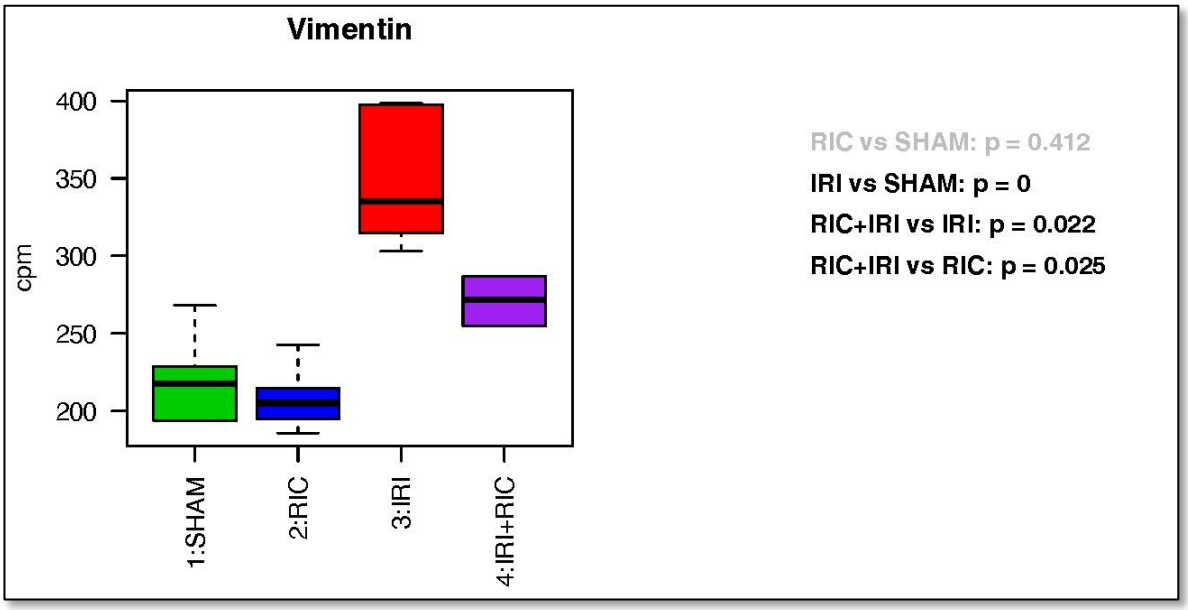


Figure 9-35 Expression pattern across the four groups for Vimentin.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

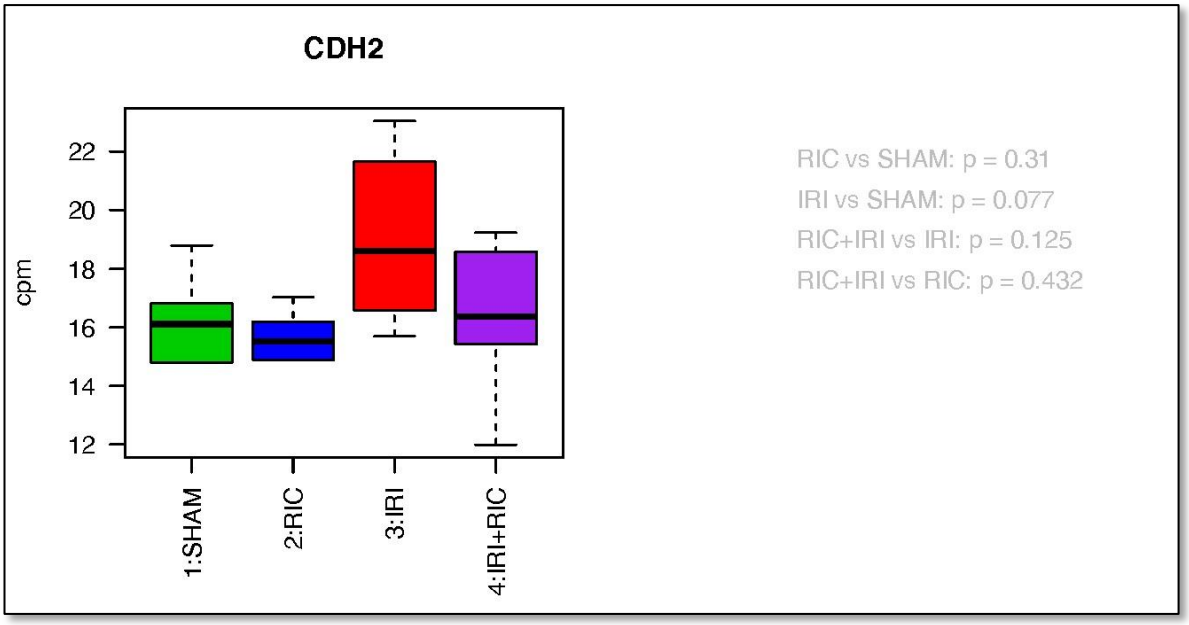


Figure 9-36 Expression pattern across the four groups for CDH2.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

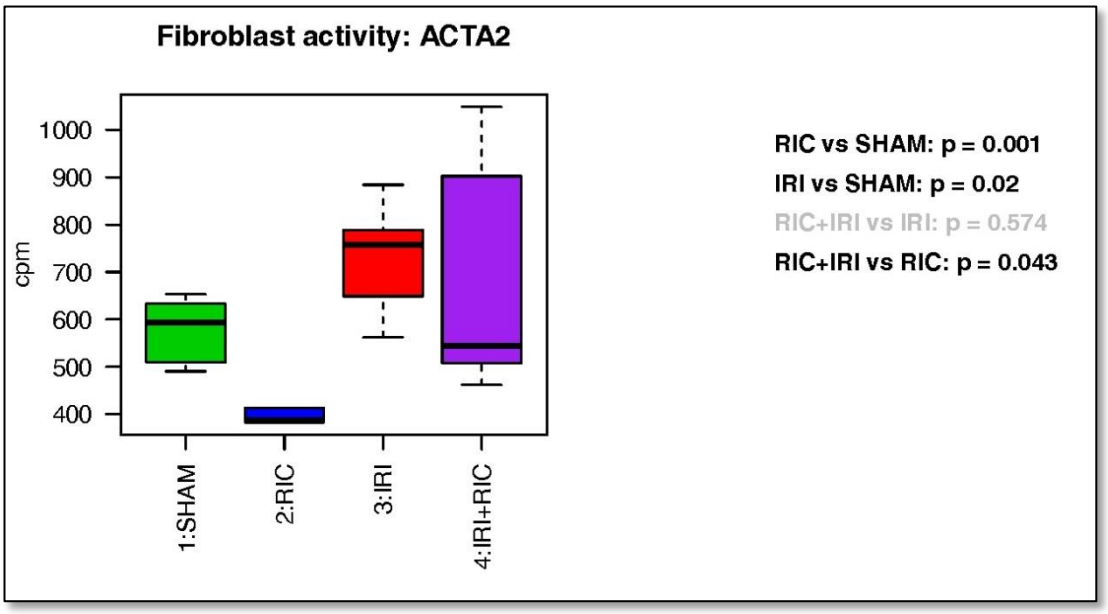


Figure 9-37 Expression pattern across the four groups for ACTA2.
Graph shows counts per million, median, interquartile range and range for each of the four groups

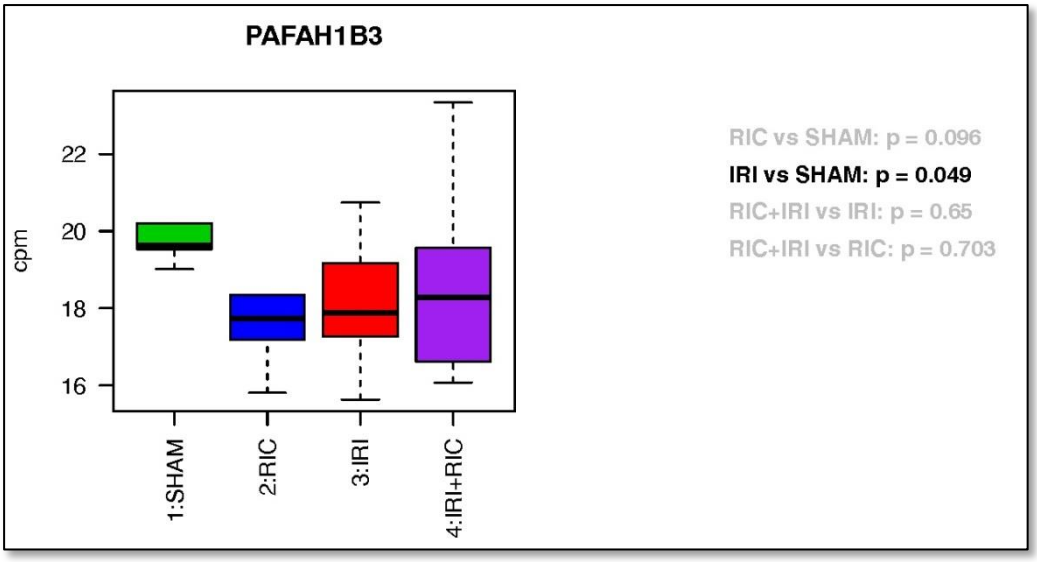


Figure 9-38 Expression pattern across the four groups for PDGFA (PAFAH1B3)
Graph shows counts per million, median, interquartile range and range for each of the four groups

9.4.3.1.9 Alpha Smooth Muscle

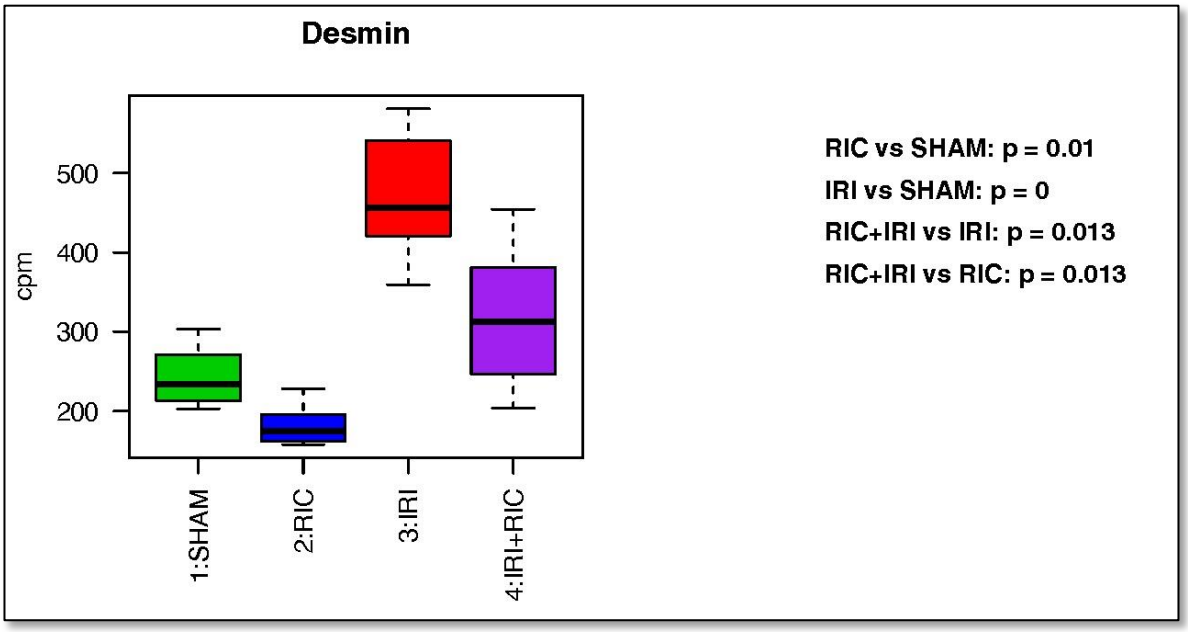


Figure 9-39 Expression pattern across the four groups for Desmin.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

9.4.3.2 Biological Processes

Targeted analysis of the change in expression pattern of selected genes important in the cell's response to hypoxia and genes that are markers of cell toxicity are shown in Table 9-9 and Figures 9-40 – 9-45.

Protein name	Expression Pattern	Figure
Hypoxia		
HIF1α	Expression *increased* in IRI relative to SHAM (p = 0.0004) *and* in IRI+RIC relative to RIC (p = 0.0005) Expression *unchanged* in RIC relative to SHAM (p = 0.08) (but with a trend towards *decreased* expression in RIC relative to SHAM) Expression *decreased* in RIC+IRI relative to IRI (p = 0.005) **HIF1α expression *increased* by IRI and *decreased* by RIC**	Figure 9-40
HIF1β	Expression *unchanged* by either RIC or IRI **Expression of patterns not affected by either IRI or RIC**	Figure 9-41
HIF2α	Expression *increased* in IRI relative to SHAM (p = 0.009) *and* RIC+IRI relative to RIC (p = 0.0001) Expression *decreased* in RIC relative to SHAM (p = 0.04) *and* RIC+IRI relative to IRI (p = 0.02) **HIF2α expression *increased* by IRI and *decreased* by RIC**	Figure 9-42
VEGF	Expression *increased* in IRI relative to SHAM (p = 0.0004) *and* in IRI+RIC relative to RIC (p = 0.0000) Expression *unchanged* in RIC relative to SHAM (p = 0.78) Expression *decreased* in RIC+IRI relative to IRI (p = 0.04) **VEGF expression *increased* by IRI and this is mitigated by RIC**	Figure 9-43
Cell Toxicity		
GZMA	Expression *unchanged* by either RIC or IRI **Expression of patterns not affected by either IRI or RIC**	Figure 9-44
GZMB	Expression is essentially unchanged but there is a trend towards higher expression in IRI which is mitigated by RIC. **GZMB expression *may be increased* by IRI and this *may be mitigated* by RIC.**	Figure 9-45

Cell Repair		
KRT-7	Expression *unchanged* in RIC relative to SHAM (p = 0.84) Expression *increased* IRI relative to SHAM (p = 0.0052) Expression *decreased* in RIC+IRI relative to IRI (p = 0.049) **KRT-7 expression *increased* by IRI and this is mitigated by RIC**	Figure 9-46
KRT-8	Expression *unchanged* in RIC relative to SHAM (p = 0.13) Expression *increased* IRI relative to SHAM (p = 0.018) Expression *unchanged* in RIC+IRI relative to IRI (p = 0.06) (but with a trend towards *decreased* expression in RIC+IRI relative to IRI) **KRT-8 expression *increased* by IRI and this is potentially mitigated by RIC**	Figure 9-47
KRT-18	Expression *unchanged* in RIC relative to SHAM (p = 0.052) (but with a trend towards decreased expression in RIC relative to SHAM) Expression *increased* IRI relative to SHAM (p = 0.00005) Expression *decreased* in RIC+IRI relative to IRI (p = 0.0033) **KRT-18 expression *increased* by IRI and this is mitigated by RIC**	Figure 9-48
KRT-19	Expression *unchanged* in RIC relative to SHAM (p = 0.13) Expression *increased* in IRI relative to SHAM (p = 0.018) Expression *decreased* in RIC+IRI relative IRI (p = 0.016) **KRT-19 expression *increased* by IRI and this is mitigated by RIC**	Figure 9-49

Table 9-9 Expression patterns of selected genes that are expected to be affected by either IRI or RIC.

9.4.3.2.1 Hypoxia

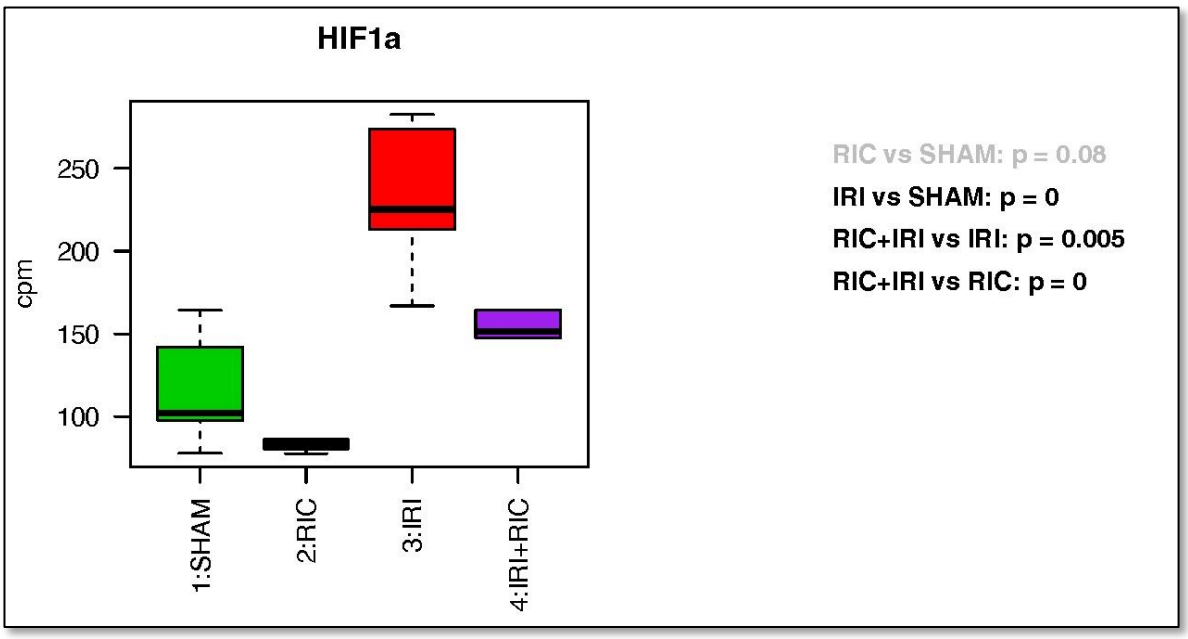


Figure 9-40 Expression pattern across the four groups for HIF-1α.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

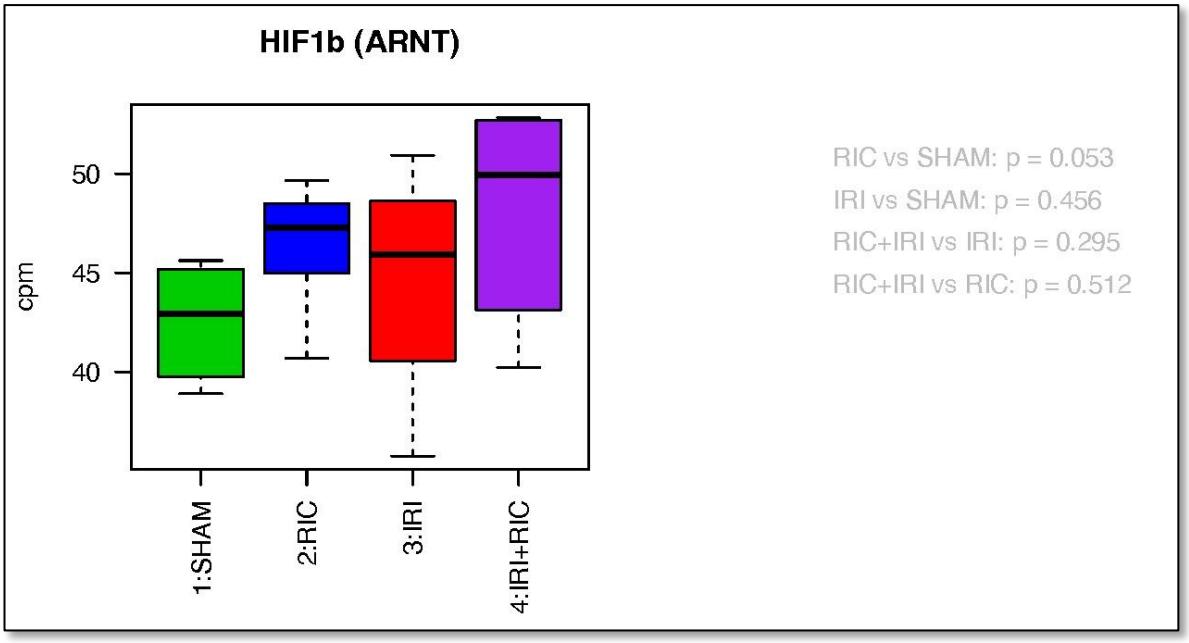


Figure 9-41 Expression pattern across the four groups for HIF-1β (ARNT).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

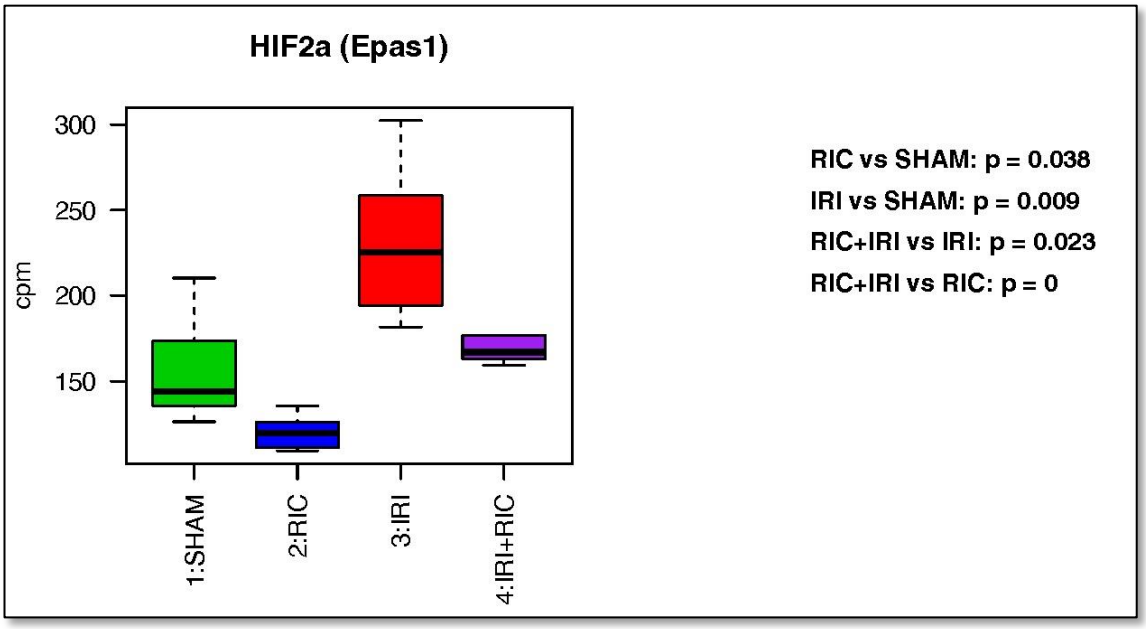


Figure 9-42 Expression pattern across the four groups for HIF-2α (EPAS1).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

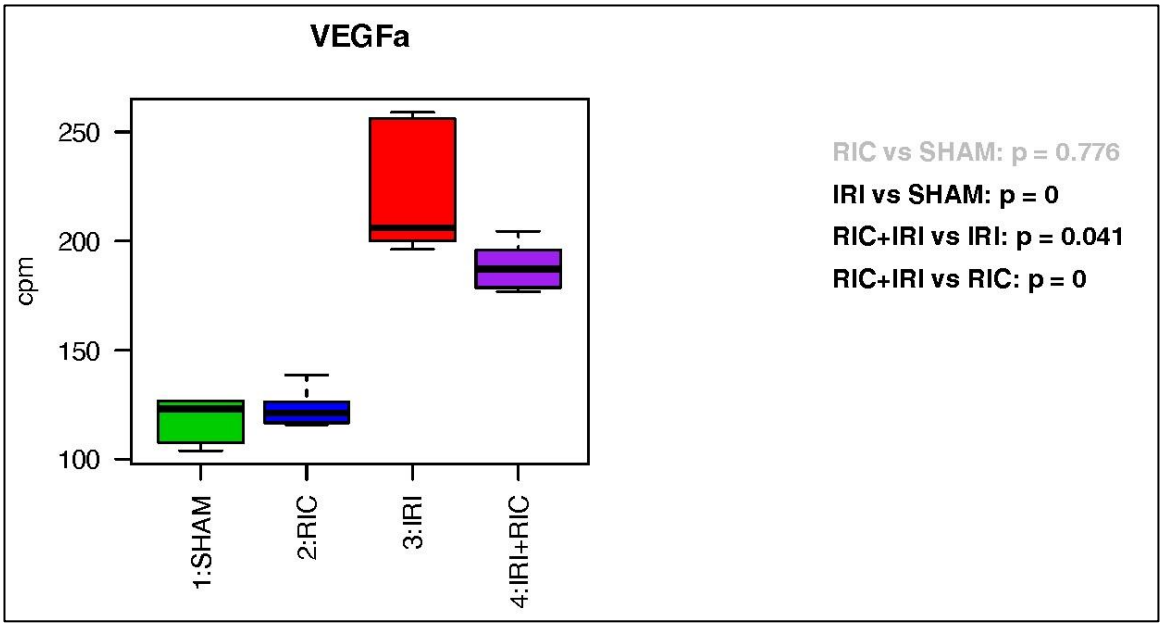


Figure 9-43 Expression pattern across the four groups for VEGFα
Graph shows counts per million, median, interquartile range and range for each of the four groups.

9.4.3.2.2 Cell Toxicity

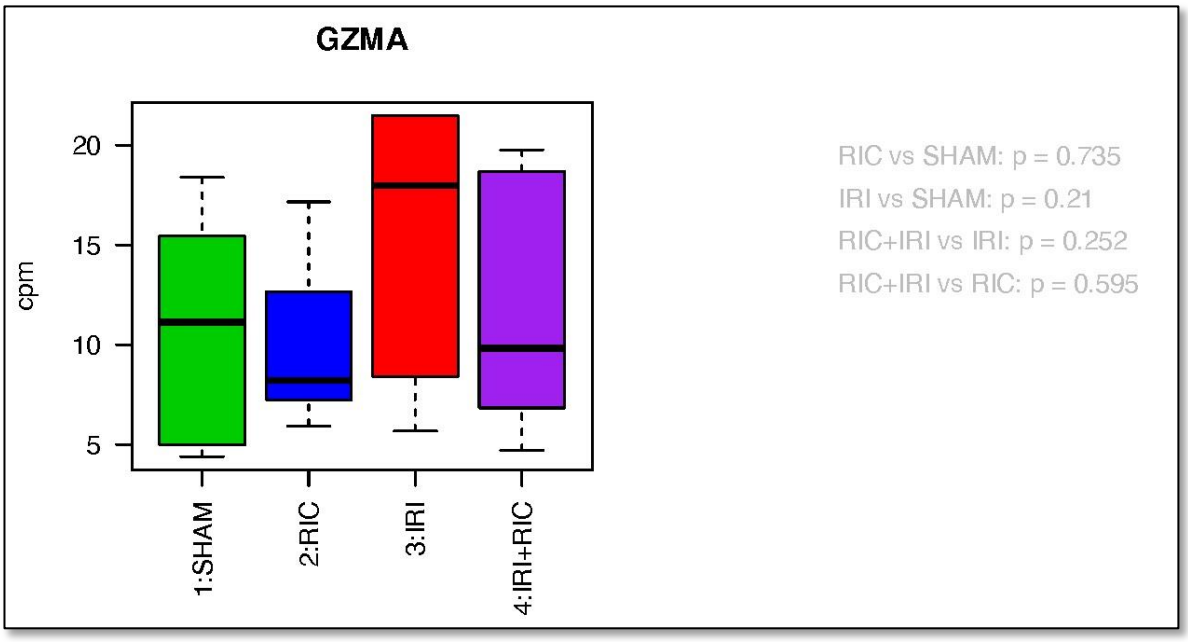


Figure 9-44 Expression pattern across the four groups for GZMα.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

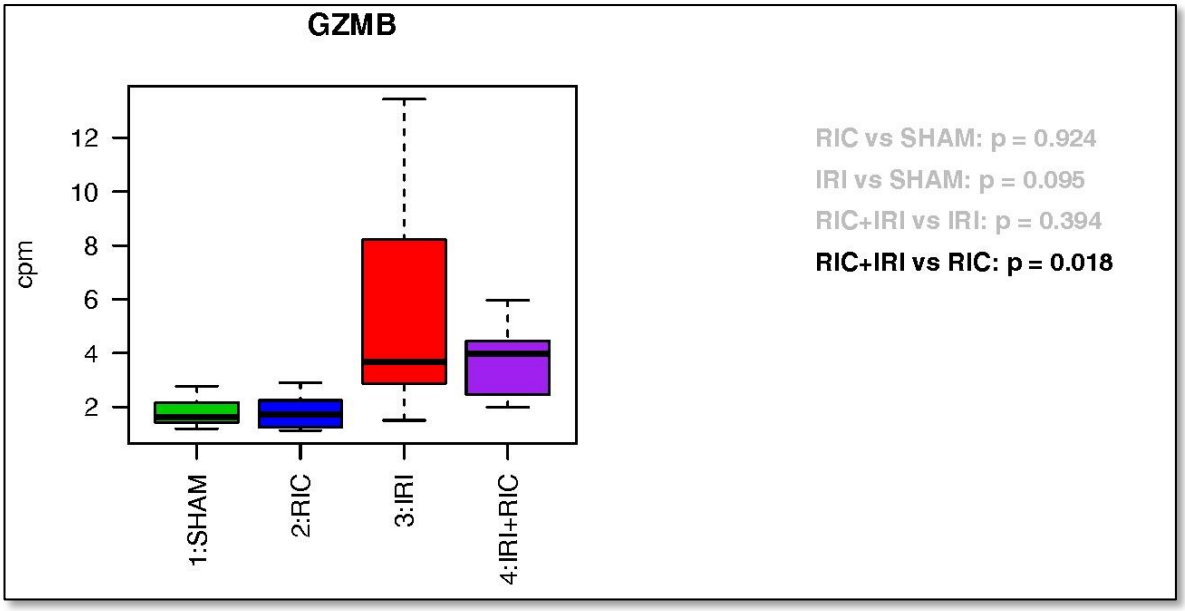


Figure 9-45 Expression pattern across the four groups for GZMβ.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

9.4.3.2.3 Cytokeratins – cytostructure and cell repair

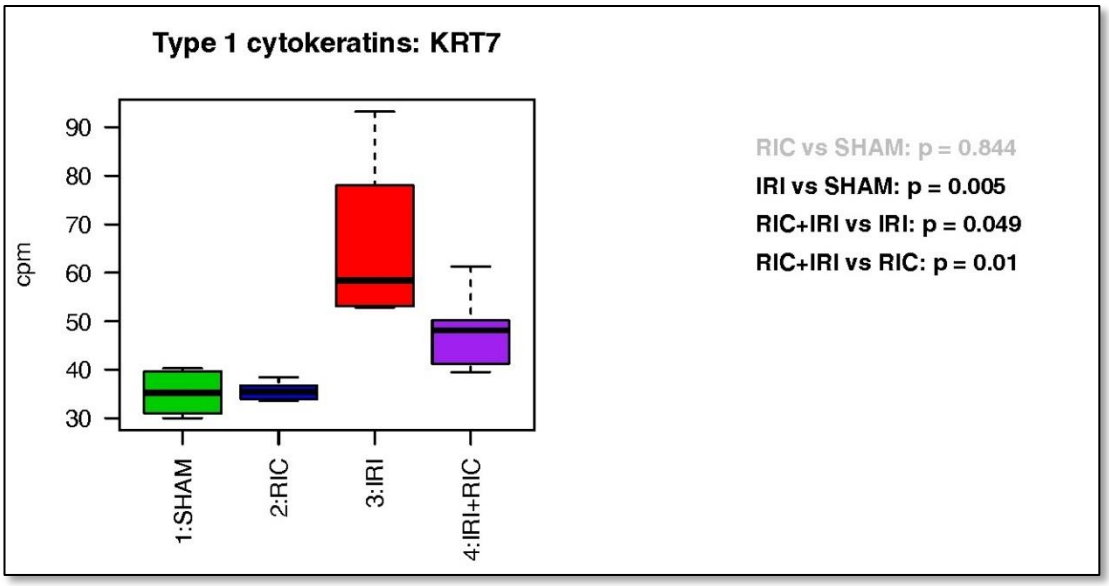


Figure 9-46 Expression pattern across the four groups for KRT-7 (CK-7).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

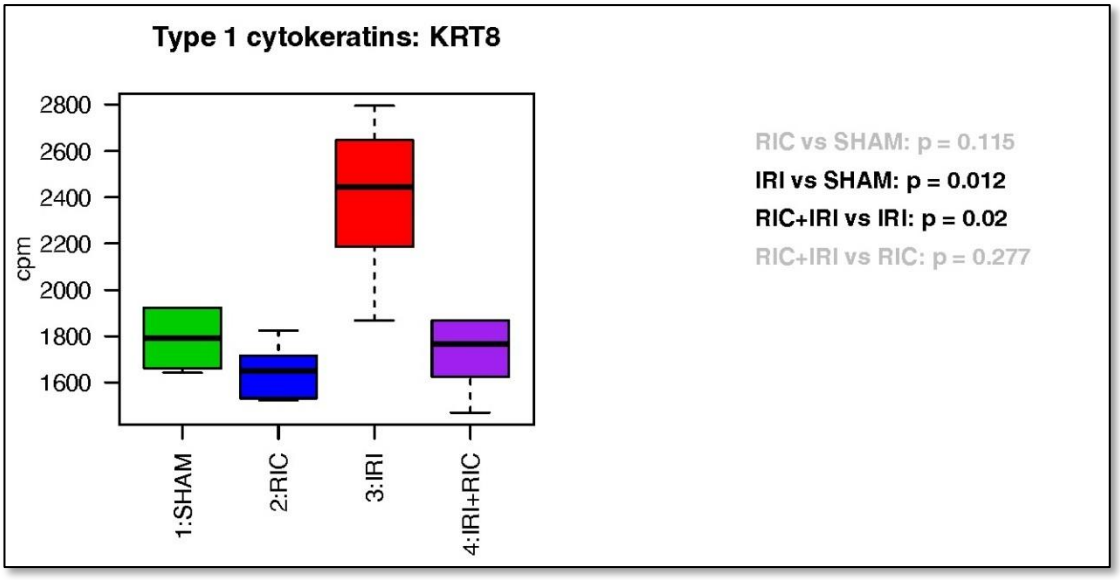


Figure 9-47 Expression pattern across the four groups for KRT-8 (CK-8).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

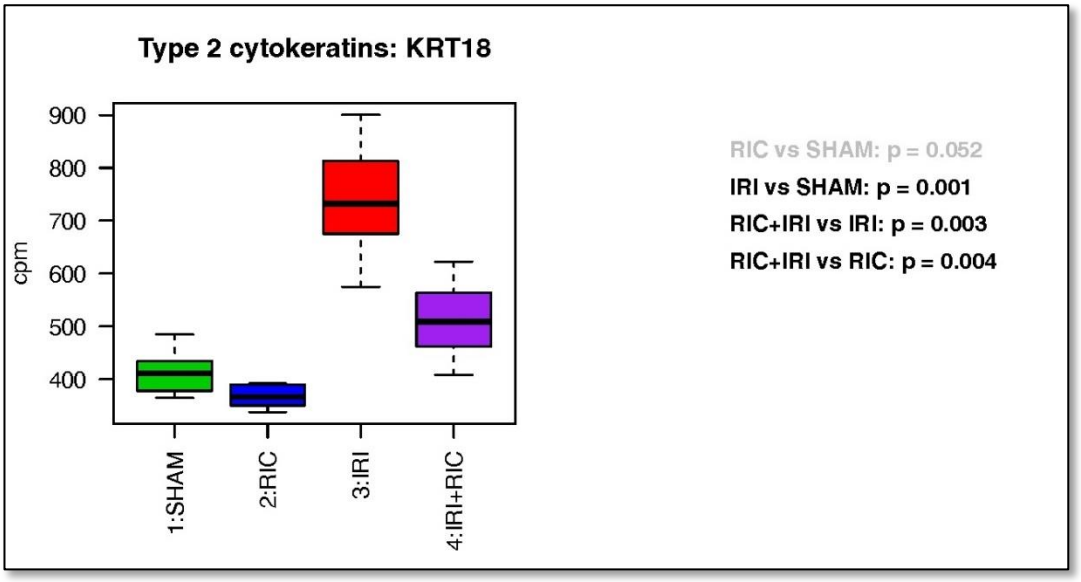


Figure 9-48 Expression pattern across the four groups for KRT-18 (CK-18).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

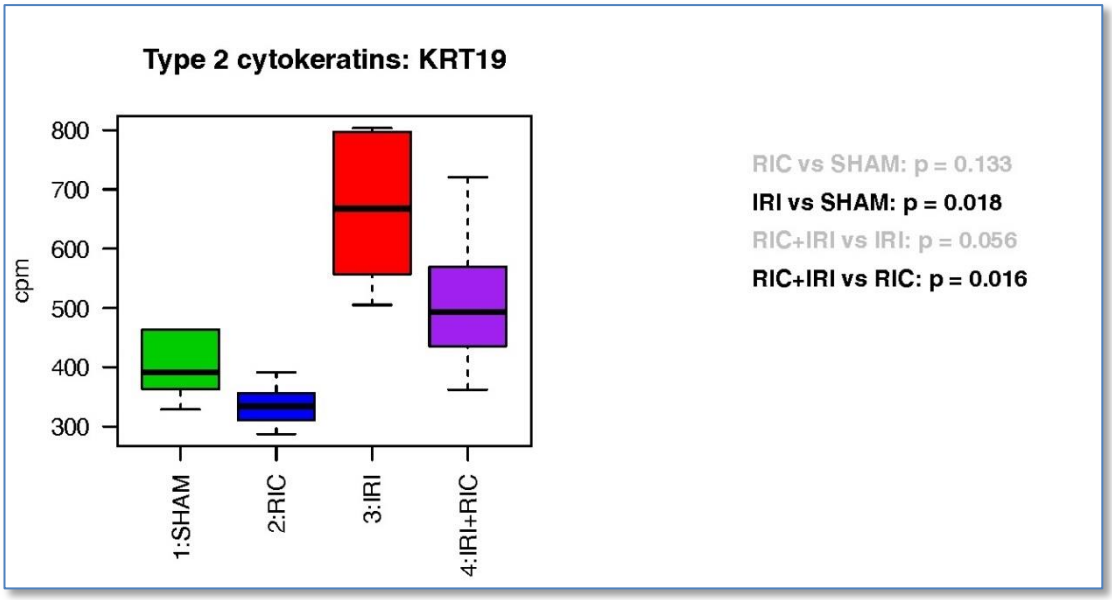


Figure 9-49 Expression pattern across the four groups for KRT-19 (CK-19).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

9.4.3.3 Necrotising Enterocolitis

Comparison of the gene counts for the genes identified as being important in NEC is summarised in Table 9-10 and Figure 9-50 to Figure 9-55. These comparisons shows that TLR-2 and TLR-4, MyD88 and NF-κβ1 are all upregulated by the application of IRI to the bowel. This supports the idea that this model is at-least in-part representing the pathophysiology of human NEC. There was no statistically significant change in the expression of i-FABP between the groups. The increased expression of TLR-2 and NF-κβ1 in IRI was mitigated by the application of RIC. They also showed reduced expression in animals exposed to RIC only compared to controls.

Protein name	Pattern of Expression	Figure
TLR2	Expression *increased* in IRI relative to SHAM (p = 0 .044) Expression *decreased* in RIC+IRI relative to IRI (p = 0.005) Expression *decreased* in RIC relative to SHAM (p = 0.005) **TLR2 *increased* by IRI and *decreased* by RIC**	Figure 9-50
TLR4	Expression *decreased* in IRI relative to SHAM (p = 0.005) *and* RIC+IRI relative to RIC (p = 0.005) Expression *unchanged* in RIC relative to (p = 0.59) *and* SHAM and RIC+IRI relative to IRI (p = 0.89) **TLR4 *decreased* by IRI and *unaffected* by RIC**	Figure 9-51
MyD88	Expression *increased* in IRI relative to SHAM(p = 0.002) and RIC+IRI relative to RIC (p = 0.0006) Expression *unchanged* in RIC relative to SHAM (p = 0.46) and RIC+IRI relative to IRI (p = 0.06) (although does show a trend to lower expression in RIC + IRI relative to IRI) **Myd88 *increased* by IRI and potentially *unaffected* by RIC**	Figure 9-52
NF-kB1	Expression *increased* in IRI relative to SHAM (p = 0.006) Expression *decreased* in RIC+IRI relative to IRI (p = 0.02) and RIC relative to SHAM (p = 0.03) but *increased* in RIC + IRI relative to RIC (p = 0.003) **NF-kB1 *increased* by IRI and *decreased* by RIC**	Figure 9-53
CD17	Expression *unchanged* by either RIC or IRI **Expression of patterns not affected by either IRI or RIC**	Figure 9-54
iFABP	Expression *unchanged* across all groups but does show a trend towards being decreased in IRI relative to SHAM(p = 0.06) **iFABP expression *may be decreased* by IRI**	Figure 9-55

Table 9-10 Differential Expression of selected Proteins shown to be important in the pathophysiology of NEC.

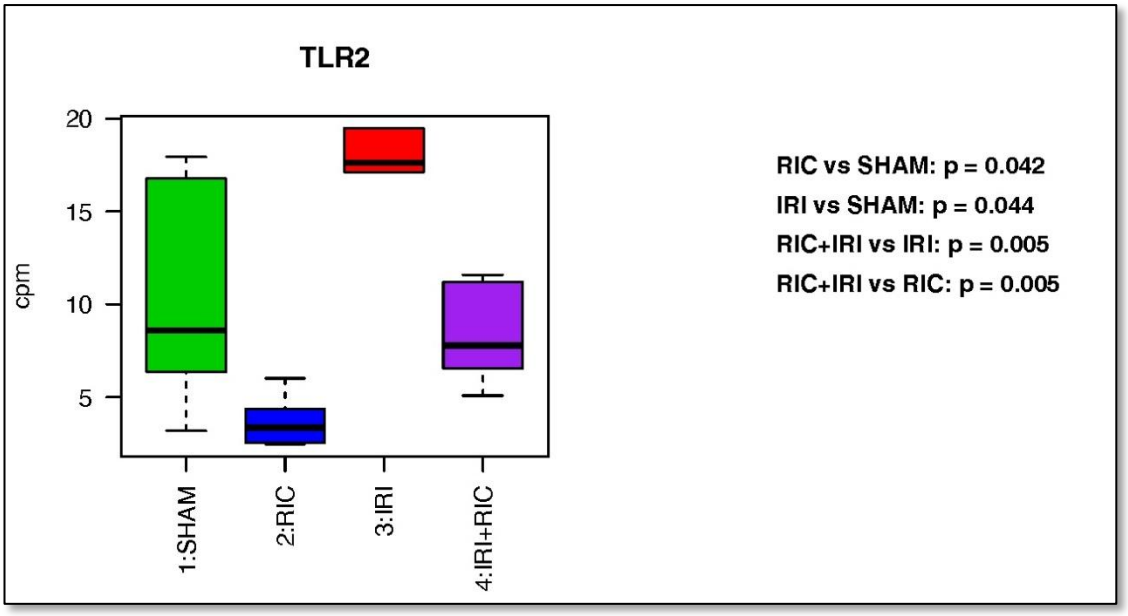


Figure 9-50 Expression pattern across the four groups for TLR2.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

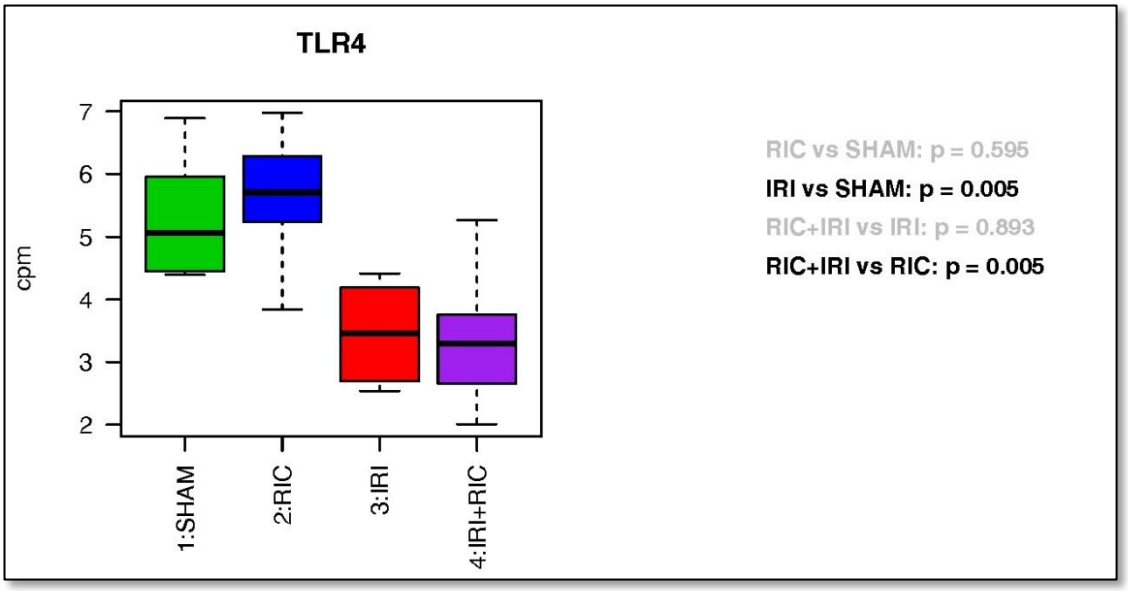


Figure 9-51 Expression pattern across the four groups for TLR4
Graph shows counts per million, median, interquartile range and range for each of the four groups.

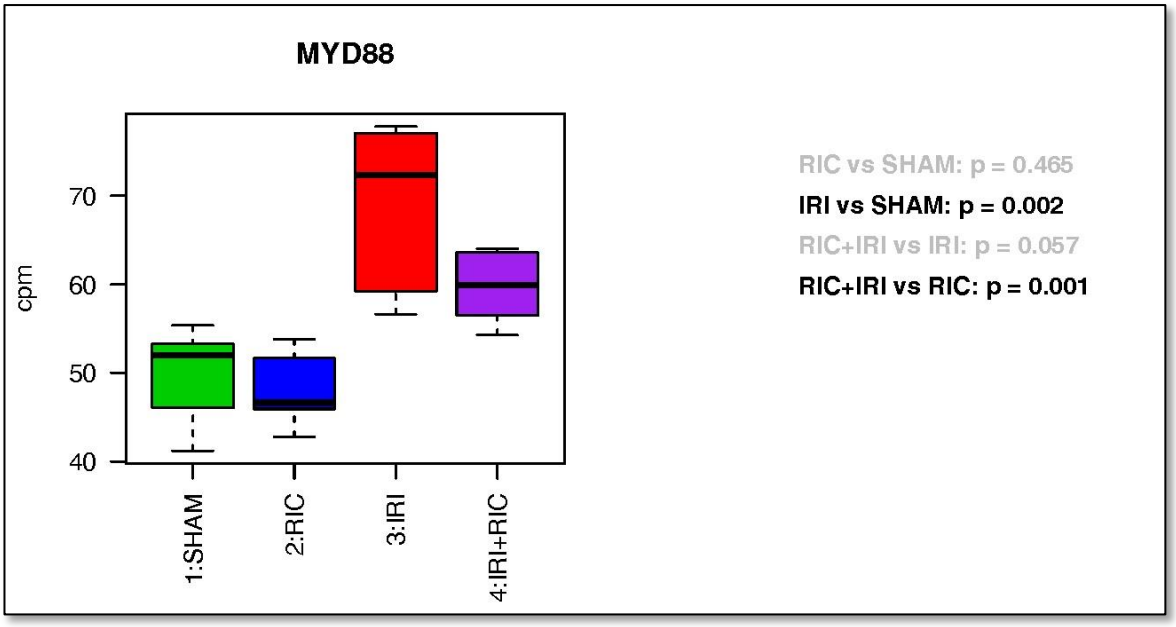


Figure 9-52 Expression pattern across the four groups for MyD88.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

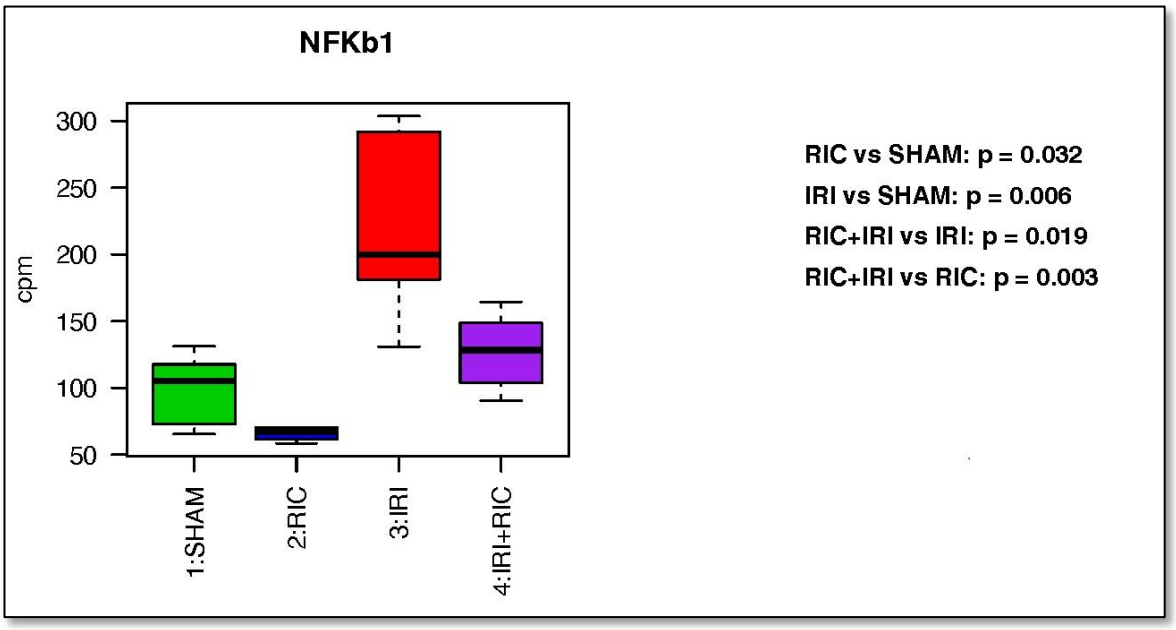


Figure 9-53 Expression pattern across the four groups for NF-κβ1
Graph shows counts per million, median, interquartile range and range for each of the four groups.

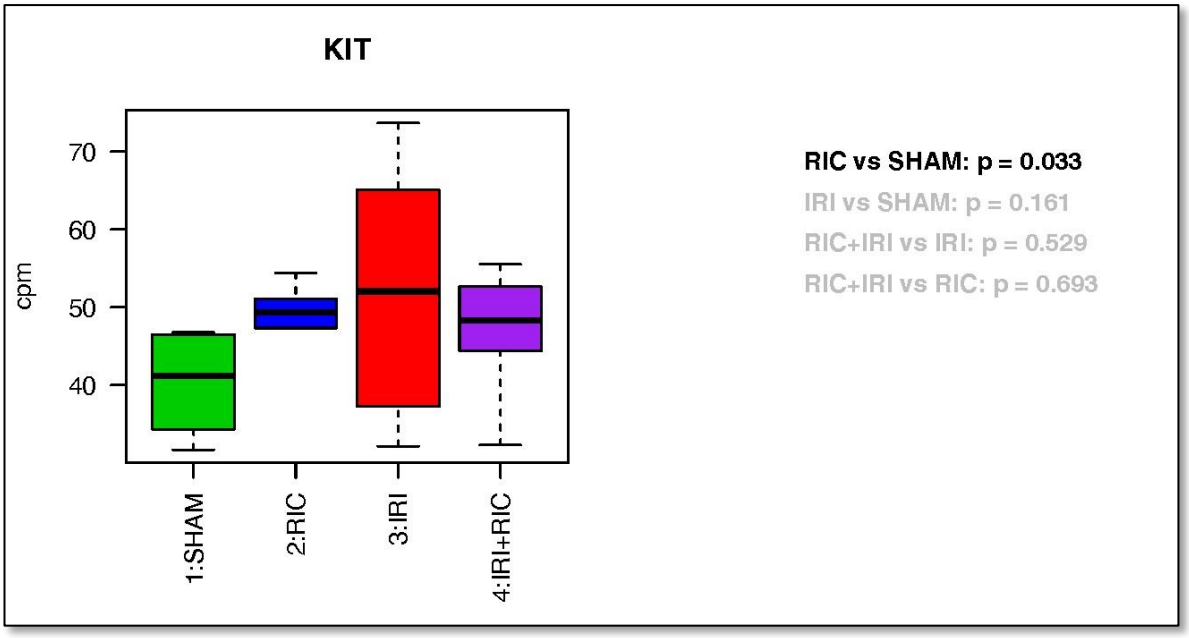


Figure 9-54 Expression pattern across the four groups for CD17 (KIT).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

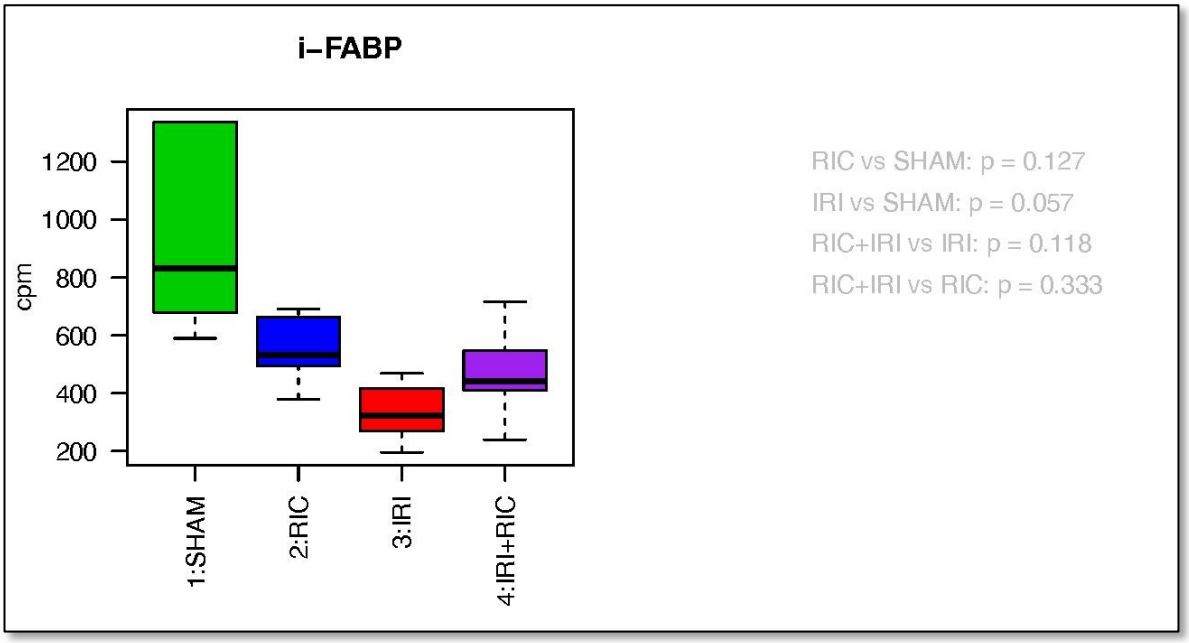


Figure 9-55 Expression pattern across the four groups for i-FABP.
Graph shows counts per million, median, interquartile range and range for each of the four groups.

9.4.3.4 Known Remote Ischaemic Conditioning Pathways

Targeted analysis of genes that have been shown to be part of RIC cellular pathways is shown in Table 9-11 and Figure 9-56 to Figure 9-60.

Protein name	Pattern of Expression	Figure
MEK 1	Expression *decreased* in IRI relative to SHAM (p = 0 .002) *and in IRI+RIC relative to RIC* (p = 0.03) Expression *unchanged* in RIC relative to SHAM (p = 0.10) and RIC+IRI relative to IRI (p = 0.44) **MEK1 *decreased* by IRI and *unchanged* by RIC**	Figure 9-56
MEK 2	Expression *decreased* in IRI relative to SHAM (p = 0.0003) *but unchanged* in *IRI+RIC relative to RIC (p = 0.08)* (although does show a trend to lower expression in RIC + IRI relative to RIC) *Expression* decreased in RIC relative to SHAM (p=0.009) *but unchanged in IRI+RIC relative to IRI* (p = 0.20) **MEK2 *decreased* by IRI and *decreased* by RIC (though to a lesser extent). Expression in animals exposed to both stimuli (IRI and RIC) is not statistically significantly different to either those who had RIC only or IRI only**	Figure 9-57
P38	Expression *decreased* in IRI relative to SHAM (p = 0 .0009) *and* in *IRI+RIC relative to RIC* (p = 0.01) Expression *unchanged* in RIC relative to SHAM (p = 0.57) and RIC+IRI relative to IRI (p = 0.56) **P38 *decreased* by IRI and *unchanged* by RIC**	Figure 9-58
PI3K	Expression *decreased* in IRI relative to SHAM (p = 0.0005) *and* in RIC+IRI relative to RIC (p=0.0001) Expression *increased* in RIC relative to SHAM (p = 0.022) *but unchanged* in RIC+IRI relative to IRI (p = 0.090) (although does show a trend to higher expression in RIC + IRI relative to IRI) **PI3K *increased by RIC* and *decreased* by IRI**	Figure 9-59
PKC	Expression *decreased* in IRI relative to SHAM (p = 0 .0003) *and* in *IRI+RIC relative to RIC* (p = 0.01) Expression *unchanged* in RIC relative to SHAM (p = 0.42) and RIC+IRI relative to IRI (p = 0.12) **PKC *decreased* by IRI and *unchanged* by RIC**	Figure 9-60

Table 9-11 Differential Expression of selected Proteins thought to be important in the protective mechanisms of RIC.

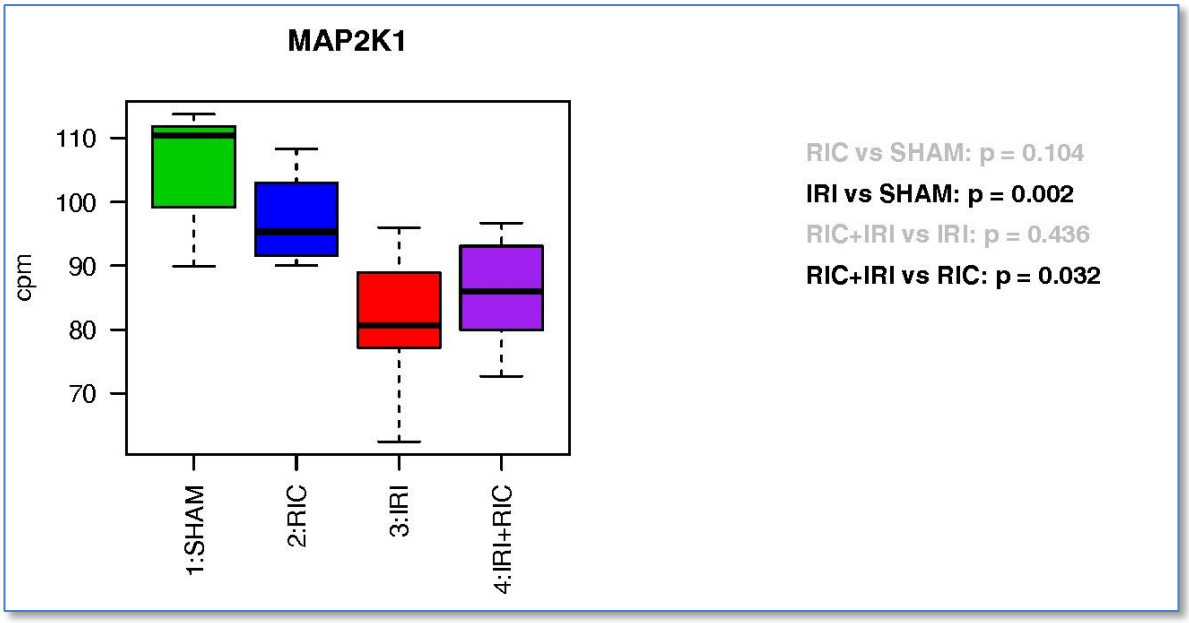


Figure 9-56 Expression pattern across the four groups for MEK1 (MAP2K1).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

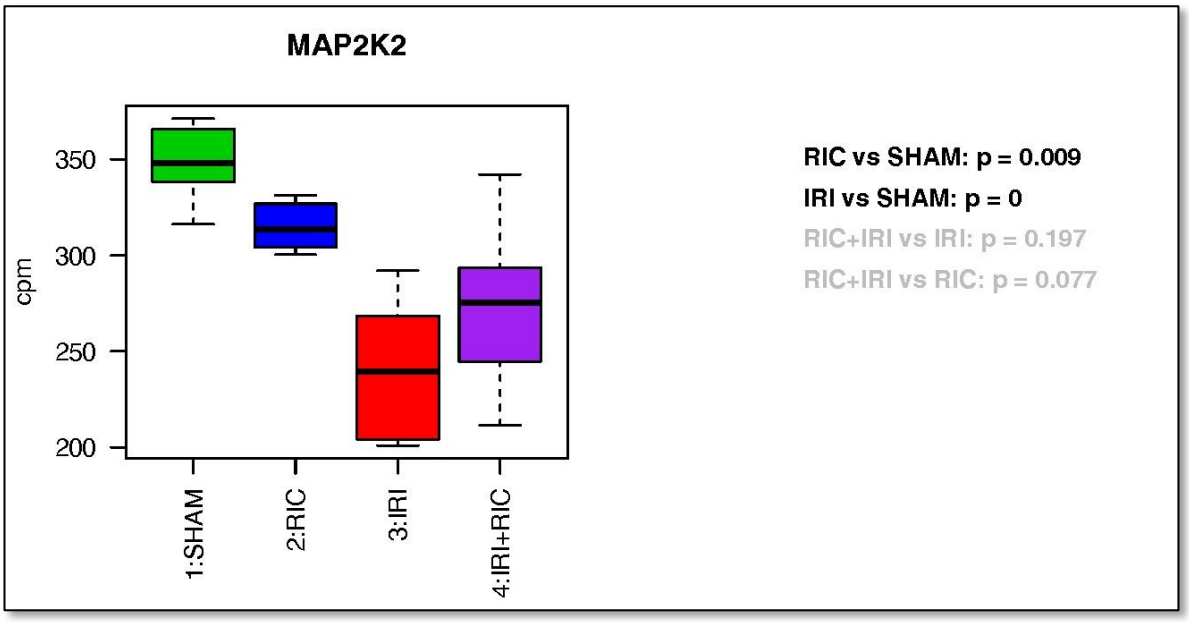


Figure 9-57 Expression pattern across the four groups for MEK2 (MAP2K2).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

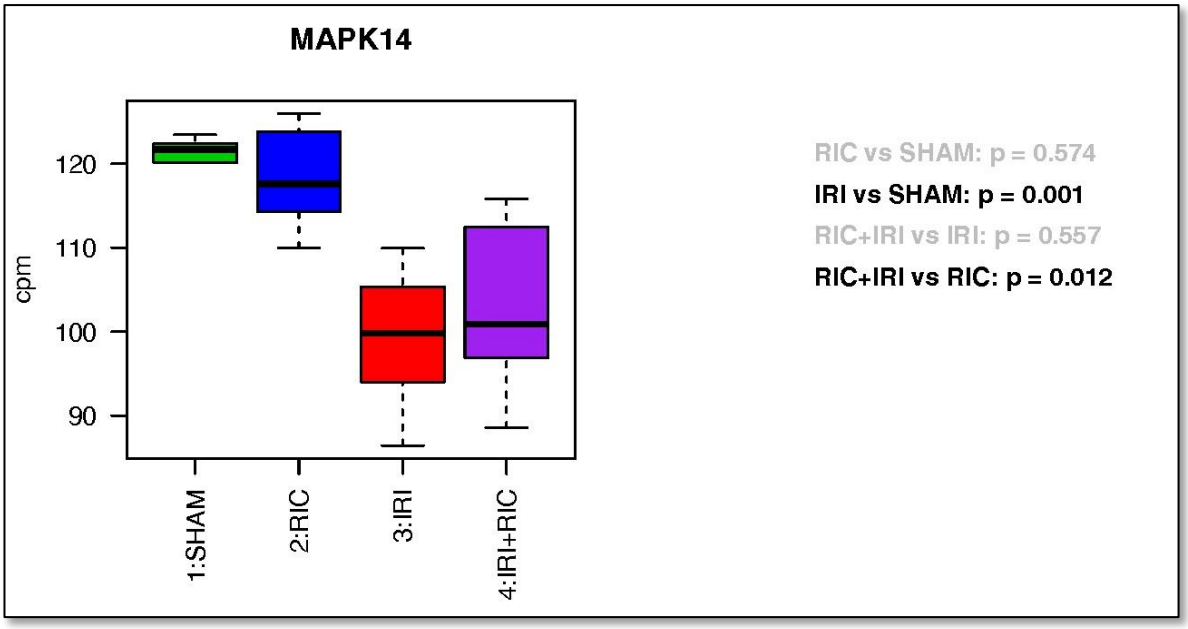


Figure 9-58 Expression pattern across the four groups for P38 (MAPK14).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

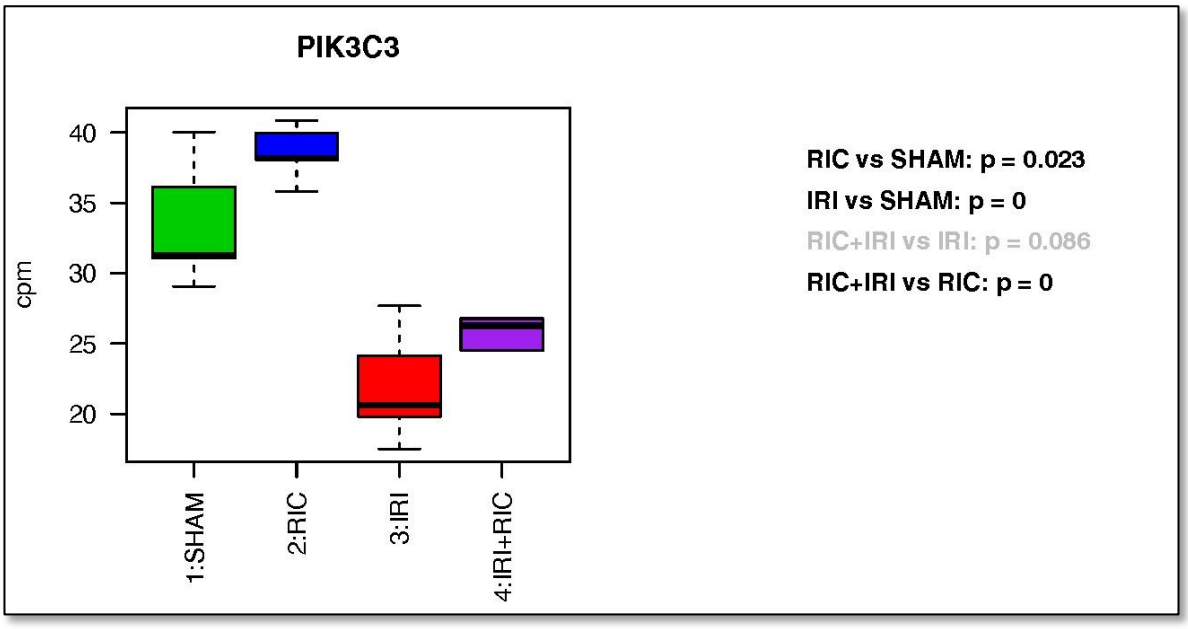


Figure 9-59 Expression pattern across the four groups for PI3K (PIK3c3).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

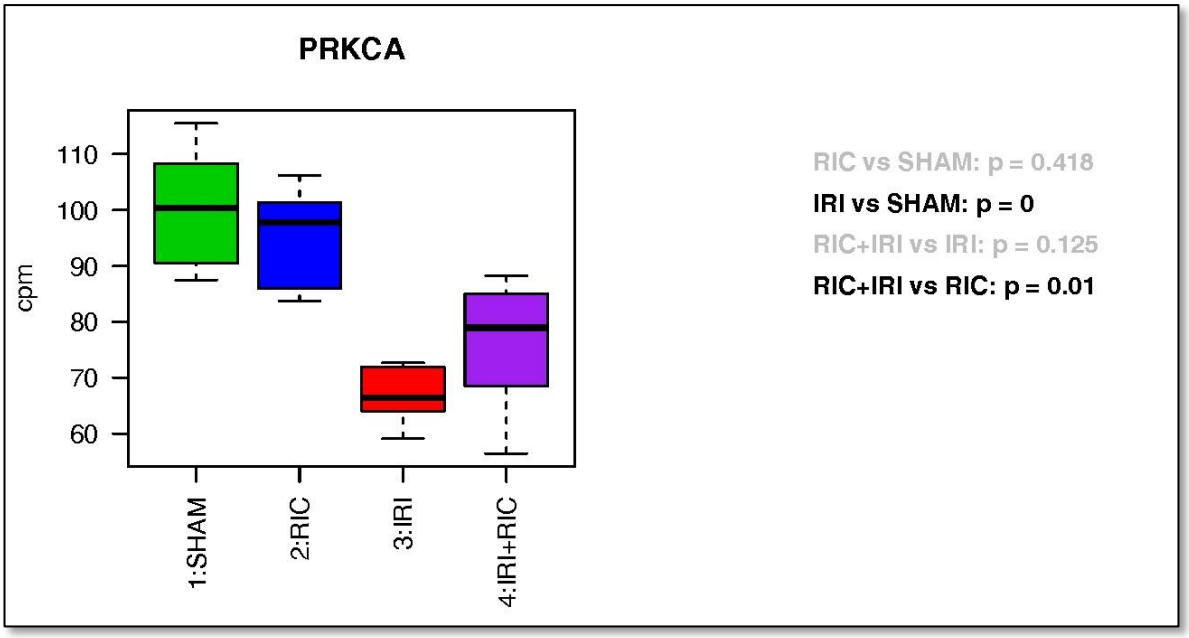


Figure 9-60 Expression pattern across the four groups for PKC (PRKCA).
Graph shows counts per million, median, interquartile range and range for each of the four groups.

9.4.4 Immunohistochemistry validation of transcriptomic results

9.4.4.1 Hypoxia-inducible Factor 1 alpha (HIF-1α).

The results of the immunohistochemistry staining for HIF-1α are reported in detail in Section 5.4.4. The median cell scores were 1 for the SHAM group, 34 for IRI and 21 for the animals that underwent RIC prior to IRI. Figure 9-61 Shows the cell counts for Chromagen staining with an anti-HIF-1α primary antibody and the counts per million from the RNA analysis. The pattern of expression in both the RNA counts per millions (cpm) and the quantitative scoring of the immunohistochemistry with the highest counts in the IRI only group, the lowest in the SHAM and an intermediate level of expression in the RIC + IRI group. (Figure 9-61).

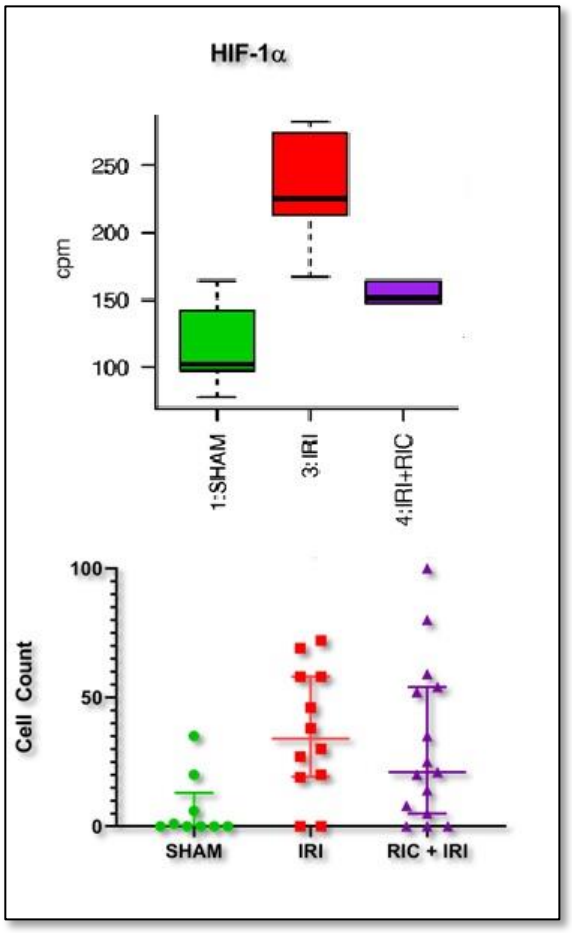


Figure 9-61 Differential expression of HIF-1α in animals exposed to IRI and RIC+IRI and the quantification of HIF-1α staining. Gene expression shown in counts per million. IHC shows quantification of staining. Graphs show median and IQR.

9.4.4.2 Vimentin, Cytokeratin-18 and Desmin

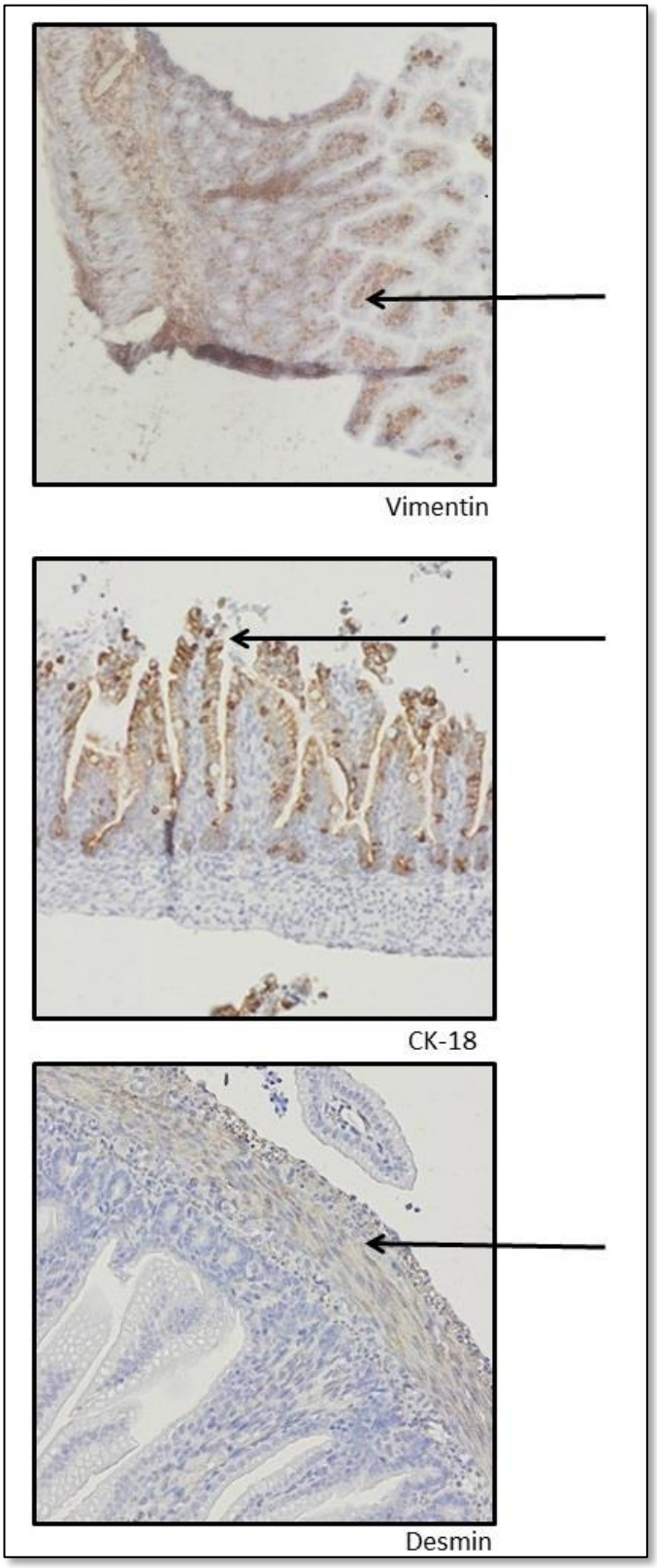


Figure 9-62 Illustrative images of Chromagen stainging for each of the chosen antibodies. Vementin shows stain within the vili but not on the apical cells. Cytokeratin-18 was confined to the apical cells and Desmin staining was seen within the smooth muscle only.

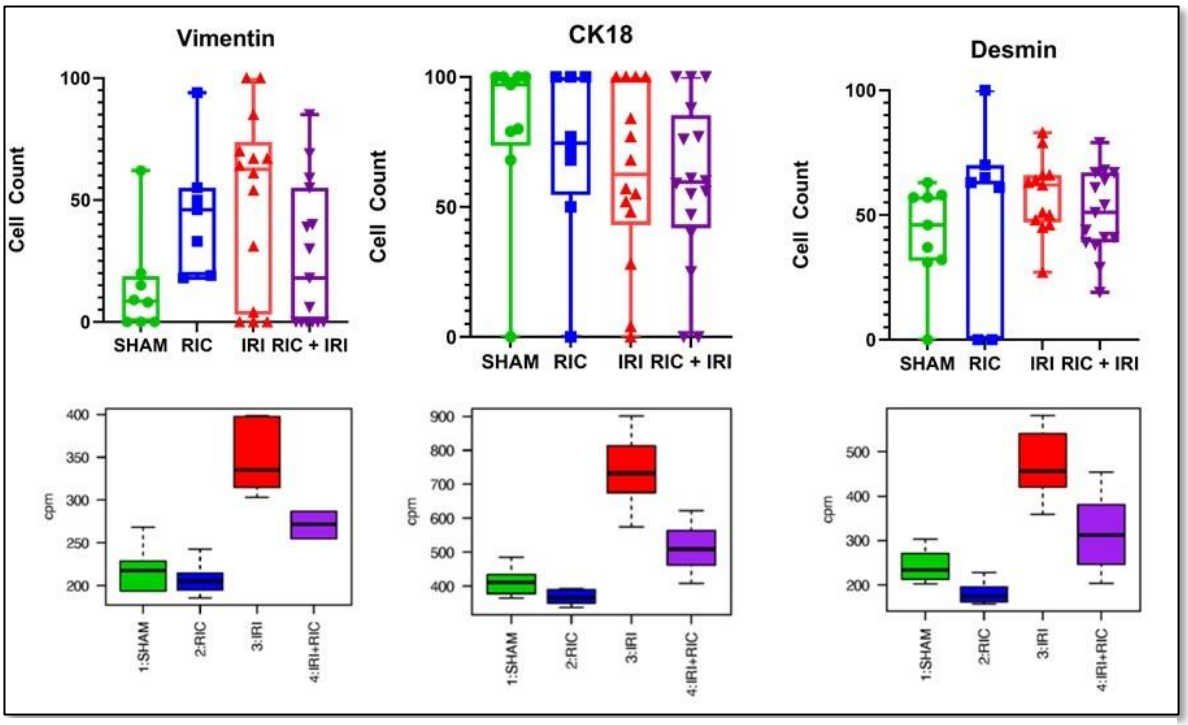


Figure 9-63 Differential expression of Vimentin, cytokeratin-18 and Desmin in animals exposed to RIC, IRI and RIC+IRI and the quantification of IHC staining.
Gene expression shown in counts per million. IHC shows quantification of staining. Graphs show median and IQR.

The staining for Vimentin, CK-18 and Desmin were quantified using the method described and are shown in Figure 9-63. In each case, the expression patterns at the protein level (as demonstrated by IHC) were not directly equivalent to the expression measured at the RNA level.

9.5 Discussion

9.5.1 Transcriptomic analysis

The initial animal experiments for this transcriptomic analysis are described in detail in Chapter 5. Careful handling of the specimens and their storage resulted in very high quality RNA for analysis. Exploration of the data revealed no outliers that needed exclusion. This provides significant confidence in the underlying material on which the various analyses were carried out.

9.5.2 Immunohistochemistry and validation of the transcriptomics

Complete validation of these transcriptomics at the protein level has not been thus far achieved. The immunohistochemistry (Section 9.4.4) showed an expression pattern at the protein level that was not entirely consistent with the RNA expression data.

The HIF-1α protein expression showed good concordance with the RNA-level expression in the three groups. This is perhaps surprising, given how HIF-1α is regulated at a post-translational level.

Conversely, the lack of concordance with the other proteins, whilst not providing the corroboration of the RNA results they might have done, are not strictly contradictory given the complex mechanisms of regulation that exist in the cell. The effects of pre- and post- translational regulation may be an explanation for these differences. Vimentin, for example is highly dynamic and constant exchanges between its soluable and polymerised forms as well as post-translational modification dictates is functional properties.[414] Similarly, post-translational regulation has been demonstrated for both CK-18[415] and Desmin[416].

Further validation of the transcriptomics is therefore desirable. However, there is some other useful correlations within this dataset. Apart from the HIF-1α results described above, the RNA analysis shows a central role for the NF-κβ pathway in promoting the protective effect of RIC. This is well-established in other organ systems.[315,361] RIC has been shown to act via a reduction in both NF-κβ[417] and consequently myeloperoxidase (MPO). This reduction in MPO due to RIC is consistent with the results described in Section 5.4.6. Similarly, as discussed in detail below, the NF-κβ pathway triggers changes in the cytokines TNF-α, IL-6 and IL-8 which are supported by the cytokines changes seen in these animals.

9.5.3 Differential expression testing vs targeted gene analysis

RNA-seq analysis on intestinal tissue in animals exposed to remote conditioning has not been previously reported. In order to investigate the differentially expressed genes, I undertook three separate approaches. The first was an analysis of the genes that show differential expression. There are several potential options for further examination of these data and indeed further analysis is clearly warranted but as an initial approach, the overlap between genes differentially expressed in both the RIC vs SHAM groups and IRI+RIC vs IRI groups was used. Arguably, genes found to be differentially expressed in both these groups are likely to be biologically significant because exposure to RIC triggers a change in the expression of these genes and this change is also seen in animals that have been exposed to injury as well. Thus the stimulus of injury does not abolish these changes suggesting they may play a role in delivering the protective effects seen.

In terms of a more directed or targeted approach, there are several logical options. The first is to look at the genes known to be important in the mechanisms of RIC in other organ systems. Much of the work on RIC has been done in cardiac tissue and the obvious question is whether bowel tissue will make use of the same or differing pathways to produce the protective effect of RIC.

The second targeted approach is to look at the known pathophysiological pathways of NEC. The significance of this analysis is that NEC is not simply an ischaemic disease. However, there is clear cross-over between anti-ischaemic mechanisms and anti-inflammatory mechanisms. The hypothesis here is that if RIC is shown to be effecting pathways known to be significant in NEC then RIC would potentially provide a protective effect against the earlier stages of NEC before ischaemic-reperfusion injury has occurred.

Given that the approach used here involved transcriptome analysis on whole tissue it is also reasonable to analyse the cell-types involved as changes in expression could represent an influx of immune cells for example.

9.5.4 Analysis of the model of NEC

Multiple pathways of NEC pathogenesis have been proposed (Section 1.5). No animal model completely replicates the human disease and this one is no exception. The importance of bacterial colonisation and invasion in the human disease is certainly not fully replicated in this (or any other) model. However, there are immune pathways that are important in NEC before it gets to the stage of necrosis. This model best mimics the common end-point of NEC with a pattern of bowel necrosis and systemic effects very akin to the human disease. Section 1.5.4 describes the state of knowledge about the immunological pathways that lead up to necrosis. The mechanisms by which RIC is protective may also function at this level – by driving an anti-inflammatory response as well as the resistance to ischaemia; if this is true then RIC would potentially provide a protective effect against NEC earlier in the pathophysiology.

Toll-like receptor 4 (TLR4) is thought to be a key part of the mechanism. The Toll-like receptors are part of the innate immune system that trigger an inflammatory response to bacterial infection by recognising common epitomes that are highly conserved in bacterial species. TLR4 activation triggers increased expression of MyD88 and NF-κβ. These data show that TLR4 expression is decreased by IRI. However, the down-stream effects of increased MyD88 and NF-κβ are seen in this model (Figure 9-52 and Figure 9-53). Moreover, both of these genes show reduced expression in response to RIC. In the case of MyD88, there is a mitigation of the effect of IRI therefore the reduction might not be realised if something other than IRI was triggering the rise. However, NF-κβ expression is suppressed independent of IRI.

This supports the concept that the model is a reasonable representation of the human disease and that there is some cross-over in the pathways such that RIC could have a protective effect against NEC both in terms of reducing the necrosis but also in terms of interrupting the pathophysiological pathways earlier.

9.5.5 Putative mechanisms of RIC

9.5.5.1 Targeted analysis

9.5.5.1.1 The Reperfusion injury salvage kinase pathway (RISK) Pathway

In the context of cardiac ischaemia-reperfusion, the so-called RISK pathway is an important part of the protective mechanism at the cellular level (Section 3.5.2.4, Figure 3-5). The simple hypothesis here is that an equivalent pathway would exist in the intestine.

These genes include MEK1, MEK2, and PI3K. In each case the expression of these genes is decreased by IRI. With MEK1, the expression is unchanged by RIC. With MEK2 (Figure 9-57), RIC decreases the expression but not be as much as IRI does. In the case of PI3K (Figure 9-59), RIC increases the expression. The RISK pathway is an anti-apoptotic cascade and works by inhibiting the opening of the mitochondrial permeability transition pore (mPTP).[308] Therefore it is not surprising that in the context of widespread ischaemia all the proteins in the pathways show reduced expression. Equally, because it is a cascade (primarily mediated by phosphorylation), it is also not surprising that there is no clear change in expression seen at the RNA level. A cascade like this works by (in this case) phosphorylation of proteins. Thus a change in the expression level of just one of the proteins involved with have a knock-on effect on all the down-stream proteins.

The increase in expression PI3K due to RIC (Figure 9-59) which is seen in both the animals exposed to IRI and those that were not ,suggesting a potential key role here for this molecule in the protective mechanism of RIC. These data show that RIC increases this expression and that if IRI occurs, expression, whilst much lower than baseline is still higher than that seen in IRI alone. PI3K expression in increased by RIC. Thus PI3K expression is increased by RIC. IRI results in a decrease in expression but this decrease is mitigated by RIC.

Phosphoinositide 3-kinases are a family of signal transducer enzymes that function by phosphorylating the 3 position hydroxyl group of the inositol ring of phosphatidylinositol.[418]

Hausenloy *et al.* (2012) studied the role of P13K in RIC in the porcine heart.[309] Their data showed that the protective effect of RIC on the heart could be abolished by administration of a blocking agent of PI3K (Wortmannin). The role of PI3K was also confirmed by Western-blotting analysis.

The increase in PI3K expression in the intestine demonstrated here would be consistent with an equivalent RISK pathway existing in the intestine.

9.5.5.1.2 P38 and Protein Kinase C

As discussed in Section 3.5.2.1, Heinen *et al.* (2011)[303] used Western Blotting analysis to study the protective pathways of ischaemic conditioning – both direct and remote. There data showed a role for P38 and PKC in direct ischaemic conditioning but in their data in the heart, the expression of P38 and PKC did not change with *remote* conditioning.[303]

These data are consistent with those results as both P38 and PKC in the intestine show altered expression with IRI but not with RIC (Table 9-11, Figure 9-59 and Figure 9-58). It is intriguing that these pathways clearly play a role is the protective effect of conditioning when the conditioning is applied directly to the target organ but seem to have little / no role in the same protective effect when the conditioning is delivered remotely. However, these results are entirely consistent with these findings in an entirely different organ system. Further supporting the hypothesis that the mechanisms in the intestine are similar to that that seen in cardiac tissue.

9.5.5.2 Analysis of gene expression changes in animals exposed to ischaemic conditioning with and without ischaemia-reperfusion injury

Differential expression was seen in 868 genes in the RIC vs SHAM group and 135 in the RIC+IRI vs IRI alone comparison. Therefore there is significant scope for further analysis. This approach here was aimed at beginning to answer the central question remains the same: *what is happening in intestinal tissue that provides this protection against IRI?* The hypothesis here is that important genes will show differential expression in both groups. That may or may not be the case, and is not specifically answered here. However, this, very simple approach identifies 25 gene candidates (Table 9-6).The known roles of these 25 are summarised in Section 9.4.2.5. Of these 25 genes, eight are known to have the sorts of functions likely to be important in the mechanisms of RIC. Table 9-7 shows these eight genes and the results from the differential expression analysis; specifically the fold change in the expression levels and the corrected p - values.

9.5.5.2.1 C-X-C Motif Chemokine ligand-1 (Cxcl-1).

Cxcl-1 is a neutrophil chemoattractant[413] and is downregulated by RIC. A Pubmed search showed no previous reports of a role of Cxcl-1 in the mechanisms of RIC. However, it has been established as an important part of the pathophysiology of brain damage after stroke. The production of Cxcl-1, through the Nfκβ-1 dependent pathway triggers neutrophil infiltration and thus leads to neuroinflammation.[419] Similarly, Cxcl-1 has been shown to recruit neutrophils in cardiac ischaemia.[420]

Reduced expression of Cxcl-1 due to RIC, leading to less neutrophil recruitment in the presence of IRI, leading to less inflammation is a very plausible mechanism.

9.5.5.2.2 Mitogen-Activated Protein Kinase Kinase Kinase 8 (MAP3k8)

As with many of the genes discussed here, Map3k8 has an important role in oncogenesis. Map3k8 also has some interesting downstream effects. It induces the production of Nfκβ as well as the production of TNF-α and IL-2.[413] Nfκβ is discussed below but this shows consistent changes in expression at three different stages in the pathway; MAP3k8 induces Nfκβ and one of the downstream effects of Nfκβ is to increases the expression of Cxcl-1. In theses data the expression of all three is suppressed.

9.5.5.2.3 Superoxide Demutase 2 (SOD2)

SOD2 is an important part of the cells response to oxidative stress and dismutates superoxide to hydrogen peroxide.[421] Hence, the reduced expression of SOD2 by RIC is at first glance surprising. However, increased production of hydrogen peroxide through the up-regulation of SOD2 stimulates pro-oxidants involved in apoptosis. Thus reduced expression of SOD2 can be said to be anti-apoptotic.[422]

9.5.5.2.4 Nuclear factor kappa-light-chain-enhancer of activated B cells pathway subunit 2 (Nf-κβ2)

NF-κβ is a family of transcription factors composed of five structurally related members including NF-κβ1 (Section 9.4.3.2.3) and NF-κβ2. [423] Functional NF-κβ2 is produced by the post-translational processing of its precursor protein p100. This may be especially important in the so-called 'non-canonical' NF-κβ pathway.[424] The precursor protein (p100) has an inhibitory function on NF-κβ[423] and thus changes in the RNA expression levels could have promoter or inhibitory effects on the whole pathway, depending on whether the p100 protein is rapidly converted to mature NF-κβ2 or not. However, the Map3k8 and Cxcl-1 expression levels along with NF-κβ2 are all reduced in RIC suggesting that the overall effect is to inhibit NF-κβ pathway.

9.5.5.2.5 Protein C Receptor (Procr)

The protein encoded by this gene is a receptor for activated Protein C known as endothelial cell protein C receptor (EPCR).[413] Activated Protein C is an important inhibitor of the clotting cascade but it is also exerts important anti-inflammatory effects.[425] The Protein C Receptor protein is found on the surface of endothelial and other cells types. Down-regulation of this receptor (seen in both RIC and RIC and IRI groups) implies a dampening down of Activated-Protein C suppression of coagulation. Coagulation is a cardinal feature of ischaemic necrosis.[7] Activated-protein C, via

the Protein C receptor, triggers anti-inflammatory downstream effects via several mechanisms including suppressing NF-κβ. [425,426] Therefore, the down-regulation of this receptor in these data is potentially surprising.

However, thrombin activation or EPCR produces a pro-inflammatory response and a disruption of the endothelial barrier.[426,427] Hence, EPCR has a pro-inflammtory mechanisms quite apart from the binding ligand that it is named for and reduced expression of this molecule would be expected to result in reduced inflammation. Although the cross-talk for this multi-ligand receptor is undoubtedly complex.[424]

9.5.5.2.6 Ubiquitin Specific Protease 36 (Usp36)

Usp36 could be a putative part of the RIC protective pathway because of its suggested role in autophagy.[413] Autophagy, a mechanism by which cells removes unnecessary or damaged components is thought to be important in maintaining cellular function in response to stress.[428] Loss of Usp36 function autonomously activates autophagy.[429] This implies that reduced expression of Usp36 would cause the cell to increase its autophagic activity which could be important in responding to the severe stress of IRI.

Conversely, work in the mouse and human kidney suggests that Usp36 interacts with SOD2 in the mechanism of acute kidney injury due to ischaemia. These data suggests that increased Usp36 expression would be protective.[430]

9.5.5.2.7 Tissue Inhibitor of Metalloproteinase 1 (TIMP1)

TIMP1 appears to have multiple functions. One such function is that it may be anti-apoptotic.[413] In these data, its expression is increased in RIC (compared to SHAM) but decreased in animals that undergo RIC and IRI. In the brains of rats, TIMP1 has been shown to be increased in response to infarction.[431] Thus the decreased expression in animals exposed to RIC and IRI compared to IRI alone may be a reflection of the reduced injury. However, the increase seen in RIC alone is less easily explained. As the name implies, TIMP proteins are named for their inhibition of metalloprotinases (MMPs) which in turn play a key role in the normal physiology and wound healing of connective tissue.[432] This inhibitory relationship is anti-inflammatory.

9.5.5.2.8 CD55

CD55 is a regulator molecule of the complement cascade. [413] Several studies looking at renal IRI have shown that over-expression of CD55 is protective against inflammation.[433] Although a specific role with respect to RIC has not been established. As with TIMP1, these results show increased expression of CD55 in the presence of RIC but in animals that underwent IRI and RIC,

the expression is reduced compared to IRI alone. Thus if CD55 is part of the protective effect of RIC (and it clearly has an anti-inflammatory role in other contexts), it modulation of inflammation (presumably via the complement cascade) is not straight-forward.

9.5.6 Nuclear factor kappa-light-chain-enhancer of activated B cells pathway

Since its discovery in 1986,[434] nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) has been extensively studied as a master regulator of pro-inflammatory genes.[423] Figure 9-64 summarises the range of downstream effects of NF-κβ. As discussed in Section 5.5.3 and Section 7.5.2, changes in the levels of TNF-α, IL-6 and IL-8 equivalent (KC/GRO) are seen in these animals. TNF-α correlates with intestinal injury, whilst IL-8 equivalent is suppressed by RIC. IL-6 seems to rise in response to RIC but as with each of these cytokines, the measurement is only on a systemic level and thus it is not clear from where they are derived.

CXCL-1 showed reduced expression in both groups, indicating a concomitant down-stream effect of NF-κβ. Further corroboration is needed to confirm this but these data implicate a key role for NF-κβ in the protective effect of RIC in the intestine.

As discussed in Section 1.5.4, Toll-like Receptor-4 (TLR-4) triggers the NF-κβ.[109] Importantly, this is an early step in the pathogenesis of NEC, prior to the development of IRI. Indeed proponents of a immunological understanding of NEC, often cite the role of TLR-4 as being central to the development of the disease. [99,101] Ultimately, IRI is the common end-point of multiple pathways in NEC (although, it also leads to a vicious cycle of further inflammation and bowel compromise) thus one might expect RIC to only be effective at this late stage in the pathogenesis. Clearly, if RIC is effective in the intestine, it would be effective at reducing the ischaemic injury but if RIC is acting on NF-κβ as these data would suggest, then it is also likely to offer a protective effect at every stage in the pathogenesis of NEC. Whilst this hypothesis is not formally proven, it does present the prospect that RIC will be protective at multiple stages in the pathogenesis of NEC and thus (assuming that RIC can be delivered safely to human infants) could be a very effective therapy.

There is undoubtedly a lot more work that can be done with these data, including detailed pathway analysis but the initial overview here shows a least one promising mechanism by which RIC is exerting its protective effect in the intestine.

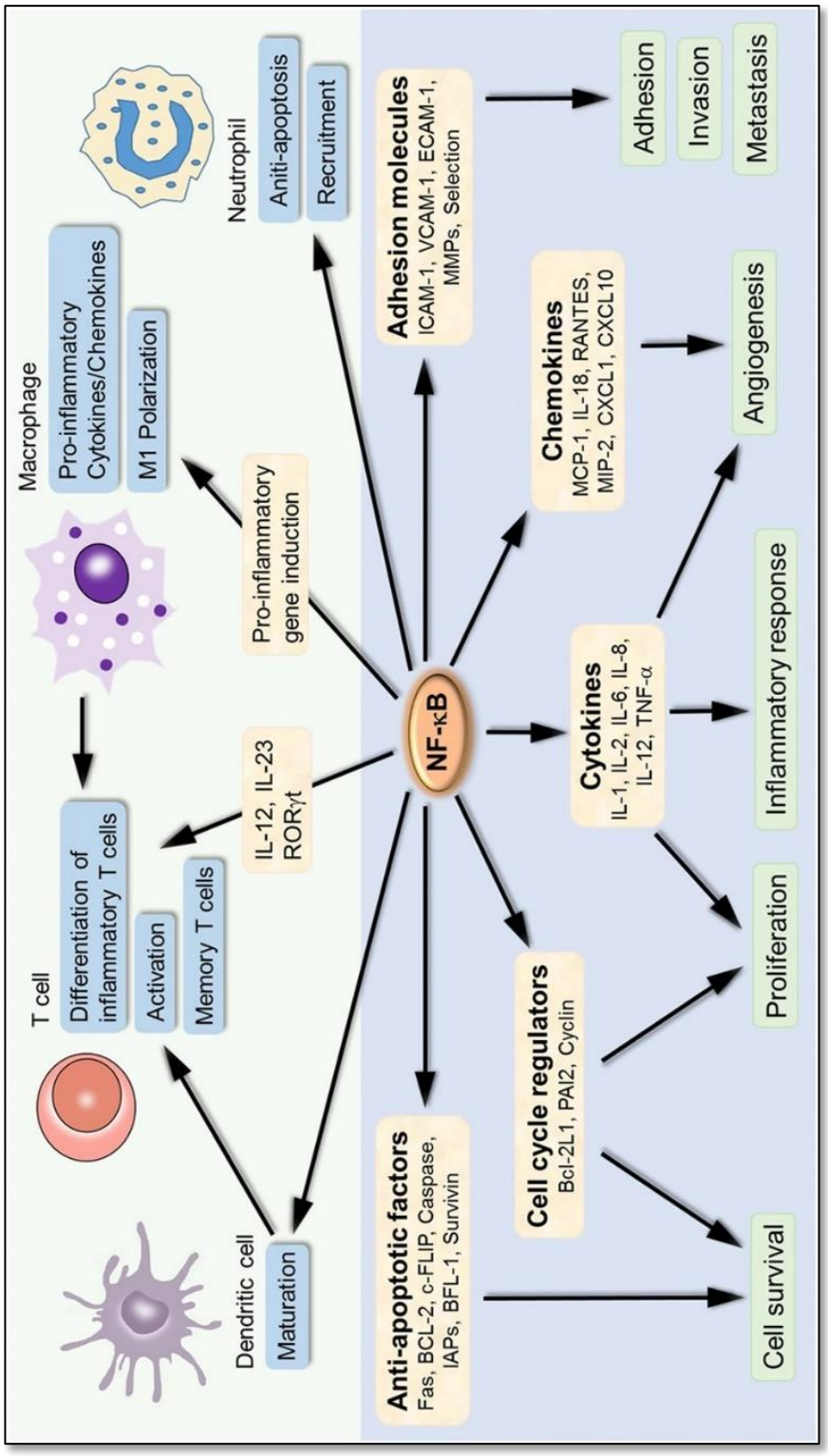


Figure 9-64 NF-κβ target genes involved in the development and progression of inflammation. NF-κβ directly increases the production of pro-inflammatory cytokines, chemokines, and adhesion molecules as well as having a role in cell proliferation, differentiation and apoptosis. *From* Liu *et al.* (2017)[423]

9.6 Conclusion

A large number of genes show changes in their expression in response to RIC. Targeted fanalysis suggests that the changes mostly reflect a change in the resident cells rather than an influx of immune cells, although marker analysis does suggest an increase in regulatory T-cells.

Multiple genes involved in inflammation are down-regulated in the intestine by application of ischaemic conditioning to the hind limb of rat pups.

Increased expression of PI3K may indicate that something similar to the RISK pathway identified in cardiac tissue is present in the intestine. The lack of a role for PKC and P38 in remote conditioning is also consistent with what is seen in cardiac ischaemic conditioning.

The expression of NF-κβ is down-regulated with RIC suggesting that the suppression of NF-κβ pathway is central to the protective mechanism in the intestine. This is especially relevant to NEC as NF-κβ is known to be important in NEC pathogenesis.

Chapter 10 Maternal Remote Ischaemic Conditioning

10.1 Abstract

10.1.1 Background

Protocol 1a (Chapter 5) demonstrated that RIC delivered by means of a ligature reduced the extent and severity of bowel injury in this IRI model of NEC. Very little work has been done on the potential of vertical transmission of the protective effect of RIC. However, there has been some work that suggests RIC may provide a protective effect to the offspring of conditioned mothers and it is biologically plausible given what is known about placental transmission and the putative mechanisms of RIC transmission.

The aim of this protocol was to investigate the effectiveness of RIC given to the pregnant mothers prior to delivery against bowel injury to the offspring.

10.1.2 Methods

Experimental animals underwent IRI by means of occlusion of the superior mesenteric artery (SMA) for 40 minutes, followed by 90 minutes of reperfusion. The comparator groups of Controls (Sham surgery) and the IRI-only (injury without RIC) were taken from protocol 1a. The pregnant dam was given three cycles of RIC on day 20 of gestation and then allowed to deliver naturally.

Bowel injury was assessed macroscopically (by measuring the amount of bowel affected graded as normal, mild or severe) and microscopically using the Chiu-Park scoring system.

10.1.3 Results

Offspring of dams that had undergone RIC showed injury to a median length of 100% of the small bowel (range 76-100%) and thus showed no protective benefit compared with the IRI alone group, (median 100%, range 0-100%, p=0.81). Similarly, the proportion of small bowel showing severe necrosis was not reduced by maternal RIC (p=0.63).

The median Chiu-Park score was 5.5 (range 0.5-7) in the area of maximal injury in the offspring of conditioned dams, compared to 5.5(4-7) in the animals who had IRI alone (p=0.87).

10.1.4 Discussion

In this model, maternal RIC shows no protective effect on the small bowel exposed to IRI.

10.2 Introduction

Chapters 5-8 outline the results of remote ischaemic pre-conditioning (pre-RIC), remote ischaemic post-conditioning (post-RIC) and the efficacy of giving RIC 48 hours prior to injury (early-pre-RIC), using this ischaemia-reperfusion model. It is unknown whether the protective effect of RIC could be passed by vertical transmission (from mother to offspring).

As described in 3.5, current evidence supports three, inter-related pathways by which the protective effect of RIC is transmitted to the target organ. Conceptually, it is easily conceivable that the same mechanisms could also act across the placenta as the transport of proteins,[435] pharmocoactive and toxic compounds[436] from maternal to foetal circulation is well established. This would theoretically allow remote conditioning of mothers prior to delivery to confer a benefit on newborns. This paradigm of maternal treatment for a benefit to the newborn infant is very-much standard practice in neonatology. Maternal corticosteroid administration promotes foetal lung development and thus when pre-term labour is suspected, this practice is routine to improve the post-natal respiratory function of the infant.[437]

The putative pathways of RIC are neuronal (Section 3.5.1.1), humeral (3.5.1.2) and systemic (3.5.1.3). The neuronal pathway includes the action of catecholamines.[259] Catecholamines undergo rapid placental transfer.[438] The work of Dickson *et al.* (1999) is described in detail in Section 3.5.1.3 but in summary it implies a molecule (or molecules) less than 15kDa in size and hydrophobic is present in plasma and conveys the protective signal from the conditioning organ to the target organ.[284] Placental transfer of multiple small molecules occurs by multiple mechanisms.[435] Various cytokines have been implicated as part of the transmission of RIC, including tumour necrosis factor alpha (TNF-α)[290] and interleukin-6 (IL-6).[292] An in-vitro study with term placentas showed bidirectional transfer of IL-6 but minimal transfer of TNF-α.[439] It is biologically plausible, therefore that all three of these transfer mechanisms for RIC that have been shown to occur could also be active across the placenta and hence allow vertical transmission of remote ischaemic conditioning. Despite this, there has been very little published research of this potential phenomenon. However, Lopes de Freitas *et al.* (2014)[440] studied the effect of maternal conditioning on the colon of rat pups submitted to hypoxic insult.

Lopes de Freitas *et al.* (2014)[440] performed global hypoxia and re-oxygenation on new-born Wistar rats on days 1, 2 and 3 of life. This was done by placement in an acrylic chamber with a supply of 100% CO_2 for ten minutes followed by 100% O_2 for ten minutes. This model produced severe histological damage to the colons of the rat pups. This damage was attenuated by exposure of the mother to a single cycle of limb ischaemic with a tourniquet for 10 minutes one day prior to delivery of the pups.

10.3 Methods

10.3.1 Experimental protocol

Figure 10-1 summarises the experimental protocol. The methodology is based on previous work (as detailed in Chapter 4) with some minor modifications.

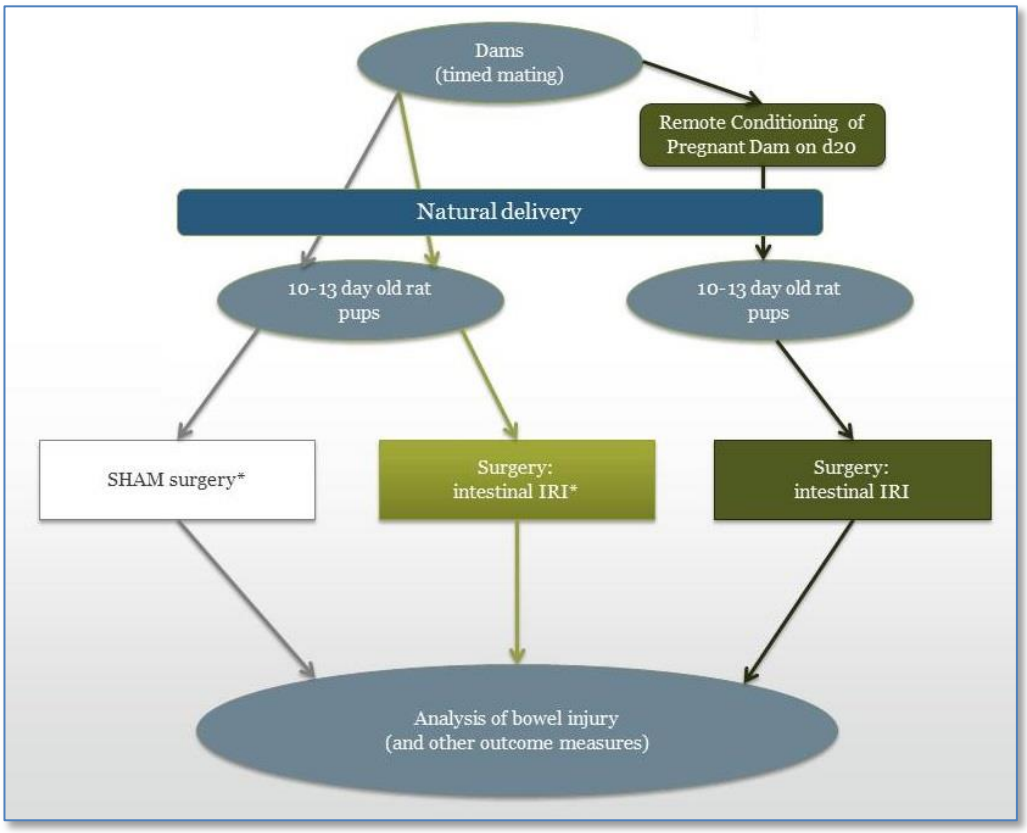


Figure 10-1 Experimental protocol for maternal pre-conditioning.
Pregnant dams underwent remote conditioning on day 20 of gestation and the pups under intestinal IRI.

The IRI was performed on the rat pups in the same way as in previous chapters, with pups aged 10-13 days undergoing IRI to induce the bowel injury. The only difference here was that the dams underwent RIC prior to delivery rather than the pups after birth.

The controls for this experimental work derive from chapter 5. This was done to minimise the number of animals used under the '3 'R's' principals. The comparator groups for this work are both the animals who underwent sham surgery ('controls') and those that underwent ischaemia-reperfusion injury only ('IRI').

10.3.1.1 Remote Ischaemic Condition of Pregnant Dams; Ethical and Legal Considerations

All animal experiments were carried out according to the Animals in Scientific Procedures Act (APSA) 1986 and revisions. Project licence: PA813F125. The original licence did not include any experimental work on the adult rats. We therefore applied for a modification to the licence in order to allow conditioning of the dams. Ethical approval was obtained from the university ethics committee prior to application for the project licence modification as per standard ASPA procedures. At each stage every effort was made to follow the principals of Replacement, Reduction and Refinement ("the 3 R's") in the use of the animals for scientific experimentation.[356]

As with the original application, I prepared (under supervision) the licence modification application.

10.3.1.2 Remote Ischaemic Condition of Pregnant Dams; Procedures

Sexually mature female rats were timed-mated using a standard protocol; they were paired with a breeder male just prior to the end of the day light cycle and then checked daily for an ejaculatory plug.[441] Normal gestation of the rat is 21 days but individual variation is common.[441] On day 20 of gestation the pregnant dams underwent 3 cycles of remote ischaemic conditioning using restraint.

As per the licensing, the females had been introduced to the restraining device to ensure familiarity and reduce stress prior to the timed mating.

The restraining device is shown in Figure 10-2. This is a modified standard rodent restraining tube. It is adjustable to allow comfortable fit of the animal and good aeration. The modification is an opening on the right side that allows access to the right hind-limb so that RIC could be performed. The restraining tube and procedure were discussed with, and observed by, the NACWO to ensure animal welfare throughout the process.

RIC was performed in an analogous way to the technique used in the pups. On day 20 of gestation, the pregnant dam was placed in the restraining tube and underwent three cycles of 5 minutes of limb ischaemic, followed by 5 minutes of limb reperfusion. Limb ischaemia was achieved by means of a ligature (Medline Medi-Loop mini vessel loop) applied to the right hind limb at the hip joint and tightened to achieve arterial obstruction. Occlusion of the arterial inflow was confirmed by the change in colour seen in the limb/paw and loss of signal on Nellcor™ OxiMax™ N-65 pulse oximeter (Tyco Healthcare Group LP (California)).

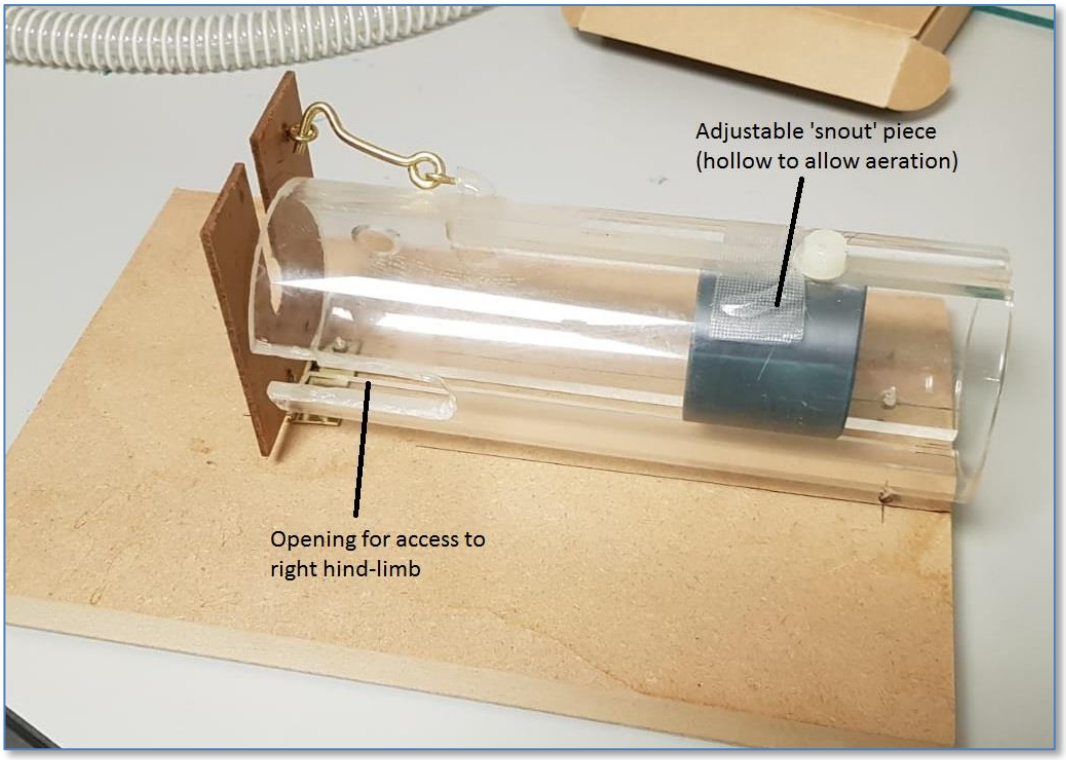


Figure 10-2 (Modified) rodent restraining tube for maternal RIC administration

Macroscopic injury in the pups was assessed as described in Section 4.7. and microscopic injury was scored with the Chiu-Park scoring system, described in Section 4.8. For each animal, a specimen was taken from the terminal ileum and from the area of maximal macroscopic injury. The median score from three independent scores, blinded to which experimental group the specimen is derived will be used for analysis.

10.3.2 Power Calculation

As described in Section 7.3.1, a power calculation showed that a sample size of 18 in each group has an 80% power to detect a difference between means of 2 with a significance level (alpha) of 0.05 (two-tailed). Given that this large group size; interim analysis of the outcomes was performed with 14 animals. At the point, the macroscopic and microscopic data showed no significant difference. In order to add to this number, a third litter (7-14 pups per litter) would be needed. This is an excessive number, unlikely to provide meaningful data and thus on the basis of minimising the number of animal used, no further experiments were performed.

10.4 Results

Two litters of rat pups were used. In the first litter, RIC was delivered to the pregnant Dam on day 20 of gestation (as determined by the timed mating – presence of plug indicating day 0). The pups

were then born on day 22. All pups (six) from this litter were included. The second litter were born on day 22 following the application of RIC to the dam on day 20. Eight pups from this litter were included.

10.4.1 Macroscopic Bowel Injury

Mild injury was seen in a median length of bowel of 26% and the median length of bowel showing severe injury was 68%. The median total injury was 100% (range 76-100%, Table 10-1). The range of total bowel harvested was 32-51cm. Compared to IRI alone the total macroscopic injury was not reduced by maternal RIC (p = 0.81). Similarly the amount of small bowel showing severe necrosis was not reduced by maternal RIC with a median of 68% vs 78% (p = 0.63).

Median length of bowel injury			
	Mild	Severe	Total
Controls*	0%	0%	0%
IRI only*	18%	78%	100%
(mat)RIC + IRI	26%	68%	100%

Table 10-1 Protocol 4: Macroscopic injury.
*Controls (Sham surgery) and IRI only taken from protocol 1a.
Full results are shown in Table B-12

Figure 10-3 and Figure 10-4 show these data plotted against the Controls and IRI only groups from protocol 1a.

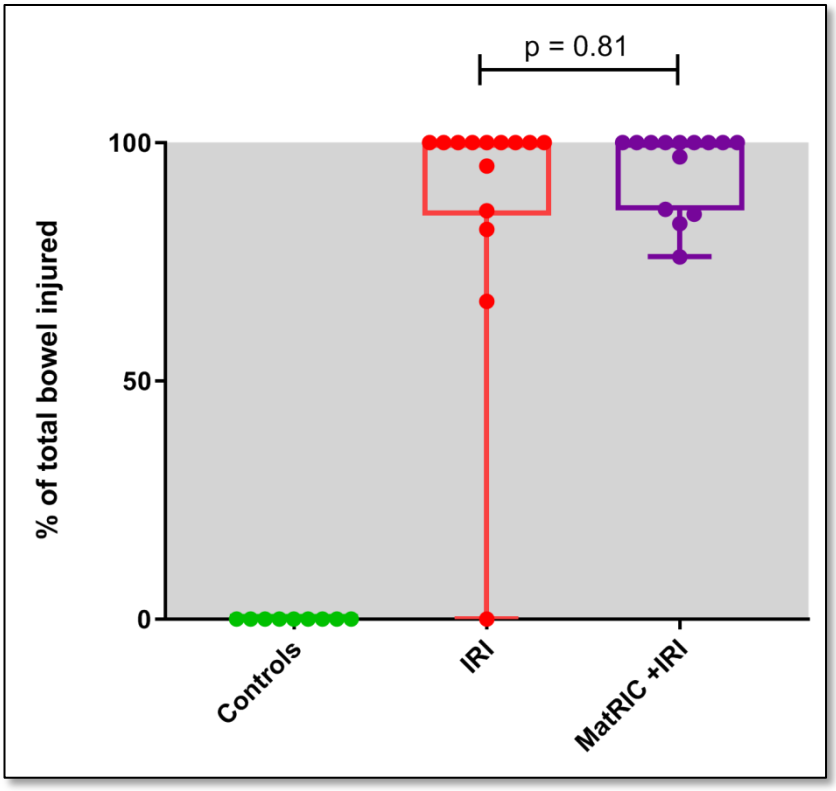


Figure 10-3 Protocol 4: percentage of total bowel showing macroscopic injury.
Graph shows each data point, inter-quartile range and median

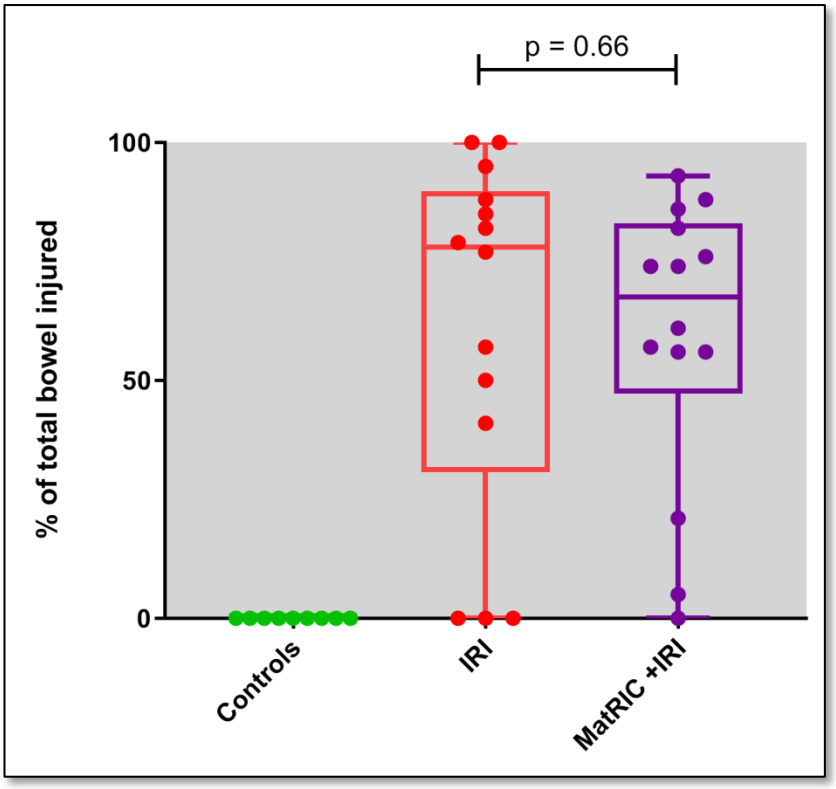


Figure 10-4 Protocol 4: percentage of total bowel showing severe macroscopic injury. Graph shows each data point, inter-quartile range and median

10.4.1 Microscopic Bowel Injury

Table 10-2 shows the Chiu-park scores for this experimental protocol.

	Controls*		IRI only*		(mat)RIC + IRI	
	TIF	MXF	TIF	MXF	TIF	MXF
Median	0	0	4	5.5	4	5.5
Range	0	0	0 – 7	4 – 7	0-7	0.5-7
IQR	0 - 0.25	0 – 1	3 – 5	4 - 6	3.25-5.5	4-6.875

Table 10-2 Protocol 4: Median Chiu-Park scores for each group.
TIF = Fixed point in terminal ileum
MXF = Area of maximal macroscopic injury
*Controls and IRI only animals from Protocol 1a.
Full results are shown in **Error! Reference source not found.**

Pups whose mother had undergone RIC prior to delivery had a median score of 4 (IQR 3.25-5.5) for the bowel specimen taken from a fixed point in the terminal ileum sample compared to 4 (3 - 5) for animals who underwent IRI without conditioning (p = 0.77). The specimens from the area of maximal injury had a median score of 5.5 (0.5 - 7) compared to 5.5 (4 - 7) for IRI only (p = 0.97). Figure 10-5 and Figure 10-6 show these groups and each of the data points.

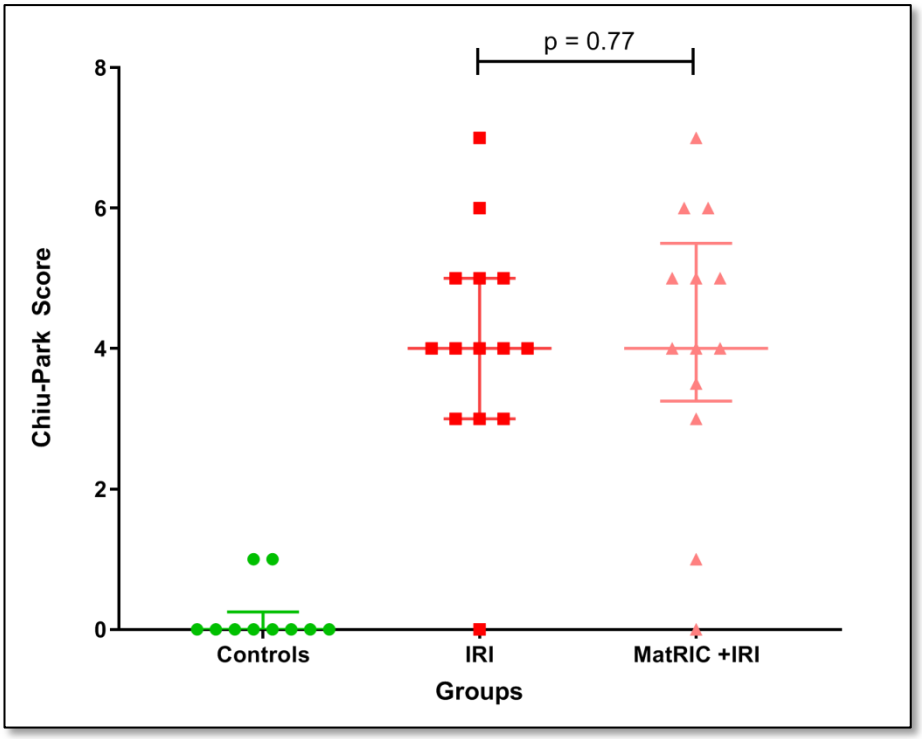


Figure 10-5 Protocol 4: Chiu-Park Scores – fixed point in the terminal ileum

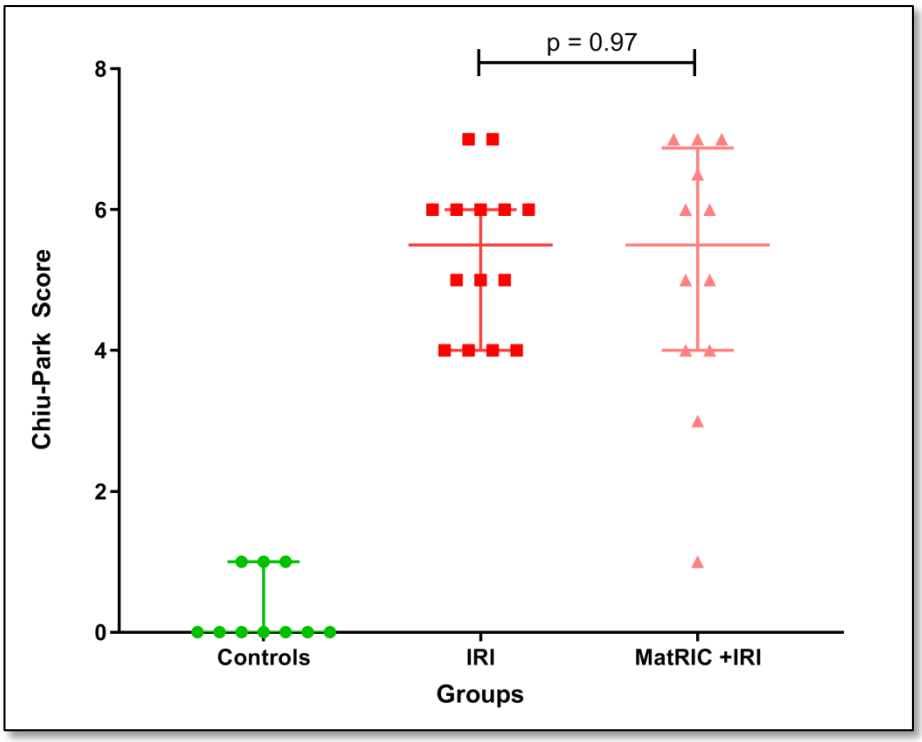


Figure 10-6 Protocol 4: Chiu-Park Scores – area of maximal macroscopic injury

10.5 Discussion

In the previous protocols, the data showed a protective effect from RIC in the rat pups. This effect is seen when the conditioning is both pre- and post- injury and indeed a (smaller) protective effect is seen when the RIC is delivered 48 hours prior to injury (Chapter 8).

At least some of the factors thought to be important for transmitting the protective effect of RIC to the target organ (such as catecholamines[259] and IL-6[292]) can be transported across the placenta [435,438]meaning that it is biologically plausible that RIC could be delivered to a mother prior to delivery and provide a protective effect to the offspring. Lopes de Freitas *et al.* (2014)[440] published the first (and so far, only) direct evidence that vertical transmission of RIC does indeed occur.

From a clinical perspective, such a mode of delivery is desirable. RIC delivered by means of a blood-pressure cuff is a minimally invasive technique that has been used in multiple studies in adults.[442] However, there remain technical and ethical challenges[443] in clinical translation of this approach to neonates. Given that the administration of maternal steroids for pre-term labour is routine practice,[437] the practicalities of instituting RIC at the same time would be straightforward.

In Lopes de Freitas *et al.*'s work, global hypoxia and reoxygenation induced atrophy and cellular necrosis in the colon, as well as showing mucosal inflammation measured by COX-2 expression. [440] There are some important differences between Lopes de Freitas and this work. Firstly, the model of ischaemic injury and end-points used to measure the impact of RIC are very different. Secondly, the timing of the interventions. In Lopes de Freitas, the insult to the bowel is from day 1 to day 3 post birth. In my model, the application of IRI to the small bowel occurs from day 10 of life to day 13. (Up to 14 days after the application of RIC). Multiple studies (including this body of work, Chapter 8) have shown RIC conferring protection for a prolonged period of time. However, in general that effect has not been shown to exist beyond approximately 48 hours. Indeed, my work showed a potential protective effect at two days but it was attenuated compared to the application of RIC immediately prior to injury. Therefore the difference in outcomes between Lopes de Freitas and this work could easily be explained by the difference in models and/or the timing of the insult after the application of maternal RIC. Unfortunately it is not technically possible to perform intestinal IRI on 2 day old pups.

10.6 Conclusion

In this model, maternal remote ischaemic conditioning does not confer any protective advantage to the gut of the offspring exposed to ischaemia-reperfusion injury at 10 days of age.

Chapter 11 Discussion

11.1 Introduction: Necrotising enterocolitis in context

Necrotising enterocolitis (NEC) is a modern disease. It emerged as an entity with the advent of effective neonatal care. In the latter part of the 20th century, premature neonates were surviving for the first time. In parallel and not directly related to this, treatments for congenital disorders (especially congenital heart disease) advanced. As a consequence there was, for the first time, populations of new born babies at risk of NEC. By the beginning of the 21st century, survival for preterm and other high-risk neonates had improved beyond all recognition, to the point that most very-premature neonates now survive and associated morbidities are much lower than previously described.

11.1.1 Contemporary outcomes

In order to properly delineate the current outcomes of NEC, I conducted a systematic review to identify the current clinical outlook, both in terms of mortality and morbidity. This published systematic review constitutes Chapter 2 of this thesis.

Important findings:

- NEC is the cause of between 10% and 21% of neonatal deaths.
- Overall mortality is around a quarter in cases of confirmed NEC (23.5%).
- Mortality for infants requiring surgery for NEC is around a third (34.5%), rising to over 50% in the subset of the smallest babies.
- The proportion of neonates that develop severe neurodevelopmental delay having had NEC is between a quarter and nearly two-thirds (24.8 and 61.1%).
- Long-term data on intestinal failure is lacking but between 15 and 35% of neonates who contract NEC will still require parenteral nutrition 90 days later.

Unequivocally, this demonstrates the clinical need: In the 21st century, NEC remains a devastating disease with high mortality and long-term sequelae for the survivors. The need for novel treatment options is clear.

11.1.2 Necrotising enterocolitis pathophysiology and remote ischaemic conditioning

The pathophysiology of NEC is complex and multifactorial (Section 1.5). It is also not fully understood. The role of several factors such as prematurity and feeding are not controversial as

the epidemiological data shows how important these are. Similarly, the importance of bacterial colonisation has been well-established. Ultimately, these factors combine to produce an ischaemia-reperfusion injury (IRI) to the intestinal mucosa leading to necrosis, bowel perforation and systemic sepsis.

In other IRI diseases, remote ischaemic conditioning (RIC) has shown great potential to reduce the injury caused by ischaemia and reperfusion (Chapter 3). Disappointingly, these very dramatic results in experimental animals and other biological models have not always translated to the clinical context. However, there are some areas whereby clinical trials have shown the potential for this technology in humans. Moreover, there is good evidence that typical comorbidities found in adult patients that are not relevant to the neonate are natural inhibitors of RIC. Therefore, it is likely that the translation from basic science to clinical practice would not be as challenging in neonates as it has been in other diseases.

11.2 Summary of Results

The main hypothesis of this thesis is that remote ischaemic conditioning reduces the extent of intestinal injury and inflammation in an experimental rat model of necrotising enterocolitis.

Using the primary outcome measure of a validated scoring system of bowel injury, this work shows that remote ischaemic conditioning does indeed significantly reduce the extent of bowel injury in this model.

11.2.1 Timing of Remote Ischaemic Conditioning and Dose effects

In this study, experimental animals were exposed to pre-RIC, (prior to injury) post-RIC (during the reperfusion phase), early-pre-RIC (48 hours prior to injury) and finally a combination of early-pre-RIC (48 hrs) and immediate pre-RIC (immediately prior to injury). Figure 11-1 shows the Chiu-Park scores for area of maximal macroscopic injury in each case. In summary this results show that:

- Immediate pre-RIC reduces the severity of the intestinal injury
- Post-RIC also reduces the severity of injury but is less efficacious than pre-RIC
- Early-pre-RIC shows a non-significant trend towards lower injury scores
- The combination of Early-pre-RIC and immediate-pre-RIC produces the biggest reduction in intestinal injury.
- Within this model there is no evidence of maternal transmission of RIC.

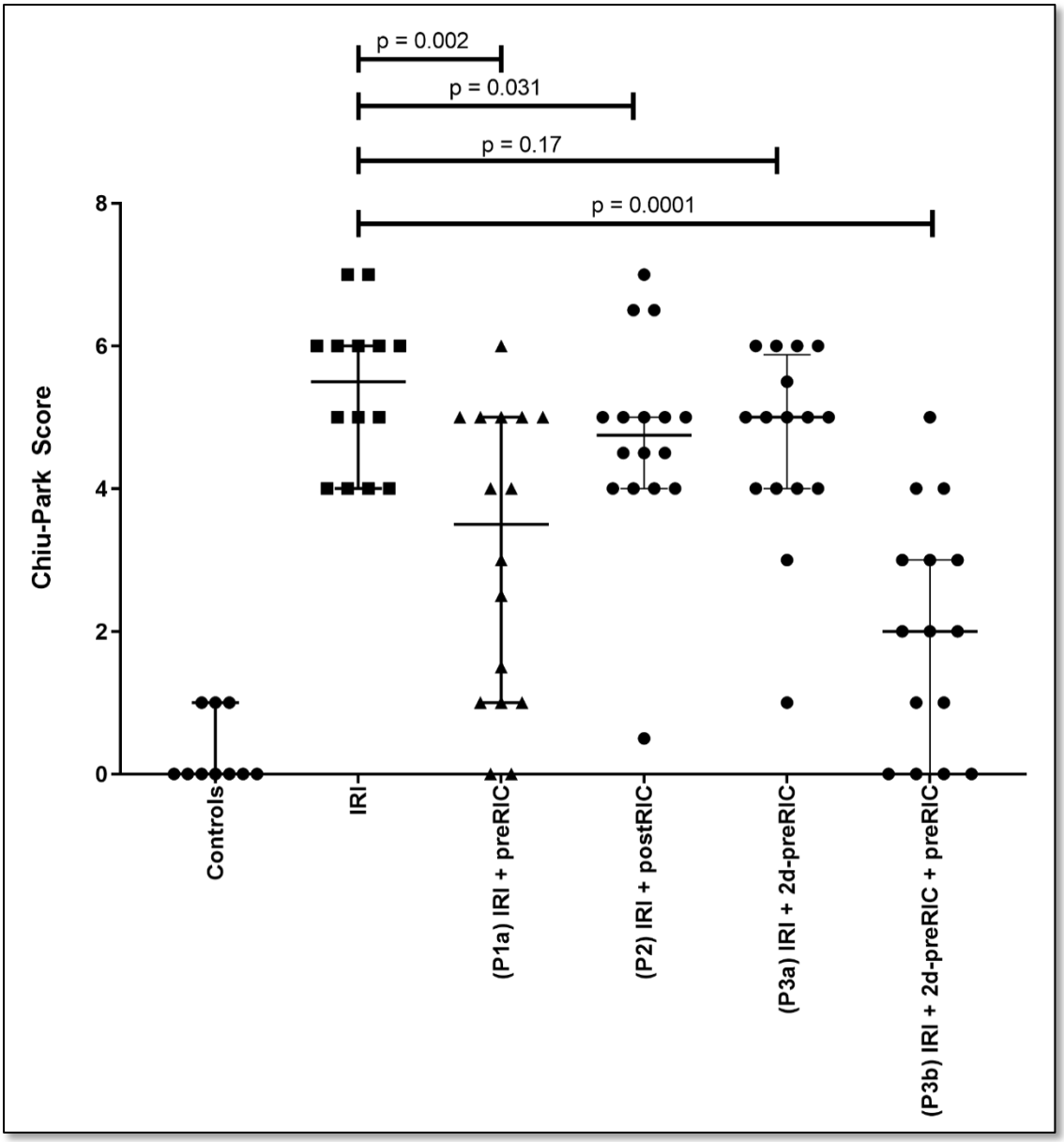


Figure 11-1 Microscopic scoring of intestinal injury in animals exposed intestinal ischaemia and reperfusion and remote ischaemic conditioning and different time points.
Each group shows each data point (individual animal), median and interquartile range. p-values calculated with Mann-Whitney Tests.

11.2.2 Cytokine analysis

Analysis of systemic cytokines revealed a rise in interleukin-10 (IL-10) in response to IRI that correlates with the intestinal injury and was consistent across the groups. A similar result has been reported by Lieu *et al.* (2019) in a murine study.[366] This rise in IL-10 also shows how the model is working; IL-10 is known to rise in response to ischaemia in general.[366] and specifically in human NEC.[99]

Interleukin-4 (IL-4), Interferon gamma (IFN-γ) and interleukin-1-beta (IL-1β) appear to be part of the pathway by which RIC is transmitted from the conditioned tissue to all other organs. This is supported by the fact that these cytokines were seen to rise in the post-RIC group (i.e. animals in which the blood was sampled only seventy minutes after exposure to RIC) and in animals that underwent RIC alone (Figure 7-11).

11.2.3 Transcriptomic Analysis

Multiple pro-inflammatory genes are down-regulated by the application of RIC. These include the chemoattractant CXCL-1, which is known to be important in ischaemic injury in multiple tissues and NF-κβ which is a central activator of pro-inflammation genes.

The role of the NF-κβ pathway in promoting the protective effect of RIC to the target organ is established in other organ systems,[315,361] so it is coherent to expect it to be key to the protective mechanisms in the intestine as well. Indeed RIC has been shown to act via a reduction in both NF-κβ [417] and myeloperoxidase which is consistent with these results (Section 5.4.6). Indeed Pearce *et al.* (2021)[315] have suggested that RIC should be thought of as a means of modulating the innate immune system. These gene expression changes would be consistent with that understanding of how RIC achieves its overall protective effect.

Increased expression of PI3K may indicate that something similar to the RISK pathway identified in cardiac tissue is present in the intestine. The lack of a role for PKC and P38 in remote conditioning is also consistent with what is seen in cardiac ischaemic conditioning

11.3 Comparable work

Since I started this work, there have been a small number of publications reporting parallel investigations. Hummitzsch *et al.* (2019)[444] studied the effect of RIC on the adult rat intestine. Using a similar protocol to this work, they reported significant reductions in intestinal injury in animals exposed to bilateral hind-limb RIC despite achieving lower injury scores (Chiu-Park) in their injury group than I observed with a longer ischaemia time. Miyake *et al.* (2020) again, using a similar model in much older rat pups showed that both pre- and post- RIC reduced bowel injury.[445] The IRI model of NEC has important limitations. Whilst it produces an intestinal injury that is similar to that seen in human disease it does so by a much simpler pathway.[353] It is important, therefore to acknowledge that the other factors implicated in the pathogenesis of NEC are lacking in this model yet it does mimic the more severe features of NEC and importantly has been used as a basis for clinical translation previously.[350,352] The model is arguably most representative of the sub-group of term infants with congenital cardiac disease who develop NEC,

as this is thought to be related primarily to poor gut perfusion.[140] However, arterial flow in the SMA of premature neonates who do not have cardiac conditions has also been shown to be an independent risk-factor for NEC.[139] Importantly, the IRI model involves arterial occlusion only, whilst venous outflow is unaffected and hence the model produces ischaemia without venous congestion. The IRI model of NEC is indeed a simplified pathway to a similar pattern of intestinal injury and systemic effects. That notwithstanding, the Transcriptomic analysis in this project shows that at least some of the pathways of NEC are affected by RIC thereby implying that as well as moderating the IRI that is the common endpoint of NEC pathophysiology, RIC would be expected to modulate the pathophysiology of NEC at several points in the pathogenic pathway (Figure 1-2). Indeed, more recently there has been interest in the so-called 'inflammatory hypothesis' of RIC focuses on the interaction between RIC and the innate immune system. [315]

Corroboration of these results in different NEC models that include other factors known to be involved in the pathogenesis of NEC, such as formula feeding, and microbial dysbiosis, is important to confirm our findings. One example of this is the recently published work of Koike *et al.* (2020)[446] Using a neonatal mouse gavage model of NEC, they showed that RIC is protective against intestinal injury. Mouse pups are formula fed and exposured to lipopolysaccharide and hypoxia to induce an NEC-like illness. Significant intestinal injury is noted from day 7 onwards. RIC performed on day 5 or day 6 attenuated the injury, whereas RIC performed on day 7 was not effective. Interestingly, Koike *et al.*'s work showed that the mechanism involved here was that it appears to improve the intestinal microcirculation.[446]

Gavage models do not have a fixed time point for insult and the progression of intestinal damage takes time. One particular advantage of the IRI model is that it gives a fixed time point for the initiation of bowel injury. Taken together the Koike work and these studies justify translation to human clinical trials.

Since NEC occurs in premature babies from around two weeks of age onwards, it would be potentially practical to use RIC prophylactically before this time. This work demonstrates that the effect of RIC can be used in this way as the protective effect may extend to 48 hrs. Moreover, the protective effect is increased by repeated applications of RIC.

11.4 Further Work

11.4.1 Laboratory studies

The mechanisms by which RIC confers such a profound protective effect on distant organs is an intriguing question. Potentially, there may be direct applications as the intermediary molecule(s)

could be mimicked with pharmacological agents. This is definitely speculative at the moment and the complexity and interactions of the mechanisms of RIC (Figure 3-4) would seem to suggest that a single agent is unlikely to be effective. In this work, the focus was very much on the mechanisms at the intestinal level. There are two reasons for this; firstly the model does not allow multiple blood sampling as the size of the rat pups mean that obtaining sufficient volume of blood is not possible before euthanasia thus by-definition only allowing sampling at a single time point. Secondly, the focus of this work was on the intestine – an organ that has received only a fraction of the research interest of other organs. The vast majority of the work has been cardiac or involving the brain or kidney. Hence the decision to focus on the mechanisms at the end-organ level in the intestine.

11.4.1.1 The role of cytokines in the transmission of remote ischaemic conditioning

Notwithstanding this, the serum collected at the end of each experiment did yield some interesting hints as to the potential mechanism with three cytokines showing increased levels in response to RIC alone (i.e. without the IRI stimulus).

Interleukin-4 (IL-4), Interferon gamma (IFN-γ) and interleukin-1-beta (IL-1β) were raised in response to RIC. This was seen in animals that underwent RIC alone (with the serum collected 130 minutes after RIC) and with animals that had IRI and post-RIC (hence the serum was collected 80 minutes after the completion of RIC). In the case of the postRIC group, the timing of the collection is relatively short but the picture is complicated by the fact that these animals were also exposed to IRI. In the RIC only group, the delay from exposure to RIC to collection of the serum significantly complicates the picture. Each of these three cytokines have previous been found to have a role in the transmission of RIC to the end-organ.[363,365,366]

The resolution of this quandary is straightforward in that the answer lies in using a model in which RIC can be given and blood samples taken at multiple time-points. This would be possible with a larger animal or even human volunteers. Several studies have considered the role of various cytokines in the transmission of RIC. For the most part, these were studies in which the cytokines changes were not the primary end-point as the focus was on RIC exerting a protective effect on a particular target organ.

11.4.1.2 Further analysis of the transcriptomics data

With 868 differentially expressed genes in the analysis that compared RIC with SHAM and 135 in the comparison between RIC+IRI and IRI alone, there is undoubtedly much more scope for exploration of these datasets. Unlike the work of Yoon *et al.* (2015),[294] this model showed a difference in the primary outcome (i.e. the RIC applied to these animals did result in a reduction in

the injury within the target organ) and the samples for analysis were collected immediately after injury rather than two days later. Having a robust set of 'wet-lab' experiments underlying the bioinformatics is a vital starting point to meaningful analysis. For this reason, all of the transcriptomics data (including the raw counts) will be made available in the repository once our group has finished with it.

11.4.2 Remote Ischaemic Conditioning and the premature neonate – a role for more than just NEC?

Central to this entire body of work is the argument that remote ischaemic conditioning provides a global protective effect to the whole organism. NEC is the focus of this work and indeed a major contributer to mortality and mobidity of premature neonates. It is not however the only condition that neonates suffer than may be ameanable to RIC as a putative therapy.

Intrapartum-related insults like as hypoxia-ischaemic brain injury, leading to neonatal encephalopathy are a major cause of global child deaths[447] and life long morbitidy.[448] Remote conditioning has naturally attracted research interest as a potential therapy.[449] Animal studies show a significant beneficial effect from the use of RIC.[450]

Translation to clinical use has the same challenges as would RIC for NEC. There is an appealing synergy here; the same trials that would establish safety for the use of the technology for one condition would inform trials for the other. Moreover, in real life, neonates are not a single organ system and those at risk of NEC are also at risk of encephalopathy, hence the potential for a therapy to prevent and treat both is very appealing.

11.4.3 Clinical translation

The most important implication of this work is that RIC shows significant promise for the prevention and treatment of NEC. The fact that RIC provides both a long-lasting protective effect and that the effect of repeated RIC is indeed additive provides the starting point for a clinical protocol for the use of RIC for premature neonates.

This body of work along with the other recent published studies collectively provide a strong empirical case that RIC is indeed a promising therapeutic option for NEC. There is probably very little more to be done in terms of animal-experiments to support this; the next step is to begin human translation of RIC.

As discussed in Section 3.6, RIC has been used safely in multiple clinical situations. The use of RIC in neonates has not been as widespread but there are studies that have used RIC in term neonates with congenital cardiac disease (and therefore different outcomes of interest).[327]

It is unlikely that RIC would cause significant harm in premature babies but the safety needs to be empirically established before study of the efficacy. However, no harms have been reported from the studies in term neonates and RIC remains significantly less invasive than many procedures routinely performed on babies within the neonatal ward. The use of RIC as a treatment for hypoxic-ischaemic injury has also been proposed; the ease of application being noted as a particular attraction.[451] Thus it is easy to envisage RIC becoming a routine part of neonatal care.

In the first instance, it could be argued that RIC should be investigated for the prevention of NEC in babies undergoing cardiac surgery. There are two reasons for this; firstly NEC in this context, is probably a simpler pathophysiology with IRI being possibly the key initiating step of the disease in way that it does not appear to be for NEC in premature babies. Therefore, this is a logical sub-group to begin with. Secondly, the development of NEC is often post-operatively (i.e. after cardiac surgery thus there is a clear window in which RIC could be effective.

Conversely, the increasing evidence for an anti-inflammatory effect of RIC (rather than just an 'anti-ischaemic injury' effect from the work of Peace *et al.* (2021)[315] and this study would justify the investigation of RIC in all forms of NEC, as would Koike *et al.*[446] data.

11.4.3.1 A putative clinical protocol

Following appropriate safety/efficacy pilot work, a putative protocol for the use of RIC in neonates based on this work would apply RIC to at-risk infants every two days. These data suggest both a prolonged protection and an additive effect from repeated cycles. Infants would undergo three cycles of limb ischaemia / reperfusion for five minutes each. Ischaemia being achieved by inflation of a cuff above systolic pressure for the allotted time. Occlusion of arterial inflow being demonstrated by the loss of a trace on pulse oximetry. This could be done with an appropriately sized manual sphygmomanometer or an automatic device equivalent to the AutoRIC™ device[452,453] that had been calibrated for neonates. This would then be repeated 48 hours later.

11.5 Conclusion

In the twenty-first century, NEC continues to be a leading cause of mortality and life-long morbidity in neonates. With the impressive improvements in outcomes for babies born prematurely, the deleterious effects of NEC are even more stark and finding new measures for the prevention and treatment of NEC is a key priority.24 The efficacy of RIC as a putative treatment

for NEC is demonstrated by this work and other similar studies. Clinical studies of RIC in the context of neonates with NEC/those at risk of NEC are now needed and there is now a sufficient evidence base to justify human trials.

Appendix A R Script for Meta-analysis (Chapter 2)

The meta-analyses for this systematic review were analyses of proportions. Many commercial statistics packages only contain the option of a meta-analysis of odd's ratios[ref]. *R* is a computer language and environment for statistical computing[454] and has the ability to perform these analysis using the packages metaphor[205] and meta.[206] Wang(2018) wrote a comprehensive guide to using *R* for meta-analysis of proportions[455].

The complete *R* script is reproduced below (Section A.1), an electronic can also be found in the additional material. The data from the various studies was compiled in a .csv file for importation into *R.* The .csv file can be found in Section A.2.

A.1 R Script for meta-analysis of proportions

/Meta_Analysis_of_NEC_mortality.R

```
#https://www.researchgate.net/publication/325486099_How_to_Conduct_a_Meta-Analysis_of_Proportions_in_R_A_Comprehensive_Tutorial

# install.packages(c("metafor", "meta")) # this is only needed once - then simply Library command
library ("metafor")
library ("meta")
setwd("~/Files/NEC review/R")
#get data from CSV file
dat=read.csv("dataTH.csv", header=T, sep=",") # opening CSV file

#Sub group sorting
#1 and 2 - neonates
neonates = dat$Population =="Neonates"
datN=dat[neonates,]
BellII = datN$NEC.defin == "Bell II+" # Only those that are also Bell II+
BellAll = datN$NEC.defin != "Bell II+" # the rest (i.e. Bell I-III or ICD9)
datN1=datN[BellII,]
datN2=datN[BellAll,]
#3 - neonates who underwent surgery
neonatessurg = dat$Population =="Neonates + Surgery"
datNS=dat[neonatessurg,]
#4 <1500g / VLBW
VLBW = dat$Population =="<1500g"
datVLBW=dat[VLBW,]
BellII = datVLBW$NEC.defin == "Bell II+" # Only those that are also Bell II+
datVLBW=datVLBW[BellII,]
#5 <1000g / ELBW
ELBW = dat$Population =="<1000g"
datELBW=dat[ELBW,]
#6 <1500g / VLBW + Surgery
```

```
VLBWS = dat$Population =="<1500g + Surgery"
datVLBWS=dat[VLBWS,]
#7 <1000g / ELBW + Surgery
ELBWS = dat$Population =="<1000g + Surgery"
datELBWS=dat[ELBWS,]
FPTitles = c("Mortality (%) - Neonates with NEC",
        "Mortality (%) - Neonates with NEC",
        "Mortality (%) - Neonates with Surgical NEC",
        "Mortality (%) - Neonates <1500g with NEC",
        "Mortality (%) - Neonates <1000g with NEC",
        "Mortality (%) - Neonates <1500g with Surgical NEC",
        "Mortality (%) - Neonates <1000g with Surgical NEC")
datalist = list(datN1,datN2,datNS,datVLBW,datELBW,datVLBWS,datELBWS) #list of defined
subgroups

pdf("Forest plots TH.pdf", width = 11, height = 5)

for(i in 1:length(datalist)){
  lb = FPTitles[i] #label for each plot
  dat = as.data.frame(datalist[i])
#loop: dat takes each group in turn then completes the forest plot construction below

#performing transformation - double ascine transformation
ies.da=escalc(xi=mortality, ni=n, data=dat, measure="PFT", add=0)
pes.da=rma(yi, vi, data=ies.da)
pes=predict(pes.da, transf=transf.ipft.hm, targ=list(ni=dat$n))
print(pes)

#test for hetrogeneity
print(pes.da)
confint(pes.da)

pes.summary=metaprop(mortality, n, authoryear, data=dat, sm="PFT",
                method.tau="DL", method.ci="NAsm")
forest(pes.summary,
     xlim=c(0,60),
     pscale=100,
     rightcols=FALSE,
     leftcols=c("studlab", "event", "n", "effect", "ci"),
     leftlabs=c("Study", "Deaths", "n", "mortality (%)", "95% C.I."),
     xlab=lb, smlab="",
     weight.study="random", squaresize=0.5, col.square="navy",
     col.square.lines="navy",
     col.diamond="maroon", col.diamond.lines="maroon",
     pooled.totals=FALSE,
     comb.fixed=FALSE,
     fs.hetstat=10,
     print.tau2=TRUE,
     print.Q=TRUE,
     print.pval.Q=TRUE,
     print.I2=TRUE,
     digits=1)
}
dev.off()

#Citations
citation(package = "metafor")
```

```
citation(package = "meta")
citation()
```

A.2 Data file (.csv)

/dataTH.csv

author	year	authoryear	mortality	n	NEC defin	Mort defin	Population
Rees	2010	Rees 2010(16)	27	211	Bell I-III	In-hospital	Neonates
Abdullah	2010	Abdullah 2010(274)	2718	20822	ICD-9	In-hospital	Neonates
Clark	2012	Clark 2012(283)	1505	7099	Bell II+	In-hospital	Neonates
Ganapathy	2013	Ganapathy 2013(287)	66	316	ICD-9	6 months	Neonates
Heida	2017	Heida 2017(289)	117	441	Bell II+	30 day	Neonates
Zhang	2011	Zhang 2011(308)	1660	5374	Surgical NEC	In-hospital	Neonates + Surgery
Choo 2011							
Murthy	2014	Murthy 2014(294)	259	753	Surgical NEC	In-hospital	Neonates + Surgery
Stey	2015	Stey 2015(301)	473	1375	Surgical NEC	In-hospital	Neonates + Surgery
Battersby	2017	Battersby 2017(279)	247	531	Surgical NEC	In-hospital	Neonates + Surgery
Allin 2017							
Allin 2018	2018	Allin 2018(277)	41	159	Surgical NEC	1 year	Neonates + Surgery
Ganapathy	2013	Ganapathy 2013*(287)	38	111	Surgical NEC	6 months	Neonates + Surgery
Hull	2014	Hull 2014(290)	4804	17156	Bell II+	In-hospital	<1500g
Autmizguine	2015	Autmizguine 2015(278)	645	2780	All NEC	In-hospital	<1500g
Youn	2015	Youn 2015(307)	63	149	Bell II+	In-hospital	<1500g
Kastenberg	2015	Kastenberg 2015(291)	411	1879	Bell II+	unclear	<1500g
Hayakawa	2015	Hayakawa 2015(288)	17	44	Bell II+	In-hospital	<1500g
Shah	2012	Shah 2012(298)	105	208	Bell II+	In-hospital	<1000g
Fullerton	2017	Fullerton 2017(37)	952	2881	Bell II+	2 years	<1000g
Kelley-Quon	2012	Kelley-Quon 2012(292)	496	1272	Surgical NEC	In-hospital	<1500g + Surgery
Fisher 2014							
Fullerton	2016	Fullerton 2016(286)	1742	4328	Surgical NEC	In-hospital	<1500g + Surgery
Hull 2014							
Autmizguine	2015	Autmizguine 2015*(278)	322	706	Surgical NEC	In-hospital	<1500g + Surgery
Youn	2015	Youn 2015*(307)	23	77	Surgical NEC	In-hospital	<1500g + Surgery
Wahawan	2014	Wadhawan 2014(306)	252	472	Surgical NEC	In-hospital	<1000g + Surgery
Tashiro	2017	Tashiro 2017(303)	522	886	Surgical NEC	2 years	<1000g + Surgery
Fisher	2014	Fisher 2014*(285)	1127	2782	Surgical NEC	In-hospital	<1000g + Surgery
Fullerton							

Appendix B Full data tables

In each results section, the summary statistics are reported. The following is the full data for each experiment.

B.1 Protocol 1a

B.1.1 Macroscopic

Controls				IRI only				RIC + IRI			
	Mild	Severe	Total		Mild	Severe	Total		Mild	Severe	Total
Median	**0%**	**0%**	**0%**		**59%**	**40%**	**100%**		**40%**	**0%**	**41%**
Unique ID				**Unique ID**				**Unique ID**			
933	0%	0%	0%	145	0%	100%	100%	092	0%	0%	0%
361	0%	0%	0%	076	59%	41%	100%	199	0%	55%	55%
847	0%	0%	0%	782	0%	100%	100%	102	0%	8%	8%
22	0%	0%	0%	290	50%	50%	100%	658	42%	18%	61%
205	0%	0%	0%	457	0%	0%	0%	426	40%	0%	40%
674	0%	0%	0%	723	29%	57%	86%	766	0%	0%	0%
701	0%	0%	0%	866	0%	95%	95%	908	100%	0%	100%
993	0%	0%	0%	017	21%	79%	100%	336	41%	0%	41%
460	0%	0%	0%	159	12%	88%	100%	865	8%	0%	8%
297	0%	0%	0%	977	67%	0%	67%	710	76%	24%	100%
				240	23%	77%	100%	976	35%	0%	35%
				038	15%	85%	100%	266	78%	22%	100%
				783	0%	82%	82%	018	66%	20%	85%
				244	100%	0%	100%				

Table B- 1 Macroscopic bowel injury for each individual animal. Summary data presented in Table 5-1. The Unique ID is used for each animal for each sample from that animal. The amount of bowel affected was assessed as either ‘mild’ or ‘severe’ injury. This was measured in cm. The data presented record these as a percentage of the total bowel extracted from that animal. (range of total bowel length: 27-41cm).

B.1.2 Microscopic

Controls			IRI only			RIC + IRI		
	TIF	MXF		TIF	MXF		TIF	MXF
Mean	**0.20**	**0.30**		**4.07**	**5.42**		**4.08**	**3.38**
Median	**0**	**0**		**4**	**5.5**		**5**	**4**
Range	**0**	**0**		**0 – 7**	**4 – 7**		**1 – 6**	**0 - 6**
IQR	**0 - 0.25**	**0 – 1**		**3 – 5**	**4 - 6**		**2.5 - 5**	**1 - 5**
Unique ID			**Unique ID**			**Unique ID**		
933	0	1	145	3	4	092	6	5
361	0	0	076	3	5	199	1	1
847	0	0	782	3	4	102	5	0
22	0	0	290	6	6	658	5	1
205	1	1	457	7	6	426	6	5
674	0	0	723	4	6	766	5	4
701	1	1	866	4	4	908	3	3
993	0	0	017	4	6	336	4	4
460	0	0	159	4	6	865	2	5
297	0	0	977	0	4	710	4	5
			240	5	7	976	2	0
			038	4	7	266	5	6
			783	5	5	018	5	5
			244	5	5			

Table B- 2 Protocol 1a: Chiu-Park Scores.

Summary data presented in Table 5-2

Animals exposed to IRI with RIC immediately prior to injury. (median score of three independent, blinded assessors)

MXF: ‘area of maximal macroscopic injury’ TIF: ‘terminal ileum (5-7cm from ileo-caecal valve). IQR: Inter-quartile range.

B.1.3 Serum Cytokine Analysis

Unique ID	IFN-γ *pg/ml*	IL-1β *pg/ml*	IL-10 *pg/ml*	IL-13 *pg/ml*	IL-4 *pg/ml*	IL-5 *pg/ml*	IL-6 *ng/ml*	KC/GRO *ng/ml*	TNF-α *pg/ml*
Controls									
Mean	**1.7**	**14.6**	**18.7**	**4.2**	**0.3**	**21.8**	**1.5**	**14.0**	**15.5**
Median	**1**	**0**	**16.5**	**4**	**0**	**10.8**	**1.1**	**13.5**	**13.4**
Range	**0 - 3 . 2**	**2.1 - 34.1**	**9.1 - 39.2**	**1.3 - 10.6**	**0.3 - 0.3**	**4.6 - 57.6**	**0.3 - 4.5**	**8.8 - 22.9**	**8.2 - 27.7**
933	1.5	-	11.2	2.6	-	7.1	0.3	13.2	15.0
361	0.7	-	22.6	2.0	-	-	1.4	13.4	10.5
847	-	-	21.6	10.6	-	-	1.6	13.6	11.7
022	-	-	10.9	1.3	-	-	0.4	14.9	8.2
205	-	7.7	39.2	2.5	-	4.6	4.5	22.9	21.3
674	3.2	34.1	14.4	3.4	-	14.5	0.9	13.9	11.7
701	1.3	2.1	11.0	5.8	-	19.1	0.4	8.8	21.8
993	-	-	18.5	4.5	-	14.6	4.1	16.9	9.5
460	1.5	-	9.1	4.6	-	34.9	0.6	9.7	17.4
297	1.9	-	28.9	5.0	0.3	57.6	1.2	12.8	27.7
IRI									
Mean	**1.5**	**12.8**	**43.3**	**3.5**	**0.3**	**46.5**	**2.1**	**23.2**	**22.3**
Median	**0.6**	**0**	**45.5**	**2.1**	**0**	**0**	**1.8**	**21.3**	**19.15**
Range	**0 - 4.3**	**0 - 33.1**	**2.2 - 74.3**	**0 – 8**	**0 - 0.3**	**0 - 72.3**	**0.3 - 6.6**	**1.5 - 39.8**	**4.7 - 42.2**
145	-	-	-	-	-	-	-	-	-
076	1.1	-	59.3	4.4	-	9.5	2.1	23.7	28.1
782	-	-	43.9	1.5	-	-	2.2	18.6	18.6
290	0.7	-	74.3	6.6	0.2	69.4	1.9	39.8	33.9
457	0.5	5.5	47.1	2.3	-	-	3.4	23.0	23.5
723	-	-	41.6	-	-	-	2.7	23.3	18.4
866	1.6	-	61.6	4.9	0.2	54.6	2.4	20.6	28.0
017	-	-	32.5	1.4	-	-	0.8	34.6	19.7
159	4.3	33.1	55.5	8.0	0.3	72.3	6.6	36.5	42.2
977	-	-	20.6	1.6	-	9.1	0.7	17.7	9.9
240	0.9	3.3	49.4	1.9	-	-	0.8	21.7	17.1
038	1.5	-	47.5	3.3	0.3	64.2	1.3	20.3	31.8
783	-	-	27.4	-	-	-	1.7	20.9	14.1
244	1.4	9.1	2.2	2.7	-	-	0.3	1.5	4.7

Unique ID	IFN-γ *pg/ml*	IL-1β *pg/ml*	IL-10 *pg/ml*	IL-13 *pg/ml*	IL-4 *pg/ml*	IL-5 *pg/ml*	IL-6 *ng/ml*	KC/GRO *ng/ml*	TNF-α *pg/ml*

RIC+IRI									
Mean	**1.4**	**5.5**	**42.6**	**3.4**	**1.8**	**26.6**	**1.8**	**21.9**	**21.8**
Median	**0.8**	**0**	**35.3**	**2.6**	**0**	**10.9**	**1.8**	**21.3**	**18.6**
Range	**0 - 3.1**	**0 - 6.5**	**27.6 - 75.8**	**0 - 9.7**	**0 - 1.8**	**0 - 70.7**	**0.8 - 2.5**	**13.4 - 29.3**	**12.6 - 41.8**
092	-	-	-	-	-	-	-	-	-
199	0.9	-	37.4	1.4	-	-	1.1	16.7	16.3
102	-	-	34.3	3.4	-	10.9	2.1	22.5	24.9
658	-	6.5	68.9	1.5	-	13.6	2.0	19.4	20.3
426	0.8	-	27.6	1.2	-	-	2.5	21.3	15.6
766	-	-	31.9	2.4	-	-	2.4	21.1	14.7
908	-	-	30.6	1.8	-	-	1.7	16.1	13.1
336	-	-	35.3	2.6	-	12.4	2.3	22.7	18.6
865	0.8	-	39.1	3.9	-	5.8	1.4	13.4	12.6
710	1.4	-	75.8	9.7	-	70.7	2.0	29.3	41.8
976	1.3	-	52.5	4.1	-	33.7	1.3	26.6	25.9
266	3.1	4.4	33.0	3.2	1.8	48.6	0.8	28.1	28.6
018	1.7	-	44.8	5.7	-	16.7	1.8	25.7	29.2

Table B- 3 Protocol 1a: Blood cytokine levels.
Summary data presented in Table 5-3
Units as indicated.
"-" indicates the cytokine was not detected.
Sufficient blood for analysis was not obtained from 092 and 145.

B.1.4 Myeloperoxidase

Controls			IRI only			RIC + IRI		
	Activity by sample weight	Activity by protein content		Activity by sample weight	Activity by protein content		Activity by sample weight	Activity by protein content
Mean	**1.2**	**12.9**		**3.58**	**26.1**		**2.38**	**23.8**
Median	**1.2**	**12.9**		**3.36**	**24.2**		**2.38**	**22.2**
Range	**0.63 - 2.06**	**6.82 - 19.5**		**1.43 - 9.77**	**12.2 - 44.0**		**1.33 - 4.19**	**14.7 - 37.2**
IQR	**0.95 - 1.53**	**9.60 - 15.69**		**2.50-3.71**	**18.4 - 33.7**		**1.62 - 2.82**	**18.2 - 28.8**
Unique ID			**Unique ID**			**Unique ID**		
933	1.88	11.0	145	5.84	37.4	092	-	-
361	2.06	13.1	076	2.79	12.2	199	2.65	20.4
847	1.07	19.5	782	2.77	21.2	102	4.19	32.2
22	0.74	6.8	290	3.51	32.7	658	2.80	20.6
205	1.01	9.9	457	3.80	44.0	426	2.72	37.2
674	1.06	17.6	723	2.80	32.8	766	2.82	29.2
701	1.24	15.0	866	1.72	19.1	908	1.47	17.5
993	1.25	15.0	017	3.30	17.5	336	1.70	25.0
460	1.41	12.6	159	1.43	18.7	865	2.08	27.8
297	0.63	8.8	977	3.42	21.6	710	1.33	16.9
			240	3.68	26.7	976	2.11	20.5
			038	1.59	13.1	266	1.59	14.7
			783	3.66	31.4	018	3.05	23.9
			244	9.77	36.5			

Table B- 4 Myeloperoxidase (MPO) activity in each sample. Summary data presented in Table 5-5 MPO activity is reported at Units/mg of sample and Units/mg of protein for each group.

B.1.5 Nitric Oxide

Controls				IRI only				RIC + IRI			
	Nitrite (pM /mg)	Nitrate (µM /mg)	Nitric Oxide (NO_2 + NO_3)		Nitrite (pM /mg)	Nitrate (µM /mg)	Nitric Oxide (NO_2 + NO_3)		Nitrite (pM /mg)	Nitrate (µM /mg)	Nitric Oxide NO_2 + NO_3)
Mean			**3.50**				**3.42**				**3.8**
Median			**2.45**				**3.67**				**3.6**
Range			**0.9 – 12.0**				**1.1 - 7.08**				**2.2 - 6.7**
IQR			**1.73 - 3.79**				**2.13 - 3.85**				**2.9 - 3.8**
Unique ID				**Unique ID**				**Unique ID**			
933	46.8	1.64	1.68	145	1935.9	2.8	4.78	92	-	-	-
361	0.0	3.51	3.51	76	43.1	1.1	1.10	199	41.7	2.3	2.33
847	383.6	2.39	2.77	782	0.0	2.1	2.07	102	0.0	3.6	3.65
022	0.0	0.89	0.89	290	1310.7	2.3	3.62	658	816.5	3.1	3.92
205	92.4	1.74	1.83	457	323.0	3.4	3.71	426	1959.5	3.6	5.58
674	567.4	4.08	4.65	723	274.7	3.5	3.75	766	0.0	6.7	6.69
701	0.0	12.0	12.00	866	0.0	1.9	1.93	908	0.0	2.8	2.76
993	77.6	3.80	3.88	17	397.7	1.9	2.33	336	329.6	2.9	3.19
460	0.0	1.70	1.70	159	2934.9	4.1	7.08	865	727.1	3.1	3.80
297	0.0	2.12	2.12	977	0.0	3.9	3.88	710	371.8	1.8	2.16
				240	214.5	4.5	4.71	976	0.0	2.9	2.90
				38	0.0	1.3	1.33	266	411.7	2.9	3.28
				783	0.0	2.3	2.32	18	417.4	2.6	3.06
				244	0.0	2.5	2.49				

Table B- 5 Nitric Oxide Levels in intestinal samples.
Summary data presented in Table 5-7.
Nitric Oxide levels estimated by adding measured nitrite and nitrate levels together. Nitrite levels in pM/mg, Nitrate in µM/mg.

B.1.6 Malondialdehyde

Controls		IRI only		RIC + IRI	
Mean	**541**		**780**		**809**
Median	**374**		**524**		**717**
Range	**47 - 1490**		**200 - 1812**		**117 - 2500**
IQR	**99 - 882**		**457 - 1137**		**415 - 920**
Unique ID		**Unique ID**		**Unique ID**	
933	964	145	1812	092	-
361	1222	076	530	199	910
847	1490	782	1418	102	2500
22	193	290	485	658	907
205	138	457	1373	426	1326
674	47	723	488	766	949
701	79	866	288	908	670
993	556	017	519	336	456
460	637	159	273	865	764
297	86	977	1102	710	143
		240	448	976	291
		038	200	266	117
		783	835	018	671
		244	1149		

Table B-6 Malondialdehyde levels in each intestinal sample.
(pmoles/mg of protein)
Summary data reported in Table 5-8

B.2 Protocol 1b

B.2.1 Macroscopic and microscopic

RIC (blood pressure cuff) + IRI					
	Mild	Severe	Total	Chiu-Park score TIF	Chiu-Park score MXF
Median	**59%**	**15%**	**68%**	**3**	**5**
Range	**0- 86%**	**0-74%**	**59-86%**	**2 – 5**	**4 – 6**
IQR	**19-71%**	**0-43%**	**62-75%**	**2.75 – 4**	**4 – 5.25**
Unique ID					
758	45%	23%	68%	4	4
741	39%	26%	66%	3	5
138	68%	15%	82%	4	5
327	63%	0%	63%	3	6
870	75%	0%	75%	3	6
902	71%	0%	71%	2	4
892	86%	0%	86%	3	5
849	0%	74%	74%	3	4
703	0%	61%	61%	2	5
837	19%	43%	62%	5	5
829	59%	0%	59%		

Table B-1 Macroscopic and microscopic bowel injury for each individual animal.

Summary data presented in Table 6-1 and Table 6-2.

The Unique ID is used for each animal for each sample from that animal. The amount of bowel affected was assessed as either ‘mild’ or ‘severe’ injury. This was measured in cm. The data presented record these as a percentage of the total bowel extracted from that animal. (range of total bowel length: 31-44cm).

B.3 Protocol 2

B.3.1 Macroscopic and Microscopic

IRI + *post*RIC						
	Mild	**Severe**	**Total**		**Chiu-Park score TIF**	**Chiu-Park score MXF**
Median	**44%**	**4%**	**68%**		**4**	**4.25**
Range	**7 - 100%**	**0 - 52%**	**37 - 100%**		**0 – 6**	**0 – 6**
IQR	**34 - 62%**	**0 - 43%**	**56 - 78%**		**3.75 – 6**	**4 – 5**
Unique ID						
967	37%	0%	37%		2	4
269	7%	43%	50%		4	4
922	32%	35%	68%		3	4
211	50%	6%	56%		2	0
87	35%	46%	81%		6	4
696	69%	0%	69%		4	6
582	61%	0%	61%		4	5
217	47%	39%	87%		3	5
969	78%	0%	78%		4	6
897	78%	0%	78%		3	4.5
822	100%	0%	100%		4	4
255	26%	52%	77%		6	5
805	56%	0%	56%		6	4
391	34%	3%	37%		5	5
556	62%	0%	62%		4	5
201	24%	48%	73%		0	0

Table B-7 Macroscopic and microscopic bowel injury for each individual animal. Summary data presented in Table 7-1 and Table 7-2.

The Unique ID is used for each animal for each sample from that animal. The amount of bowel affected was assessed as either ‘mild’ or ‘severe’ injury. This was measured in cm. The data presented record these as a percentage of the total bowel extracted from that animal. (range of total bowel length: 29-41cm).

B.3.2 Serum Cytokine Analysis

Unique ID	IFN-γ *pg/ml*	IL-1β *pg/ml*	IL-10 *pg/ml*	IL-13 *pg/ml*	IL-4 *pg/ml*	IL-5 *pg/ml*	IL-6 *ng/ml*	KC/GRO *ng/ml*	TNF-α *pg/ml*
postRIC									
Mean	**1.9**	**25.2**	**34.4**	**4.9**	**0.5**	**42.1**	**3.7**	**29.9**	**27.7**
Median	**1.9**	**27.8**	**26.3**	**5.0**	**0.2**	**44.7**	**3.3**	**26.8**	**25.3**
Range	**1.1 - 2.5**	**0 - 56.7**	**26.0 - 74.9**	**0 - 8.4**	**0 - 1.4**	**26.7 - 52.2**	**1.0 - 7.7**	**17.1 - 58.8**	**13.9 - 63.9**
Unique ID									
967	2.2	-	47.4	3.8	-	32.0	1.0	26.0	21.9
269									
922	1.1	34.7	26.5	-	-	26.7	5.0	17.1	33.7
211	1.9	-	34.7	4.7	-	31.6	4.0	32.6	19.5
087									
696	1.7	20.0	53.7	6.0	0.7	31.9	7.7	55.8	32.6
582	1.2	21.9	74.9	4.3	1.0	46.3	3.2	46.1	27.2
217	2.0	42.2	16.5	5.2	1.4	50.1	1.2	19.8	63.9
969	1.9	37.4	16.0	8.4	0.5	52.2	2.2	26.7	27.1
897	2.5	33.7	20.1	4.9	-	50.4	2.2	27.0	13.9
822	2.4	6.9	26.1	5.2	-	43.1	7.1	25.6	20.5
255	2.5	47.1	25.1	6.0	1.0	51.9	4.6	30.6	23.6
805									
391									
556	1.2	2.1	48.3	4.5	1.0	38.6	2.8	26.9	27.9
201	1.7	56.7	23.7	6.1	-	49.8	3.5	25.0	20.1

Table B-8 Protocol 2: Blood cytokine levels.
Summary data presented in Table 7-3
Units as indicated.
"-" indicates the cytokine was not detected.
Sufficient blood for analysis was not obtained from 269, 087, 805 and 391

B.4 RIC only

B.4.1 Serum Cytokine Analysis

Unique ID	IFN-γ *pg/ml*	IL-1β *pg/ml*	IL-10 *pg/ml*	IL-13 *pg/ml*	IL-4 *pg/ml*	IL-5 *pg/ml*	IL-6 *ng/ml*	KC/GRO *ng/ml*	TNF-α *pg/ml*
RIC									
Mean	**2.9**	**20.7**	**17.8**	**4.3**	**1.2**	**32.9**	**1.7**	**11.2**	**13.1**
Median	**2.3**	**18.4**	**14.3**	**4.1**	**1.0**	**38.1**	**1.5**	**10.9**	**12.5**
Range	**1.9 - 4.6**	**7.7 - 38.1**	**10.5 - 39.2**	**1.3 - 10.6**	**0.6 - 2.1**	**4.6 - 48.1**	**0.4 - 4.5**	**1.2 - 22.9**	**7.4 - 21.3**
Unique ID									
714	-	-	21.6	10.6	-	-	1.6	13.6	11.7
785	-	-	10.9	1.3	-	-	0.4	14.9	8.2
329	-	7.7	39.2	2.5	-	4.6	4.5	22.9	21.3
949	1.9	18.7	13.7	4.0	0.6	48.1	2.0	7.8	19.1
971	2.2	-	14.9	4.5	1.6	46.0	1.2	8.6	14.7
307	4.6	38.1	18.7	4.6	2.1	44.2	2.0	13.1	13.3
251	2.3	18.2	10.5	3.1	0.8	22.7	0.8	1.2	9.1
518	3.6	-	13.1	4.2	1.0	32.0	1.3	7.0	7.4

Table B-9 RIC only: Blood cytokine levels.
"-" indicates the cytokine was not detected.

B.5 Protocol 3a

B.5.1 Macroscopic and Microscopic

IRI + early preRIC						
	Mild	Severe	Total		Chiu-Park score TIF	Chiu-Park score MXF
Median	**50%**	**3%**	**68%**		**4**	**5**
Range	**0-76%**	**0-86%**	**39-100%**		**2 – 6**	**1 – 6**
IQR	**34-63%**	**0-42%**	**58-77%**		**3.75 - 5.25**	**4 - 5.875**
Unique ID						
486	66%	0%	66%		3	5
530	52%	6%	58%			6
694	48%	32%	81%		1	5
280	43%	0%	43%		4	5
450	57%	0%	57%		4	6
581	36%	30%	67%		3	5
277	0%	79%	79%		4	5
972	66%	0%	66%		6	4
985	9%	60%	69%		6	6
273	28%	49%	77%		3	4
107	54%	0%	54%		3	1
614	39%	0%	39%		4	6
465	14%	86%	100%		3	2
689	69%	0%	69%		3	5
871	76%	0%	76%		2	4
941	63%	0%	63%		3	4
835	60%	17%	76%		3	6
841	48%	40%	88%		3	4

Table B-10 Macroscopic and microscopic bowel injury for each individual animal.

Summary data presented in Table 8-1 and Table 8-2.

The Unique ID is used for each animal for each sample from that animal. The amount of bowel affected was assessed as either ‘mild’ or ‘severe’ injury. This was measured in cm. The data presented record these as a percentage of the total bowel extracted from that animal. (range of total bowel length: 28-43cm).

B.6 Protocol 3b

B.6.1 Macroscopic and Microscopic

IRI + early preRIC + immediate preRIC					
	Mild	Severe	Total	Chiu-Park score TIF	Chiu-Park score MXF
Median	***64%***	***0%***	**76%**	**2.5**	**3**
Range	**11 – 100%**	**0 – 74 %**	**46 - 100%**	**0 – 4**	**0 – 6**
IQR	**40 – 74%**	**0 – 39%**	**62 – 91%**	**1 – 3**	**1 – 3**
Unique ID					
880	74%	74%	74%	5	6
326	11%	11%	85%	4	3
171	16%	16%	56%	4	2
36	78%	78%	78%	1	3
440	34%	34%	93%	2.5	4
47	71%	71%	71%	2.5	2
532	38%	38%	86%	1	3
862	73%	73%	73%	3	1
754	100%	100%	100%	3	3
740	50%	50%	50%	0.5	0
974	46%	46%	46%	1	2
362	97%	97%	97%	2	3
927	68%	68%	86%	1	1
202	62%	62%	100%	3.5	6
56	60%	60%	60%	0	0
986	66%	66%	69%	3	5

Table B-11 Macroscopic and microscopic bowel injury for each individual animal. Summary data presented in Table 8-3 and Table 8-4

The Unique ID is used for each animal for each sample from that animal. The amount of bowel affected was assessed as either ‘mild’ or ‘severe’ injury. This was measured in cm. The data presented record these as a percentage of the total bowel extracted from that animal. (range of total bowel length: 28-46cm).

B.7 Protocol 4

B.7.1 Macroscopic and Microscopic

Maternal RIC					
	Mild	Severe	Total	Chiu-Park score TIF	Chiu-Park score MXF
Median	***26%***	***68%***	***100%***	**4**	**5.5**
Range	**0-100%**	**0-93%**	**76-100%**	**0-7**	**1-7**
IQR	**13-52%**	**47-83%**	**86-100%**	**3.25-5.5**	**4-6.875**
Unique ID					
503	0%	76%	76%	3.5	4
734	81%	5%	86%	3	6.5
501	26%	57%	83%	1	3
886	18%	82%	100%	7	6
010	13%	88%	100%	5	
150	39%	61%	100%	0	1
753	76%	21%	97%	4	6
084	29%	56%	85%	5	7
051	44%	56%	100%	6	7
984	26%	74%	100%	4	5
180	26%	74%	100%	4	5
274	8%	93%	100%	5	7
563	14%	86%	100%		
724	100%	0%	100%	6	4
503	0%	76%	76%	3.5	4

Table B-12 Macroscopic and microscopic bowel injury for each individual animal.

Summary data presented in Table 10-1 and Table 10-2.

The Unique ID is used for each animal for each sample from that animal. The amount of bowel affected was assessed as either 'mild' or 'severe' injury. This was measured in cm. The data presented record these as a percentage of the total bowel extracted from that animal. (range of total bowel length: 32-51cm).

Appendix C Chiu-Park Scoring: Comparison between Scorers

The Chiu-Park scoring system is well-established and validated for ischaemic bowel injury as the scoring indicates a progressive scale of injury.[355,358] The following analysis shows the variance in scoring between the three independent scorers.

	Scorer 1 (DT)	Scorer 2 (NH)	Scorer 3 (IJ)
Scorer 1(DT)		0.76 (0.68 – 0.82) P < 0.0001	0.72 (0.63 – 0.82) P < 0.0001
Scorer 2 (NH)	0.76 (0.68 – 0.82) P < 0.0001		0.78 (0.73 – 0.83) P < 0.0001
Scorer 3 (IJ)	0.72 (0.63 – 0.82) P < 0.0001	0.78 (0.73 – 0.83) P < 0.0001	

Table C-1 Spearman R coefficient, 95% confidence intervals and p – values comparing the independent scores.

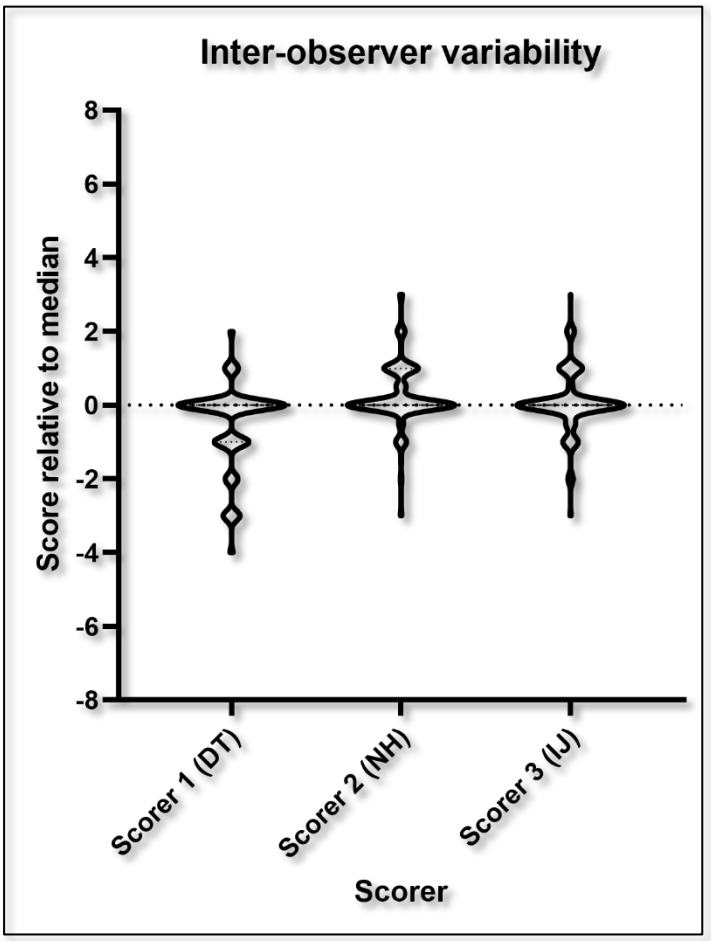


Figure C-1 Violin plot of each individual blind score against the median score of all three scorers.

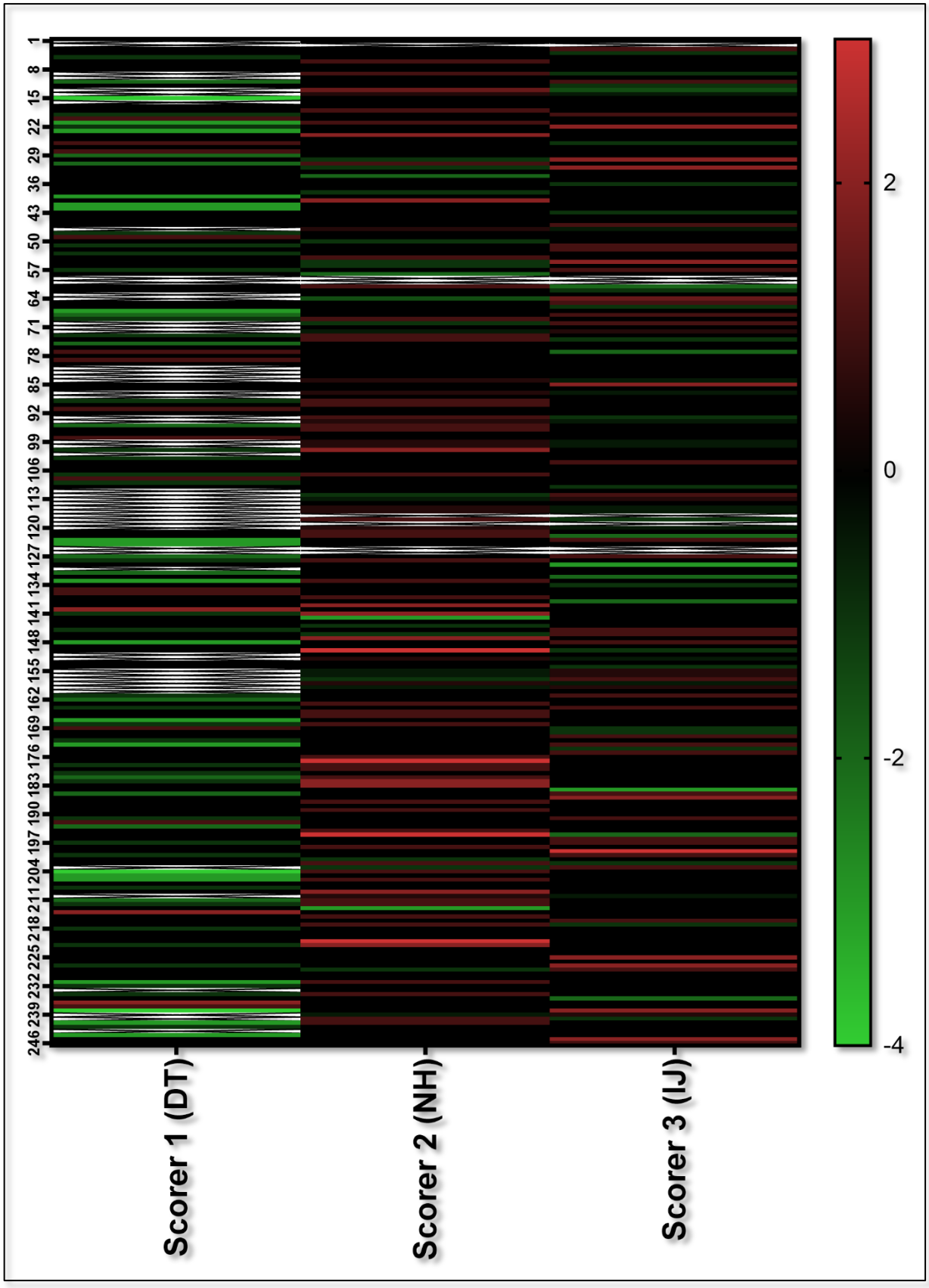
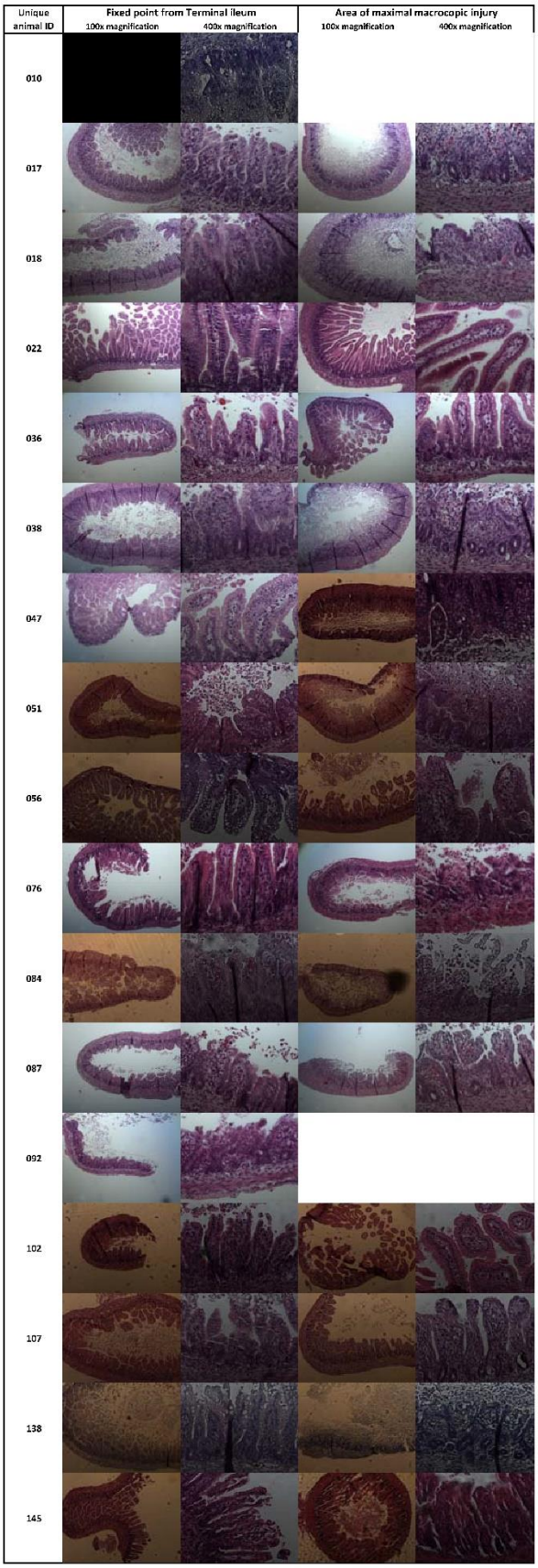
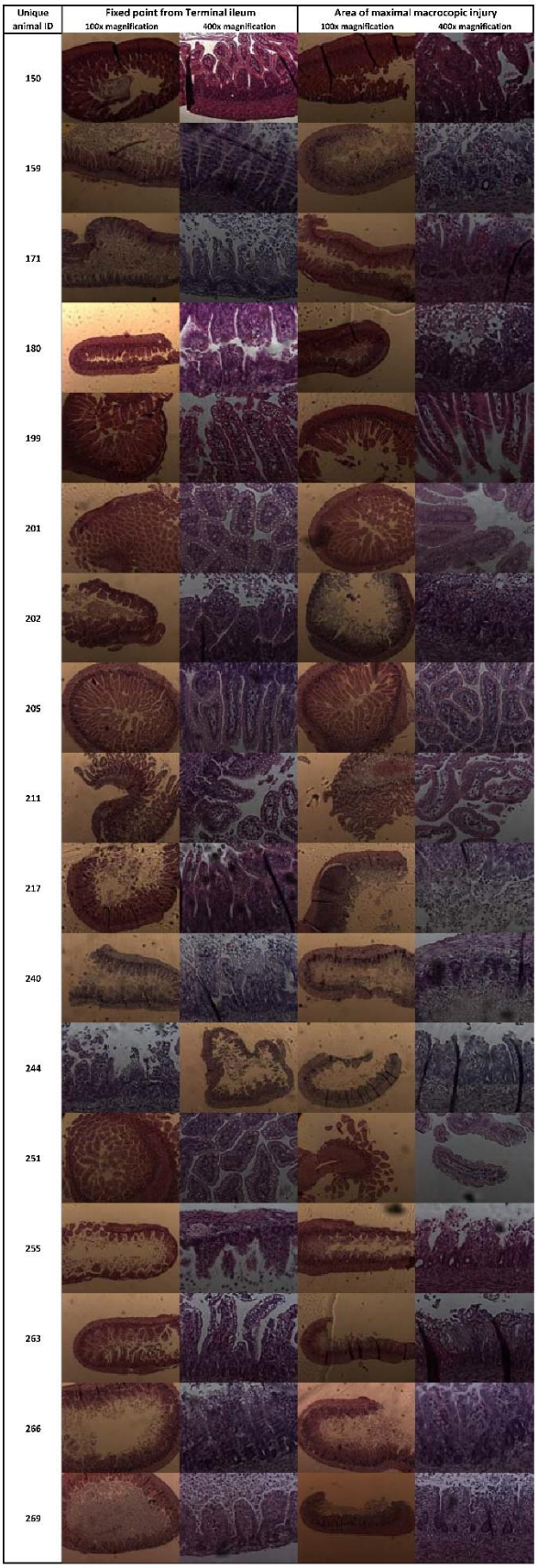


Figure C-2 Heatmap of all blinded scores against median score.

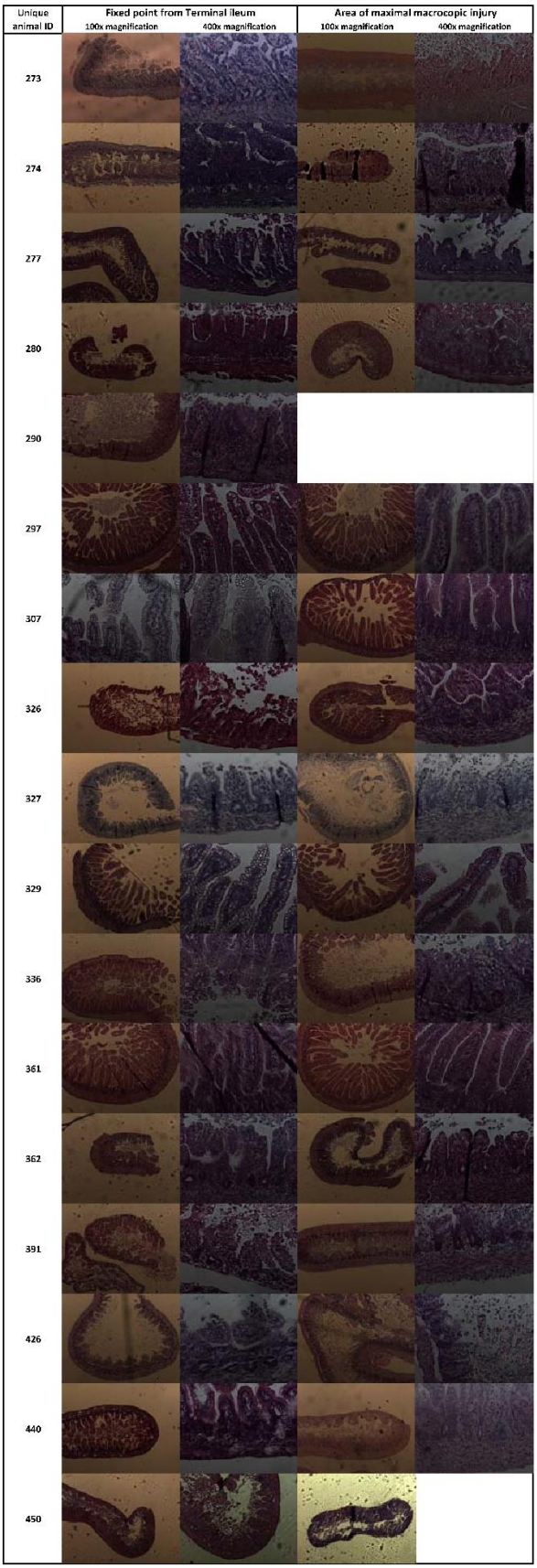
Appendix D Haematoxylin and Eosin stained Bowel Specimens used for Chiu-Park scoring Images

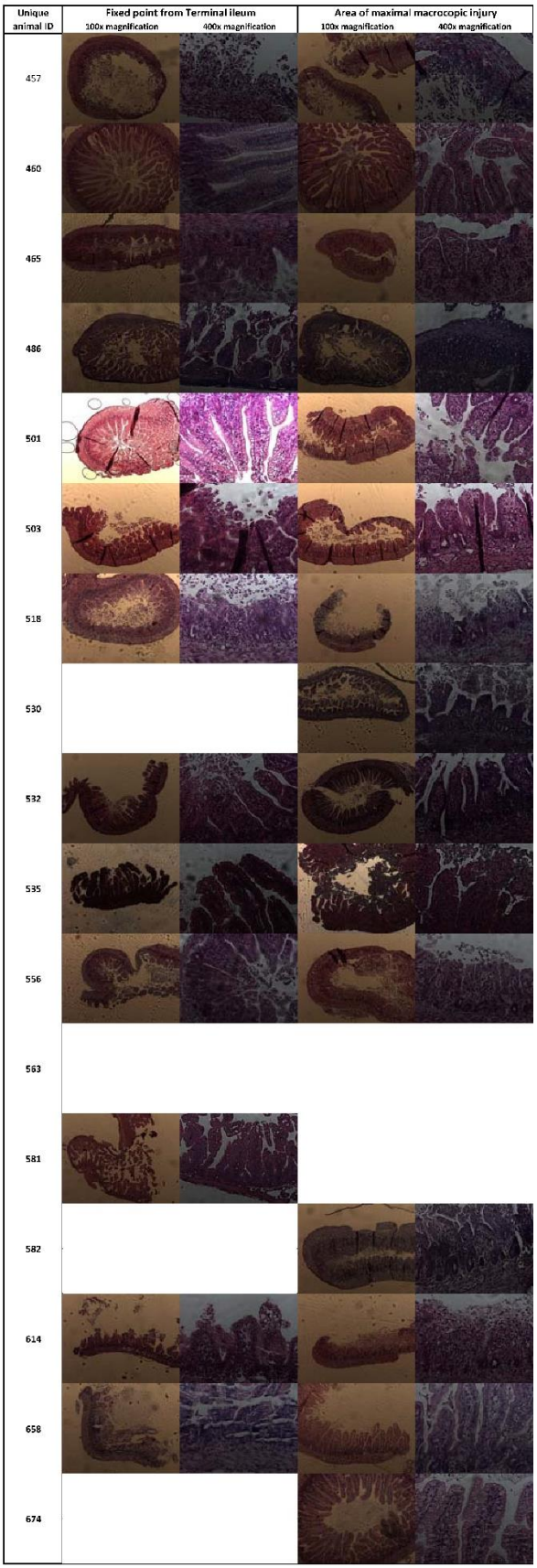
These images are reproduced for reference only. All the assessment and scoring of the specimens was done using light microscopy at 100x, 200x and 400x magnification. Each specimen is shown at 100x and 400x magnification. The bowel specimens were collected from a fixed point in the terminal ileum and from the point of maximal macroscopic injury. They were then immediately fixed in formalin and after embedding in was, 5 µm sections were cut. The sections underwent a dewaxing procedure before staining with haematoxylin and eosin using a standard technique, as described in Sections 4.6 and 4.8.

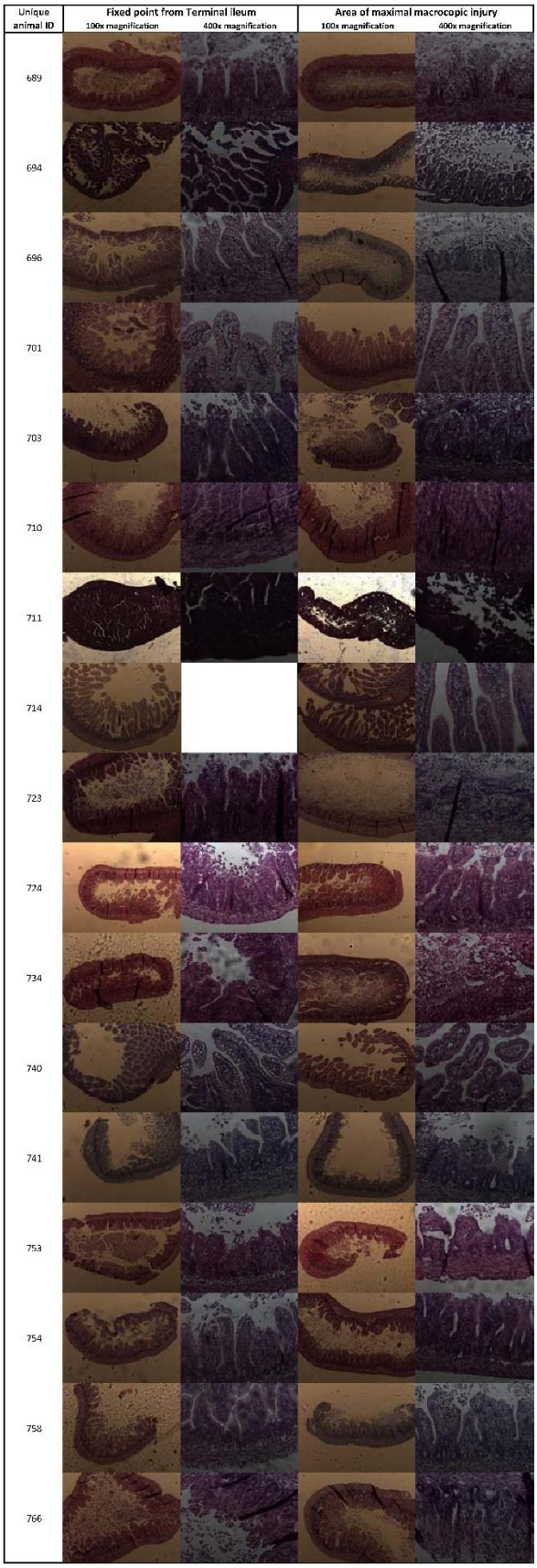

Unique animal ID
Fixed point from Terminal ileum
100x magnification
400x magnification
Area of maximal macrocopic injury
100x magnification
400x magnification
010
017
018
022
036
038
047
051
056
076
084
087
092
102
107
138
145

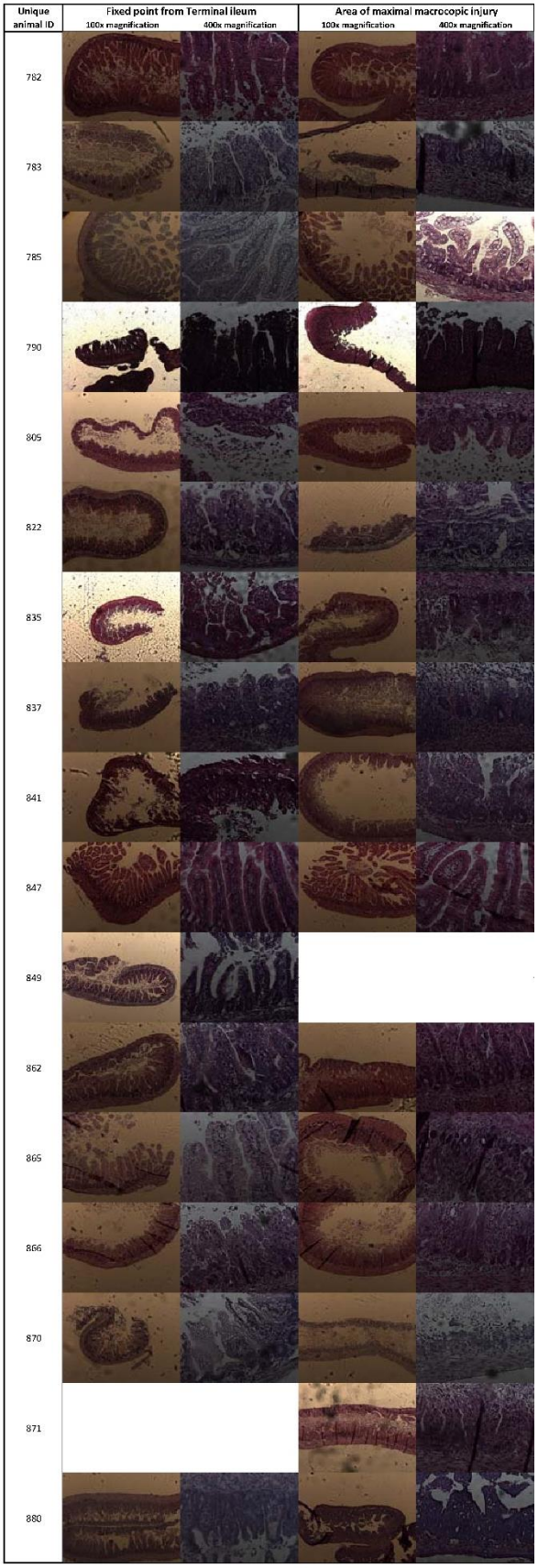

Unique animal ID
Fixed point from Terminal ileum
100x magnification
400x magnification
Area of maximal macrocopic injury
100x magnification
400x magnification
150
159
171
180
199
201
202
205
211
217
240
244
251
255
263
266
269

Appendix D

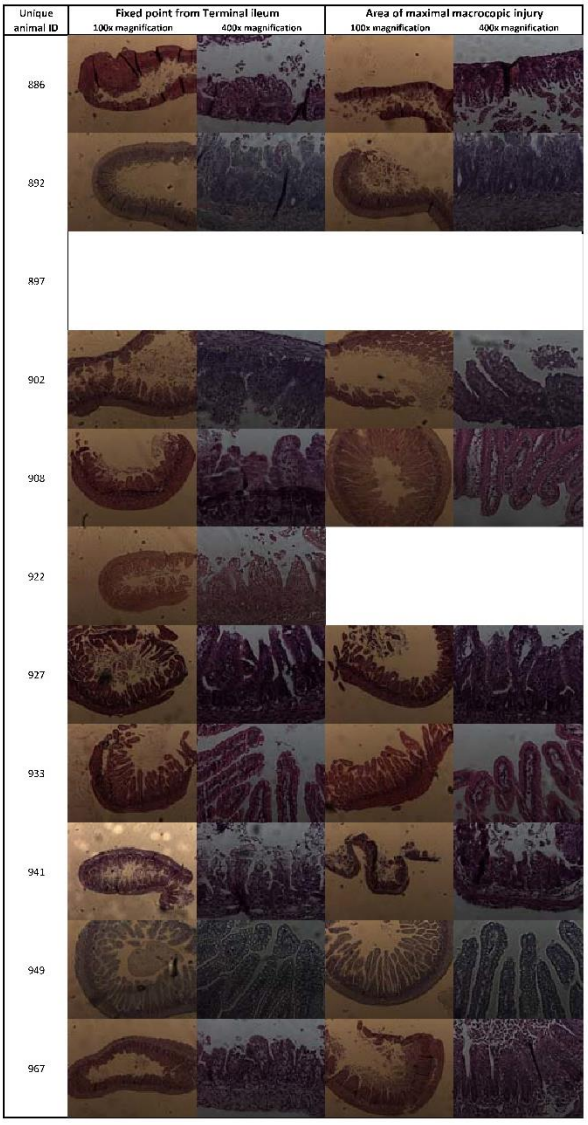

Unique animal ID
Fixed point from Terminal ileum
100x magnification
400x magnification
Area of maximal macrocopic injury
100x magnification
400x magnification
273
274
277
280
290
297
307
326
327
329
336
361
362
391
426
440
450

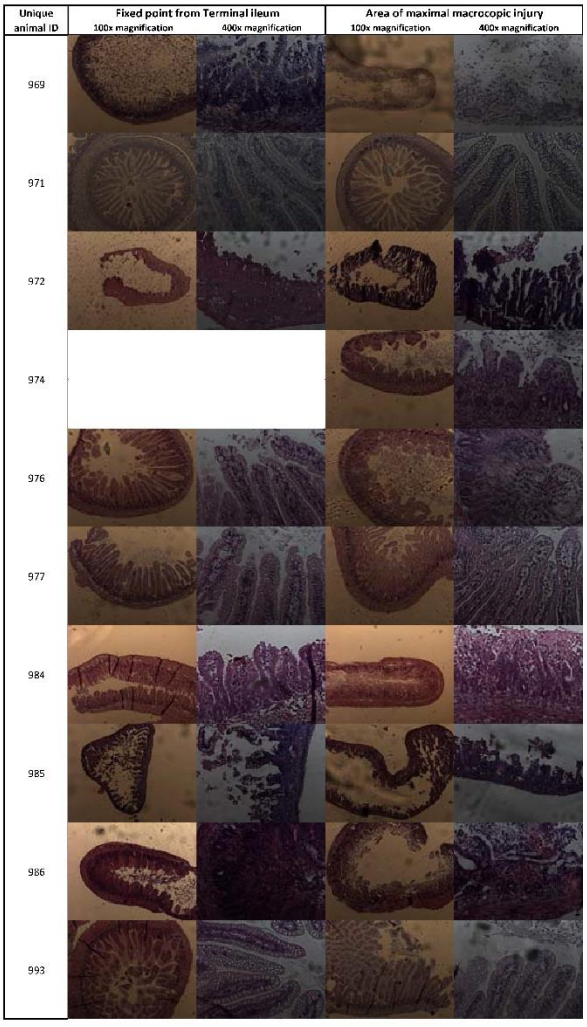

Unique animal ID
Fixed point from Terminal ileum
100x magnification
400x magnification
Area of maximal macrocopic injury
100x magnification
400x magnification
457
460
465
486
501
503
518
530
532
535
556
563
581
582
614
658
674


Unique animal ID
Fixed point from Terminal ileum
100x magnification
400x magnification
Area of maximal macrocopic injury
100x magnification
400x magnification
689
694
696
701
703
710
711
714
723
724
734
740
741
753
754
758
766


Unique animal ID
Fixed point from Terminal ileum
100x magnification
400x magnification
Area of maximal macrocopic injury
100x magnification
400x magnification
782
783
785
790
805
822
835
837
841
847
849
862
865
866
870
871
880

Appendix D


Unique animal ID
Fixed point from Terminal ileum
100x magnification
400x magnification
Area of maximal macrocopic injury
100x magnification
400x magnification
886
892
897
902
908
922
927
933
941
949
967


Unique animal ID
Fixed point from Terminal ileum
100x magnification
400x magnification
Area of maximal macrocopic injury
100x magnification
400x magnification
969
971
972
974
976
977
984
985
986
993

Appendix E Qiagen wet lab (Chapter 9)

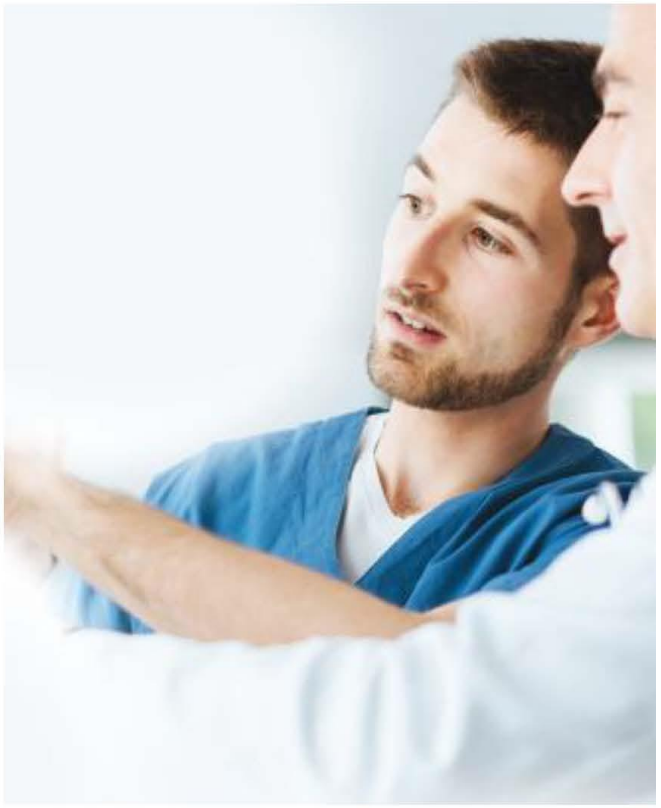
Chapter 9 describes the Transciptomic analysis I performed using RNAseq. The RNA extraction, library preparation and next generation sequencing was performed by Qiagen Genomic Services (Hilden, Germany). The following pages are the formal report they produced on the labarotory processing they performed.

Project QNS20743
post sequencing consultation

Dr. rer. nat. Michael Gombert

Project overview

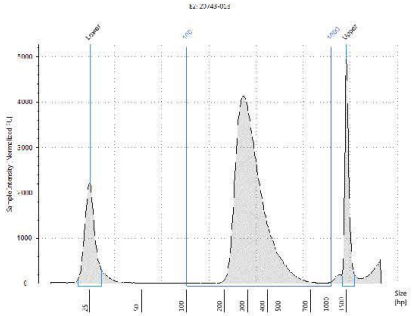
- 24 samples of rattus norvegicus origin
- Cells were provided
- RNA extraction and QC
- Library preparation and QC
- Sequencing
- Data analysis
- Online data delivery

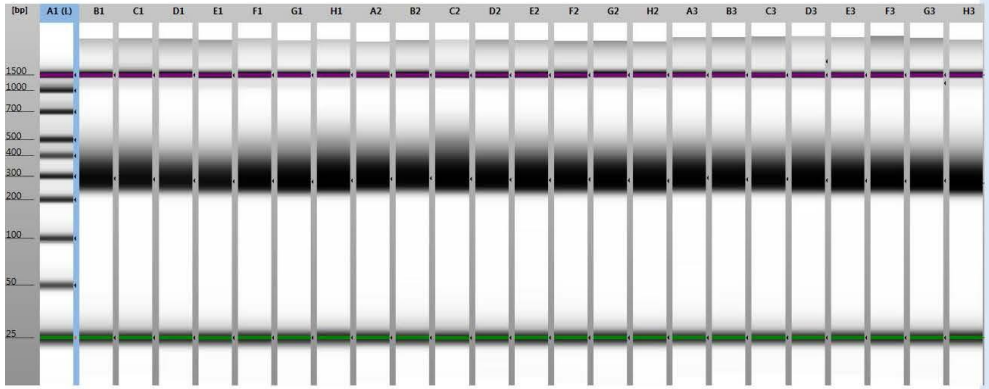


Sample	Sample ID	Conc. (ng/µl)	RIN
1	017	1990	8.7
2	518	2460	9.4
3	038	2940	8.3
4	251	2540	9.2
5	535	3520	8.1
6	711	2920	9.1
7	971	2760	9.5
8	297	3780	9.0
9	976	2220	9.4
10	307	2760	9.5
11	266	3360	7.4
12	329	2780	9.3
13	240	2800	8.1
14	993	2980	9.5
15	790	2220	9.5
16	460	5060	8.2
17	701	4500	8.9
18	018	4540	8.2
19	674	3600	9.5
20	244	1620	9.3
21	949	2860	9.7
22	159	3900	9.1
23	205	2820	10.0
24	783	2800	8.9

Sample QC

Sample prep yielded sufficient amounts of RNA in excellent quality



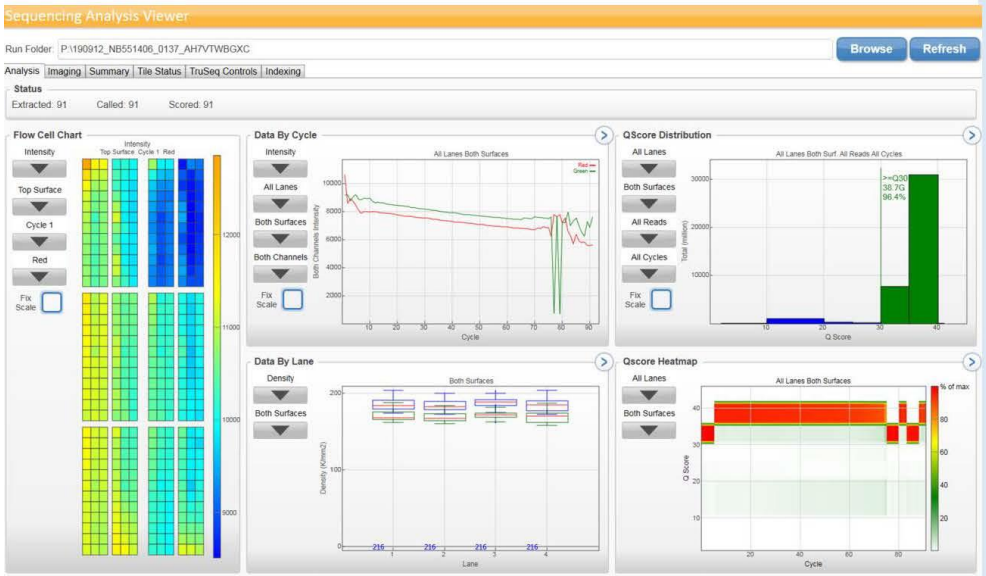


Library QC

Top: Example library pool from the protocol

Bottom: Example libraries from your project

Libraries look good, no overamplification humpbacks, no primer/adapter dimer peaks



Sequencing QC

Intensities look as expected (upper middle)

Loading density good, but with room for more clusters (lower middle)

Quality scores along the read excellent (upper and lower right)

Sequencing QC

Intensities look as expected (upper middle)

Loading density good, but with room for more clusters (lower middle)

Quality scores along the read excellent (upper and lower right)

381M reads yield

2.84 reads per UMI (not oversequenced)

20743-001_1_S1_R1_001.fastq.gz	35,448,682
20743-002_1_S2_R1_001.fastq.gz	35,824,804
20743-003_1_S3_R1_001.fastq.gz	36,151,504
20743-004_1_S4_R1_001.fastq.gz	36,351,389
20743-005_1_S5_R1_001.fastq.gz	35,935,406
20743-006_1_S6_R1_001.fastq.gz	35,468,326
20743-007_1_S7_R1_001.fastq.gz	35,813,369
20743-008_1_S8_R1_001.fastq.gz	35,272,964
20743-009_1_S9_R1_001.fastq.gz	35,975,890
20743-010_1_S10_R1_001.fastq.gz	36,398,930
20743-011_1_S11_R1_001.fastq.gz	35,959,430
20743-012_1_S12_R1_001.fastq.gz	36,034,973
20743-013_1_S13_R1_001.fastq.gz	36,359,659
20743-014_1_S14_R1_001.fastq.gz	36,150,402
20743-015_1_S15_R1_001.fastq.gz	36,177,348
20743-016_1_S16_R1_001.fastq.gz	35,851,446
20743-017_1_S17_R1_001.fastq.gz	35,651,672
20743-018_1_S18_R1_001.fastq.gz	36,191,488
20743-019_1_S19_R1_001.fastq.gz	36,242,953
20743-020_1_S20_R1_001.fastq.gz	35,895,126
20743-021_1_S21_R1_001.fastq.gz	35,742,467
20743-022_1_S22_R1_001.fastq.gz	35,931,799
20743-023_1_S23_R1_001.fastq.gz	35,735,846
20743-024_1_S24_R1_001.fastq.gz	35,448,319

Sequencing QC

Intensities look as expected (upper middle)

Loading density good, but with room for more clusters (lower middle)

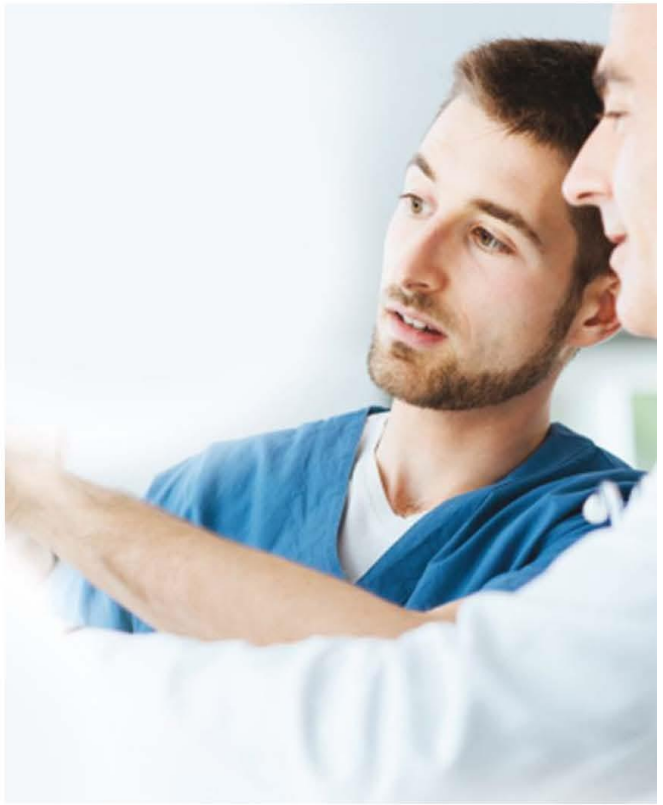
Quality scores along the read excellent (upper and lower right)

median 35.9M reads/sample, 35.2M reads min, 35.9M reads max

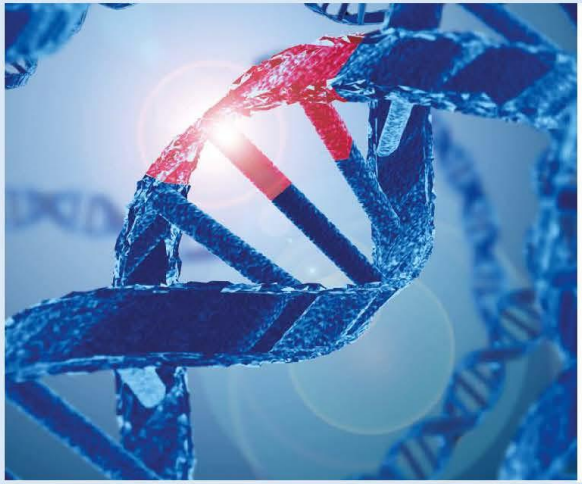
Conclusion

- EXCELLENT input material
- Library construction successful
- Sequencing worked within expected parameters

- 35.xM reads provided per sample



Contact information



Michael Gombert

QIAGEN Genomic Services
QIAGEN Str. 1
40724 Hilden
Germany

Phone: +49 2103 29 12632
Mail: Genomic.Services@QIAGEN.com

Appendix F R Scripts for RNA analysis (Chapter 9)

The transciptomics was performed as described in detail in chapter 9. The raw count matrix derived from the next-generation sequencing and genome alignment was analysed using *R* (R version 3.6.1 (2019-07-05) -- "Action of the Toes" Copyright (C) 2019 The R Foundation for Statistical Computing). The following is the scripts used, An electronic can also be found in the additional material.

F.1 Script for analysis of differentially expressed genes

/R_code_gene_expression.R

```
#Loading packages at the start.
library(EnhancedVolcano)
library(gplots)
library(RColorBrewer)
library(edgeR)
library (biomaRt)

#citations

citation ("EnhancedVolcano")
citation ("gplots")
citation ("amap")
citation ("dendextend")
citation ("RColorBrewer")
citation ("edgeR")
citation (package="biomaRt")


pheno<-read.table("Sample phenotype.csv", header=TRUE, row.names=1, sep=',')
counts<-read.table("Raw_Counts_All_Samples.csv", header=TRUE, sep=',', row.names=1,check.names =
FALSE)
#Reads in raw counts and the phenotype information

# Exploring the Raw data (QC)

# Merging Pheno and Count data together to keep only samples that we have both for and make sure that
the samples are in the same order within the files.
t_counts<-t(counts)
combined<-merge(pheno, t_counts,by="row.names", all=FALSE)
dim(t_counts)
dim(pheno)
dim(combined)

row.names(combined)<-combined$Row.names
combined<-combined[,-1]

save(combined,file="Counts_pheno_combined.RData")
```

```
########################################
#
#
# Starting from here read in combined (load above saved file)
# Data merged in "Counts_pheno_combined"
#
#
########################################

load("Counts_pheno_combined.RData")

values<-combined[,12:length(colnames(combined))] #takes gene counts in 'values'
info<-combined[,1:8] #takes phenotypic info into 'info'

# Boxplot
pdf("Raw_Boxplot_mRNA_RNA_Counts.pdf")
par(cex=0.3, mar=c(25,10,10,15))
boxplot(t(values), outline=FALSE, las=2, col="gray85", main="Raw Sample Counts mRNA Hits", cex.main=5,
label=row.names(values))
boxplot(t(values), outline=TRUE, las=2, col="gray85", main="Raw Sample Counts mRNA Hits", cex.main=5,
label=row.names(values))
#raw counts whiskers are 1.5xIQR
dev.off()


##### THERE ARE SOME LOWLY EXPRESSED SMALL RNAs
# Trying filtering out lowly expressed genes just to see if this changes the plots.
# Removing small RNAs that are not expressed in at least half of all samples (45)
values<-t(values)
Filter <-6 #limit of a gene being expressed in how many samples... random number to remove of v.v.lowly
expressed genes
#this is artbitary can be stricter or less strict.
# if filter is 6: 18040  - if 10: 17154 - if 2: 19251
keep_tags<-rowSums(values>1)>=Filter
values_filtered<-values[keep_tags,]
#save(values_filtered, file="Raw_Filtered_mRNA_All_Samples.RData")
dim(values)
#dim(values)
#[1] 30967    24
print (Filter)
dim(values_filtered)
#dim(values_filtered)
#[1] 18040   24
#### PLOTTING FILTERED SAMPLES
# Boxplot
pdf("Raw_Filtered_Boxplot_mRNA_Only_RNA_Counts.pdf")
par(cex=0.3,mar=c(25,10,10,15))
boxplot(values_filtered, outline=FALSE, las=2, col="gray85", main="Raw Filtered Sample Counts mRNA
Hits", cex.main=5)
#Same as last charts with very lowly expressed removed.
dev.off()


#Hierachical Clustering
values_filtered<-t(values_filtered)
```

```
distance_euc<-dist(values_filtered,method="euclidean")
#distance_pear<-dist(values_filtered,method="pearson")

hc_euc_av<-hclust(distance_euc, method="average")
hc_euc_comp<-hclust(distance_euc, method="complete")
#hc_pear_av<-hclust(distance_pear, method="average")
#hc_pear_comp<-hclust(distance_pear, method="complete")
hc_euc_ward.D<-hclust(distance_euc, method="ward.D")
#hc_pear_ward<-hclust(distance_pear, method="ward")



pdf("Clustering_Raw_mRNA_All_Data_Filtered.pdf", w=18)
par(cex=0.5)
plot(hc_euc_av, main="genes_filtered Euclidean Average", hang=-1)
plot(hc_euc_comp, main="genes_filtered Euclidean Complete", hang=-1)
#plot(hc_euc_ward, main="genes_filtered Euclidean Ward", hang=-1)
#plot(hc_pear_av, main="genes_filtered Pearson Average", hang=-1)
#plot(hc_pear_comp, main="genes_filtered Pearson Complete", hang=-1)
#plot(hc_pear_ward, main="genes_filtered Pearson Ward", hang=-1)
dev.off()

#PCA Analysis
#library(rgl)
pcs<-prcomp(values_filtered)
PC1<-pcs$x[,1]
PC2<-pcs$x[,2]
PC3<-pcs$x[,3]
PCA_details<-cbind(PC1,PC2,PC3)
pca<-merge(PCA_details,info,by="row.names",all=FALSE)

row.names(pca)<-pca$Row.names
pca<-pca[,-1]

PC1<-pca$PC1
PC2<-pca$PC2
PC3<-pca$PC3

plot(PC1,PC2,main="PCA Raw Counts RNA All Data")

pdf("PCA_Raw_Counts_mRNA_All_Data_Filtered.pdf")
#par(mfrow=c(3,2))
plot(PC1,PC2,main="PCA Raw Counts RNA All Data")
plot(PC1,PC2,main="PCA Raw Counts RNA All Data")
text(PC1,PC2,labels=row.names(pca),cex=0.3)
dev.off()

#IQR/Median Plotting
# Generating IQR and Median plots
# margin 1 = rows, 2 = columns
IQR_samples<- apply(values_filtered, 1, IQR)
Median_samples<- apply(values_filtered, 1, median)

SD_IQR<-sd(IQR_samples)
SD_Median<-sd(Median_samples)
Mean_IQR<-mean(IQR_samples)
Mean_Median<-mean(Median_samples)
```

```
SD_1_IQR<-c((Mean_IQR - SD_IQR), (Mean_IQR + SD_IQR))
SD_1_Median<-c((Mean_Median - SD_Median), (Mean_Median + SD_Median))

SD_2_IQR<-c((Mean_IQR - (2 * SD_IQR)), (Mean_IQR + (2 *SD_IQR)))
SD_2_Median<-c((Mean_Median - (2 * SD_Median)), (Mean_Median + (2 * SD_Median)))

pdf("IQR_RAW_mRNA_All_Samples_Filtered.pdf")
plot(Median_samples, IQR_samples, main="IQR Median Plot of the Raw Counts mRNA All Samples",
xlim=c(300,420),ylim=c(1000,1300))
points(SD_1_Median, SD_1_IQR, pch=3, col="firebrick1", cex=1)
points(SD_2_Median, SD_2_IQR, pch=3, col="firebrick3", cex=1)

plot(Median_samples, IQR_samples, main="IQR Median Plot of the Raw Counts mRNA All
Samples",xlim=c(300,420),ylim=c(1000,1300))
points(SD_1_Median, SD_1_IQR, pch=3, col="firebrick1", cex=1)
points(SD_2_Median, SD_2_IQR, pch=3, col="firebrick3", cex=1)

text(Median_samples,IQR_samples,labels=row.names(values_filtered),cex=0.3)


dev.off()

#### ONLY NEEDED IF REMOVING OUTLIERS

##### Outlier Removal:
#outliers<-c("SampleID_22.H.AN")
# Removing the outliers from filtered Matrix
#values_filtered<-t(values_filtered)
#values_filt_out_rem <- values_filtered[!row.names(values_filtered) %in% outliers,]
#info_out_rem<-info[!row.names(info) %in% outliers,]
#save(values_filt_out_rem,
file="./Data/Raw_Filtered_Out_Removed_mRNA_Only_RNA_All_Samples_Macrophages_Exosomes_Applie
d.RData")
#write.table(info_out_rem,
file="./Data/Phenotype_Data_Outliers_Removed_mRNA_Only_Final_Macrophages_Exosomes_Applied.csv"
, sep=',', col.names=NA)

##### PCA Plot to explore the variation of the datasets:
### ONLY NEED TO REDO PLOTS ONLY IF OUTLIERS ARE REMOVED
#pcs<-prcomp(values_filt_out_rem)
#PC1<-pcs$x[,1]
#PC2<-pcs$x[,2]
#PC3<-pcs$x[,3]
#PCA_details<-cbind(PC1,PC2,PC3)
#write.table(PCA_details, "./Data/PCA_Raw_Filtered_NEW_Outlier_Removed_mRNA_Only_RNA.csv",
sep=",")

# Add Demo Data for colouring.
#pca <-
read.table("./Data/PCA_Raw_Filtered_NEW_Outlier_Removed_mRNA_Only_RNA.csv",header=TRUE,
sep=",",row.names=1)
#PC1<-pca$PC1
#PC2<-pca$PC2
#PC3<-pca$PC3

### Plotting As A Continous Colour Palette Legend is Min and Max
# Optionally set colours using RColorBrewer
```

```
cols = brewer.pal(4, "Blues")
#To view the colorbrewer palletes in R:
#display.brewer.all(5)

# Define colour pallete
#pal = colorRampPalette(c("blue", "red"))
# Use the following line with RColorBrewer
pal = colorRampPalette(cols)

pdf("Colour_coded_for_known_variables.pdf")
par(mfrow=c(3,2))

for (i in 6:length(colnames(pca))){
  print(i)
  print(colnames(pca[i]))
  pca_current<-pca
  # Rank variable for colour assignment
  pca_current$order = findInterval(as.numeric(pca_current[,i]), sort(as.numeric(pca_current[,i])))

  # Make plot
  plot(PC1,PC2, pch=19, col=pal(nrow(pca_current))[pca_current$order], main=paste("PCA Coloured By
",colnames(pca[i]),sep=""))
  # Add a simple legend
  legend("topleft", col=pal(2), pch=19,
         legend=c(round(range(as.numeric(pca_current[,i])), 1)))
  rm(pca_current)
}

dev.off()


plot3d(PC1,PC2,PC3, xlab="PC1", ylab="PC2", zlab="PC3", box=FALSE, col=as.integer(pca$Condition),
type='s', size=1)
plot(PC1,PC2,main="PCA Raw Counts Small RNA All Data", col=as.factor(pca$Group))
text(PC1,PC2,label=pca$Donor)

pdf("PCA_2D_Raw_Filtered_Counts_mRNA_All_Data.pdf")
par(mfrow=c(2,2))

for (i in 6:length(colnames(pca))){
  print(i)
  print(colnames(pca[i]))
  plot(PC1,PC2, main=paste("PCA Coloured By ",colnames(pca[i]),sep=""), col=as.factor(pca[,i]))
}
dev.off()

pdf("PCA_2D_Raw_Filtered_Counts_mRNA_All_Data_TEXT.pdf")
for (i in 6:length(colnames(pca))){
  print(i)
  print(colnames(pca[i]))
  plot(PC1,PC2, main=paste("PCA Coloured By ",colnames(pca[i]),sep=""), col=as.factor(pca[,i]))
  text(PC1,PC2,labels=pca[,i],cex=0.8)

}
dev.off()
```

```
#### Variances explained by the Principal Components:
test<-data.frame(summary(pcs)$importance)
prop<-test[3,]

pdf("PCA_Variance_Explained_Filtered_Out_Rem_mRNA.pdf")
barplot(as.numeric(prop),names.arg = colnames(prop), las=2, cex.names=0.7, xlab="Principal Components",
ylab="Percentage of Variance Explained", main="PCA Percentage of Variance Explained")
dev.off()
write.table(prop, file="PCA_Variance_Explained_Filtered_Out_Rem_mRNA.csv", sep=',')


########################################
#
#
# Starting from here read in combined (load above saved file)
# Data merged in "Raw_Filtered-mRNA_All_samples.RData"
#
#
########################################

load("Raw_Filtered_mRNA_All_Samples.RData")
pheno<-read.table("Sample phenotype.csv",row.names=1, sep=',', header=TRUE)

#Save each analysis in its own subfolder - create the subfolder ID to access later
#These subfolders need to be created in the workingdir
#'subdir' is a vector of the four subdirectory names relative to the working directory
subdir <-c("./results_IRI_vs_SHAM/",
        "./results_RIC_vs_SHAM/",
        "./results_RIC+IRI_vs_IRI/",
        "./results_RIC+IRI_vs_SHAM/",
        "./results_RIC+IRI_vs_RIC/"
        )

values_filtered<-t(values_filtered)
combined<-merge(pheno, values_filtered, by="row.names",all=FALSE)
row.names(combined)<-combined$Row.names
combined<-combined[,-1]
values_filtered<-combined[,12:length(colnames(combined))]
pheno<-combined[,1:8]


row.names(values_filtered) == row.names(pheno)


#Set up the experimental Design
#Covariate Model

Condition<-as.factor(pheno$Group)
#OTHERFACTOR<-as.factor(pheno$factor)


design<-model.matrix(~0+Condition) #OTHERFACTOR: condition+OTHERFACTOR

#Read values (genes) into edgeR.
eds<-t(values_filtered)
eds_filtered<-DGEList(counts=eds, genes=rownames(eds))
```

```
#Creates rownames required by EdgeR

### NORMALIZE THE DATA
#Account for different library sizes etc.
eds_filtered<-calcNormFactors(eds_filtered,method="TMM")

#Calculating Differentially Expressed Genes:
eds_filtered<- estimateDisp(eds_filtered, design)

# Saving a normalized / filtered cpm matrix
counts.per.m <- cpm(eds_filtered, normalized.lib.sizes=TRUE)

#export Normalized counts table to a file
write.table(counts.per.m, file="filtered_matrix_counts_per_million.csv", sep = ",", col.names=NA)
#create the matricies of counts per million for the 2-way comparisons:
IRI<-counts.per.m[,1:6]
RIC<-counts.per.m[,7:12]
RICIRI<-counts.per.m[,13:18]
SHAM<-counts.per.m[,19:24]
IRIvsSHAM<-cbind(IRI,SHAM)
RICIRIvsIRI<-cbind(RICIRI,IRI)
RICvsSHAM<-cbind(RIC,SHAM)
RICIRIvsSHAM<-cbind(RICIRI,SHAM)
RICIRIvsRIC<-cbind(RICIRI,RIC)
rm(IRI)
rm(RIC)
rm(SHAM)
rm(RICIRI)


comp<-c("IRI_vs_SHAM",
      "RIC_vs_SHAM",
      "RIC_and_IRI_vs_IRI",
      "RIC_and_IRI_vs_SHAM",
      "RIC_and_IRI_vs_RIC"
) #Filenames for 5 groups in each of the subfolders

#for test runs of the loop:
#    IRI vs SHAM = 1
#    RIC vs SHAM = 2
#    RIC and IRI vs IRI = 3
#    RIC and IRI vs SHAM = 4
#    RIC and IRI vs RIC = 5

#write these five matricies to csv files
write.table(IRIvsSHAM, file=(paste0(subdir[1],"filtered_matrix_counts_per_million_",comp[1],".csv")), sep =
",", col.names=NA)
write.table(RICvsSHAM, file=(paste0(subdir[2],"filtered_matrix_counts_per_million_",comp[2],".csv")), sep
= ",", col.names=NA)
write.table(RICIRIvsIRI, file=(paste0(subdir[3],"filtered_matrix_counts_per_million_",comp[3],".csv")), sep =
",", col.names=NA)
write.table(RICIRIvsSHAM, file=(paste0(subdir[4],"filtered_matrix_counts_per_million_",comp[4],".csv")),
sep = ",", col.names=NA)
write.table(RICIRIvsRIC, file=(paste0(subdir[5],"filtered_matrix_counts_per_million_",comp[5],".csv")), sep
= ",", col.names=NA)
```

```
fit <- glmQLFit(eds_filtered, design)
#Changes column names
colnames(design)<-c("ConditionIRI","ConditionRIC","ConditionRICIRI","ConditionSHAM")

my.comparisons<-makeContrasts(
  IRI_vs_SHAM = ConditionIRI - ConditionSHAM,
  RIC_vs_SHAM = ConditionRIC - ConditionSHAM,
  RIC_and_IRI_vs_IRI = ConditionRICIRI - ConditionIRI,
  RIC_and_IRI_vs_SHAM = ConditionRICIRI - ConditionSHAM,
  RIC_and_IRI_vs_RIC = ConditionRICIRI - ConditionRIC,
  levels=design)

filteredresults<-paste0(subdir,"filtered_matrix_counts_per_million_",comp,".csv") #creates the vector to
open each the data for each comparison
readtbls<-paste0(subdir,comp,"_DE.csv") # this selects only differentially expressed genes for heatmap
readtbls_topx<-paste0(subdir,comp,"_t50d_t50U.csv") #this selects only top x of differentially expressed
genes for heatmap


#filteredresults<-
c("filtered_matrix_counts_per_million_IRI_vs_SHAM.csv","filtered_matrix_counts_per_million_RIC_vs_SHA
M.csv","filtered_matrix_counts_per_million_IRI_vs_IRIRIC.csv","filtered_matrix_counts_per_million_IRI_vs
_SHAM.csv")
#readtbls<-
c("IRI_vs_SHAM_DE.csv","RIC_vs_SHAM_DE.csv","RIC_and_IRI_vs_IRI_DE.csv","RIC_and_IRI_vs_SHAM_DE.
csv")

for (i in 1:5) {
   qlf <- glmQLFTest(fit, contrast=my.comparisons[,comp[i]])
   #Writing out all genes so that I can pull out log FC and convert to gene symbol for NPA
   all_genes<-as.data.frame(topTags(qlf, n=nrow(eds_filtered)))
   #Sort genes based on their significance and then pull the counts per million expressi n level data from
eds_filtered for this ordered list.
   all_results<-(all_genes)
   #Extract only the genes differentially expressed with an FDR corrected p-value <0.05
   DE_results<-all_results[all_results["FDR"]<=0.05,]
   #Nom_sig<-all_results[all_results["PValue"]<=0.01,]

   #Add gene names to DE_results file
   ensembl = useEnsembl(biomart="ensembl", dataset="rnorvegicus_gene_ensembl", mirror = "uswest") #
load rat genome
   mart <- useMart(biomart = "ensembl", dataset = "rnorvegicus_gene_ensembl") #mart
   gene.name.search.matrix <-as.matrix(row.names(DE_results))
   SearchList = gene.name.search.matrix

   gene.name<-getBM(attributes = c("ensembl_gene_id","external_gene_name"),
            filters = "ensembl_gene_id",
            values = SearchList,
            mart = ensembl)

   DE_results <- cbind(DE_results,gene.name)

   ### Save the differential testing - ALL genes and only the significant ones (miRNAs in this case).
   #write.table(Nom_sig, file="./Results/DEG_res_NOM_SIG_Block_Design_Tumour_Healthy.csv", sep=",",
col.names=NA)

   fn<-paste0 (subdir[i],comp[i],"_DE.csv")
```

```
  write.table(DE_results, file=fn, sep=",", col.names=NA)
  fn<-paste0 (subdir[i],comp[i],"_all_results.csv")
  write.table(all_results, file=fn, sep=",", col.names=NA)

## Volcano Plots

#colnames(all_results)
#Standard volcano
data<-all_results
data$Colour<-"black"
data$Colour[abs(data$logFC) > 1 & data$FDR < 0.05]="firebrick2"
data$Colour[abs(data$logFC) < 1 & data$FDR < 0.05]="darkorange1"
fnp<-paste0(subdir[i],comp[i],"_Volcano_plots.pdf")
pdf(fnp)
mtitle<-paste(comp[i],"volcano plot")
plot(data$logFC, -log2(data$FDR), ylim=c(0,20), xlab="Log Fold Change",
   ylab="Negative Log2 False Discovery Rate Corrected P Value", main=mtitle,
   col = data$Colour, pch=20)
legend("topleft", c("< 0.05 Q Val", "< 0.05 P Val and > 1logFC"), col=c("darkorange1", "firebrick2"),
pch=c(20,20))
dev.off()
#Enhanced volcano
fnp<-paste0(subdir[i],comp[i],"_Enhanced_Volcano_plots.pdf")
pdf(fnp)
mtitle<-paste(comp[i],"enhanced volcano plot")
EnhancedVolcano(data,
            lab=NA,
            x='logFC',
            y='FDR',
            ylim = c(0,10),
            title = mtitle,
            pCutoff= 0.05,
            FCcutoff=2,
            pointSize = 1.5,
            labSize = 1
)
dev.off()
#Reorder differential expression by logFC

fn<-paste0(subdir[i],comp[i], "_DE.csv")

dat<-read.csv(fn, header=T, sep=",") # opens differential expression file
#dat<-dat[,2:7] #removes duplicated column
dat2 <-dat[order(dat$logFC),] #reorders according to logFc
neg <- dat2$logFC <0 #downregulated genes only
pos <- dat2$logFC >0 #upregulated genes only
datneg <- dat2[neg,] #creates dataframe of downregulated genes (biggest change at the top)
datpos <- dat2[pos,] # creates dataframe of upregulated genes
datpos <-datpos[order(datpos$logFC,decreasing=TRUE),] #reorders upregulated so biggest change at the
top)
fnU<-paste0(subdir[i],comp[i], "_DE_UpReg.csv")
fnD<-paste0(subdir[i],comp[i], "_DE_DwnReg.csv")
fnC<-paste0(subdir[i],comp[i],"_t50D_t50U.csv") #change this to make more accurate

write.csv(datpos,fnU) #Up regulated genes
write.csv(datneg,fnD) #Down regulated genes
```

```
 #Selection of most DE gene subset.
 #Set the value of x for the number of pos AND neg genes (i.e. total = 2x)
 x <-25 #change this number for number of selected genes i.e. 50 most neg and 50 most pos changed
 #because there might not be enough down or up regulated genes:
 neg_count<-nrow(datneg)
 if (neg_count > x) {neg_count<-x}
 pos_count<-nrow(datpos)
 if (pos_count > x) [419]

 datcombined <-rbind(datpos[1:pos_count,],datneg[1:neg_count,]) #top x up and down regulated
 write.csv(datcombined,fnC) #Top x of both up and down regulated


 #Heatmaps

 c<-rep(1:500,each=1)
 cpms<-read.table(filteredresults[i], header=TRUE, row.names=1, sep=',')
 DeGene<-read.table(readtbls[i], header = TRUE, row.names = 1, sep=",")
 TopGene<-read.table(readtbls_topx[i],header=TRUE, row.names=2, sep=",")
 matrix<-as.matrix(cpms[row.names(cpms) %in% row.names(DeGene),]) #Takes the cpms for differentially
expressed genes
 gene.name.search.matrix <-as.matrix(row.names(matrix))
 SearchList = gene.name.search.matrix
 gene.name<-getBM(attributes = c("ensembl_gene_id","external_gene_name"),
          filters = "ensembl_gene_id",
          values = SearchList,
          mart = ensembl)
 row.names(matrix) <- gene.name[,2]

 matrix_topx<-as.matrix(cpms[row.names(cpms) %in% row.names(TopGene),]) #Takes the cpms for most
differntially expressed genes
 gene.name.search.matrix <-as.matrix(row.names(matrix_topx))
 SearchList = gene.name.search.matrix
 gene.name<-getBM(attributes = c("ensembl_gene_id","external_gene_name"),
          filters = "ensembl_gene_id",
          values = SearchList,
          mart = ensembl)
 row.names(matrix_topx) <- gene.name[,2]


 colour<-c(rep("dodgerblue1",6),rep("firebrick",6))
 htmpname<-paste0(subdir[i],"heatmap_of_DEGS_scaled_by_row_",comp[i],".pdf")
 pdf(htmpname)
 heatmap.2(matrix, col=bluered(100), trace="none",scale="row",dendrogram = "row", Colv = FALSE,
ColSideColors = colour, sepwidth=c(0.00001,0.00001),cexRow=0.5,cexCol=0.3) # Conv=false for no column
dendrogram reordering
 heatmap.2(matrix, col=bluered(100), trace="none",scale="row",Colv = TRUE, ColSideColors = colour,
sepwidth=c(0.00001,0.00001),cexRow=0.5,cexCol=0.3) # Conv=True column dendrogram reordered
#complete Eculidian clustering
 heatmap.2(matrix_topx, col=bluered(100), trace="none",scale="row",dendrogram = "row", Colv = FALSE,
ColSideColors = colour, sepwidth=c(0.00001,0.00001),cexRow=0.5,cexCol=0.3) # Conv=false for no column
dendrogram reordering
 heatmap.2(matrix_topx, col=bluered(100), trace="none",scale="row",Colv = TRUE, ColSideColors = colour,
sepwidth=c(0.00001,0.00001),cexRow=0.5,cexCol=0.3) # Conv=True column dendrogram reordered
#complete Eculidian clustering

 dev.off()
```

```
}
```

F.2 Script used to interrogate Ensembl Database for gene names

Ensembl is a genome browser for vertebrate genomes that supports research in comparative genomics, evolution, sequence variation and transcriptional regulation.[399] Ensembl annotate genes, computes multiple alignments, predicts regulatory function and collects disease data. Ensembl tools include BLAST, BLAT, BioMart and the Variant Effect Predictor (VEP) for all supported species

http://www.ensembl.org/index.html

/Gene_Names.R

```
#Gene names to Emsembl ID

library (biomaRt)

genes<-read.table("Genes_of_interest.csv", header=TRUE, sep=',',check.names = FALSE)

ensembl = useEnsembl(biomart="ensembl", dataset="rnorvegicus_gene_ensembl", mirror = "uswest") #
load rat genome
mart <- useMart(biomart = "ensembl", dataset = "rnorvegicus_gene_ensembl") #mart

#Emsembl search for gene -> gene ID
gene.code.search.matrix <-as.matrix(genes$`Ensemble name`)

SearchList <- gene.code.search.matrix

gene.code<-getBM(attributes = c("ensembl_gene_id","external_gene_name"),
          filters = "external_gene_name",
          values = SearchList,
          mart = ensembl)
write.csv(gene.code, "genes.csv")
```

F.3 Script used to for targeted analysis of differential expression

/Targeted_Gene_Expression_Comparison_(t-Tst).R

```
library(FSA)

data<-read.table("Gene Counts_IJ2.csv", sep=',', header=TRUE, row.names=1, check.names=FALSE)


group<-data[46,]
boxplot_data<-data[1:45,]
table(names(group) == colnames(boxplot_data))
```

```
#boxplots

pdf("./Boxplots_for_individual_genes (t-Tests).pdf")
tTsts_columnnames <-c ("Gene name",  "SHAM vs RIC, p =", "SHAM vs IRI p =","IRI vs RIC+IRI p =", "IRI vs
RIC+IRI p =")
tTsts_results_df <- tTsts_columnnames
par(mfrow=c(2,2))

for (i in row.names(boxplot_data)) {

  kdata <- as.numeric(boxplot_data[i,])
  SHAM <- kdata [1:6]
  RIC <- kdata [7:12]
  IRI <- kdata [13:18]
  RICIRI <-kdata [19:24]
  RvS <- t.test(RIC, SHAM, alternative = "two.sided", var.equal = FALSE)
  IvS <- t.test(IRI, SHAM, alternative = "two.sided", var.equal = FALSE)
  RIvI <- t.test(RICIRI, IRI, alternative = "two.sided", var.equal = FALSE)
  RIvR <- t.test(RICIRI, RIC, alternative = "two.sided", var.equal = FALSE)
  boxplot(as.numeric(boxplot_data[i,]) ~as.factor(data[46,]), col=c("green3","blue", "red","purple"),
outline=FALSE, main=paste(i), las=2,xlab="", ylab="cpm")

  tTsts_matrix <- (c(i, RvS$p.value, IvS$p.value, RIvI$p.value, RIvR$p.value))

  tTsts_results_df <- rbind(tTsts_results_df, tTsts_matrix)

  Comp1 <- paste0("RIC vs SHAM: p = ", round(RvS$p.value,4))
  Comp2 <- paste0("IRI vs SHAM: p = ", round(IvS$p.value,4))
  Comp3 <- paste0("RIC+IRI vs IRI: p = ", round(RIvI$p.value,4))
  Comp4 <- paste0("RIC+IRI vs RIC: p = ", round(RIvR$p.value,4))

  plot(NA, xlim=c(0,20), ylim=c(0,4), bty='n',
       xaxt='n', yaxt='n', xlab='', ylab='')
  #text(1,4,kwtext, pos=4, cex=1)
  text(1,3.5,Comp1, pos=4, cex=1)
  text(1,3,Comp2, pos=4, cex=1)
  text(1,2.5,Comp3, pos=4, cex=1)
  text(1,2,Comp4, pos=4, cex=1)
}
dev.off()

write.csv (tTsts_results_df, "./t_Test_results_for_individual_genes.csv", row.names = FALSE)
```

F.4 Data file (.csv)

/Gene counts_IJ2.csv

Name	297	993	460	701	674	205	518	251	971	307	329	949	17	38	240	244	159	783	535	711	976	266	790	18
Fibroblast	653.0882	490.2029	632.7467	618.9959	508.9152	568.4983	331.1843	491.4265	412.2919	388.2881	382.5533	386.1339	751.3512	648.8764	884.0953	789.083	562.2473	762.0806	902.419	578.9005	461.5819	508.3544	507.8702	1048.729
Pafah1b3	22.06223	20.20501	19.54663	19.53217	19.01708	19.69902	18.34097	17.52273	15.80636	17.94364	22.11631	17.19011	17.26801	17.907	19.16951	17.84813	20.74081	15.63533	17.86892	16.07621	23.33668	18.69984	19.5734	16.61316
Pdgfra	195.2103	187.1663	184.9071	173.817	166.3426	163.0573	175.1116	202.6245	191.2683	186.9162	227.0411	161.7915	170.2133	121.0872	181.5789	183.6012	132.8963	173.1623	179.4627	158.4879	177.9662	108.2082	139.3211	205.2149
Ptafr (Plat	30.84092	27.76261	30.38796	32.02503	30.45763	24.96025	25.90225	26.42773	28.99727	23.6019	26.12075	29.1929	22.27882	27.85118	24.45056	21.54101	24.08873	17.98253	19.95749	20.07566	25.18209	24.89511	18.95813	21.65973
alpha smc	303.2498	202.4743	232.4639	271.1298	213.0898	235.2055	157.6625	228.0469	195.2862	167.1123	182.6983	161.7915	475.9881	436.7962	540.8272	581.2482	359.0446	420.564	380.5847	289.0974	203.3406	336.293	246.648	454.4491
B Cells: Cd	0.731557	2.891939	1.773303	8.277001	0.189413	3.507486	2.248998	1.903084	5.420405	3.216679	7.972157	3.824232	1.040706	0.560762	8.813176	6.641811	2.613015	9.048051	2.823444	0.431313	5.613107	8.969841	1.345912	2.615375
B Cells: M	0.269521	1.002539	1.088163	2.70743	0.265178	1.46825	1.318378	1.292661	2.956585	1.937759	4.041185	1.817457	0.385447	0.411225	3.566421	2.764429	1.184022	3.52079	1.121642	0.078421	2.575878	2.964609	0.730638	0.736725
Cell toxici	18.39273	15.43579	4.409243	5.000507	12.84719	8.940889	7.253262	7.618891	12.67294	17.15667	5.944599	19.81196	5.682389	8.401666	43.69234	16.16803	21.46546	19.76411	8.783102	18.68473	6.841404	10.88266	4.715043	1.989159
Cell toxici	1.578624	2.776261	1.652396	1.199005	1.43954	2.16159	2.248998	1.256753	1.667817	2.906638	1.800164	1.135911	8.210014	2.878578	1.508871	13.42723	4.368634	2.990778	3.867731	4.077869	5.95912	4.446913	2.461096	1.989159
Endotheli	26.02804	44.92145	56.02025	10.09485	28.52562	40.33609	29.08187	22.58565	21.45419	28.79509	28.98632	19.19689	32.99424	26.50535	16.11748	16.29936	29.68222	29.15062	25.75909	34.26978	41.40627	22.69066	23.99569	23.57521
Fibroblast	18.78947	16.8118	14.79096	16.05119	16.17588	14.80485	12.75725	17.02003	14.89664	16.19966	16.01779	15.03188	17.19092	15.70134	23.04457	21.64871	16.57631	20.02686	18.56511	16.82121	11.99513	15.43117	15.92022	19.22853
Fibroblast	228.7849	193.6442	218.882	268.1516	193.4666	216.2814	185.7362	242.7688	203.2083	194.5897	206.7617	214.6871	303.1153	317.4287	398.5133	397.6829	314.6642	352.9118	281.8802	261.7286	198.381	286.8829	254.9542	356.2068
Hypoxia: F	39.77362	45.61551	45.17892	38.90964	41.63301	44.21064	49.67184	47.03848	44.99315	47.51384	48.49423	40.70346	43.59402	40.56179	35.76709	48.61087	48.21829	50.91895	50.89934	52.81624	46.98012	43.13886	52.68284	40.22521
Hypoxia: F	144.5018	143.363	210.2573	135.6422	126.3628	173.539	124.9745	114.5082	109.3178	135.6819	126.085	111.0542	302.3058	194.3227	181.6817	244.6699	258.3619	206.2501	176.8713	162.8011	159.3584	200.3391	164.5089	169.3732
Hypoxia: \	97.87467	77.96667	97.97498	164.3023	105.9956	142.0532	80.61495	83.37661	77.78092	100.2984	84.0934	86.25348	273.6671	213.0522	166.9017	282.5103	223.8619	226.4663	122.259	150.1754	164.241	195.7782	147.5504	152.5758
Immune c	64.26154	53.36591	73.59207	72.63648	42.16336	58.56686	54.44126	49.55199	65.99248	61.13217	72.44743	58.34794	70.07421	42.35623	83.77661	114.8854	64.87626	112.3245	75.65282	49.2089	74.316	58.5302	44.60737	59.82211
Macropha	5.082398	11.1436	13.70279	10.1722	7.728056	7.34125	13.53276	18.09725	19.52104	16.43219	12.34398	15.44838	7.51621	2.80381	10.93931	8.9446	7.063306	8.442323	12.106	6.626537	6.804931	5.891209	6.383468	6.114821
Macropha	661.0968	602.3716	605.6232	669.8569	661.2034	689.792	594.86	509.2724	659.5079	568.5771	581.9307	650.4981	581.9474	622.1842	557.219	514.507	732.2975	582.9746	614.8145	632.1089	620.7481	673.0802	607.6216	458.7221
Macropha	6.738028	5.012694	6.770793	9.476005	6.743107	5.220445	4.420444	4.703848	5.951074	6.045807	6.318944	5.225189	7.631844	6.579608	7.51006	8.113779	6.940821	6.360136	7.580753	3.999448	6.497363	6.765389	6.075831	6.888383
Macropha	28.91577	8.637257	14.79096	23.36125	17.76695	12.23542	4.110238	6.140138	4.927641	8.9912	9.588631	5.868871	29.48667	20.52389	12.75681	31.09085	18.45442	20.67044	12.64748	15.72332	18.80006	18.20574	19.07349	14.43982
Macropha	25.75852	32.54395	43.00259	41.84913	28.94233	28.75323	27.87982	34.2196	37.07102	37.90256	36.29719	30.89677	35.49964	16.33687	27.53689	35.57856	24.9053	25.81912	32.02481	21.84012	26.48925	18.39578	17.22767	27.25884
NEC: Kit	43.70093	46.50238	46.79101	31.71561	38.75393	34.34074	47.34529	51.096	50.94423	47.78513	54.44579	39.71901	65.0634	32.15036	37.27596	73.67024	42.1757	61.93561	52.63982	51.40467	55.51593	32.30663	44.41509	45.27178
NEC: Myd	52.40261	51.55363	55.37541	46.10367	41.2163	53.26485	51.6494	46.75122	42.79467	53.83093	46.51037	45.92865	77.82168	75.77764	56.61694	77.0809	68.87744	59.24769	57.04903	54.26702	62.78221	64.00514	56.5283	63.57941
NEC: Nfkb	117.5497	72.56838	131.0229	112.281	65.30965	97.9649	65.02706	70.05502	58.37359	86.50154	69.43491	61.49063	292.0144	181.014	130.7916	303.7641	200.1406	199.625	144.9626	103.4759	111.5317	148.4585	90.32992	164.3634
NEC: Tlr2	17.94241	6.362265	16.76577	10.01749	3.182141	7.178111	2.920429	2.944394	2.46382	6.007051	4.33509	3.748505	17.692	19.47713	9.087516	25.27478	17.55619	17.11179	8.315622	6.548116	7.227836	11.59238	5.076011	11.19823
NEC: Tlr4	6.89204	5.20549	4.916885	5.956346	4.394385	4.445535	5.234737	6.283767	6.974507	3.836762	5.65766	5.75528	3.931556	4.187023	2.537646	4.415906	2.694672	2.990778	2.01122	3.058402	3.537026	3.762772	2.653369	5.267587
Neutroph	108.6555	127.1682	129.2093	136.2611	119.7849	135.4461	133.8929	130.7023	143.7734	125.7218	119.6558	135.7792	51.99676	61.12306	66.18455	41.43054	93.007	68.52289	95.26221	88.06628	122.1812	85.93564	91.06056	56.10164
NK Cells: I	0.077006	0.077118	0.040302	0.038678	0	0.122354	0.193879	0.035907	0.037905	0	0.146952	0.037864	0.115634	0.074768	0.102878	0	0.151432	0.038677	0.156841	0.076892	0.038008	0	0	0
NK Cells: I	37.34793	40.91129	38.52903	33.76552	39.58734	37.84822	47.53916	54.04039	49.57965	43.36704	47.5023	47.10243	42.51477	39.06642	55.34812	46.92349	41.6041	44.52095	50.82198	54.65913	38.90729	41.65655	46.76083	50.39202
RIC: Map2	89.90455	99.0971	111.839	113.7121	109.4429	111.2607	108.2621	91.59937	96.73338	90.06702	102.94	93.86408	81.48343	88.89947	62.48096	79.77353	95.94665	77.19236	93.05761	79.94975	96.69153	84.07326	87.86882	72.7148
RIC: Map2	341.2522	316.301	338.3381	354.9828	371.3255	365.7982	300.3576	320.4721	304.3008	326.8999	306.6525	331.1558	224.6769	292.1196	254.1075	200.9776	268.5689	204.2437	342.2555	244.7898	289.1519	261.3036	293.5626	211.4034
RIC: Mapk	122.2856	122.3869	123.4461	110.6952	121.1486	120.111	117.3357	114.3286	117.8843	125.9543	123.8072	109.994	103.6852	109.9467	95.98474	86.45124	105.3372	94.00129	104.5061	97.2807	112.4544	115.8098	96.8672	88.5544
RIC: Pik3c	29.06978	40.02443	36.1512	31.05809	31.32893	31.20031	40.83095	38.06167	38.28398	38.05758	39.971	35.81905	19.81196	21.38373	17.52347	19.85363	27.68163	24.15337	26.08851	26.78062	30.33384	24.51503	26.45678	19.74424
RIC: Prkca	108.2705	87.45223	115.4662	93.71576	106.9048	90.50113	106.1294	83.66387	101.282	98.01183	86.03977	97.46113	64.06124	71.92708	72.66584	59.16597	67.40762	65.4564	88.26162	82.06711	68.47221	84.98545	75.79407	56.50684
TCells: Cd	31.8805	34.16944	31.0731	50.89969	48.60841	51.02169	26.9492	24.52464	30.17232	26.39227	29.75782	28.66281	52.18948	41.08516	53.56491	62.0881	52.46444	49.06391	30.90317	31.83874	45.866	57.99991	29.64852	41.44081
TCells: Cd	8.54767	11.37496	12.45342	9.940136	7.841704	11.46051	10.27559	12.81888	10.765	9.727548	13.2257	10.37465	12.6812	6.542224	14.43715	12.38608	12.24851	12.72027	14.92944	8.626261	12.22581	8.627771	9.80593	9.835285
TCells: Cd	3.003235	3.894478	4.916885	2.900818	1.969897	3.181208	3.295945	2.08262	2.729155	3.100414	3.343162	2.650458	7.554755	6.430071	11.48799	13.21182	6.695851	10.18379	6.111015	5.136546	4.72885	8.969841	5.691285	8.288161
TCells: Cd	6.699525	11.45208	13.66249	4.796019	4.773211	10.35932	10.77968	8.58183	7.960035	12.32414	10.94794	7.572737	9.096541	6.392687	9.567611	13.06821	8.818926	13.74244	16.05108	9.645728	8.496552	5.169061	9.113746	7.625108
TCells: Itg	15.97875	23.01983	24.98745	10.90708	16.66836	19.73981	20.43486	18.06134	16.79188	25.65592	24.83492	17.34157	29.48667	13.64521	20.02683	30.40872	21.63903	23.24478	24.05729	22.23223	18.4156	11.51636	24.14951	19.81791
Type 1 cyt	30.03235	35.59013	30.9925	34.84849	40.26923	39.60197	36.1003	34.68639	36.76778	38.48388	33.61532	34.00159	77.97586	60.03892	53.08481	93.23666	52.87273	56.82479	41.26869	39.52396	50.1335	47.88983	48.41437	61.29556
Type 1 cyt	1642.693	1782.128	1661.061	2348.464	1923.642	1800.238	1710.441	1532.7	1715.691	1523.427	1590.574	1824.424	2646.284	2186.561	2437.272	2795.161	2450.314	1867.987	1737.617	1470.817	1867.012	2534.018	1796.869	1624.701
Type 2 cyt	377.7531	397.3524	433.8547	364.3427	423.7551	484.7672	391.5583	337.564	350.7722	382.3973	389.8274	350.0119	813.1768	775.9077	675.2196	900.9936	688.5703	574.6837	407.8909	461.0344	523.5952	622.3778	563.5525	493.5692
Type 2 cyt	417.4112	328.5242	363.1241	725.2045	463.153	364.4523	310.9821	330.311	356.3822	317.9862	287.2915	391.5105	708.9521	626.2216	797.2666	803.5155	556.6539	505.0251	435.6225	362.1853	484.7264	720.3619	500.91	569.0467
i-FABP	941.9378	678.0247	589.5426	2810.428	1336.423	718.9123	493.5775	538.2495	662.1991	523.8149	378.5121	691.5802	282.4939	468.498	361.0659	196.0232	416.5309	268.4886	547.4	410.8061	470.386	716.181	412.6566	239.7673
Group	1:SHAM	1:SHAM	1:SHAM	1:SHAM	1:SHAM	1:SHAM	1:SHAM	2:RIC	2:RIC	2:RIC	2:RIC	2:RIC	2:RIC	3:IRI	3:IRI	3:IRI	3:IRI	3:IRI	4:IRI+RIC	4:IRI+RIC	4:IRI+RIC	4:IRI+RIC	4:IRI+RIC	4:IRI+RIC

Appendix G Lists of differentially expressed genes, fold change and p-values from RNA analysis (Chapter 9)

G.1 Ischaemia reperfusion injury vs SHAM

Over 6000 genes showed differential expression. For this reason, the 200 with the greatest statistical significance (Table G - 1) and the 200 with the greatest fold-change in expression are reported below. (Table G - 2). The complete datasets are included in the additional material.

	Gene	LogFC	FDR		Gene	LogFC	FDR		Gene	LogFC	FDR
1	Elac1	2.17643	1.86E-09	71	Mfap3	1.696538	1.16E-06	141	Abca5	2.577279	3.46E-06
2	Pigv	1.727601	1.49E-08	72	Ptgs2	-0.73053	1.16E-06	142	Zbtb18	2.980444	3.46E-06
3	Rnf19b	1.317808	1.49E-08	73	Socs1	-0.71162	1.17E-06	143	RGD156396	1.031053	3.47E-06
4	Phyhipl	1.856258	1.97E-08	74	Sox9	2.861273	1.25E-06	144	Dglucy	2.306498	3.63E-06
5	Ddx50	1.178725	2.75E-08	75	Rap1gap2	1.488197	1.25E-06	145	Ptprr	0.893686	3.63E-06
6	Cep85l	1.840383	2.75E-08	76	Lamc2	0.753596	1.25E-06	146	Commd5	2.559542	3.73E-06
7	Rxrb	2.036708	3.22E-08	77	Mtmr1	3.327457	1.25E-06	147	Myc	1.781125	3.73E-06
8	B3galt4	1.869033	3.51E-08	78	Areg	0.925762	1.25E-06	148	Cacnb1	2.05801	3.81E-06
9	Ppard	1.470781	5.17E-08	79	Ereg	1.068621	1.26E-06	149	Il1a	0.894257	3.91E-06
10	Srsf3	2.170614	5.56E-08	80	Cxcl2	-0.63328	1.30E-06	150	Rnf145	-0.61356	4.00E-06
11	Cdkn1a	4.509469	5.56E-08	81	Cxcl1	0.892171	1.30E-06	151	Rnd3	-1.54079	4.03E-06
12	Arid5b	5.057314	5.56E-08	82	Rlim	1.272234	1.44E-06	152	Slc34a2	1.323124	4.13E-06
13	Zfp365	1.777873	5.56E-08	83	Klhl20	-0.51589	1.47E-06	153	Thbd	-0.77875	4.22E-06
14	Egr2	2.361699	7.10E-08	84	Mnt	1.598721	1.54E-06	154	Mapkapk2	0.8372	4.22E-06
15	Tmem119	1.884074	7.62E-08	85	Plekha6	1.87881	1.54E-06	155	Stac2	-0.6015	4.22E-06
16	Znrd1	2.102177	9.13E-08	86	Adora2b	1.239885	1.61E-06	156	Arf2	-1.48787	4.35E-06
17	Man1a1	2.99078	1.17E-07	87	Uap1	1.589169	1.61E-06	157	Sema6d	1.701224	4.36E-06
18	Hsf2	2.417614	1.17E-07	88	Ppl	2.203609	1.61E-06	158	Dyrk3	1.476212	4.49E-06
19	RGD130295	1.454789	1.27E-07	89	Socs3	1.222389	1.61E-06	159	Map4k5	1.679052	4.60E-06
20	Ppp1r18	2.374408	1.27E-07	90	Itpkb	1.106516	1.70E-06	160	Cdkn2aipnl	0.871662	4.67E-06
21	Ier3	2.046304	1.27E-07	91	Plcd3	1.867119	1.81E-06	161	Mia2	0.835305	4.70E-06
22	Gpank1	2.397634	1.33E-07	92	Tmem39a	-1.42904	1.81E-06	162	Rprm	1.916396	4.70E-06
23	Rabgef1	3.223347	1.33E-07	93	Arhgap31	1.348174	1.84E-06	163	Tfap4	3.071683	4.75E-06
24	Hsph1	1.526775	2.02E-07	94	Acbd4	1.459117	1.86E-06	164	Azin1	-0.62025	4.80E-06
25	Mcoln1	1.779176	2.02E-07	95	B4galt4	0.611723	1.86E-06	165	Rassf5	0.966829	4.89E-06
26	Pnpla6	1.21738	2.02E-07	96	Mat2b	1.486083	1.94E-06	166	Dhrs7b	2.204222	4.91E-06
27	Ptcd1	4.468139	2.02E-07	97	Pcp4l1	-1.10148	2.07E-06	167	Gna15	0.878588	4.91E-06
28	Tsc22d1	2.540983	2.02E-07	98	Trim16	1.634532	2.07E-06	168	Rassf3	7.541044	4.91E-06
29	Ddx55	0.830175	2.02E-07	99	Cant1	-0.73775	2.07E-06	169	Ngfr	0.580567	4.91E-06
30	Snrnp35	2.041079	2.06E-07	100	Wdr81	1.631054	2.07E-06	170	Clint1	1.3243	4.91E-06
31	RGD156348	2.788684	2.36E-07	101	Sde2	3.149626	2.07E-06	171	Odc1	0.816954	4.97E-06
32	Mphosph9	3.247179	2.48E-07	102	Mpzl1	4.616436	2.07E-06	172	Hpcal1	1.181946	5.12E-06
33	Stard13	1.947567	2.85E-07	103	Btg2	2.467452	2.07E-06	173	Adamts8	1.567928	5.12E-06
34	Wsb2	3.296561	2.92E-07	104	Pmp22	4.377986	2.07E-06	174	Brinp2	1.891085	5.12E-06
35	Taok3	0.92114	2.92E-07	105	Rasd1	1.71599	2.09E-06	175	Nr4a2	1.475957	5.12E-06
36	Hnf1a	0.795183	2.97E-07	106	Hs3st3b1	1.532241	2.12E-06	176	Gadd45a	-0.47596	5.17E-06
37	Sik1	2.524787	3.10E-07	107	Zfp330	0.81507	2.17E-06	177	Fam110c	0.483206	5.24E-06
38	Cstb	1.385274	3.15E-07	108	Prkca	-0.77292	2.18E-06	178	Art4	0.801748	5.24E-06
39	Itgb2	2.136755	3.22E-07	109	Rufy1	1.980563	2.25E-06	179	Arfgef1	1.085113	5.26E-06
40	Ppp1cc	1.209684	3.22E-07	110	Adamts4	-0.98552	2.29E-06	180	Birc3	1.172781	5.26E-06
41	Tmem184a	1.075936	3.24E-07	111	Tnfrsf12a	1.546103	2.29E-06	181	Tafa4	0.996227	5.54E-06
42	Mafk	2.091602	3.32E-07	112	Pou2f1	1.894494	2.29E-06	182	Pde4b	1.212536	5.67E-06
43	Prmt2	2.169816	3.32E-07	113	Zfp354a	8.435065	2.30E-06	183	Nr4a3	-0.80445	5.68E-06
44	Zfp113	1.518035	3.56E-07	114	Rgs2	-3.06066	2.33E-06	184	Irak4	-0.56665	5.75E-06
45	Cyp3a62	0.896573	3.57E-07	115	Prox1	3.005981	2.33E-06	185	Oxsm	1.422091	5.75E-06
46	Ywhag	0.939512	4.27E-07	116	Atf3	1.260581	2.34E-06	186	Ctnna2	-0.69447	5.78E-06
47	Nup153	3.13598	4.35E-07	117	Angel2	1.268782	2.37E-06	187	Phc2	0.968337	5.87E-06
48	Castor2	2.242782	4.35E-07	118	Gpr65	0.882227	2.39E-06	188	Tubgcp6	1.023451	5.90E-06
49	Adamts1	1.790636	4.35E-07	119	Sat1	-0.68743	2.39E-06	189	Snap25	0.995182	5.94E-06
50	Ripk4	2.807838	4.37E-07	120	Rgs1	0.834731	2.39E-06	190	Hk2	0.93757	5.95E-06
51	Zbtb21	2.812989	4.37E-07	121	Tmco1	-0.55069	2.41E-06	191	Ngly1	2.268313	6.12E-06
52	Ets2	-1.01364	4.75E-07	122	AABR07020	3.038716	2.55E-06	192	Nipal4	0.661908	6.12E-06
53	Vps26c	1.172741	5.05E-07	123	Gosr1	1.023002	2.55E-06	193	Slc38a2	0.956273	6.18E-06
54	Fam43a	3.501506	5.54E-07	124	Dusp1	1.72971	2.57E-06	194	Ptges	0.908027	6.22E-06
55	Heg1	1.548363	6.26E-07	125	Tmem19	1.239253	2.59E-06	195	Peli1	1.215211	6.22E-06
56	Rcan1	1.000743	6.42E-07	126	Adcy3	-1.45855	2.78E-06	196	Elovl5	-0.76315	6.22E-06
57	Dnajc28	1.783099	6.71E-07	127	Dusp10	-1.0125	2.78E-06	197	Saysd1	-0.62016	6.24E-06
58	Lrrc8c	2.400895	6.71E-07	128	Phlda1	1.665842	2.80E-06	198	Tbc1d14	1.907548	6.27E-06
59	Cds1	-0.87519	6.71E-07	129	Snx12	1.739933	2.84E-06	199	H3f3a	-0.81015	6.27E-06
60	Exoc1	2.545935	7.04E-07	130	Baiap2	-0.8284	2.88E-06	200	Htr3a	2.42644	6.29E-06
61	Pkd2	1.633756	7.04E-07	131	Trib1	1.236753	3.05E-06				
62	Nmu	3.617471	7.04E-07	132	Ubtd2	1.514977	3.11E-06				
63	Tbc1d1	2.148026	7.04E-07	133	Ndel1	0.961019	3.13E-06				
64	Rell1	1.147372	7.75E-07	134	Cdk17	1.261865	3.15E-06				
65	Adcy5	1.172993	8.35E-07	135	Slc35e4	-0.7303	3.25E-06				
66	Slc49a4	2.006955	8.44E-07	136	Btg1	0.719352	3.25E-06				
67	Nup54	1.688668	8.94E-07	137	Gtf2a1	0.988682	3.38E-06				
68	Hnrnpdl	0.926804	9.37E-07	138	Timp3	0.973175	3.41E-06				

Table G - 1 Ischaemia-reperfusion injury vs SHAM, differentially expressed genes. (200 with lowerest corrected p-values)

1	Rbm38	-9.45746	1.55E-02	71	Mtmr1	3.327457	1.25E-06	141	Ndor1	2.630037	1.43E-05
2	Zfp354a	8.435065	2.30E-06	72	Rassf1	3.321232	6.43E-05	142	Zbtb41	2.623175	1.61E-05
3	Rassf3	7.541044	4.91E-06	73	Wsb2	3.296561	2.92E-07	143	Tmem86b	-2.60595	1.08E-04
4	AC130970.1	5.882656	2.27E-03	74	Cadm2	3.285679	5.45E-03	144	Cdkl5	2.601925	9.87E-04
5	Prrg2	-5.77212	4.72E-03	75	Nr4a1	3.283438	7.46E-06	145	Lamtor5	2.586081	1.65E-03
6	Adamts9	5.676753	1.86E-03	76	Ccdc181	3.271309	1.69E-04	146	Unc45a	-2.57805	2.81E-04
7	Mfn1	-5.47227	3.63E-03	77	Synj2	3.267144	3.73E-04	147	Abca5	2.577279	3.46E-06
8	Tmem37	-5.30195	1.25E-02	78	AABR07028	3.25927	4.56E-05	148	Popdc2	2.568125	2.70E-02
9	Map2k1	5.232036	1.25E-03	79	Mphosph9	3.247179	2.48E-07	149	Kmt2c	2.565067	2.74E-02
10	AABR07035	5.191964	1.35E-04	80	Rabgef1	3.223347	1.33E-07	150	Commd5	2.559542	3.73E-06
11	Ina	5.080146	4.56E-04	81	Tmem87b	-3.20564	3.63E-02	151	Meltf	-2.55496	9.15E-04
12	Arid5b	5.057314	5.56E-08	82	Opa1	3.196092	2.84E-03	152	Akap12	2.547067	5.25E-05
13	AABR07071	4.953074	6.89E-04	83	Swap70	3.192848	1.24E-05	153	Atp10d	2.546757	9.57E-07
14	Tmem80	4.932277	4.26E-05	84	Aasdh	-3.19186	2.88E-03	154	Exoc1	2.545935	7.04E-07
15	Trpm4	4.816162	2.11E-02	85	Atp5f1c	-3.15027	3.71E-02	155	Tsc22d1	2.540983	2.02E-07
16	Nrxn3	4.768465	5.83E-03	86	Sde2	3.149626	2.07E-06	156	Nol11	2.539732	4.65E-02
17	Dnpep	-4.64562	1.73E-03	87	Nup153	3.13598	4.35E-07	157	Abcc10	2.539209	3.70E-02
18	Glt8d2	4.619089	4.20E-02	88	Tfap4	3.071683	4.75E-06	158	Rab9a	2.534784	4.10E-02
19	Map1lc3b	-4.61765	4.38E-03	89	Rgs2	-3.06066	2.33E-06	159	Ap5b1	2.529183	5.43E-04
20	Mpzl1	4.616436	2.07E-06	90	Cldn3	3.060101	1.14E-04	160	Ciao3	-2.52581	2.04E-02
21	Prcp	-4.57634	3.54E-03	91	Eef1a2	3.055643	3.77E-03	161	Sik1	2.524787	3.10E-07
22	Ptpmt1	4.56663	3.41E-03	92	AABR07050	3.053782	4.69E-02	162	Dbt	2.508597	2.72E-05
23	Vwa7	4.557482	1.53E-04	93	AABR07020	3.038716	2.55E-06	163	Psd3	2.481056	1.44E-03
24	Hagh	-4.51089	3.50E-02	94	S100a16	3.027711	1.78E-02	164	Spata13	-2.47585	1.82E-02
25	Cdkn1a	4.509469	5.56E-08	95	Tm7sf3	3.025336	1.41E-02	165	Ercc3	2.475846	2.92E-04
26	Ptcd1	4.468139	2.02E-07	96	Ppil4	3.018281	3.23E-05	166	Hunk	-2.46854	6.97E-03
27	Schip1	4.40305	1.13E-05	97	Prox1	3.005981	2.33E-06	167	Btg2	2.467452	2.07E-06
28	Pmp22	4.377986	2.07E-06	98	Dscaml1	3.005006	3.58E-05	168	Stx11	2.448588	2.68E-05
29	Nat8f4	4.344325	1.32E-02	99	Chd1	2.99919	2.50E-05	169	Plpp6	2.447701	3.38E-04
30	Gmpr	-4.34295	4.31E-03	100	Bdkrb2	2.993835	1.16E-04	170	Csnk1e	2.444078	2.86E-04
31	Cx3cl1	4.265021	3.62E-04	101	Man1a1	2.99078	1.17E-07	171	Mboat2	2.442972	4.95E-02
32	AY172581.9	4.21891	5.89E-04	102	Skiv2l	2.988772	2.70E-03	172	Map3k7cl	2.442501	2.87E-02
33	Galnt12	4.168881	1.60E-02	103	Zbtb18	2.980444	3.46E-06	173	AC091481.3	-2.44004	1.50E-04
34	Letmd1	4.137936	2.11E-03	104	Gdnf	2.979274	2.09E-05	174	Htr3a	2.42644	6.29E-06
35	Nell1	-4.13579	1.50E-03	105	Cdc42bpg	2.961052	2.03E-03	175	Stx7	2.426349	9.28E-03
36	AABR07028	-4.12877	1.36E-02	106	Sfi1	2.946021	4.52E-05	176	Klrb1b	2.423465	6.94E-06
37	Nudt18	4.070589	1.84E-05	107	Ppp2r2c	2.939511	2.56E-03	177	RGD130606	2.421431	5.63E-03
38	Stc1	-4.01191	2.72E-05	108	Ankrd22	-2.93831	4.31E-04	178	Hsf2	2.417614	1.17E-07
39	Dock5	-3.97546	3.94E-02	109	Thap6	2.928907	1.33E-02	179	Acvr2a	2.411255	7.65E-03
40	Tiparp	3.924836	1.65E-05	110	Trim36	2.926538	3.77E-05	180	Tmem127	2.406897	4.93E-03
41	Klf6	-3.92132	3.82E-05	111	Dock6	2.925111	1.73E-02	181	Vegfa	2.406629	5.25E-05
42	Stx4	-3.89033	2.02E-02	112	Gaa	-2.9104	2.55E-02	182	Lrrc8c	2.400895	6.71E-07
43	Smim24	3.887851	7.11E-04	113	Nefm	2.907452	3.00E-04	183	Gpank1	2.397634	1.33E-07
44	Pak7	-3.82189	3.15E-03	114	Zfp133	2.897487	5.57E-04	184	Sh2b3	-2.38529	1.15E-02
45	Rilp	3.818027	3.01E-02	115	Tpp1	2.878409	4.31E-04	185	Pgf	2.381036	1.86E-04
46	Baiap2l1	3.804165	2.84E-02	116	Plekhg4	2.86476	4.18E-03	186	Mob3a	-2.37733	2.01E-02
47	Hist1h2bo	3.767879	1.30E-02	117	Sox9	2.861273	1.25E-06	187	Emc7	-2.37512	1.54E-02
48	Tnr	3.761706	2.89E-03	118	Gjb2	-2.85789	3.25E-02	188	Ppp1r18	2.374408	1.27E-07
49	Casp7	-3.75107	6.25E-03	119	Fbxw8	2.84346	6.74E-03	189	Arvcf	-2.37078	2.89E-02
50	Hs6st1	3.68687	3.49E-02	120	Rhot2	-2.81434	1.04E-02	190	Egr2	2.361699	7.10E-08
51	Cage1	3.68138	2.24E-02	121	Zbtb21	2.812989	4.37E-07	191	Cebpb	2.358975	1.37E-04
52	Disp3	3.6668	2.29E-02	122	Tra2b	2.811383	2.88E-02	192	LOC100911	2.352617	5.91E-03
53	Nmu	3.617471	7.04E-07	123	Ripk4	2.807838	4.37E-07	193	Pla2g12b	2.34894	6.61E-04
54	Il6st	3.590611	9.00E-03	124	AABR07073	-2.79201	2.70E-02	194	Ippk	2.347928	3.45E-04
55	Fbxl7	-3.57606	2.24E-02	125	RGD156348	2.788684	2.36E-07	195	Mthfd2	2.339707	3.59E-03
56	Moxd1	-3.57575	2.99E-05	126	Abcg5	-2.78575	3.15E-03	196	Pnkp	2.337314	2.09E-02
57	Rprd2	3.541542	2.49E-03	127	Dynlt3	-2.78341	2.98E-03	197	AABR07044	2.333689	8.58E-04
58	Dnah12	-3.53227	6.32E-03	128	Rnps1	2.761915	3.33E-03	198	AABR07050	2.333593	4.72E-02
59	Med31	-3.52878	9.09E-03	129	Sphk1	2.743825	1.44E-05	199	Lsm10	-2.3182	1.98E-03
60	B4galt5	-3.51935	8.16E-03	130	Fgf2	2.736237	1.59E-03	200	AABR07043	2.314747	4.85E-02
61	Tango2	-3.50967	9.07E-05	131	Cttn	-2.73309	2.60E-02				
62	Fam43a	3.501506	5.54E-07	132	LOC685203	2.715982	2.25E-03				
63	Psen2	3.493733	1.70E-04	133	Zfp385d	2.711562	1.85E-02				
64	Mfsd9	3.464116	4.90E-04	134	Slc38a1	-2.71002	3.09E-02				
65	Myh14	3.406141	1.76E-03	135	Ctnnal1	2.703451	1.27E-03				
66	RGD130622	-3.40098	9.79E-03	136	Tldc2	2.684346	3.13E-02				
67	Atg14	3.387723	2.74E-04	137	Mrpl50	2.676929	2.64E-02				
68	Cdh5	3.376356	8.85E-03	138	Ccdc158	-2.6623	2.88E-03				
69	Phpt1	3.369095	9.53E-03	139	Cebpg	2.640664	1.08E-02				

Table G - 2 Ischaemia-reperfusion injury vs SHAM, differentially expressed genes. (200 with greatest fold change (stat. sig. only.)

G.2 Remote ischaemic conditioning vs SHAM

Over 800 genes showed differential expression. For this reason, the 200 with the greatest statistical significance (**Table** G - 3) and the 200 with the greatest fold-change in expression are reported below. (Table G - 6). The complete datasets are included in the additional material.

	Gene	LogFC	FDR		Gene	LogFC	FDR		Gene	LogFC	FDR
1	Tmcc2	-1.60466	0.007948	71	Serpinb2	0.816314	0.013307	141	Crebbp	-0.7219	0.019052
2	Crp	-2.83052	0.008598	72	Ugdh	-2.33252	0.014397	142	Azin1	0.976827	0.019052
3	Tmed5	-0.64042	0.008598	73	Utp18	-0.84689	0.014397	143	Nol10	0.575792	0.019052
4	Pigv	-3.46436	0.008598	74	Hgd	-0.28387	0.014397	144	Fjx1	-0.48555	0.019052
5	Dnajb5	1.474968	0.008598	75	Cxcl2	-0.77709	0.014397	145	Pwp1	-0.40037	0.019052
6	Plxdc2	-1.0524	0.008598	76	Selp	1.029977	0.014397	146	Odc1	-0.2965	0.019052
7	Csf2rb	1.288028	0.008598	77	Cxcl1	-0.59385	0.014521	147	Rbm28	-0.86452	0.019052
8	Ccl7	-1.00437	0.008598	78	Gfpt2	-1.75454	0.014897	148	Grm3	-0.3874	0.019204
9	Fam13c	0.897808	0.008598	79	LOC24906	-0.40907	0.014897	149	Pno1	-0.42308	0.019204
10	Sesn1	-1.05143	0.008598	80	Wfikkn2	-0.53514	0.014897	150	Phospho1	0.464499	0.019204
11	Sec63	-3.24919	0.008598	81	Cxcl6	-1.68547	0.014897	151	Adamts8	0.621567	0.019204
12	Numa1	-5.09896	0.008598	82	Gfap	0.853229	0.014897	152	Zbtb44	-0.8648	0.019204
13	Col11a2	1.139432	0.008598	83	Uap1	-1.09931	0.014897	153	Ar	-2.8665	0.019204
14	Rxrb	-2.32047	0.008598	84	Socs3	-0.47841	0.014897	154	Ndufa5	0.777788	0.019204
15	Ppa1	-2.28351	0.008598	85	Ndp	-0.38648	0.01499	155	Cpsf2	-0.90209	0.019204
16	Sgpl1	-0.60694	0.008598	86	Ttc19	-1.17079	0.015134	156	Stx17	-0.2757	0.019204
17	Pcbd1	0.741574	0.008598	87	Tsr1	0.941297	0.015136	157	Lyz2	-2.30789	0.019204
18	Cdk19	-0.31508	0.008598	88	Bst1	-0.87588	0.015682	158	Il1rn	0.956807	0.019204
19	Rpf2	1.194135	0.008598	89	Zfp496	-1.34697	0.015682	159	Pde4b	0.532748	0.019204
20	Rhobtb1	0.485832	0.008598	90	Helz	-0.56744	0.015682	160	Nr4a3	-1.35726	0.019351
21	Tpst2	-2.3755	0.009563	91	Mnda	-0.53432	0.015682	161	Gpr176	0.45007	0.019362
22	Cmklr1	-1.04635	0.009816	92	Gosr2	-0.67913	0.016188	162	Cd44	-0.53929	0.019448
23	Abcf1	0.57537	0.009816	93	Lrrc59	-0.48119	0.016349	163	Lztfl1	-0.39768	0.019448
24	Ier3	-1.41487	0.009816	94	Adamts4	-2.42432	0.016349	164	Zbp1	1.349442	0.019448
25	Slc7a1	-0.85971	0.009816	95	Tnfrsf12a	-1.82938	0.016349	165	Grpel1	-1.30295	0.019562
26	Slc15a4	-1.03618	0.009816	96	Atp5pd	0.712578	0.016349	166	Thrb	-0.78812	0.019562
27	Zfp358	0.699281	0.009816	97	Hnrnpab	-1.10377	0.016349	167	Fabp1	1.155041	0.019562
28	Bud31	1.463603	0.009816	98	Ufc1	0.858696	0.016349	168	Snu13	1.194017	0.019562
29	Rilpl2	-0.60233	0.009816	99	Atf3	-0.40545	0.016349	169	Hdac11	0.990464	0.019562
30	Cers4	1.193191	0.009816	100	Rgs1	-0.82038	0.016591	170	Mrpl19	0.699883	0.019562
31	Arl6ip4	-0.45357	0.009816	101	Uck2	0.386631	0.016591	171	Ano6	-0.31055	0.019562
32	Sppl3	-1.1991	0.009816	102	Cd55	0.487938	0.016591	172	Timm17a	0.227785	0.019732
33	Oasl	-0.99881	0.009816	103	Psmc1	0.668068	0.016591	173	Rraga	-0.49834	0.019732
34	Cstb	-0.36394	0.010015	104	Spata7	-2.4429	0.016591	174	Plin2	1.673176	0.019732
35	Rrp1	-1.11993	0.010015	105	Tnpo2	0.723115	0.016591	175	Gpn2	-1.24724	0.019773
36	Zcchc8	3.34358	0.010015	106	Pfn1	2.234915	0.016986	176	Xpot	-0.45394	0.019773
37	Eif3b	-2.04549	0.010015	107	Pik3r6	0.683451	0.016986	177	Strap	-1.47951	0.019773
38	Pcnt	0.475073	0.010015	108	Chmp6	-0.37711	0.016986	178	Anxa7	-0.35732	0.019773
39	Pdgfa	-1.32647	0.010015	109	Phlda1	-2.66257	0.017252	179	Ccl2	-0.56129	0.019773
40	Naa25	1.046524	0.010015	110	Tmem94	-0.38165	0.017252	180	Yars1	-1.7622	0.019773
41	Serpine1	1.115246	0.01027	111	Pms1	-0.25849	0.017252	181	Cdkn1b	-0.42966	0.019773
42	Vgf	-0.85684	0.01027	112	Tcaim	0.695648	0.017443	182	Ttc9	0.85598	0.019773
43	Rasa4	0.367603	0.01027	113	Trib1	2.869476	0.017443	183	Necab1	-1.23868	0.019773
44	Tmem120a	-0.80904	0.010327	114	Glrx5	-1.03875	0.017499	184	Atp1a2	-0.42614	0.019773
45	Psmb1	-1.17579	0.010381	115	Ptrh2	0.599539	0.018352	185	Ascc2	0.764686	0.01995
46	Tfg	-0.35847	0.010381	116	Dcaf13	0.615712	0.018393	186	Rnf144a	-0.52941	0.01995
47	Kcnj15	-0.56601	0.01074	117	Vipr2	1.841465	0.019052	187	Zfp691	0.999468	0.01995
48	Opa1	-1.61316	0.01074	118	Deptor	1.579497	0.019052	188	Zfp41	-3.40671	0.019966
49	Ppp1r2	0.690118	0.011562	119	Chrdl1	1.088018	0.019052	189	Ndufaf4	-0.66877	0.020157
50	Eif4g1	0.583668	0.011749	120	Slc25a32	-6.24469	0.019052	190	Bmf	-0.65721	0.020157
51	Tm4sf19	-0.61593	0.012038	121	Map2k6	-0.80063	0.019052	191	Hoxb9	0.645843	0.020157
52	Tfrc	-0.87064	0.012038	122	Ppargc1a	1.07136	0.019052	192	Ehd4	-1.41871	0.020157
53	Tnk2	-0.76695	0.012038	123	Ublcp1	1.082306	0.019052	193	Tcp11l2	-0.47435	0.020157
54	Sspn	-2.30232	0.012038	124	Bdkrb1	0.779841	0.019052	194	Rnf43	-0.40997	0.020157
55	Masp1	0.328201	0.012038	125	Scin	-3.25215	0.019052	195	Hoxb7	-0.32037	0.020157
56	Ube2v2	-2.99936	0.012038	126	Myc	1.681409	0.019052	196	Cacnb4	0.788024	0.020157
57	Ergic2	0.462755	0.012038	127	Desi2	-0.38622	0.019052	197	Smu1	0.547282	0.020157
58	Dgcr6	0.62185	0.012038	128	RGD156368	-0.99968	0.019052	198	Gria3	1.258749	0.020157
59	Ugt2a3	-0.53369	0.012143	129	Pbx1	-0.39815	0.019052	199	Usp20	-0.76995	0.020157
60	Tmem50b	-0.51545	0.012143	130	Chp1	-3.16311	0.019052	200	Zmynd19	-0.98072	0.020157
61	Evi5	-0.83958	0.012143	131	Cytip	0.36128	0.019052				
62	Fras1	-2.07162	0.012143	132	Arf2	0.448583	0.019052				
63	Urb1	-1.99273	0.012143	133	Sos2	0.952228	0.019052				
64	Lrrc8c	-0.59303	0.012663	134	Mpped2	-0.43074	0.019052				
65	Fryl	-1.36556	0.012663	135	Pa2g4	-3.0179	0.019052				
66	Hnrnpd	-0.56796	0.012683	136	Fbxl20	-0.50342	0.019052				
67	Morf4l2	1.308821	0.012753	137	Cebpz	-0.79291	0.019052				
68	Eprs	-0.91816	0.012966	138	Slc12a6	-0.39893	0.019052				

Table G - 3 Remote ischaemic conditioning vs SHAM, differentially expressed genes. (200 with lowerest corrected p-values)

	Gene	LogFC	FDR		Gene	LogFC	FDR		Gene	LogFC	FDR
1	Slc25a32	-6.24469	0.019052	71	Tnfrsf21	-2.02055	0.024224	141	Arf5	1.288571	0.020157
2	AABR0702	-5.18396	0.039828	72	Urb1	-1.99273	0.012143	142	Csf2rb	1.288028	0.008598
3	Numa1	-5.09896	0.008598	73	Nop14	-1.9926	0.025414	143	LOC10091	1.287218	0.048147
4	Fzd2	-5.01463	0.03669	74	Slc25a25	-1.9807	0.027165	144	Gria3	1.258749	0.020157
5	Cd3g	-3.74787	0.02972	75	Rab28	-1.95561	0.031593	145	Nolc1	1.255884	0.033722
6	LOC68975	-3.64927	0.043307	76	Ubxn2b	-1.94211	0.020582	146	Lilrb4	-1.25321	0.039828
7	Chuk	3.593205	0.036918	77	Psma4	-1.89942	0.026966	147	Gpn2	-1.24724	0.019773
8	Epb42	-3.53547	0.02473	78	Zdhhc1	-1.89262	0.031537	148	Necab1	-1.23868	0.019773
9	Pigv	-3.46436	0.008598	79	Yif1a	-1.85243	0.035341	149	Cnot11	-1.23083	0.037482
10	Ngp	-3.45758	0.037634	80	Bcl2a1	1.845386	0.045514	150	Pard6g	1.225924	0.048635
11	Fgd5	-3.45504	0.021415	81	Vipr2	1.841465	0.019052	151	Ppif	1.225851	0.021863
12	Cap1	-3.44732	0.026936	82	Tnfrsf12a	-1.82938	0.016349	152	Fam204a	-1.21237	0.020755
13	Bola3	-3.43994	0.03669	83	Ncoa2	-1.82223	0.020245	153	Sppl3	-1.1991	0.009816
14	Zfp41	-3.40671	0.019966	84	Olr35	-1.79226	0.041347	154	Rpf2	1.194135	0.008598
15	Rere	-3.3661	0.032286	85	Pla2g4e	-1.78582	0.037847	155	Snu13	1.194017	0.019562
16	Zcchc8	3.34358	0.010015	86	Yars1	-1.7622	0.019773	156	Cers4	1.193191	0.009816
17	Scin	-3.25215	0.019052	87	RT1-M2	-1.7617	0.045876	157	Psmb1	-1.17579	0.010381
18	Sec63	-3.24919	0.008598	88	Gfpt2	-1.75454	0.014897	158	Ttc19	-1.17079	0.015134
19	Chp1	-3.16311	0.019052	89	Dusp6	-1.74198	0.037634	159	Ltv1	1.169508	0.028882
20	Slco2b1	3.123587	0.032286	90	Mmp8	-1.73317	0.020755	160	Fabp1	1.155041	0.019562
21	Anxa13	-3.10868	0.020245	91	LOC10369	-1.68746	0.048799	161	Psma3l	-1.154	0.020245
22	Peli2	-3.092	0.025648	92	Cxcl6	-1.68547	0.014897	162	Coq10b	-1.14996	0.027351
23	Ndrg2	-3.06104	0.021415	93	Myc	1.681409	0.019052	163	Heatr3	-1.14541	0.029261
24	Pa2g4	-3.0179	0.019052	94	Fpr1	-1.67885	0.024195	164	Col11a2	1.139432	0.008598
25	Cnppd1	-3.01205	0.033224	95	Abcc5	-1.67748	0.040093	165	Cul2	1.131425	0.029261
26	Ube2v2	-2.99936	0.012038	96	Plin2	1.673176	0.019732	166	Srp54a	-1.12826	0.042156
27	LOC10091	-2.95753	0.045514	97	Fbn2	-1.66031	0.043882	167	Tbrg4	-1.12433	0.047043
28	Ccl17	-2.89661	0.030387	98	AABR0703	-1.6591	0.046389	168	AABR0703	-1.12115	0.048799
29	Pgp	-2.89561	0.02064	99	Slc2a12	-1.65148	0.023966	169	Aldh1a7	1.120599	0.032286
30	Plcg1	-2.89048	0.046389	100	Crocc	-1.63677	0.020245	170	Rrp1	-1.11993	0.010015
31	Trib1	2.869476	0.017443	101	Hoxc5	1.633584	0.030738	171	Serpine1	1.115246	0.01027
32	Ar	-2.8665	0.019204	102	Mxd4	-1.63076	0.028498	172	Slc35e2b	-1.11114	0.031608
33	Rpl7l1	-2.84868	0.030387	103	Khdrbs1	1.627419	0.044592	173	Lrp8	-1.10919	0.026771
34	Crp	-2.83052	0.008598	104	Tubb4b	-1.62016	0.021243	174	Mycbp	-1.10573	0.032069
35	AABR0703	2.824782	0.049703	105	Opa1	-1.61316	0.01074	175	AABR0706	-1.10561	0.045648
36	Ctdsp2	2.805202	0.045448	106	Polr3e	-1.60842	0.031537	176	Hnrnpab	-1.10377	0.016349
37	Map3k8	-2.7615	0.030387	107	Tmcc2	-1.60466	0.007948	177	Wdr97	-1.10195	0.042415
38	Tpm4	-2.75128	0.029261	108	Tcea3	-1.58646	0.024942	178	Uap1	-1.09931	0.014897
39	Wfdc2	-2.67766	0.028138	109	Eif4e	1.580712	0.046852	179	Dhx29	1.092511	0.021415
40	Tomm20	-2.67087	0.035145	110	Deptor	1.579497	0.019052	180	Chrdl1	1.088018	0.019052
41	Phlda1	-2.66257	0.017252	111	Dcun1d5	-1.52932	0.020245	181	Sphk1	-1.08704	0.022956
42	Akap17b	-2.64533	0.020755	112	Pla2g7	-1.52459	0.038454	182	Eef1akmt4	-1.08568	0.04022
43	Ripor3	-2.56943	0.023177	113	Zfp532	-1.51563	0.031872	183	Ublcp1	1.082306	0.019052
44	Hspa14	-2.54684	0.028827	114	Arhgef18	-1.50228	0.040016	184	Ppargc1a	1.07136	0.019052
45	Rpp38	-2.48935	0.043578	115	Dpep3	-1.498	0.035036	185	Slc17a2	-1.06923	0.032069
46	Spata7	-2.4429	0.016591	116	Strap	-1.47951	0.019773	186	Trmt6	-1.06754	0.035449
47	Kiz	2.42999	0.038012	117	Sod2	-1.47528	0.033794	187	Sec23b	1.066737	0.020245
48	Adamts4	-2.42432	0.016349	118	Dnajb5	1.474968	0.008598	188	Guca2b	-1.06581	0.020582
49	Tpst2	-2.3755	0.009563	119	Map1lc3a	-1.471	0.038273	189	RGD13115	-1.0658	0.038978
50	Sgk2	-2.36319	0.042473	120	Bud31	1.463603	0.009816	190	Plxdc2	-1.0524	0.008598
51	Ugdh	-2.33252	0.014397	121	Gdpd1	1.431825	0.049703	191	Sesn1	-1.05143	0.008598
52	Rxrb	-2.32047	0.008598	122	Ehd4	-1.41871	0.020157	192	LOC10091	1.050809	0.045514
53	Cyp26b1	-2.31953	0.028498	123	Ier3	-1.41487	0.009816	193	Smo	-1.04858	0.020245
54	Lyz2	-2.30789	0.019204	124	Pik3ip1	-1.41138	0.033733	194	Naa25	1.046524	0.010015
55	Sspn	-2.30232	0.012038	125	Ulk1	-1.38859	0.04353	195	Cmklr1	-1.04635	0.009816
56	LOC68923	-2.29807	0.042156	126	Pgam5	1.376741	0.043477	196	Glrx5	-1.03875	0.017499
57	Ctu2	2.284427	0.046389	127	Fryl	-1.36556	0.012663	197	Slc15a4	-1.03618	0.009816
58	Ppa1	-2.28351	0.008598	128	Nr4a3	-1.35726	0.019351	198	AABR0705	-1.03117	0.03669
59	Snx18	-2.25082	0.023905	129	Zbp1	1.349442	0.019448	199	Selp	1.029977	0.014397
60	Pfn1	2.234915	0.016986	130	Zfp496	-1.34697	0.015682	200	Slc10a3	-1.02583	0.049703
61	Zfp39	-2.20407	0.028001	131	Rubcn	-1.32761	0.04947				
62	Iqsec2	2.141694	0.049111	132	Pdgfa	-1.32647	0.010015				
63	Tfdp2	-2.12906	0.024195	133	Stn1	-1.31943	0.035449				
64	Emilin2	-2.11321	0.028351	134	Ptx3	1.314483	0.025414				
65	Insr	2.110614	0.040453	135	Morf4l2	1.308821	0.012753				
66	Chd6	-2.08587	0.030936	136	Polr2d	1.304547	0.02984				
67	Fras1	-2.07162	0.012143	137	Grpel1	-1.30295	0.019562				
68	Inha	-2.05752	0.035341	138	Capns1	-1.29644	0.043882				

Table G - 4 Remote ischaemic conditioning vs SHAM, differentially expressed genes. (200 with greatest fold change (stat. sig. only.)

G.3 Remote ischaemic conditioning and ischaemia reperfusion injury vs ischaemia reperfusion injury alone

The differentially expressed genes in this analysis are presented by lowest p-value (Table G - 5) and greatest fold change (Table G - 6). The complete datasets are included in the additional material.

Table G - 5 Ischaemia-reperfusion injury + remote ischaemic conditioning vs ischaemia-reperfusion injury, differentially expressed genes. (order by lowerest corrected p-values)

	Gene	LogFC	FDR		Gene	LogFC	FDR
1	Rnf19b	-1.1509	0.013525	71	Arhgef3	-0.47007	0.040327
2	Rabgef1	-1.49639	0.013525	72	Coq10b	-1.20453	0.040327
3	Tsc22d1	-1.25752	0.014013	73	S1pr3	-1.31827	0.040327
4	Ets2	-0.58584	0.014013	74	Ifitm3	-0.6236	0.040327
5	Lrrc8c	-1.08023	0.016462	75	Gem	-0.77664	0.041194
6	Nmu	-0.5791	0.016462	76	Tgif1	-0.78968	0.041194
7	Mfap3	-1.23666	0.016462	77	Pdp1	-0.80755	0.041194
8	Flt4	-0.71916	0.016462	78	Ipp	-0.67598	0.041804
9	Ugdh	-0.71264	0.016462	79	Map3k8	-0.75824	0.042433
10	Cxcl1	-0.73582	0.016462	80	Enc1	-0.67996	0.042669
11	Bmerb1	-0.71124	0.016462	81	Plppr4	-1.94046	0.042669
12	Pcp4l1	-0.78617	0.016462	82	Zfp652	-0.60495	0.042669
13	Btg2	-0.99358	0.016462	83	Rnf214	-0.85132	0.042669
14	Adamts4	-1.06986	0.018664	84	Syt4	-0.92757	0.042669
15	Cd55	-0.56804	0.020411	85	Itih3	-0.55502	0.042669
16	Trib1	-1.31366	0.020411	86	Stk25	-0.59753	0.043396
17	Commd5	-0.64919	0.020843	87	Bok	-0.88085	0.043591
18	Ngfr	0.675728	0.020843	88	Vip	1.641125	0.043591
19	Adamts8	-0.9328	0.02288	89	Pfkfb3	-0.74986	0.043591
20	Birc3	-0.47237	0.025208	90	Sod2	-0.98012	0.043591
21	Pde4b	-1.17434	0.026514	91	Nfkb2	-0.49015	0.043961
22	Ptges	-0.98134	0.026994	92	Procr	-1.34504	0.044383
23	Htr3a	-0.72811	0.026994	93	Smim3	-0.87396	0.044383
24	Scn9a	-1.22387	0.026994	94	Akap12	-0.45748	0.044383
25	Rdh10	-0.72757	0.026994	95	Lmna	-0.8307	0.044383
26	Mmp19	-0.93577	0.026994	96	Nfkbie	0.462819	0.044458
27	Nrdc	-0.52751	0.026994	97	Th	-0.39327	0.044458
28	Tac1	0.527713	0.026994	98	Coq8b	-0.52121	0.045352
29	Nfkbia	-0.90983	0.028591	99	AABR0702	-0.5921	0.045492
30	Brd3	-0.54672	0.028591	100	Cadm4	-0.80501	0.045492
31	Sema7a	-0.70069	0.031478	101	Pwwp3a	-0.4027	0.045492
32	Amer1	-1.22165	0.031478	102	Lrrd1	-1.67306	0.045492
33	Jdp2	-1.41978	0.031888	103	Lypd8	-0.63394	0.045492
34	RGD15633	0.392442	0.031888	104	Usp36	-1.60342	0.045566
35	Fli1	-1.46852	0.031888	105	Tceal8	0.645753	0.045566
36	Ets1	-1.12489	0.031888	106	Nfkbiz	-0.79103	0.045566
37	Penk	-0.8265	0.03249	107	Scn11a	-0.93852	0.045566
38	Malsu1	-0.63484	0.032836	108	Relb	-0.32418	0.045566
39	Zc3h12a	-1.38056	0.033916	109	Acer3	-0.64572	0.045566
40	Gnl2	-0.63195	0.033916	110	Kdm6b	-1.43678	0.045566
41	Hcn4	-0.79341	0.033916	111	Ctla2a	-0.66992	0.045566
42	Snai1	0.299532	0.034972	112	Ms4a18	3.335701	0.045566
43	Arih1	-0.85413	0.036712	113	Arrdc3	-0.34889	0.045566
44	Spry2	-0.73446	0.036712	114	Icos	-1.51527	0.045566
45	Timp1	-1.08748	0.036933	115	Krt18	-0.44685	0.045566
46	Slc34a3	-0.88717	0.036933	116	Tcim	-0.85017	0.045566
47	Mmp7	-1.62232	0.038373	117	Kcne4	0.813913	0.045566
48	Tfpi2	1.073292	0.038977	118	Tnfaip3	-0.5739	0.045566
49	Plau	0.506001	0.039144	119	Glul	-0.63216	0.045566
50	Birc2	-3.08967	0.040327	120	Gstm5	-1.05623	0.045566
51	Elmsan1	-0.92208	0.040327	121	Slc6a17	0.302439	0.045566
52	Noct	-1.71534	0.040327	122	Bod1l1	0.30224	0.045566
53	Ipo5	0.398426	0.040327	123	LOC10091(	-0.68261	0.045566
54	Calcb	-0.62897	0.040327	124	Ifrd1	-0.8579	0.045566
55	Zbtb41	-0.94917	0.040327	125	Azin2	-1.22982	0.045566
56	Tiparp	0.401806	0.040327	126	Faim2	-0.64994	0.045566
57	Prag1	-0.75859	0.040327	127	RGD15646	-1.27982	0.045566
58	LOC10090	-0.59884	0.040327	128	Ttf2	-1.29497	0.045566
59	Gldc	0.494114	0.040327	129	Zfp36	-0.65613	0.045566
60	F3	-1.10019	0.040327	130	Slc66a2	-0.72455	0.046365
61	Epor	-2.07416	0.040327	131	Ier5	-0.51366	0.047677
62	Bin1	-0.68043	0.040327	132	AABR0704	-0.36097	0.048539
63	Tmem184	-1.24346	0.040327	133	Dll1	-0.76357	0.048746
64	Fgl2	-0.57937	0.040327	134	LOC10091	0.312427	0.048746
65	Abca7	-0.82235	0.040327	135	AC111884	-0.81902	0.04931
66	Mat2a	-0.831	0.040327				
67	Ctrc	0.353223	0.040327				
68	Igsf10	-1.24847	0.040327				

Table G - 6 Ischaemia-reperfusion injury + remote ischaemic conditioning vs ischaemia-reperfusion injury, differentially expressed genes. (order by greatest fold-change)

	Gene	LogFC	FDR		Gene	LogFC	FDR
1	Ms4a18	3.335701	0.045566	71	Pfkfb3	-0.74986	0.043591
2	Birc2	-3.08967	0.040327	72	Cxcl1	-0.73582	0.016462
3	Epor	-2.07416	0.040327	73	Spry2	-0.73446	0.036712
4	Plppr4	-1.94046	0.042669	74	Htr3a	-0.72811	0.026994
5	Noct	-1.71534	0.040327	75	Rdh10	-0.72757	0.026994
6	Lrrd1	-1.67306	0.045492	76	Slc66a2	-0.72455	0.046365
7	Vip	1.641125	0.043591	77	Flt4	-0.71916	0.016462
8	Mmp7	-1.62232	0.038373	78	Ugdh	-0.71264	0.016462
9	Usp36	-1.60342	0.045566	79	Bmerb1	-0.71124	0.016462
10	Icos	-1.51527	0.045566	80	Sema7a	-0.70069	0.031478
11	Rabgef1	-1.49639	0.013525	81	LOC100910	-0.68261	0.045566
12	Fli1	-1.46852	0.031888	82	Bin1	-0.68043	0.040327
13	Kdm6b	-1.43678	0.045566	83	Enc1	-0.67996	0.042669
14	Jdp2	-1.41978	0.031888	84	Ipp	-0.67598	0.041804
15	Zc3h12a	-1.38056	0.033916	85	Ngfr	0.675728	0.020843
16	Procr	-1.34504	0.044383	86	Ctla2a	-0.66992	0.045566
17	S1pr3	-1.31827	0.040327	87	Zfp36	-0.65613	0.045566
18	Trib1	-1.31366	0.020411	88	Faim2	-0.64994	0.045566
19	Ttf2	-1.29497	0.045566	89	Commd5	-0.64919	0.020843
20	RGD15646	-1.27982	0.045566	90	Tceal8	0.645753	0.045566
21	Tsc22d1	-1.25752	0.014013	91	Acer3	-0.64572	0.045566
22	Igsf10	-1.24847	0.040327	92	Malsu1	-0.63484	0.032836
23	Tmem184	-1.24346	0.040327	93	Lypd8	-0.63394	0.045492
24	Mfap3	-1.23666	0.016462	94	Glul	-0.63216	0.045566
25	Azin2	-1.22982	0.045566	95	Gnl2	-0.63195	0.033916
26	Scn9a	-1.22387	0.026994	96	Calcb	-0.62897	0.040327
27	Amer1	-1.22165	0.031478	97	Ifitm3	-0.6236	0.040327
28	Coq10b	-1.20453	0.040327	98	Zfp652	-0.60495	0.042669
29	Pde4b	-1.17434	0.026514	99	LOC100909	-0.59884	0.040327
30	Rnf19b	-1.1509	0.013525	100	Stk25	-0.59753	0.043396
31	Ets1	-1.12489	0.031888	101	AABR0702	-0.5921	0.045492
32	F3	-1.10019	0.040327	102	Ets2	-0.58584	0.014013
33	Timp1	-1.08748	0.036933	103	Fgl2	-0.57937	0.040327
34	Lrrc8c	-1.08023	0.016462	104	Nmu	-0.5791	0.016462
35	Tfpi2	1.073292	0.038977	105	Tnfaip3	-0.5739	0.045566
36	Adamts4	-1.06986	0.018664	106	Cd55	-0.56804	0.020411
37	Gstm5	-1.05623	0.045566	107	Itih3	-0.55502	0.042669
38	Btg2	-0.99358	0.016462	108	Brd3	-0.54672	0.028591
39	Ptges	-0.98134	0.026994	109	Tac1	0.527713	0.026994
40	Sod2	-0.98012	0.043591	110	Nrdc	-0.52751	0.026994
41	Zbtb41	-0.94917	0.040327	111	Coq8b	-0.52121	0.045352
42	Scn11a	-0.93852	0.045566	112	Ier5	-0.51366	0.047677
43	Mmp19	-0.93577	0.026994	113	Plau	0.506001	0.039144
44	Adamts8	-0.9328	0.02288	114	Itpkc	-0.49773	0.040327
45	Syt4	-0.92757	0.042669	115	Gldc	0.494114	0.040327
46	Elmsan1	-0.92208	0.040327	116	Nfkb2	-0.49015	0.043961
47	Nfkbia	-0.90983	0.028591	117	Birc3	-0.47237	0.025208
48	Slc34a3	-0.88717	0.036933	118	Arhgef3	-0.47007	0.040327
49	Bok	-0.88085	0.043591	119	Nfkbie	0.462819	0.044458
50	Smim3	-0.87396	0.044383	120	Akap12	-0.45748	0.044383
51	Ifrd1	-0.8579	0.045566	121	Sdc4	-0.45024	0.040327
52	Arih1	-0.85413	0.036712	122	Krt18	-0.44685	0.045566
53	Rnf214	-0.85132	0.042669	123	Pwwp3a	-0.4027	0.045492
54	Tcim	-0.85017	0.045566	124	Tiparp	0.401806	0.040327
55	Mat2a	-0.831	0.040327	125	Ipo5	0.398426	0.040327
56	Lmna	-0.8307	0.044383	126	Th	-0.39327	0.044458
57	Penk	-0.8265	0.03249	127	RGD15633	0.392442	0.031888
58	Abca7	-0.82235	0.040327	128	AABR0704	-0.36097	0.048539
59	AC111804	-0.81902	0.04931	129	Ctrc	0.353223	0.040327
60	Kcne4	0.813913	0.045566	130	Arrdc3	-0.34889	0.045566
61	Pdp1	-0.80755	0.041194	131	Relb	-0.32418	0.045566
62	Cadm4	-0.80501	0.045492	132	LOC100911	0.312427	0.048746
63	Hcn4	-0.79341	0.033916	133	Slc6a17	0.302439	0.045566
64	Nfkbiz	-0.79103	0.045566	134	Bod1l1	0.30224	0.045566
65	Tgif1	-0.78968	0.041194	135	Snai1	0.299532	0.034972
66	Pcp4l1	-0.78617	0.016462				
67	Gem	-0.77664	0.041194				
68	Dll1	-0.76357	0.048746				
69	Prag1	-0.75859	0.040327				
70	Map3k8	-0.75824	0.042433				

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