Inflammation in type 2 diabetes and the
neuroendocrine role of IL-18

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Abstract:

Cardiovascular disease is one of the leading causes of morbidity and mortality in type 2 diabetes, and yet, recent randomised controlled trials investigating the effect of different regimens of blood glucose control failed to show any benefit of more intensive control on rates of cardiovascular mortality, and minimal benefit on levels of morbidity. This thesis examines the theoretical understanding of type 2 diabetes, including the historical process which led to the above trials being conducted. A review of the historical and modern evidence surrounding the role of chronic inflammation suggests that this may be a common pathogenic alteration in obesity, diabetes and cardiovascular disease which may contribute to the development of cardiovascular disease in persons with type 2 diabetes, and even the pathogenesis of type 2 diabetes itself. This thesis therefore investigates the role of inflammation in type 2 diabetes, by a two-pronged approach: one investigates the effects of a dietary intervention on levels of chronic inflammation in participants with type 2 diabetes in the Lifestyle Over and Above Drugs in Diabetes (LOADD) randomised controlled trial. In this trial, chronic inflammation is assessed by a range of inflammatory markers and adipokines including leptin, neopterin, tumor necrosis factor α and its soluble receptors, the interleukins 1, 6, 10, and 18, and interleukin 1 receptor antagonist. The dietary intervention group exhibited a significant reduction in circulating levels of interleukin-18 (IL-18) and neopterin relative to a 'care as usual' control group; reductions which further analyses suggest were unrelated to concomitant changes in adiposity or glycaemia. In the second branch of the thesis, the neuroendocrine effects of IL-18 are further investigated in rodents, as previous evidence has linked this cytokine with effects on appetite, body weight, and blood glucose control. Clear evidence of central production of IL-18 in the medial habenula is presented, with transport along fibres in the fasciculus retroflexus terminating in the interpeduncular nucleus (IPN). However, no evidence for a corresponding IPN focus of expression of the genes encoding the IL-18 receptor was found. In light of this discrepancy, further investigations were conducted to assess other potential receptors for central IL-18 action; bioinformatics approaches suggest that the interleukin-1 receptor accessory protein like (IL1RAPL) receptors may form alternative central receptors for IL-18, and immunohistochemistry experiments localize IL1RAPL1 in the interpeduncular nucleus. A model is thus proposed whereby habenula IL-18 expression may influence appetite via actions on IL1RAPL1 in the IPN. The thesis concludes with a discussion of the hypothesis of inflammation as a cause of diabetes and cardiovascular disease, and the potential for an improved understanding of the pathological processes involved in type 2 diabetes to produce greater improvements in clinical outcomes than has been attained thus far.
Acknowledgements:

It is difficult to know where to start when it comes to writing an acknowledgements section, as not only have so many assisted during the process of completing this thesis itself and the research contained therein, but also all the others who have provided guidance, mentoring and just simply bouncing-of-ideas in the years beforehand while working as a young scientist, have all in some way or other contributed to the current thesis in your hands now.

As little science can be done without money these days, I should perhaps first thank the funders, especially the National Heart Foundation of New Zealand who provided me with a postgraduate scholarship without which, quite simply, this thesis never would have been completed. In many ways it's quite a strange experience to have an organisation you've never previously been involved with contribute a sizeable financial sum to allow you to pursue a higher education and spend your days in research and contemplation, and I am extremely grateful for having had the opportunity to do so. The Southern Trust, Lotteries New Zealand, and Health Research Council of New Zealand have all contributed to the funding of the LOADD study at various stages, and the Maurice and Phyllis Paykel Trust provided pilot funding to pursue our ideas around investigating the neuroendocrine role of IL-18, which led to further funding from the Health Research Council, and these funders jointly contributed to the completion of the work included here.

I obviously owe many thanks to my supervisors Jim and Dave. Jim, it's been a long time since I first started working at the Edgar National Centre for Diabetes Research shortly after its formation and had but a vague idea that there was a 'type 1' and 'type 2' diabetes, but your mentoring and inspiration along the way has been invaluable, and I certainly wouldn't have got so far as to contemplate a thesis on diabetes without it. It's a rare experience to work with someone with such a wealth of experience and perspective, and I'm sure your talk of things such as seeing fibre come in and out of nutrition 'fashion' has rubbed off in some way in my appreciation of the historical diabetes literature and thinking of longer-term timescales of research. To Dave, after discussing the problems of obesity and diabetes over many an ale-based beverage I remember coming to you with a paper from Nature Medicine and some vague ideas of trying to figure out what IL-18 was doing in the brain. Maybe you should have said 'aim for something a little less ambitious son!', but I'm glad that instead you were on board with giving it a go and were willing to put your name out there as PI on a project which could well have failed (and did seem like it was for a while). With a bit of
hindsight maybe I can say that like any good discovery science project, it led us down completely different lines of thought than when we started, and yet despite ending up outside the lab’s comfort zone with completely different receptors and brain regions than we’d usually investigate, you’ve stayed supportive throughout. Many thanks.

To all the team involved in the LOADD study: Kirsten, Minako, Sue, Alex, Ashley, Sheila, Margaret, and many others, thanks for all your input and help, you’ve all certainly made my job the easy one in this project. Thank you Sheila for your additional help in the statistical analysis and interpretation, and I’d especially like to thank Ashley, Holly and Michelle, from the Lipid Laboratory for help with all the LOADD study assays, as well as Drs Sarah Young and Clare Fitzpatrick for their assistance in planning and conducting the multiplex analyses. Many thanks as well to the wider ENCDOR team for their support.

There are so many to thank from the 'Grattan lab' that I can’t name you all, but special thanks to Ilona, Sharon, Jet and Rachael Augustine who took the time to teach me various hands-on lab procedures during the course of this PhD. To all the wider members of the lab and CNE group, many thanks for providing a great 'science geek' working environment and comraderie in general.

I’d also like to thank the members of my PhD committee, who took the time to read over various reports and updates, and provide feedback during our annual meetings: Marilyn, Kirsten, Sheila, Jim, Dave and, for a spell, Dr Alex Tups.

Y por último, gracias a mi amor Paulina por soportar mi ausencia durante las noches y fines de semana mientras que estaba ocupado haciendo las investigaciones y escribiendo este pequeño libro. Tal vez es más duro la vida de la pareja de un científico, que la del dicho científico. Entonces espero que valdría la pena para ti también, aunque no puedo garantizar que en el futuro cuando me preguntés en que pienso, que igual no voy a responder 'trabajo'. Gracias por tu apoyo.
Table of contents:

Preliminaries

Abstract ................................................................................................................................. i
Acknowledgements ............................................................................................................... ii
Table of contents .................................................................................................................. iv
List of tables ........................................................................................................................ viii
List of figures ......................................................................................................................... x
List of biological and chemical abbreviations ..................................................................... xiv
List of trial and organisation acronyms ................................................................................ xvi
Interleukin-1 family nomenclature ..................................................................................... xvii
References for list of figures and interleukin-1 family nomenclature table ......................... xviii

Section 1: Introduction

Chapter 1: Introduction ......................................................................................................... 3

1.1 Diabetes: the glucose disease .......................................................................................... 4
1.2 The (attempted) prevention of diabetes complications by glucose lowering therapy .......................................................... 6
1.3 End of the glucose apotheosis? ..................................................................................... 11
1.4 The pathogenesis of diabetes and its complications revisited ......................................... 12
1.5 Lifestyle effects on inflammatory markers ..................................................................... 27
1.6 Inflammation and diabetes: a key role of NF-κB ............................................................. 37
1.7 A role for IL-18 in metabolic disease ............................................................................ 39
1.8 Patterns and dynamics of cytokine release .................................................................... 43

Section 2: Inflammatory marker and adipokines in the Lifestyle Over and Above Drugs in Diabetes (LOADD) Study

Chapter 2: Overview and methods of the LOADD study

2.1 The LOADD study: rationale ....................................................................................... 47
2.2 Inflammatory markers and adipokines of interest ......................................................... 50
2.3 Overview of the LOADD study .................................................................................... 54
2.4 Summary of previously published LOADD study findings ................................. 56
2.5 The current work: measurement of inflammatory markers and adipokines........ 59
2.6 Statistical analysis .................................................................................................................. 62

Chapter 3: Results
3.1 Effect of dietary intervention on inflammatory markers and adipokines .......... 66
3.2 Correlation of inflammatory markers and adipokines with anthropometric
variables and glycaemic control ................................................................................................. 72

Chapter 4: Discussion
4.1 Discussion of LOADD study findings ................................................................................. 77
4.2 Interpreting the effects of the LOADD study dietary intervention; theoretical
considerations of establishing cause and effect in studies of metabolic disease ............ 85

Section 3: Animal studies into the biological role of IL-18

Chapter 5: Introduction
5.1 CNS control of appetite and blood glucose ................................................................. 89
5.2 Architecture of the hypothalamus ..................................................................................... 91
5.3 Inflammation in the hypothalamus .................................................................................... 94
5.4 Key questions regarding the biological actions of interleukin-18: how are the
central effects of IL-18 mediated? ......................................................................................... 95
5.5 Hypotheses .......................................................................................................................... 96

Chapter 6: General laboratory methods
6.1 Animals ................................................................................................................................. 97
6.2 Reverse transcription polymerase chain reaction (RT-PCR) ........................................ 97
6.3 Gel electrophoresis: visualization of PCR products ....................................................... 100
6.4 Product sequencing: verification of amplification of desired gene products .......... 101
6.5 In situ hybridisation methods ............................................................................................ 101
6.6 Immunohistochemistry ..................................................................................................... 105

Chapter 7: Brain–derived IL-18 as a mediator of the metabolic effects of IL-18
7.1 Overview ............................................................................................................................... 109
7.2 Methods ............................................................................................................................... 110
7.3 In situ hybridisation to examine IL-18 mRNA expression ............................................. 111
7.4 Immunohistochemistry to examine the distribution of IL-18 protein ......................... 113
7.5 Examining expression of an IL-18 splice variant in the rat habenula .......................... 120
Section 5: Appendices

Appendix 1: Chapter 3, LOADD study supplementary information ............................................. 289
Appendix 2: Section 3 supplemental information, detecting IL-18 system splice variants by
RT-PCR ............................................................................................................................... 317
Appendix 2.1: Establishing methodology to examine expression of an IL-18 splice
variant ............................................................................................................................. 318
Appendix 2.2: Establishing methodology to examine expression of an IL-18r1
splice variant .................................................................................................................. 322
Appendix 2.3: Establishing methodology to examine expression of sIL-18rap ...... 326
References, Appendix 2 ..................................................................................................... 329
Appendix 3: Section 3 supplementary information, images of micropunch procedure .......... 331
Appendix 4: Chapter 8 supplementary information: IL1RAPL1 and IL1RAPL2 bioinformatics
comparisons ...................................................................................................................... 337
References, Appendix 4 ..................................................................................................... 350
List of tables:

Preliminaries: Nomenclature

Table i Nomenclature of IL-1 and IL-18 family members.............................................. xvi

Section 1: Introduction

Chapter 1: Introduction

Table 1 Research into the effects of salicylates in diabetes and insulin resistance over the 19th and 20th centuries................................................................. 22

Section 2: Inflammatory markers and adipokines in the Lifestyle Over and Above Drugs in Diabetes (LOADD) Study

Chapter 2: Methods

Table 2a Measured intra- and inter-assay coefficients of variation (CV) for RIAs and multiplexes........................................................................................................... 64

Table 2b Calculated intra- and inter-assay coefficients of variation (CV) for ELISAs.... 65

Chapter 3: Results

Table 3 Comparison of effect sizes of control and dietary interventions on adipokines and inflammatory markers................................................................. 68

Table 4 Pairwise correlation coefficients for Δ BMI, HbA1c, IL-18 and neopterin...... 70

Table 5 Intervention effect sizes for IL-18 and neopterin with additional adjustment for changes in HbA1c and BMI................................................................. 71

Table 6 Pearson correlation coefficients of adipokines and inflammatory markers with anthropometric, clinical and biochemical measures at baseline............... 74

Table 7 Partial correlation coefficients for leptin when adjusting for BMI.............. 76

Section 3: Animal studies into the biological role of IL-18

Chapter 6: General Laboratory Methods

Table 8 Primer sequences for use in generating in situ hybridisation probes for use in rat and mouse tissues................................................................. 102

Chapter 8: Central expression of IL-18 receptors
Table 9  Primer sequences for amplification of IL-18 system transcripts in rat hypothalamic micropunch samples…………………………………….. 127
Table 10 Primers used for amplifying IL-18 receptor transcripts in brain micropunches……………………………………………………………………………………………….. 128
Table 11 PCR settings for amplifying IL-18 and receptor transcripts from brain micropunches……………………………………………………………………………………………….. 130

Chapter 9: Examining potential unknown mediators of IL-18 signalling in the CNS
Table 12 BLAST search results using amino acids encoding the extracellular portion of IL-18r1 as a query against reference protein sequence databases for rat, mouse and human species……………………………………………………………………………………… 151
Table 13 BLAST search results using IL-18BP protein sequence as a query against reference protein sequence databases for rat, mouse and human species........... 153
Table 14 Primer combinations and reaction conditions for RT-PCR of IL-18r1, IL1RAPL1, IL1RAPL2 and Actb transcripts across various rat tissues........... 174

Appendices
Appendix 1: Chapter 3, LOADD study supplementary information
Appendix 1, Table 1 Baseline characteristics of LOADD study participants……………….. 291
Appendix 1, Table 2 Dietary intake across intervention and control groups……………….. 292
Appendix 1, Table 3 Pairwise correlation coefficients of Δ BMI, HbA1c, IL-18, etc……… 293
Appendix 1, Table 4 Pearson correlation coefficients, p-values, and number of observations for correlations shown in Table 6, Chapter 3………… 300
Appendix 2: Section 3 supplemental information, detecting IL-18 system splice variants by RT-PCR
Appendix 2, Table 1 Primer details for establishing methodology for amplifying an IL-18 splice variant and examining its expression in the habenula…… 319
Appendix 4: Chapter 8 supplementary information, IL1RAPL1 and IL1RAPL2 bioinformatics
Appendix 4, Table 1 Consensus amino acid residue codes…………………………………….. 338
Appendix 4, Table 2 Reference protein and nucleotide sequences used for multiple sequence alignment in Figures 23 and 24…………………………………….. 339
Appendix 4, Table 3 Sources of amino acid sequences for 23 species used in multiple sequence alignment of IL1RAPL, IL-18BP and IL-18 proteins....... 340
List of figures:

Section 2: Inflammatory markers and adipokines in the Lifestyle Over and Above Drugs in Diabetes (LOADD) Study

Chapter 2: Overview and methods of the LOADD study

Figure 1 LOADD study participant flow chart ............................................................... 58

Chapter 3: Results

Figure 2 Forest plot of LOADD dietary intervention effect sizes on adipokines and inflammatory markers ................................................................. 69

Figure 3 Scatter plots of change in IL-18 levels versus change in neopterin levels ....... 70

Section 3: Animal studies into the biological role of IL-18

Chapter 5: Introduction

Figure 4 Schematic diagram showing the position of hypothalamic nuclei in sagittal section in the rat brain ................................................................. 92

Chapter 6: General Laboratory Methods

Figure 5 Schematic diagram showing sagittal rat brain with approximate plane of sectioning of coronal oblique sections ............................................. 107

Chapter 7: Brain-derived IL-18 as a mediator of the metabolic effects of IL-18

Figure 6 In situ hybridisation for expression of the IL-18 gene in the rat brain .......... 112

Figure 7 Immunohistochemistry for IL-18 in the rat brain showing coronal sections focused on the habenula and surrounding brain regions .................. 114

Figure 8 Sagittal views of IL-18 immunostaining in the rat brain ......................... 117

Figure 9 Staining of ependymal cells surrounding the 3rd ventricle during immunohistochemistry for IL-18 ......................................................... 119

Figure 10 RT-PCR for IL-18 splice variants in the rat habenula ....................... 121

Chapter 8: Central expression of IL-18 receptors

Figure 11 Schematic diagram of positions of primers used to amplify IL-18r1 splice variants ......................................................................................... 131

Figure 12 Schematic diagram of positions of primers used to amplify IL-18rap splice variants ......................................................................................... 132
Figure 1: Expression of IL-18 signalling components in rat hypothalamic micropunches .......................................................... 134

Figure 14: In situ hybridisation for IL-18r1 expression in the mouse brain .......................................................... 136

Figure 15: In situ hybridisation for IL-18rap expression in the mouse brain .......................................................... 136

Figure 16: RT-PCR for the detection of IL-18r1 transcripts in rat brain micropunches .......................................................... 138

Figure 17: RT-PCR for the detection of IL-18rap transcripts in rat brain micropunches .......................................................... 141

Chapter 9: Examining potential unknown mediators of IL-18 signalling in the CNS

Figure 18: Alignment of rat IL-18r1 vs IL1RAPL1 proteins .......................................................... 156

Figure 19: Alignment of rat IL-18BP vs IL1RAPL2 proteins .......................................................... 157

Figure 20: Alignment of members of the human IL-1 receptor family with human and viral IL-18BP .......................................................... 160

Figure 21: Manual alignment of multi-species consensus sequences for IL-18BP, IL1RAPL1 and IL1RAPL2 .......................................................... 165

Figure 22: Immunostaining for IL-18 and IL1RAPL1 in sagittal sections through the interpeduncular nucleus .......................................................... 167

Figure 23: Immunostaining for IL1RAPL1 in the cortex and hippocampus .......................................................... 169

Figure 24: Immunostaining for IL1RAPL1 in the cerebellar cortex with and without primary antibody preabsorption .......................................................... 170

Figure 25: RT-PCR of IL1RAPL1, IL1RAPL2 and IL-18r1 expression across rat tissues .......................................................... 176

Figure 26: In situ hybridisation for IL1RAPL1 expression in the mouse brain from Carrié et al. 1 .......................................................... 179

Chapter 10: A model of habenula IL-18 function

Figure 27: A model of IL-1RacP function as proposed by Yoshida et al. 2 .......................................................... 194

Figure 28: A model of IL-1RacPb function .......................................................... 195

Figure 29: A model of IL-18 and IL1RAPL function .......................................................... 196

Figure 30: Schematic diagrams depicting a model of habenula function governing food intake .......................................................... 199

Appendices

Appendix 1: Chapter 2, LOADD study supplementary information

Appendix 1, Figure 1 Scatter graphs showing the correlation of change in neopterin levels with various dietary components .......................................................... 294

Appendix 1, Figure 2 Scatter graphs showing the correlation of change in IL-18 levels
with various dietary components........................................... 296

Appendix 1, Figure 3  Distribution of baseline measures of adipokines and inflammatory markers in the LOADD study........................................... 298

Appendix 1, Figure 4  Scatter plots of statistically significant pairwise correlations between inflammatory markers & adipokines and age & sex........ 306

Appendix 1, Figure 5  Scatter plots of statistically significant pairwise correlations between inflammatory markers & adipokines and smoking.......... 308

Appendix 1, Figure 6  Scatter plots of statistically significant pairwise correlations between inflammatory markers & adipokines and anthropometric measures................................................................. 309

Appendix 1, Figure 7  Scatter plots of statistically significant pairwise correlations between inflammatory markers & adipokines and markers of cardiovascular risk................................................................. 311

Appendix 1, Figure 8  Scatter plots of statistically significant pairwise correlations between inflammatory markers & adipokines and markers of glycaemia................................................................. 313

Appendix 1, Figure 9  Scatter plots of statistically significant pairwise correlations between inflammatory markers and adipokines......................... 314

Appendix 2: Chapter 6, Detecting IL-18 system splice variants by RT-PCR, supplemental information

Appendix 2, Figure 1  Schematic diagram showing location of primers designed to amplify rat IL-18 splice variants........................................... 320

Appendix 2, Figure 2  RT-PCR testing the amplification of IL-18 transcripts.................... 321

Appendix 2, Figure 3  Alignment of rat and mouse intronic sequences transcribed in the IL18r1 type II splice variant........................................... 323

Appendix 2, Figure 4  Sequencing results for rat IL-18r1 type II..................................... 324

Appendix 2, Figure 5  Sequencing results for rat sIL-18rap........................................... 327

Appendix 3: Chapter 6 supplementary information, images of micropunch procedure

Appendix 3, Figure 1  Micropunches of rat brain habenula and arcuate nuclei.............. 332

Appendix 3, Figure 2  Micropunches of rat brain habenula and ventromedial hypothalamic nuclei................................................................. 333

Appendix 3, Figure 3  Micropunches of rat brain paraventricular hypothalamic nuclei..... 334

Appendix 3, Figure 4  Micropunches of rat brain interpeduncular nuclei and ventral
**List of biological and chemical abbreviations:**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2 (R)</td>
<td>angiotensin 2 (receptor)</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>adr</td>
<td>adrenal</td>
</tr>
<tr>
<td>ApoE</td>
<td>apolipoprotein E</td>
</tr>
<tr>
<td>arc</td>
<td>arcuate nucleus of the hypothalamus</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>CART</td>
<td>cocaine and amphetamine regulated transcript</td>
</tr>
<tr>
<td>CDK-5</td>
<td>cyclin-dependent kinase 5</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CIMT</td>
<td>carotid intimal medial thickness</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>dipeptidyl-peptidase IV</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>FBG</td>
<td>fasting blood glucose</td>
</tr>
<tr>
<td>GIP</td>
<td>gastrointestinal inhibitory peptide</td>
</tr>
<tr>
<td>GLP</td>
<td>glucagon-like peptide</td>
</tr>
<tr>
<td>HbA1c</td>
<td>haemoglobin A1c, also known as glycated haemoglobin</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>hyp</td>
<td>hypothalamus</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitor of kappa beta kinase</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>IL-10</td>
<td>interleukin-10</td>
</tr>
<tr>
<td>IL-18</td>
<td>interleukin-18</td>
</tr>
</tbody>
</table>
IL-18 BP........ interleukin-18 binding protein
IL-1ra........ interleukin-1 receptor antagonist
IL-6........... interleukin-6
IL1RAPL(1/2) interleukin-1 receptor accessory protein-like (1/2)
IPN........... interpeduncular nucleus
IκB............ inhibitor of kappa beta
Kir6.2........ inwards rectifying potassium channel family member, encoded by KCNJ11 gene
LDL............ low density lipoprotein
LPS............ lipopolysaccharide
MAPK.......... mitogen activated protein kinase
MRI............ magnetic resonance imaging
NF-κB.......... nuclear factor kappa beta
NIDDM......... non-insulin dependent diabetes mellitus
NPY............ neuropeptide Y
NSAID......... non-steroidal anti-inflammatory drug
PCI............ percutaneous coronary intervention
PCR............ polymerase chain reaction
POMC.......... proopiomelanocortin
PPAR-γ......... peroxisome proliferator activated receptor gamma
PVN.......... paraventricular hypothalamus
RT............ reverse transcription/reverse transcriptase
SGLT2.......... sodium glucose co-transporter 2
spl............ spleen
SUR1.......... sulphonylurea receptor, encoded by ABCC8 gene
TCF7L2......... transcription factor 7-like 2 gene
% TE........... percent of total energy intake
TIR............ toll/interleukin-1 receptor
TLR............ toll-like receptor
TNFa.......... tumour necrosis factor alpha
VMH.......... ventromedial hypothalamus
VTA.......... ventral tegmental nucleus
List of trial and organisation acronyms:

ACCORD........... Action to Control Cardiovascular Risk in Diabetes
ADVANCE........... Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation
DCCT.............. Diabetes Control and Complications Trial
DEHKO............. Development Programme for the Prevention and Care of Diabetes (acronym refers to Finnish translation)
DIGAMI........... Diabetes Mellitus, Insulin Glucose infusion in Acute Myocardial Infarction
EASD............... European Association for the Study of Diabetes
GWAS.............. Genome-Wide Association Studies
HEART2D........... Hyperglycemia and Its Effect After Acute Myocardial Infarction on Cardiovascular Outcomes in Patients With Type 2 Diabetes Mellitus
JUPITER........... Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin
LOADD............. Lifestyle Over and Above Drugs in Diabetes
NZGG.............. New Zealand Guidelines Group
PROactive.......... PROspective pioglitAzone Clinical Trial In macroVascular Events
Steno-2........... Refers to a study conducted at Steno Diabetes Centre, Copenhagen
UGDP.............. University Group Diabetes Program
UKPDS............ United Kingdom Prospective Diabetes Study
VADT............. Veteran’s Affairs Diabetes Trial
WCRF.............. World Cancer Research Fund
WHO.............. World Health Organisation
**Interleukin-1 family nomenclature:**

A number of different names have been proposed for members of the interleukin-1 family and are utilised in the existing literature. For clarity, the range of different naming practices are summarized below in Table i. Names in the left hand column will be used in this thesis.

**Table i: Nomenclature of IL-1 and IL-18 family members.**

<table>
<thead>
<tr>
<th>Ligand or receptor</th>
<th>Other names</th>
<th>New IL-1 family naming system</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Interleukin 1 beta</td>
<td>IL-1F2</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>Interleukin 1 receptor antagonist</td>
<td>IL-1F3</td>
</tr>
<tr>
<td>IL-18</td>
<td>Interleukin 18</td>
<td>IL-1F4</td>
</tr>
<tr>
<td>IL-18s</td>
<td>Interferon gamma inducing factor (IGIF)</td>
<td></td>
</tr>
<tr>
<td>IL-18r1</td>
<td>IL-18Rα - Interleukin-18 receptor α subunit</td>
<td>IL-1R5</td>
</tr>
<tr>
<td></td>
<td>IL-1Rrp - Interleukin-1 Receptor related protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-18r1 type I</td>
<td>IL-1R7</td>
</tr>
<tr>
<td>IL-18rap</td>
<td>IL-18Rβ - Interleukin-18 receptor β subunit</td>
<td>IL-1R8</td>
</tr>
<tr>
<td></td>
<td>IL-18RII – Interleukin-18 receptor II</td>
<td></td>
</tr>
<tr>
<td>IL-18r1 type II</td>
<td>(truncated IL-18r1 splice variant described in Alboni <em>et al.</em> 6)</td>
<td>IL-1R9</td>
</tr>
<tr>
<td>sIL-18rap</td>
<td>truncated IL-18rap</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-18rap short</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sIL-18Rβ 8,9</td>
<td></td>
</tr>
<tr>
<td>IL1RAPL1</td>
<td>Interleukin-1 receptor accessory protein like 1</td>
<td>IL-1R8</td>
</tr>
<tr>
<td>IL1RAPL2</td>
<td>Interleukin-1 receptor accessory protein like 2</td>
<td>IL-1R9</td>
</tr>
</tbody>
</table>
References for list of figures and interleukin-1 family nomenclature table:


Section 1:

Introduction
Chapter 1. Introduction

“...we need to return to science to understand the metabolic pathways underlying the development of diabetes and cardiovascular disease”.

K. Borch-Johnsen & N. Wareham, "The rise and fall of the metabolic syndrome". *Diabetologia*, 2010

Rates of obesity, cardiovascular disease and type 2 diabetes are steadily on the rise throughout the world, and New Zealand is no exception. Data from the latest New Zealand Health Survey show that 1 in 4 New Zealand adults are obese, 1 in 20 have been diagnosed with ischaemic heart disease, and 1 in 20 with diabetes. That the prevalence of these three conditions should rise simultaneously is no surprise; obesity and overweight are major risk factors for the development of both heart disease and diabetes, and diabetes itself is a major risk factor for heart disease. This thesis examines the link between these three diseases, and focuses in particular on evidence that chronic sub-clinical inflammation could play a role in the pathogenesis of all three. The principal focus will be on the role of inflammation in diabetes, both as a mechanism by which diabetes may increase the risk of cardiovascular diseases, and as a cause of diabetes pathogenesis itself.

The experimental work in the thesis takes a two-pronged approach to examining the role of inflammation: on the one hand describing the effects of dietary intervention in people with type 2 diabetes on a range of inflammatory markers which have been linked to cardiovascular risk and insulin resistance; on the other detailing investigations in animals into the physiological role of one inflammatory marker in particular, interleukin-18 (IL-18), focusing on potential mechanisms by which it could influence appetite and glucose homeostasis.

To begin, it is necessary that we cast our eyes back somewhat, into the history of obesity, heart disease and diabetes and the scientific and clinical thinking as to their cause and pathogenesis, in order to arrive at the modern-day picture of the evidence linking inflammation with each, and to the future promise of addressing chronic inflammation in order to curb the increasing prevalence of these conditions.
1.1 Diabetes: the glucose disease

Diabetes is a disease which has been recognised throughout the course of human history. In former times the major cause for concern was the wasting, lethargy, high urine production and thirst characteristic of what is now called type 1 diabetes; as a physician in the ancient Roman empire noted in the second century AD “a melting down of the flesh and limbs into urine”\(^1\). Historically, dietary treatment aimed to avoid ketoacidosis in those with ‘severe diabetes’\(^*\) by prescribing an extremely low carbohydrate, or even "starvation", diet (thus ameliorating the post-prandial rise in blood glucose); however, the dietary regimens were harsh and mortality rates high\(^12,13\). In 1921–22 Banting and Best performed their famous experiments involving ligating the pancreatic ducts of dogs\(^14,15\), leading to the discovery of insulin, which was heralded as “a miraculous development in the treatment of diabetes”\(^16\), having remarkable effects on glycosuria and blood glucose.

However, subsequent to the introduction and increasingly widespread use of insulin, it became apparent to physicians of the time that other problems were becoming manifest; Elliot Joslin is said to have noted: "The era of coma as the central problem of diabetes has given way to the era of complications. People with diabetes are at increased risk for the development of serious complications, including blindness, kidney failure, heart disease, stroke, and amputations"\(^16\). A link between diabetes and cardiovascular disease had been previously recognized in the late 1800’s (prior to the introduction of insulin)\(^17\). Yet after the introduction of insulin in the early 20th century, rates of death from arteriosclerosis amongst those with diabetes rapidly rose to become a leading cause of mortality. As Joslin was to note in 1954: "insulin hid the tragic symptoms and signs of the disease and thus allowed to burrow, unobserved, the slowly developing arteriosclerosis that finally killed the patient"\(^18\), and Ewen Downie, in 1955, foresaw the growing problem of diabetes-related cardiovascular disease: “the grim realization that long-standing diabetes predisposes to premature

\(^*\) Part of the difficulty of interpreting the historical literature relating to diabetes is the changing definitions of ‘diabetes’ which have been used a different times. It is likely that what is referred to as ‘severe diabetes’ in childhood is what is now known as type 1 diabetes; in older patients it could be inferred that they were probably in the later stages of what is now diagnosed as type 2 diabetes, with insulin insufficiency due to pancreatic degradation seen in the advanced stages. Along with changing classification systems and glycaemic thresholds for diagnosis, the word ‘diabetes’ across different time periods has referred to disease definitions which capture differing groups of patients. Nevertheless, preventing the complications of diabetes, be it in what is now termed ‘type 1’ or ‘type 2’ diabetes, has remained a challenge throughout history.
Arteriosclerosis has posed a problem second only to that of the causation of diabetes” [19]. Indeed, these words still ring true over 50 years later. In particular, given that the vast majority of cases of diabetes in the world are what is now classified as ‘type 2’ diabetes, it is the complications of this type of diabetes which form much of the diabetes-related health-care, mortality, and morbidity burden facing modern societies, and the costs of type 2 diabetes management are well documented as placing an incredible strain on health systems worldwide [20].

The question thus arose as to how to prevent the onset of these diabetes complications, particularly the rates of early death due to cardiovascular causes. An obvious suspect was the elevation of blood glucose seen in diabetes, and with the widespread availability of insulin and increasing lengths of survival of people with diabetes, the question of whether lowering blood glucose levels in people with diabetes could influence the development of these longer-term complications came to be the foremost question in subsequent diabetes research. However, during much of the 20th century, evidence addressing this issue consisted mainly of expert opinion or limited retrospective reviews of patient records. It is only in recent times that larger, well designed, randomised controlled trials have been conducted investigating this question; these have typically involved a prospective comparison of treatments aimed at the sustained lowering of blood glucose to different levels and follow-up of participants for micro- and/or macro-vascular end points, with the aim of comparing rates of disease across different levels of blood glucose control or different treatment strategies.
1.2 The (attempted) prevention of diabetes complications by glucose lowering therapy

In the context of type 1 diabetes, exogenous doses of insulin were shown in the Diabetes Complications Control Trial (DCCT) to result in impressive long-term improvements in diabetes microvascular outcomes in the range of 40% - 70% risk reductions with a more rigorous ‘intensive’ treatment schedule being more efficacious (at the inevitable trade-off of more dose-related adverse effects, i.e. hypoglycaemic episodes) 21 b.

Insulin resistance is a characteristic feature of type 2 diabetes and thus in a similar fashion treatment of type 2 diabetes with exogenous doses of insulin, thereby providing greater amounts of insulin than the body can produce, represented a logical progression in evaluating the potential prevention of complications in this second, more abundant, form of diabetes. Indeed the first study to examine this question was published just a few years after the original DCCT report 22; the small Kumamoto study in Japanese participants with type 2 diabetes (then known as NIDDM c) published by Ohkubo et al., which showed a more intensive insulin treatment regimen to be associated with lower rates of microvascular complications 23.

In terms of studies examining the prevention of macrovascular complications of diabetes, the first prospective study to examine the potential of blood glucose lowering to reduce mortality in individuals with type 2 diabetes was the University Group Diabetes Program (UGDP) 24. Here, the use of a variable-dose insulin regimen provided the best long-term fasting blood glucose control, and yet neither it nor a fixed-dose insulin regimen provided any significant reduction in cardiovascular mortality compared to a placebo group treated with diet only. While this may certainly not be a fault of insulin therapy but a beacon pointing towards the potential of dietary treatment of diabetes, the study received extensive criticism of its randomisation, conduct, and analysis 25–37, as well as coverage in the lay media (discussed in 38). One memorable result of the study was that the use of

b It should be noted that the paper cited here is a re-analysis of the DCCT data published 13 years after the original report, in which it was shown that a number of the results of the original analysis were in fact an artifact of the statistical modeling originally used (see in particular pages 996 & 997 of the paper cited here).

c Abbreviation: NIDDM – non-insulin dependent diabetes mellitus
tolbutamide (the sulphonylurea used in the study) resulted in significantly greater cardiovascular mortality than either placebo or insulin treatment groups, *despite equivalent or better fasting glucose control* than fixed-dose insulin treatment, *and better postprandial glucose control* than either fixed-dose or variable insulin treatment for the majority of the study. Likewise, in analyses of the two insulin treatment and placebo groups “in spite of differences in blood glucose levels among the treatment groups, there were only minor differences in the occurrence of fatal or nonfatal events” 40. The authors noted that, despite the study’s weaknesses, “the possibility that control of blood glucose levels will not alter the course of vascular disease in the adult-onset diabetic should not be overlooked as one possible explanation” 40 e.

Perhaps the most well-known study of blood glucose lowering and its effect on the incidence of diabetes complications is the United Kingdom Prospective Diabetes Study (UKPDS). This study and its subsequent analyses have not escaped their share of controversy either, with various criticisms of study design and analysis 42-45. However, a beneficial effect of intensive glucose lowering on the incidence of retinopathy was reported in the key publications describing the results of the study 46 (that this was coupled with a suspicious lack of effect on other microvascular complications and could have been due to physician behaviour given the lack of blinding was subsequently noted by others 42-44). A later epidemiological analysis of the mean HbA1c over a period of 10 years of follow-up showed that higher mean glycaemia was associated with an increased incidence of microvascular and macrovascular disease 47. However, this finding does not delineate that blood glucose itself is the causative mechanism, as the intervention could bring about changes which result in a lower incidence of diabetes complications and lower blood glucose without the former arising from the latter. Yet despite these limitations the UKPDS has been cited in many position statements and

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4 The variable insulin regimen group had the highest proportion of patients classified as exhibiting ‘good’ control based on fasting glucose, and the lowest fasting blood glucose levels over the course of the study. However, one hour glucose tolerance measures in the tolbutamide group were clearly lower than other groups for most of the follow-up period, only surpassed by the variable insulin regimen towards the end of the study 39.

e Another treatment arm using the biguanide phenformin, which was started 18 months after the initiation of the trial, was also terminated early due to toxic side effects. In the wake of the trial the drug’s manufacturer attempted to obtain extensive records from the UGDP researchers via a Freedom of Information Act request 41.
treatment guidelines as providing evidence of good glycaemic control reducing microvascular complications in type 2 diabetes \[48\].

More recently, three separate studies have examined approaches of intensive blood glucose control compared with ‘normal’ or ‘conventional’ blood glucose control and studied their effects on the incidence of macrovascular endpoints – the ACCORD \[49,50\], ADVANCE \[51\] and VA Diabetes Trial \[52\]. Contrary to expectation, however, these found either no difference or higher levels of cardiovascular morbidity and mortality in the treatment (lower average blood glucose) arms, prompting a large amount of media coverage \[53-56\], and comment, criticism and discussion in various medical journals \[42,57-68\]; repercussions now–familiar from the earlier release of similar findings from the UGDP and UKPDS studies. These studies would result in the unprecedented release of a joint position statement from respected diabetes and cardiovascular disease authorities reproduced in three high impact journals \[69-71\] discussing the results of these and earlier studies.

Meta-analyses of trials involving intensive glucose lowering and follow–up for cardiovascular endpoints have been undertaken analysing the combined UKPDS, ACCORD, ADVANCE, VA Diabetes Trial \[72\], and PROactive studies \[73-77\]. These analyses come to similar conclusions that targeting intensive vs. conventional glycaemic control in these studies reduces incident cardiovascular events and myocardial infarctions in the range of 11–15 % (depending on outcome and meta-analysis), however, produces no reduction in cardiovascular mortality nor all-cause mortality. Thus, we are left with the somewhat odd conclusion that the effect of ‘tight’ control of blood glucose (to the extent of the reduction in HbA1c achieved in these studies and by way of the medication regimens they employed) appears to be that it avoids only non-fatal cardiovascular events; in any event it does not save lives, and the question of whether the reduction in non-fatal events is worthwhile from the perspective of adverse events, quality of life, or healthcare costs is another matter entirely, which has not yet been addressed.

Indeed, to date, the only trial which has conclusively shown a reduced incidence of macrovascular events in people with type 2 diabetes involving blood glucose lowering is the Steno-2 study. However, while the Steno-2 intervention included blood glucose lowering, this was in fact a

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\( ^{1} \) Trial abbreviations: ACCORD - Action to Control Cardiovascular Risk in Diabetes; ADVANCE - Action in Diabetes and Vascular Disease-Preterax and Diamicron Modified Release Controlled Evaluation; VA Diabetes Trial - Veterans Affairs Diabetes Trial.
multifactorial intervention involving ACE (angiotensin converting enzyme) inhibitors, aspirin use, diet therapy and glycaemic control in which a number of cardiovascular risk factors were modified. Given the known benefit of controlling other traditional cardiovascular risk factors on macrovascular disease outcomes (even in diabetes) it cannot be known from the design of this trial whether lower glycaemia in the treatment group was responsible for any of the beneficial effect. Of note, the proportion of patients receiving different forms of oral hypoglycaemic agents did not differ between treatment groups, nor the proportion taking insulin, nor mean insulin dose, and thus it is likely that the lower glycaemia in the treatment group resulted from the documented change in dietary behaviour.

1.2.1 Could future trials redeem glycaemic control as a strategy for cardiovascular benefit?

Despite the lacklustre support from the above trials for the hypothesis that glucose lowering can reduce cardiovascular events, some have attempted to salvage the hypothesis with a revised ‘glycaemic memory’ theory whereby initial poor control of blood glucose produces longer term detrimental effects on the pathogenesis of diabetes (which are seemingly irreversible given the lack of success or even harm in the above trials), and some simply downplay the results and question the early termination of the ACCORD study (with notable drug company conflicts of interest). One might muse that perhaps glucose lowering does have a beneficial effect on cardiovascular disease in type 2 diabetes but that, however, due to various factors such as study weaknesses, lower than expected cardiovascular event rates, or the rapidity of glucose lowering, this beneficial effect has not been evident in trials thus far, and indeed various commentators have suggested that this could be the case. Thinking along such lines is a worthwhile endeavour as it presents us with considerations to take heed of in pursuing further the 'glucose-lowering as cardiovascular benefit' hypothesis. If we assume that indeed glucose lowering can in fact improve cardiovascular outcomes in diabetes, the following questions come to light:

i. What would it take to prove it?

Clearly, at least another study would be necessary, of sufficient duration (at least 5 years) and sufficient size (in the thousands if not tens of thousands) to observe the presumed cardiovascular benefit. Such a study would incur a substantial cost and also involve hard ethical questions as to whether participants should be subjected to such glucose-lowering treatment when there are clear risks of hypoglycaemia, which analyses from both the previous ADVANCE and ACCORD studies.
suggest is associated with increased risks of mortality. Power calculations based on the expected benefit level would be greatly problematic given the already largely negative results from the existing studies, and additionally in light of these prior studies one trial with a ‘positive result’ would most likely not be deemed sufficient to prove the hypothesis, necessitating further studies.

**ii. Would it change clinical practice?**

The mainstay of diabetes treatment around the world is currently glycaemic control. Even in the absence of substantial supportive evidence for a beneficial effect of good glycaemic control on macrovascular and microvascular endpoints, good glycaemic control has been pursued as a means to reduce their incidence. In addition, more attention has been given in recent years to states of ‘pre-diabetes’, in recognition of blood glucose being a continuous variable upon which the diagnosis of diabetes is made by a pre-defined cut-off. Thus, the thrust of clinical practice is currently to identify individuals with, or at risk of, diabetes as early as possibly in the disease process and establish good glycaemic control. It is unlikely that clinical practice would, or even could, greatly change in a hypothetical future in which glycaemic control was also shown to produce cardiovascular benefit.

**iii. Does it offer anything new?**

The current pharmacopeia of diabetes drugs is exclusively devised around lowering blood glucose levels. Proving a cardiovascular benefit of glucose lowering would not offer any new drug targets, neither would it offer any hope to those already with diabetes, given not only that their clinical treatment would be unlikely to change, but also that data from current trials would suggest that for those with established diabetes there is no benefit of good glycaemic control on cardiovascular mortality.

Perhaps the best way to close this part of the discussion on whether glucose control exerts any benefit on cardiovascular disease in those with type 2 diabetes is the words of Jay Skyler *et al.* in the release of joint position statements from the American Diabetes Association, American Heart Association and American College of Cardiology Foundation: “It is likely that a real benefit of glucose lowering on CVD in type 2 diabetes, even if it could be proven, is modest compared with and incremental to treatment of other CVD risk factors” 69–71.
1.3 End of the glucose apotheosis?

“The history of modern knowledge is concerned in no small degree with man’s attempt to escape
from his previous concepts”


Einstein famously noted: “it can scarcely be denied that the supreme goal of all theory is to make the
irreducible basic elements as simple and as few as possible without having to surrender the adequate
representation of a single datum of experience” \(^8^5\), often paraphrased as ‘things should be made as
simple as possible, but not simpler’. However, to the detriment of advancement in the understanding
and treatment of diabetes, it appears that in the present day state of affairs this dictum has been
breached (one wonders possibly in the hope of profits from drug revenue, or due to the ever-
increasing importance and centrality in scientific and medical circles of the prestige of ‘high impact’
publications, rather than the primacy of advancing scientific understanding) and thus despite
evidence to the contrary, the populist view of diabetes pathogenesis has surrendered ‘the adequate
representation of a single datum of experience’, and we are left with various oversights in the widely
accepted theory of diabetes pathogenesis; of particular relevance to the current discussion is that the
focus on the symptom of elevated blood glucose results in a simplification which largely casts aside
the plethora of concomitant comorbidities characteristic of this disease (e.g. insulin resistance,
hyperuricaemia, ectopic lipid deposition, alterations in circulating adipokines, elevated reactive
oxygen species, endoplasmic reticulum stress, chronic subclinical inflammation) as curiosities or
epiphenomena of little importance.

In the absence of a clear rationale for focusing on reducing blood glucose levels in the future to
control the macrovascular complications of type 2 diabetes, it is starkly clear that if we are to
improve the burden of cardiovascular disease in individuals with type 2 diabetes, and the wider
impact this has on society (especially in the face of projected increases in the prevalence of obesity
and type 2 diabetes in the immediate and medium-term future \(^8^6\text{--}^8^9\)), then new avenues and
hypotheses need to be explored.
1.4 The pathogenesis of diabetes and its complications revisited

“…we see around us every day the growth of new beliefs, which think themselves new; and which are yet but the old, which pretend to be young”

the character Van Helsing, from Bram Stoker’s ‘Dracula’

“It is certainly a sobering thought for those entering this area of research to learn that little is truly new: rediscovery and explanation of previously believed but unproven hypotheses are often the rule”

A. Boulton, “Why ‘diabetes and history’?”, Diabetic Medicine, 1994

1.4.1 The obesity, cardiovascular disease and diabetes trinity

As stated in the opening paragraph of this thesis, there is a rising incidence of obesity, diabetes and heart disease worldwide, and thus considerable interest in identifying key pathophysiological components which could form a common ground for their development. A number of metabolic abnormalities exist in type 2 diabetes (listed on the previous page), and these same aberrations are often also observed in obese individuals. Far from the state of despair raised by some commentators in the wake of the failure of blood glucose lowering treatments to prevent cardiovascular disease in diabetes, the array of metabolic abnormalities in the diseases of obesity and diabetes could be viewed as offering an abundance of potential therapeutic targets, and in addition, open the way for the advancement of alternative theories of the underlying pathogenesis of diabetes and its complications; theories in which blood glucose need not be a central player.

Theories regarding a common predisposing factor underlying these three diseases are often referred to as a ‘common soil’ hypothesis, and evidence of this idea is found mentioned throughout the modern history of diabetes: cf. “Perhaps it would be more nearly correct to regard the problem in the light of an abiotrophy affecting both the insulin-producing tissues and the vascular systems alike, and to suggest that a condition is present which requires merely an additional stimulus to bring about arterial lesions of pathogenic proportions, just as it requires an additional stimulus, such as obesity, overfeeding, or an infectious illness, for diabetes to become manifest” (Dry and Hines, 1941); and: “Is it the high blood sugar? The elevated lipids? Or may there be a common factor which produces both the diabetes and the complications as two branches of the same tree?” (Ricketts, 1947). Early studies of retinopathy in diabetes noted a surprising correlation with the presence of elevated blood pressure, with however, a higher prevalence also seen in those with diabetes than those without in the absence of hypertension, leading the authors to conclude: “the very existence of
retinitis in cases in which patients have no other signs of vascular disease must mean that diabetes alone does something to injure the finer arterioles or venules of the retina” ⁹³. Notably, they also raised the possibility that other complications of diabetes could all arise from the same phenomena, stating: “The possibility occurs to us that lesions like those observed in the retina may also occur in the peripheral nerves and thus account for the high incidence of peripheral neuritis among patients with retinitis. Also, similar lesions… might contribute to the atherosclerosis of the larger arteries”.

One of the most well-known theories regarding the link between cardiometabolic diseases is the hypothesis from Gerald Reaven that insulin resistance could be a central underlying causative factor⁹⁴. He proposed that various surrogate measures of risk of diabetes and heart disease could be grouped together as one 'syndrome'. The so-called 'metabolic syndrome/syndrome X' has been highly controversial and the clinical utility of the syndrome later questioned by the author himself⁹⁵, who is, however, still supportive of the role of insulin resistance as an underlying cause of these conditions ⁹⁵,⁹⁶.

Although the plethora of metabolic alterations in diabetes and obesity are numerous, and any of these could be potentially causative or merely associative, there is a growing pool of evidence suggesting that chronic activation of the innate immune system and the resulting low grade systemic inflammation associated with obesity, diabetes and heart disease may be a major contributing factor to the joint pathophysiology of these conditions ⁹⁷–⁹⁹. Hints of this hypothesis can be seen much earlier, with Himsworth noting in 1939, for example: “diet is not the only factor influencing insulin-sensitivity. It is probable that sepsis and obesity have a similar influence, and for this reason they also may precipitate diabetes” ¹⁰³. The role of inflammation in the development of insulin resistance and pathogenesis of diabetes, and the relevance of inflammatory processes as a link between obesity, diabetes and cardiovascular disease is the focus of the remainder of this thesis ⁹. The evidence linking inflammation of the innate immune system to each of these diseases is discussed below, along with

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⁹ This state has been termed ‘metaflammation’ by some ⁹⁷,¹⁰⁰–¹⁰².

⁹ This is also a wealth of evidence that obesity is a risk factor for many cancers ¹⁰⁴,¹⁰⁵, increasing recognition of a link between diabetes and certain cancers ¹⁰⁶–¹⁰⁸, and evidence that chronic inflammation may play a role as a mediator between these diseases and risk of cancer ¹⁰⁹–¹¹⁵. However, an additional examination of the link between inflammation, metabolic diseases and cancer, while clinically relevant, is outside the scope of this thesis.
the existing evidence as to whether targeting inflammation in these conditions can reduce the risk of cardiovascular disease, or improve diabetes control or the development of diabetes complications.

1.4.2 Inflammation and obesity

There is emerging evidence of a central role of adipose tissue biology, including appropriate adipocyte differentiation in the pathogenesis of obesity-related insulin resistance. Adipocyte size has been shown to relate to degree of insulin resistance, and, in a study in Pima Indians, was shown to predict incident diabetes better than percent body fat. Adipose tissue function impacts on the release of pro-inflammatory cytokines and adipokines which act locally via paracrine actions, and systemically via release into the circulation. Obesity has been shown to be associated with increased macrophage infiltration into adipose tissue, and higher levels of circulating inflammatory markers, and thus obesity-associated inflammation could provide a mechanism by which obesity increases the risk of type 2 diabetes and heart disease.

1.4.3 Inflammation and cardiovascular disease

The development of atherosclerosis is a multi-factorial process dependent on genetic influences and nutrition and lifestyle effects, although the key chain of events is thought to be similar in all cases: circulating lipoproteins accumulate in the vascular endothelial wall and these, in turn, cause the release of chemoattractant factors to cause monocyte accumulation, their differentiation into macrophages, and subsequent uptake of embedded lipoproteins into the macrophage. These heavily lipid-laden macrophages are classified as foam cells and, primarily due to their size, become embedded in the vascular wall. Accumulated monocytes, macrophages and foam cells themselves also secrete chemoattractant and pro-inflammatory cytokines and thus the process becomes self-amplifying, until such point as the blood vessel becomes occluded, and depending on the concentration of other thrombolytic factors such as matrix metalloproteinases (which degrade the vascular wall), the atheroma either bursts causing thrombosis or becomes so occluded as to limit blood flow, with the small vessels supplying the heart itself often ironically the first to fail, resulting in the typical symptoms of angina and ‘heart attack’. Thus, current theory is that as well as traditional factors such as lipoprotein concentration (commonly, lipid or cholesterol levels), blood pressure and family history of disease, inflammatory factors also contribute to the pathogenesis of cardiovascular disease, and thus a clear mechanism exists by which the subclinical inflammation associated with obesity and type 2 diabetes may contribute to heart disease.
Given the acknowledged role of inflammation on the pathogenesis of atherosclerosis and cardiovascular disease there are surprisingly few studies specifically examining anti-inflammatory medications and cardiovascular outcomes. In part this may represent the relatively recent nature of the hypothesis of cardiovascular disease as an inflammatory disorder; while the origins of the hypothesis can be traced as far back as the works of Virchow \textsuperscript{129,130}, an association of cardiovascular disease with elevated levels of CRP was shown in the 1950’s \textsuperscript{131–137}, modern reviews on the pathogenesis of atherosclerosis begin to discuss inflammatory mechanisms in the 1980’s \textsuperscript{138}, and the major modern work affirming the hypothesis of atherosclerosis as an inflammatory disorder was published in 1999 \textsuperscript{139}.1

Low dose aspirin is a recommended treatment for reducing cardiovascular risk as both a primary and secondary prevention measure, however this has entered clinical practice more as an anti-platelet rather than an anti-inflammatory agent. In addition, the data from studies examining the use of aspirin specifically in patients with type 2 diabetes are inconsistent \textsuperscript{140–146}, and a recent meta-analysis on the use of aspirin for the primary prevention of cardiovascular events in people with type 2 diabetes found a relative risk (95 % CI) of events of 0.91 (0.79 to 1.05) \textsuperscript{140}. Combined with an increased risk of gastrointestinal and retinal bleeding with aspirin therapy it appears that its use as a cardioprotective agent in type 2 diabetes is thus not warranted. Whether this difference of an apparent lack of effect in those with type 2 diabetes compared to benefits in those without arises from a greater level of ‘aspirin resistance’ in people with type 2 diabetes is a possibility \textsuperscript{147}, although it is not certain that this underlies the discrepancy. In addition, whether the low doses of aspirin used for their anti-platelet effects are sufficient to bring about anti-inflammatory effects \textsuperscript{148} is not clear.

Another major class of cardioprotective agents is the 'blockbuster' statins (hydroxymethylglutaryl-coenzyme A reductase inhibitors). Although designed to inhibit a specific enzyme in the cholesterol synthesis pathway, they show pleiotropic effects, including anti-inflammatory properties \textsuperscript{149}. Thus,

\textsuperscript{1} The relatively ‘young’ age of this hypothesis still does not provide a great deal of assurance as regards the paucity of studies examining anti-inflammatory medications and cardiovascular disease outcomes; notably the work by Ross in 1999 has been cited some 11,310 times [source: Scopus database, 28-11-11] showing an intense interest in this hypothesis, which would be expected to translate into experimental works testing it. The possibility exists therefore that publication bias may be present and that studies have been undertaken which have had ‘negative’ findings and remain unpublished.
while they show cardioprotective effects (in those with diabetes as well as those without \textsuperscript{150,151}) the extent to which the observed cardioprotective benefits can be ascribed to their anti-inflammatory properties cannot be easily ascertained \textsuperscript{1}. One study has examined their use more specifically as an anti-inflammatory agent, the JUPITER trial \textsuperscript{k}, by targeting individuals with moderate cholesterol levels but C-reactive protein (CRP) \textsuperscript{l} levels in the ‘intermediate’ risk range or above \textsuperscript{154}. The study reported an impressive level of benefit, with almost half the odds of the primary end point in those randomised to rosuvastatin. The study has raised a large amount of controversy regarding the stated benefits, early termination and external validity of the trial \textsuperscript{155–161} (also including the role of drug company ties and the lead author possessing a patent on the measurement of CRP). One commentary analysing data in the published reports comes to the conclusion that rosuvastatin in fact produced no difference in cardiovascular mortality \textsuperscript{162}. The wider adverse effects of statins in general must also be taken into account, with their cardioprotective benefit counterbalanced by the findings of a meta-analysis on statin use and incident diabetes which revealed a significant 9 % increase in risk of diabetes in those randomised to statin therapy \textsuperscript{163}. Given acknowledged anti-inflammatory effects of statins, this increase in risk is in fact in the opposite direction from that expected by the hypothesis of inflammation as a cause of insulin resistance; however, the mechanism by which statins elevate diabetes risk is unclear. There is also a certain cruel irony that a cardioprotective agent raises the risk of a disease which has been branded a ‘cardiovascular disease equivalent’ \textsuperscript{164}. Given the pleiotropic nature of statins and controversy regarding this study, the findings do not provide clear evidence regarding the hypothesis of whether inhibiting inflammation produces cardiovascular benefit.

In contrast to the studies of aspirin and statins, which exert effects via diverse pathways not limited to anti-inflammatory effects, a recent trial investigated the cardioprotective benefit of a medication specifically targeting p38-MAPK (mitogen activated protein kinase) \textsuperscript{165,166}, which is activated by a

\textsuperscript{1} While statins have become a mainstay treatment for the prevention of cardiovascular events, two recent meta-analyses of their use in primary prevention have described rather modest effect sizes on risk of all-cause mortality, with one reporting a non-significant reduction in risk of all-cause mortality of 9 % \textsuperscript{152}, and another a significant 12 % reduction in odds of all-cause mortality \textsuperscript{153}.

\textsuperscript{k} Abbreviation: JUPITER – Justification for Use of statins in Prevention: an Intervention Trial Evaluating Rosuvastatin

\textsuperscript{l} Many articles talk of high sensitivity CRP, or hs-CRP, however the ‘high sensitivity’ prefix refers to the properties of the assay used to measure CRP, rather than any difference in the properties of the CRP protein itself. Thus, only CRP is used to describe this protein throughout the text.
number of pro-inflammatory pathways. This study examined the potential of blocking p38-MAPK activation specifically after percutaneous coronary intervention (PCI, or ‘stent’ implantation), which is typically accompanied by a post-surgical rise in inflammatory markers. Working on the hypothesis that post-PCI increases in inflammation could contribute to stent restenosis, 93 patients were randomised to receive a p38-MAPK inhibitor or placebo for 28 days following PCI. The study was designed principally as a test of efficacy (as measured by post-PCI increases in inflammatory markers) and safety, however longer term follow up at 30 months post-trial was conducted in some individuals and showed promising results in terms of lower cardiovascular event rates among participants who received p38-MAPK inhibitor treatment.

Chronic inflammation is also a recognized hallmark feature of rheumatoid arthritis, with a number of anti-inflammatory agents now used regularly as part of an evidence-based treatment regimen. A pilot study involving the use of the anti-IL-6 antibody, Tocilizumab, showed improvements in endothelial function and arterial stiffness across a period of 6 months in patients with rheumatoid arthritis, suggesting that modification of the underlying inflammatory process may translate into cardiovascular benefit for patients with this disease.

In summation, while there is a great amount of interest in the role of inflammation in the development of cardiovascular disease, there are currently few trials examining whether inhibition of inflammation provides cardioprotective benefit; interest in this research question appears to have only arisen in recent times.

### 1.4.4 Inflammation and diabetes

While inflammation is recognised to play a role in the pathogenesis of heart disease, the possibility of a link to the pathogenesis of type 2 diabetes has received much less attention. However, this does not imply that such evidence does not exist. Higher levels of inflammatory markers have been observed in persons with diabetes than in those without, even at comparable levels of adiposity; such cross-sectional analyses raise the question as to whether inflammation plays a role in the development of diabetes. However, in cross-sectional data the direction of causation cannot be verified. Longitudinal analyses would overcome this weakness and provide a better test of the hypothesis that inflammation contributes to insulin resistance.
1.4.4.1 Inflammatory markers and worsening glycaemic control

If a factor were to be a causal variable in the development of a disease, it would logically follow that elevations of that variable would be associated with higher incidences of disease. In the case of inflammation as a potential cause of diabetes, various studies have identified inflammatory markers in non-diabetic individuals as being independently predictive of onset of new diabetes or worsening glycaemic control.

CRP has typically been the most intensively studied marker of sub-clinical inflammation, and high levels of CRP have been associated with the incidence of type 2 diabetes or deterioration in glycaemic control in various studies and population groups \(^{179-182}\), including a meta-analysis \(^{183}\). Other inflammatory markers have also been reported to have positive associations with the incidence of diabetes or declining glycaemic control: including interleukin-6 (IL-6) \(^{184-186}\), interleukin-1 beta (IL-1β) \(^{185}\); tumour necrosis factor alpha (α) \(^{187}\); interleukin-18 (IL-18) \(^{188,189}\); and interleukin-1 receptor antagonist \(^{190,191}\). Not all studies have reported positive associations however, with a study in Pima Indians, for example, showing no association between IL-6 or CRP levels and incidence of type 2 diabetes \(^{192}\).

In addition, while the above experiments examine a more long-term timeframe of prediction, more immediate short-term elevations of inflammatory markers occur in infectious illness, and in established diabetes the presence of concomitant illness and infection has long been recognized to cause hyperglycaemia, with this statement written in the British Medical Journal at around the time of the discovery of insulin: “It is of interest to note that often in these infections the return of glycosuria occurs before the signs and symptoms of the disease [infection] are manifest or before the temperature rises. This is not difficult to understand when we remember that the injection of a few million dead microbes into a diabetic will cause a temporary hyperglycaemia” (O. Leyton, 1923) \(^{193}\). Later works imply that such knowledge had been widespread for many decades, with a physician noting in 1937: “That an infection in a diabetic patient upsets the diabetic state, raises the blood sugar and brings on acidosis is a well-known fact” \(^{194}\). In the present day, the problem is noted in diabetes treatment guidelines, with those from the American Diabetes Association stating: “The stress of illness, trauma, and/or surgery frequently aggravates glycemic control and may precipitate diabetic ketoacidosis” \(^{195}\). Thus, acute illness, which is associated with short-term elevations of pro-inflammatory cytokines, exacerbates the glucose intolerance of diabetes, and this has been well-recognized for some time. The same response can also be observed in non-diabetic individuals, with Emil Schnée providing an extensive review of observations of fever and acute illness associated with
glycosuria in his work of 1889 (e.g.: "In malaria, according to Verneuil, and according to Bourdel, glycosuria is caused during the fever-stage" - E Schnée, 1889). He also quotes the system of classifications of diabetes mellitus devised by the physician Frerichs, in which one class of diabetes is listed as "diabetes in consequence of infectious diseases". Modern studies involving experimental induction of endotoxemia by injection of LPS in non-diabetic individuals have shown (albeit with more 'advanced' techniques) similar effects as those noted above by Leyton, with LPS administration causing insulin resistance.

Evidence therefore suggests that elevated inflammatory markers can predict deterioration of glycaemic control, whether it be in non-diabetic individuals progressing to overt diabetes, or in those with established diabetes progressing further into higher levels of glycaemia. However, analyses of this nature are not sufficient to delineate causation, as despite providing predictive ability, the factor of interest may be a risk marker rather than a risk factor directly involved in the causal pathway of disease. Other evidence from the 'natural experiment' of infectious illness shows that a state of high levels of inflammation is associated with changes in glycaemia, and while recent studies of LPS administration allow this relationship to be ascribed cause-and-effect, rather than simply association, such changes could represent a physiological response which in some way assists in alleviating the short-term challenge of infectious disease, rather than a pathological process. Emil Schnée, for example, in his work on diabetes, went to great lengths to dissociate short-term glycosuria from what he saw as 'true' diabetes. Another 'natural experiment' is to examine variation in genes encoding inflammatory markers and incidence of diabetes, and indeed various studies have reported that polymorphisms in genes encoding inflammatory markers show relationships with the incidence of diabetes, the metabolic syndrome, or insulin resistance, including: IL-6 promoter polymorphisms; promoter polymorphisms of the TNFα gene; IL-18 polymorphisms; IκB genotype, and the interleukin-1 (IL-1) gene family. A recent study using the genetic approach of Mendelian randomisation concluded that although CRP was associated with the incidence of type 2 diabetes, analysis of genetic variation in the gene encoding CRP

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n Abbreviation: LPS - lipopolysaccharide; a component of the bacterial cell wall which is recognized by the immune system and initiates an immune response.

n Abbreviation: IκB – inhibitor of kappa beta, involved in the nuclear factor kappa beta signalling pathway discussed further on in the text.
suggests that CRP itself is not the causative factor involved in this association \(^{205,206}\) and the authors conclude that the causative factor must lie upstream in the inflammatory pathway \(^*\).

With the above studies suggesting that increases in inflammatory markers, or variation in inflammatory marker genes, are associated with changes in glycaemia, the question which arises then is whether targeting inflammation can improve diabetes control or the development of diabetes complications in those with established diabetes.

1.4.4.2 Inflammation and diabetes: early works on the effects of salicylates

As far back as 1874 the German physician Wilhelm Ebstein published two case reports on the use of sodium salicylate in the treatment of diabetes, where “in the first case, sodium salicylate completely got rid of the diabetic symptoms, and in the second case helped the patient significantly improve” \(^{210}\). Furthermore, he stated “it must be left to further research to learn about cases of diabetes mellitus in which the application of sodium salicylate… has prospects for success. In any case, it appears to me to be worth including sodium salicylate in the group of medications which deserve to be tested in the treatment of diabetes mellitus. Prompting [interest] in this direction has been the purpose of this text” \(^{210}\). Sadly this call went largely unheard and but a scattering of papers appeared over the following 80 years which investigated salicylate use in diabetes. Indeed, over the 140-odd years since the publication of this work, it is obvious that research into the effects of salicylates in diabetes and insulin resistance has occurred in bursts, until recently characterized by a small number of interested researchers during each phase. These works are summarized in Table 1 and briefly described below. A number of research articles appeared by German physicians and academics (cited in \(^{211}\)), including a report by another physician four years after Ebstein’s account \(^{212}\). Another German physician, Emil Schnée, discusses their use by prominent diabetes physicians around the turn of the century \(^{196}\). Interest in the salicylates as a treatment for diabetes appears not to have spread further afield, however, and but four articles appeared in the British Medical Journal addressing their potential for glucose lowering in the following quarter century \(^{213-216}\).

Reports suggest that the clinical use of aspirin as a treatment for diabetes was more widespread in the early 1900's, but appears to have been abandoned even prior to the introduction of insulin due to the severity and toxicity of side effects \(^{211,217}\) (no doubt arising from the doses used, quoted as being

\(^*\) It is noted that the concept of Mendelian randomisation is not without its limitations, see for example discussions by Sheehan \(et\ al.\) \(^{207}\), Lawlor \(et\ al.\) \(^{208}\), and Little and Khoury \(^{209}\).
as high as 14-16 g/day \(^{215}\). The 1950s-60s marked the period of greatest interest and research with studies in humans and animals into the mechanisms by which salicylates influence glucose metabolism and insulin action \(^{217-231}\). Although studies from this era were in general small and typically intervention-only studies lacking control groups or placebo use, they consistently observed that high dose aspirin or salicylate had the ability to lower blood glucose levels and reduce glycosuria and ketonuria \(^{217-223}\). The efficacy of salicylates was observed with either oral or intravenous administration \(^{219}\), and most studies reported an effect of improving blood glucose levels in persons with diabetes but lesser or no effect in those without \(^{219,221}\), and also noted an absence of hypoglycaemic episodes with salicylate therapy \(^{217}\). Case reports described the use of aspirin for periods of up to 28 months with durable clinical efficacy \(^{220}\), and dose equivalents to replace insulin in diabetes treatment were even investigated \(^{223}\).

Adverse effects however were common and severe, including hearing problems such as tinnitus \(^{217,218}\) and deafness \(^{217}\), nausea and vomiting \(^{217,218}\), hyperventilation \(^{223}\), dehydration \(^{223}\), and neurological symptoms such as headache \(^{218}\) and confusion \(^{223}\). As noted by researchers at the time: “These toxic effects of aspirin, particularly the early transient symptoms due to overdosage with the drug, present a real problem, and they will have to be avoided if aspirin is to find a place in the treatment of diabetes” (Reid and Lightbody \(^{223}\)).

Again, scant research occurred for the following decades until investigations into the effects of aspirin or salicylates on insulin action surface in the literature again, principally driven from a group led by D. Giugliano from the University of Naples \(^{232-241}\). These document improvements in glucose-stimulated insulin release with the administration of lysine acetylsalicylate in patients with diabetes, showing that these effects were additive to the improvement of insulin release seen with sulphonylurea administration (suggestive of differing modes of action), and also showing the potential for salicylates to normalise blood glucose levels in response to hypoglycaemia. These studies hypothesized a potential role for endogenous prostaglandins in exerting a deleterious effect on glucose tolerance.

A more recent proof-of-principle study established that a short course of high-dose aspirin improved surrogate biomarkers of diabetes and cardiovascular disease, including insulin sensitivity as measured
Table 1: Research into the effects of salicylates in diabetes and insulin resistance over the 19th and 20th centuries. Note how the literature falls into bursts of research activity, typically with multiple works emanating from a small number of interested researchers within each period of activity.

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<th>Year</th>
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<tbody>
<tr>
<td>1876</td>
<td>Ebstein W.</td>
<td>On the therapy of diabetes mellitus, in particular on the application of sodium salicylate. (Reprinted in part in English)</td>
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Table 1 cont:

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**Late 1970’s - 1980’s:**

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by a euglycaemic hyperinsulinaemic clamp. Another recent small study in healthy men showed that aspirin was able to ameliorate the insulin resistance caused by acute lipid infusion, without altering insulin sensitivity prior to lipid infusion. In terms of primary prevention of type 2 diabetes, a recent analysis of the Physicians' Health Study examining self-reported use of aspirin and NSAIDs and the incidence of diabetes suggested a protective effect of NSAID use.

Despite these promising results, however, the tendency for high dose aspirin to cause major gastrointestinal bleeding obviously limits its application for widespread use. Another compound named salsalate (a dimer of salicylic acid, marketed under the trade names Disalcid, Amigesic, and Salflex), which unlike aspirin does not influence gastrointestinal prostaglandins, cause gastropathy, nor alter normal platelet function, is currently undergoing clinical trials to determine efficacy in the treatment of type 2 diabetes and reducing cardiovascular and diabetes risk.

1.4.4.3 Inflammation and diabetes: current studies on salsalate, related salicylates, and other anti-inflammatory agents

One study has examined different doses of salsalate as an add-on therapy in 104 adults with type 2 diabetes, showing mean decreases of 0.4% in HbA1c and 1.8 mmol/l in fasting blood glucose over 14 weeks with the lowest dose studied (3.0 g/day). Another small randomised controlled trial including 17 people with type 2 diabetes showed improvements in glycaemic control and insulin measures over a 4 week period, and another conducted in treatment-naïve newly diagnosed patients with type 2 diabetes showed improvements in fasting and postprandial glucose levels over 3 months of treatment compared to placebo. Three trials in obese but otherwise healthy volunteers have been completed and published: a one month course of salsalate resulted in improved measures of glycaemia and inflammation; another double-blind, randomised cross-over trial of a salicylate derivative triflusal resulted in a dose-dependent increase in insulin secretion, which was also observed in vitro in human and mouse islets; and a randomised trial of one week's salsalate

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* Notably at the time of writing this study has been cited by subsequent publications 273 times, despite offering very little in the way of new findings over previous studies in the 1970's - 1980's. Source: Scopus database, 10-1-11.

* Especially in view of the evidence discussed earlier regarding the utility of glucose lowering as a prevention for diabetes complications, it could be argued that examining the potential of anti-inflammatory medications as diabetes therapies and evaluating their efficacy by measuring the extent to which they decrease blood glucose is a rather illogical approach. Only studies with clinical endpoints will reveal whether anti-inflammatory medications are truly of use in the treatment of diabetes.
therapy in obese individuals without diabetes showed improved glucose disposal, due to lower insulin clearance as revealed during a hyperglycaemic-euglycaemic clamp 254. A study of the effects of 4 days of salsalate administration in healthy volunteers showed no change in lipid-induced insulin resistance, but increased basal insulin levels, and elevated resting energy expenditure and insulin-stimulated carbohydrate oxidation 255.

At least thirteen clinical trials are currently registered for the examination of salsalate in metabolic and cardiovascular disorders, including: as a treatment in people with type 2 diabetes [www.clinicaltrials.gov registration numbers NCT00392678, NCT00799643] 256,257; to reduce diabetes and cardiovascular risk in individuals with impaired glucose tolerance [NCT00330733] 258 or overweight [NCT00258115] 259; to examine its effect on coronary artery calcification in subjects with the metabolic syndrome [NCT00624923] 260; for effects on vascular function and insulin sensitivity in healthy volunteers [NCT00553995] 261; for effects on glucose disposal and flow-mediated vasodilation in lean and obese individuals without diabetes [NCT00837590] 262; for effects on insulin-mediated and endothelium-dependent vasodilation in subjects with metabolic syndrome [NCT00762827] 263 or atherosclerosis [NCT00760019] 264; to counteract the free-fatty acid induced impairment of blood pressure and endothelial function in obese normotensive subjects [NCT00721617] 265; and on insulin resistance and endothelial dysfunction in people with HIV [NCT01046682] 266. A new trial currently registered is also investigating the potential of different doses of aspirin on markers of diabetes control in individuals with type 2 diabetes [NCT00898950] 267.

The adverse effects of aspirin and salicylates, reported most notably in the studies of the 1950s and 60s, although seen also as far back as the original report by Ebstein 210, remain a serious barrier to the effective use of salicylate-based therapies as a modern day diabetes medication. Textbooks from the same era as many of these early studies describe extensive monitoring and studies into toxicities from salicylate-based medications, also noting that some individuals exhibit allergic reactions to aspirin 211. Recent reviews document the same array of adverse effects noted above, including life-threatening consequences 268. Although it is difficult to discern to what extent adverse effects may differ between aspirin and salsalate (given that the former possesses an acetyl group and differing chemical properties), since salsalate is a dimer of salicylic acid it seems inevitable that the same range of adverse effects and difficulties noted by researchers in the 1950s-60s will again arise in the current clinical trials. Indeed, in the recent studies to report adverse effects, two of eight patients randomised to salsalate therapy reported tinnitus, dizziness or headache in a study of obese but otherwise healthy volunteers 252 (although so did one of eight patients randomized to placebo), and
subjects with type 2 diabetes randomised to differing doses of salsalate were more likely to report nausea, vomiting, diarrhoea, heartburn and tinnitus in another \(^{249}\). The only consolation in this instance is that salsalate has been available as a therapy for rheumatoid arthritis for decades, and thus has a well-established safety profile which is unlikely to result in the kind of surprise adverse effects seen in recently introduced diabetes and obesity medications \(^{269-274}\). Whether other salicylate-based therapies such as nitric oxide releasing aspirins \(^{275-278}\), will fare any better is yet to be seen. Trials utilising other anti-inflammatory medications such as diclofenac [NCT00221052] \(^{279}\) and diflunisal [NCT00506298] \(^{280}\) with a view to improve glucose metabolism are also registered and currently underway.

Other recent randomised controlled trials specifically target the inhibition of interleukin-1 with the use of subcutaneous injections of recombinant interleukin-1 receptor antagonist (IL-1ra) in participants with type 2 diabetes, and show improvements in markers of diabetes control such as fasting blood glucose, HbA1c, and pro-insulin to insulin ratio for up to 39 weeks of therapy \(^{281,282}\).

### 1.4.5 Targeting inflammation in cardiovascular disease and type 2 diabetes

Surprisingly, given the amount of literature linking inflammation to obesity, type 2 diabetes and cardiovascular disease there is but a small body of evidence that targeting inflammation can improve the outcomes of these diseases, at least as far as studies conducted in humans are concerned. The historical literature described above forms perhaps the largest pool of evidence that inflammation may be a viable target for medical therapy in diabetes, and currently registered clinical trials (mentioned above) should soon provide more definitive evidence – given the lack of randomised controlled trials in the historical literature – of whether anti-inflammatory medications can form part of the diabetes pharmacopeia. Thus, evidence that specifically targeting inflammation via the use of medications produces benefit in cardiovascular disease or type 2 diabetes appears in its infancy.

However, just as the known risk factors for obesity, type 2 diabetes and heart disease include lifestyle-associated variables such as diet and exercise, there is evidence that levels of inflammation in obesity and type 2 diabetes can be modified by these same factors, raising the possibility that inflammation could be targeted by diet and exercise strategies.
1.5 Lifestyle effects on inflammatory markers

In order for a factor such as inflammation to be plausibly related to the pathogenesis of diabetes and cardiovascular disease, changes in that factor must also be in alignment with what is already known regarding established risk factors for these diseases. As discussed above, obesity is now well recognized as a state of chronic sub-clinical inflammation, such that obesity-associated elevations in inflammation are in agreement with the role of obesity as a risk factor for cardiometabolic diseases. Given the established role for lifestyle modification in the prevention and treatment of diabetes and cardiovascular disease, changes in inflammatory markers would also be expected, therefore, to show changes in a similar direction as the change in risk of cardiometabolic disease elicited by lifestyle intervention.

1.5.1 Weight loss:

There are a large number of studies which have examined changes in levels of inflammatory markers with lifestyle intervention where the intervention produced weight loss, and it appears from the bulk of evidence that weight loss in the obese or those with type 2 diabetes has the ability to reduce circulating inflammatory markers or measures of activation of inflammatory pathways. Reviews of trials and systematic reviews and meta-analyses of weight loss studies attest to the relationship between weight loss and inflammation, at least as judged by the inflammatory marker CRP. The importance of calorie restriction in controlling levels of inflammation is also underscored by a small study showing that the increases in inflammatory markers typically observed with inactivity can be ameliorated via reducing energy intake in healthy volunteers. These results are also in agreement with studies showing the opposite phenomenon of increases in levels of inflammation with short-term overfeeding in healthy humans or the consumption of a high fat diet in laboratory animals. Clearly then, in terms of dietary studies conducted in obesity and type 2 diabetes, weight loss is a potential confounding factor which makes ascribing cause-and-effect to specific dietary factors difficult.

1.5.2 Individual dietary components:

There are many studies which have examined the association of specific dietary components to circulating inflammatory markers or the potential effects of altering dietary components on markers of inflammation, with a large proportion examining the relationship with obesity-related inflammation. Particularly in depth reviews have been conducted by Calder et al., Basu et al., and Hamer and Steptoe. Below, with a focus on randomised controlled trials conducted in
participants with metabolic diseases such as obesity, the metabolic syndrome, or type 2 diabetes, a brief review is made of the current status of the literature regarding trials which have involved altering dietary intake and examining inflammatory markers as outcomes.

n-3 fatty acids:

Although there have been a number of animal and in vitro studies into the possible anti-inflammatory role of dietary n-3 fatty acids (see, for example, discussions by Calder\(^\text{305}\) and Oliver \textit{et al.}\(^\text{306}\)), there have been few randomised controlled trials in humans assessing whether n-3 fatty acid supplementation can influence circulating inflammatory markers in type 2 diabetes. In a small study in women with type 2 diabetes, dietary supplementation with n-3 fatty acids (given as fish oil) reduced circulating PAI-1 levels, as well as reducing in the expression of inflammation-related genes in subcutaneous adipose tissue\(^\text{307}\). One study of omega-3 supplementation through capsules showed reductions in circulating TNF-\(\alpha\) and IL-2 levels, with no change in CRP levels\(^\text{308}\). Similarly, another randomised controlled trial of fish oil supplementation in type 2 diabetes reported no change in circulating CRP\(^\text{309}\), and another no change in CRP, IL-6 or TNF-\(\alpha\) levels with n-3 fatty acid supplementation\(^\text{310}\). In obese, non-diabetic subjects, supplementation with fish oil showed no effect on CRP levels in one study\(^\text{311}\) or IL-6, CRP, sTNF-R1, sTNF-R2, and PAI-1 levels in another\(^\text{312}\). However, one study of n-3 fatty acid supplementation in men at high risk of cardiovascular events reported a reduction in IL-18\(^\text{307}\). These findings suggest that \textit{in vivo} in states of obesity, type 2 diabetes, or high cardiovascular risk - which are associated with chronic, low-grade inflammation - that n-3 fatty acids have little ability to alter CRP levels. The results for other circulating inflammatory markers are less concordant, which could relate either to between-study differences in participants, or distinct effects on individual inflammatory markers which may be of relevance to their particular biological roles.

n-6 fatty acids:

A systematic review published in 2012 of randomised controlled trials in humans examining the influence of dietary n-6 fatty acids on markers of inflammation concluded that the current body of evidence did not suggest an influence of n-6 fatty acids on circulating markers of inflammation\(^\text{313}\). Similarly, a randomised controlled trial published the same year comparing an n-6 fatty acid and a saturated fat diet found no differences in circulating IL-1ra or sTNF-R2 between groups\(^\text{314}\).
**Soy:**

A randomised cross-over trial in post-menopausal women with the metabolic syndrome comparing responses to control (DASH diet), soy nut or soy protein dietary regimens reported reductions in circulating CRP with both soy-based diets, with the effect greater after the soy nut diet, which additionally produced significant reductions in IL-18 and TNFα \(^{315}\). However, another study with overweight and obese participants found no effect of a soy diet on a range of inflammatory markers \(^{316}\). Trials in post-menopausal women without diabetes suggest that soy has no effect on circulating inflammatory markers \(^{317-322}\), including the post-exercise increase in inflammatory markers \(^{323}\). Similarly a trial in middle-aged men and women examining diets with differing isoflavone content showed little effect on circulating inflammatory markers, which the exception of an increase in IL-6 in women only \(^{324}\), and another in hypercholesterolaemic subjects found no effect on circulating CRP levels but a reduction in TNFα \(^{325}\). In terms of studies involving participants with type 2 diabetes, one randomised controlled trial has been conducted in individuals with type 2 diabetes and nephropathy, finding a significant decrease in CRP with a high soy protein diet \(^{326}\).

**Nuts:**

A randomised cross-over trial in Chinese participants with type 2 diabetes compared isocaloric diets where 20% of the total energy intake was substituted with almonds, finding almond intake produced a significant decrease in IL-6, TNFα, and CRP measures \(^{327}\). This appears to be the only trial in subjects with type 2 diabetes. Other studies enrolling participants with the metabolic syndrome have compared a walnut, cashew nut, or control diet and report no effect of either nut diet on CRP levels \(^{328}\), and another including a comparison of a mixed nut diet (consisting of walnuts, hazelnuts and almonds) with a control diet in participants with the metabolic syndrome observed no change in circulating CRP, PAI-1 or IL-18 levels, but a decrease in IL-6; however, this was attenuated after adjustment for weight loss \(^{329}\). Studies in hypercholesterolaemic subjects report differing effects, with decreases in circulating CRP \(^{330}\) and TNFα \(^{331}\), as well as reduced production of IL-1β, IL-6 and TNFα by peripheral blood mononuclear cells \(^{331}\) with diet characterized by a high intake of walnuts, and in contrast no change in CRP with a walnut diet \(^{332}\). A study of pistachio nut supplementation in healthy young men observed a decrease in IL-6 but no change in CRP or TNFα levels \(^{333}\). On the whole, these works paint a picture that nuts may reduce circulating inflammatory markers, but effects appear to vary greatly across different studies; at any rate, no studies have reported worsening levels of inflammation with diets involving high levels of nut consumption.
Dietary advanced glycation endproducts (AGEs):

Two studies have examined the effect of dietary AGEs in randomised controlled trials in subjects with type 2 diabetes with small sample sizes: one study including a 2-week randomised cross-over design study in 11 subjects and one 6-week randomised parallel trial in 13 subjects (Vlassara et al. \(^{334}\)), and another a metabolic study assessing the effect of acute intake after a high or low AGE meal involving 20 subjects (Negrean et al. \(^{335}\)). In both studies, diets were balanced for macronutrient composition but altered dietary AGE content via different cooking methods. Across both trials reported by Vlassara et al., higher dietary AGE content produced higher measures of inflammatory markers or inflammatory gene expression in peripheral blood mononuclear cells. In that of Negrean et al., post-meal levels of circulating CRP and IL-6 did not differ between high and low AGE content meals.

Macronutrient composition:

One study with a low total fat composition (based on the National Cholesterol Education Program stepped diet) found little effect of a low fat dietary intervention on circulating CRP levels, with a significant effect of diet limited only to female participants with the metabolic syndrome \(^{336}\). One study in participants with type 2 diabetes showed that a high protein diet reduced circulating measures of CRP and IL-6, but not a low calorie diet based on liquid meal replacements, despite a greater weight loss on the latter \(^{337}\). In a randomised controlled trial in participants with type 2 diabetes, a 6-month low fat dietary regimen produced a significant reduction in circulating CRP levels \(^{338}\), which was not observed on a low carbohydrate diet, despite similar reductions in body weight in both dietary arms.

Alcoholic beverages:

With particular regard to type 2 diabetes, a study of secondary prevention (enrolling subjects post-myocardial infarction) randomised participants to moderate red wine consumption for a year, finding significant reductions in CRP, IL-6, TNF\(\alpha\) and IL-18 after red wine consumption \(^{339}\). This appears to be the only randomised controlled trial investigating the effect of alcohol intake on inflammation in type 2 diabetes.

Studies in healthy subjects are generally supportive of an effect of moderate alcohol consumption on circulating inflammatory markers. In a randomised cross-over study of moderate wine consumption in healthy women, CRP, IL-6, ICAM-1 and CD-40 ligand were all reduced by consumption of either white or red wine \(^{340}\). Similar effects have been reported by moderate alcohol consumption in men...
with a study by Vázquez-Agell *et al.* showing reduced CRP, IL-6, ICAM-1, and CD-40 ligand after moderate consumption of white wine for 3 months. A randomised cross-over trial of healthy individuals involving moderate red wine consumption found decreased CRP, but increased PAI-1 after 4 weeks of intake, with another reporting no change in CRP levels with 3 weeks of moderate red wine intake. A similar randomised cross-over study in healthy men compared moderate red wine intake with gin (matched for total alcohol consumption) finding reductions in ICAM-1, VCAM-1, and CRP with wine intake.

There is suggestion that some of the effects observed with wine consumption could arise from other, non-alcoholic components of the wine, as studies which have involved matched alcohol intake from gin have generally not observed the same anti-inflammatory effects as wine. In addition, a randomised controlled trial in male smokers involving supplementation with flavanols extracted from grapes produced significant decreases in the expression of inflammation-related genes in leukocytes, however, in the absence of a change in circulating CRP.

**Non-alcoholic beverages:**

One RCT has found no effect of green tea consumption on CRP in participants with type 2 diabetes or states of ‘prediabetes’. These results are supported by another RCT finding no effect of green tea on CRP or IL-6 levels in subjects with type 2 diabetes, or another finding no effect on CRP, IL-1β or IL-6 in subjects with the metabolic syndrome. In contrast, a study involving obese participants with hypertension found a green tea extract produced significantly lower CRP and TNFα than placebo. An RCT in healthy men found no effect of green tea extract supplementation on CRP and IL-6. Less research appears to have been done into the effects of black tea, however one RCT in healthy men reported a significantly lower CRP with randomisation to black tea vs placebo.

In a randomised controlled trial of coffee consumption, 4 weeks of coffee consumption resulted in lower IL-18 levels compared to no coffee in subjects at risk of type 2 diabetes, with no differences in IL-1ra, IL-6 or CRP levels. A study of acute caffeine administration (in the form of tablets) in people with coronary artery disease or healthy controls found a post-coffeeine decrease in CRP levels in both groups, suggesting that caffeine itself may contribute to the effects of tea and coffee. However, another acute study in healthy men found no effect of caffeinated or de-caffeinated coffee on serum IL-6 or IL-18 levels.
Dietary fibre:

With regard to the effects of dietary fibre on inflammation, a cross-sectional relationship between higher habitual dietary fibre intake and lower CRP levels has been shown in many studies but not all. These findings are suggestive of an anti-inflammatory effect of dietary fibre; an effect is also supported by data from one longitudinal study. With regard to intervention studies, a systematic review conducted in 2009 noted that trials involving an increase in dietary fibre generally resulted in a decrease in circulating CRP levels, however, with the caveat that interventions which modulated dietary fibre often also reported changes in carbohydrate and saturated fat intakes, or failed to report whether changes in dietary macronutrient composition had occurred. Such factors limit the ability to draw conclusions regarding cause and effect of specific dietary components, and indeed, reflect an intractable difficulty when conducting dietary intervention studies; when conducting isocaloric alterations in dietary components, a reduction in one component must be accompanied by an increase in another to maintain the same overall calorie intake. As such, while questions along the lines of “does dietary factor ‘x’ influence markers of inflammation?” are easy to ask, in reality discerning cause and effect becomes much more difficult once put into practice, especially in the case of the link between obesity and inflammation where changes in body weight may mask or confound relationships between dietary factors and inflammatory markers. In response to such problems, and out of a growing acknowledgement of real life eating habits, where people usually do not set out with the aim of ‘increasing dietary β-carotene intake’, but increase their dietary fruit and vegetable intake (as a hypothetical example), many research studies now do away with attempting to identify specific cause-and-effect relationships from a reductionist perspective, but focus more holistically on dietary patterns and their effects on surrogate markers of metabolic health. (These problems in ascribing cause and effect in dietary interventions are expanded upon further in Chapter 4, Section 4.2).

1.5.3 Dietary patterns:

‘Mediterranean’ diet:

In such works examining dietary patterns and markers of inflammation, many intervention studies have focused on the so-called ‘Mediterranean diet’, which has been shown to improve a number of circulating inflammatory markers in elderly subjects at increased risk of coronary heart disease, participants with the metabolic syndrome, obesity, hypercholesterolaemia, and reduce NF-κB gene expression in peripheral blood mononuclear cells in elderly subjects. However, not all
studies report improvements in inflammatory markers with a Mediterranean diet, such as two studies conducted in participants at a high risk of cardiovascular events showing no improvement in CRP\(^{366,367}\), or another observing no effect in healthy subjects\(^{368}\). While the disparate results of these studies may reflect differences in diet composition or study duration, they may also point to differences in effect dependent on the metabolic health of enrolled participants; indeed, it would be perfectly reasonable to expect that the potential anti-inflammatory effect of a dietary intervention would depend on the initial inflammatory status of the participants involved.

**Other dietary patterns:**

Similar to studies involving the Mediterranean diet, other dietary regimens involving the alteration of multiple components have been investigated. An improvement in CRP levels was reported in participants with impaired glucose tolerance following a diet characterized by fish, bilberries and wholegrains\(^{369}\), and likewise reduced CRP levels in adults with hyperlipidaemia have been observed with a diet high in plant sterols, almonds, viscous fibre and soy protein\(^{370}\).

Investigations into the DASH (Dietary Approaches to Stop Hypertension) diet have demonstrated the potential for this dietary pattern to influence circulating cytokine measures, with a study in participants with type 2 diabetes showing a reduction in CRP during a randomised cross-over study\(^{371}\). Although cross-over arms were only of an 8-week duration, significant differences in body weight were produced from the DASH diet relative to control, and thus it is difficult to decipher whether responses were due to changes in diet or body composition.

A randomised controlled trial in persons with type 2 diabetes (n = 162) who managed their blood glucose with diet only examined the effect of a low vs. high glycaemic index diet on inflammation. Importantly, the dietary group exhibited no significant differences in body weight or HbA1c at the completion of the study, but the low glycaemic-index diet group showed reduced CRP levels\(^{372}\), which suggests that in type 2 diabetes dietary glycaemic index in itself may modulate inflammation in the absence of changes in body weight.

Although not a ‘dietary pattern’ study *per se*, Bakker *et al.* conducted a randomised controlled trial involving supplementation with a number of substances simultaneously, on the hypothesis that each had shown promise as anti-inflammatory agents found in different foods. Overweight men with mildly elevated CRP were randomised to receive supplementation containing resveratrol, tomato extract, green tea extract, \(\alpha\)-tocopherol, fish oil and n-3 fatty acids, cellulose, and soy lethicin. No
change was observed in circulating CRP levels, although reductions in circulating IL-18 and sTNF-R2 were observed.

### 1.5.4 Dietary patterns and components - summary

One aspect of the above studies which becomes evident is the diversity of effects reported. As mentioned throughout, the different findings of these works could easily be due to factors such as study design, duration, or differences in the dietary modifications employed. In addition, for purely statistical reasons, the effect of dietary factors on inflammatory markers would be easier to detect in individuals with higher baseline levels of inflammation. Furthermore, there may be biological factors which influence the effect of specific dietary alterations on levels of inflammatory markers, such that alterations, for example, as increasing fibre intake may exert an anti-inflammatory effect in obesity-related inflammation, but not in healthy individuals. One finding which is evident is that there is little evidence that implementing dietary changes such as those reviewed above would result in increases in markers of inflammation.

### 1.5.5 Exercise:

Regarding the specific effects of exercise, a number of studies report cross-sectional relationships between levels of inflammatory markers and measures of fitness or physical activity, and that physical activity lowers levels of inflammatory markers in obesity, type 2 diabetes and heart disease. Not all interventions have shown reductions, however, and the effects of exercise on inflammation appear complex; in the short term exercise induces increases in a number of inflammatory markers, and different types of exercise or physical activity may exert differing influences on chronic inflammation, with genotype interactions also operating.

### 1.5.6 Clinical outcomes:

Studies examining the influence of lifestyle intervention on chronic low-grade inflammation typically have only gone so far as to investigate whether lifestyle interventions can influence markers of inflammation, and not tied the reduction in inflammation to solid clinical outcomes such as event rates of cardiovascular disease or diabetes complications. Clearly the effects of diet and exercise are diverse, and if future studies do arise which follow up participants for clinical events, the extent to which the anti-inflammatory effects of lifestyle modifications underlie a potential clinical benefit versus other metabolic effects of the interventions will be extremely difficult to
quantify. One study which is an exception in this regard is an analysis of exercise and cardiovascular events in the Women’s Health Initiative, which followed 27,055 women over a 10-year period and attempted to quantify the effect of baseline measures of cardiovascular risk on cardiovascular event rates by regression modelling adjusting for different sets of *a priori* risk factors. They estimated that between approximately 20 - 30% of cardiovascular events could be explained by haemostatic or inflammatory pathways, evaluated in their modelling by the grouping of baseline C-reactive protein, fibrinogen and sICAM-1 (soluble intercellular adhesion molecule-1) measures.

### 1.5.7 Lifestyle effects on inflammation in type 2 diabetes:

With particular relevance to the emphasis of this thesis on inflammation in type 2 diabetes, there is a paucity of studies examining changes in inflammatory markers with lifestyle intervention in people with type 2 diabetes. A study comparing lifestyle intervention and insulin treatment in type 2 diabetes by Aas et al. (n = 28) showed that while both were able to give similar reductions in glycaemia, only lifestyle intervention reduced circulating inflammatory markers, although to what extent this was caused by differences in body weight with insulin vs. lifestyle therapy is unknown. As mentioned above, a low-glycaemic index diet was investigated for changes in CRP in participants with type 2 diabetes (n = 162) by Wolever et al., however, this only included patients whose diabetes was managed by diet and not medications. One study of 33 women with type 2 diabetes compared the effects of diet, exercise, or diet plus exercise interventions on selected inflammatory markers, and included subjects whose diabetes was treated with either diet only, metformin, or sulphonylureas (Giannopoulou et al.); they found a significant reduction in CRP levels in all three treatment groups. A small randomised cross-over trial (n = 12) by Bozzetto et al. compared the acute effects of a meal high in monounsaturated fatty acids (MUFA) or one high in fibre and low in glycaemic index on circulating inflammatory markers, finding a significant post-meal decrease in CRP after a MUFA diet but not a high fibre/low glycaemic index diet. Two randomised controlled trials have been published which exclusively enrolled subjects with type 2 diabetes and modified one specific dietary variable: that of Azadbakht et al. in 41 people with type 2 diabetes and nephropathy, which examined the effect of soy protein, finding a significant decrease in CRP with a high soy protein diet; and that of Kabir et al., which enrolled 27 women with type 2 diabetes who were randomised to 2 months of n-3 fatty acid supplement, finding a reduction in

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1 The LOADD study, which forms part of this thesis, also examines only surrogate markers of diabetes and cardiovascular risk. In the event of a follow up study involving clinical endpoints, this same difficulty of ascribing causation will apply.
circulating PAI-1. There have been some studies of the effect of dietary alteration on levels of inflammatory markers in obese individuals which have included some individuals with type 2 diabetes, but these have generally only included small subsets of people with type 2 diabetes, such as Bastard et al. (n = 7), Monzillo et al. (n = 8), and Bjermo et al. (n = 9).

Thus, only the studies of Wolever et al. (examining the effect of glycaemic index), Giannopoulou et al. (diet and/or exercise) and Aas et al. (lifestyle and/or insulin), Azadbakht et al. (soy protein in individuals with nephropathy), and Kabir et al. (n-3 fatty acid supplementation) have examined whether long-term lifestyle changes cause alterations in circulating inflammatory markers in individuals with type 2 diabetes. The studies of Aas et al. and Giannopolou et al. consisted solely of individuals on oral hypoglycaemic agents, while that of Wolever et al. enrolled participants treated with diet only; thus, the subjects enrolled were not reflective of the broader population of individuals with type 2 diabetes who often receive a more intensive regimen of concurrent diabetes pharmacotherapy. That of Kabir et al. consisted of an intervention involving solely of supplementing one specific dietary component. The study of Azadbakht et al. is interesting: on the one hand it enrolled solely participants with nephropathy, which may limit the ability to generalize results to other patients with type 2 diabetes, and involved a highly prescriptive dietary regimen of increased soy protein intake; on the other, enrolled participants were treated with any form of diabetes medication, and additionally followed subjects for 4 years, far longer than the other works mentioned here. They found 4-year levels of circulating CRP were lower in those randomised to soy protein intake.

Given the hypothesized role of inflammation in the development of cardiovascular disease, and even in the pathogenesis of diabetes itself, an experiment to add to these works was therefore undertaken as part of the current thesis; an investigation into whether dietary modification alters selected adipokines and inflammatory markers in individuals with type 2 diabetes who are treated according to current therapeutic guidelines. This study and analysis form Section 2 of the current thesis, as part of the Lifestyle Over and Above Drugs in Diabetes (LOADD) study. This study was a randomised controlled trial of a 6-month dietary intervention in participants with type 2 diabetes who were concomitantly treated with a range of cardioprotective, renoprotective, and hypoglycaemic medications according to the current New Zealand Guidelines Group recommendations.
1.6 Inflammation and diabetes: a key role of NF-κB

As evidenced from the discussion above, many different inflammatory markers have been implicated or associated with changes occurring in obesity and type 2 diabetes. In general these highlight a shift towards a more pro-inflammatory phenotype in these disease states. One key downstream effect of a number of these inflammatory pathways is the activation of the transcription factor ‘nuclear factor kappa beta’ (NF-κB). NF-κB is present in the cell cytoplasm and is typically bound to members of an inhibitory protein family IκB (inhibitor of kappa beta). NF-κB translocates to the nucleus and influences gene transcription upon activation from a range of pro-inflammatory stimuli, including IL-1, IL-18, and toll-like receptor (TLR) signalling.\(^{401,402}\) Research predominantly conducted over the course of the last decade has implicated the activation of NF-κB as a key event in the development of obesity-related insulin resistance and shown inhibition of NF-κB to be a potential target for the treatment of insulin resistance. Various animal studies have demonstrated that manipulation of NF-κB signalling by a variety of means improves or exacerbates insulin resistance\(^{403-410}\), and that NF-κB is upregulated in the liver\(^{408}\) and hypothalamus\(^{300}\) during a high-fat diet. Likewise studies in humans have shown that hepatic NF-κB binding activity correlates with insulin resistance\(^{411}\) and weight loss in obese individuals has been shown to downregulate NF-κB activity in mononuclear cells.\(^{294}\) Genetic polymorphisms of the gene encoding NF-κB have been shown to be associated with risk of type 2 diabetes in Czech and German subjects\(^{203}\). In in vitro studies, activation of NF-κB has been shown to induce insulin resistance in muscle cells.\(^{412,413}\)

In 1959, Reid and Lightbody concluded the discussion of one of their studies on the glucose lowering effects of aspirin by stating: “These results place salicylate in the class of promising oral antidiabetic substances, and should stimulate investigations to remove its undesirable effects and to find out how it acts.”\(^{223}\). Thirty-five years later, a study in Science revealed that aspirin and salicylates inhibit NF-κB\(^{148}\), which combined with the data outlined above for a role of NF-κB in insulin signalling, suggest that this could indeed be the mechanism of their anti-diabetic actions. Sadly, however, ‘removing the undesirable effects’ of salicylates has yet to be achieved; indeed whether the typical symptoms of ‘salicylism’ observed with high doses of salicylate-based medications\(^{268}\) can be avoided, and mechanistically separated from their glucose-lowering properties, is currently unknown. Later studies also confirmed that salicylates inhibit the NF-κB pathway\(^{408,414-417}\), and in addition, that other diabetes medications such as metformin and pioglitazone also exhibit NF-κB inhibiting properties\(^{416,418}\).
1.6.1 Interleukin-18 as an upstream activator of NF-κB: links with obesity, diabetes and cardiovascular disease

Exactly how NF-κB activity is upregulated in obesity and diabetes is not known, or at least, given that many possible pathways exist including the direct activation of toll-like receptors by saturated fatty acids, and the influence of elevated circulating inflammatory factors in obesity and diabetes, the relative contributions of each to the increased NF-κB activity seen in these conditions is difficult to disentangle. While a number of pro-inflammatory cytokines induce NF-κB activity, at the outset of the investigations involved in this thesis, interleukin-18 (IL-18) was regarded as an inflammatory marker of interest as it activates the NF-κB signalling pathway, is elevated in obesity and type 2 diabetes, released from fat tissue, and evidence existed suggestive of an active role in the pathogenesis of heart disease; prerequisite qualities necessary to contribute to a possible link between inflammation and the triad of metabolic diseases of obesity, diabetes and heart disease.
1.7 A role for IL-18 in metabolic disease

IL-18 was originally described as interferon-γ (IFN-γ) inducing factor (IGIF), and given a recognised role for IFN-γ in the pathogenesis of atherosclerosis, a plausible biological role for IL-18 exists in the pathogenesis of cardiovascular disease via induction of downstream IFN-γ, if not additionally by other mechanisms. Moreover, IL-18 is involved in the hypothalamo-pituitary-adrenal stress axis, and there is abundant evidence of stress exerting detrimental effects on health outcomes associated with obesity, diabetes and cardiovascular disease. A number of animal in vivo, and human in vitro, or ex vivo studies suggest that IL-18 plays a causative role in the development of atherosclerosis.

Human endothelial cells, smooth muscle cells, and mononuclear phagocytes have been shown to express the IL-18 receptor, with increased receptor and ligand levels in atherosclerotic plaques, and with significantly higher levels in symptomatic or ulcerated plaques than in asymptomatic or unulcerated plaques. Various animal experiments in ApoE-/- mice, a highly utilised rodent model of atherosclerosis, have shown that IL-18 contributes to the pathogenesis of atherosclerosis and that its reduction results in improved outcomes. Evidence for a direct effect of IL-18 on the myocardium also comes from studies in rats where improvements in left ventricular ejection fraction were seen after myocardial infarction followed by treatment with the natural endogenous inhibitor of IL-18, IL-18 binding protein (IL-18 BP). A study in a rodent model of the metabolic syndrome also showed that adenoviral-induced IL-18 overexpression resulted in increased expression of cellular adhesion molecules in the aorta, greater aortic thickness and insulin resistance. Studies of tissue from human subjects document higher IL-18 and IL-18 receptor gene expression and protein levels, and lower levels of IL-18 BP gene expression in ventricular tissue from heart failure patients versus control tissue from organ donors, with evidence of higher IL-18 and IL-18 receptor levels in nonrheumatic aortic valve stenosis (with the caveat of comparison to tissue extracted from cadavers, which may be a poor control).

However, other work in vivo in humans has been inconsistent regarding a causative role for IL-18 in atherosclerosis. High IL-18 levels have been observed in individuals with cardiovascular disease, and elevated IL-18 levels have been reported after recent myocardial infarction. A number of studies have documented IL-18 as being predictive of future cardiovascular events, but with associations being no longer significant after adjustment for traditional cardiovascular risk factors, or of borderline significance. Others report that associations of IL-18 with future cardiovascular events remain significant after adjustment for multiple confounders, while...
others find no association at all. However, while a number of individual prospective studies have not shown a statistically significant relationship of baseline IL-18 levels with the development of cardiovascular disease, a meta-analysis of prospective cohort studies found an increased risk of developing cardiovascular disease or coronary heart disease with elevated baseline IL-18 levels, with a relative risk (95% CI) of 1.34 (1.17, 1.53) after adjustment for age, sex, classical cardiovascular risk factors and other inflammatory markers. In studies which have focused on patients recruited after myocardial infarction or percutaneous coronary intervention, IL-18 has been shown to be predictive of future mortality although not cardiovascular mortality, future adverse coronary events, and IL-18/IL-10 ratio predictive of future adverse coronary events.

Studies of subclinical atherosclerosis have produced heterogeneous results for an association of IL-18 independent of traditional cardiovascular risk factors. Two studies examining the association of IL-18 with carotid intimal medial thickness (CIMT) found that IL-18 was associated with CIMT, with one study reporting that this remained significant after adjustment for traditional risk factors, and one that the association was no longer significant. Another study similarly reports that associations with coronary artery calcium were no longer significant after adjustment for traditional risk factors. Other studies have reported positive associations for IL-18 and coronary artery disease severity after adjustment for confounders and in other studies with incomplete adjustment for other cardiovascular risk factors positive associations are reported for IL-18 and pulse wave propagation time, thin plaque fibrous cap thickness, or coronary plaque area.

Various works have examined genetic polymorphisms of genes in the IL-18 system and risk of cardiovascular disease. Multiple studies have been published examining polymorphisms of the IL-18 gene. These studies show associations of polymorphisms of the gene encoding IL-18 with sudden cardiac death, coronary artery disease, myocardial infarction, cardiovascular disease and mortality, the metabolic syndrome, insulin sensitivity, higher levels of inflammation and longer hospital after percutaneous coronary intervention, post-prandial increase in triglycerides, and BMI. In terms of studies examining other components of the IL-18 signalling system, two studies have examined polymorphisms of genes encoding the IL-18 receptor and neither found any association with cardiovascular events or death. One of these also examined polymorphisms of the IL-18 BP gene, although only found one polymorphism of low frequency (less than 2% of participants), which was therefore not examined further for associations with cardiovascular events.
In terms of evidence linking IL-18 with diabetes pathogenesis, IL-18 has been shown in various cross-sectional analyses to show a positive correlation with blood glucose levels and the metabolic syndrome. Two prospective studies report baseline IL-18 levels to be predictive of new-onset type 2 diabetes even when controlling for multiple risk factors and other inflammatory markers and adipokines. A decrease in IL-18 was also observed to be an independent predictor of improvement in insulin sensitivity in patients treated with rosiglitazone or metformin. As far as links with type 1 diabetes and β-cell failure are concerned, evidence also exists that IL-18 plays a role in β-cell apoptosis induced by streptozotocin, a common animal model of type 1 diabetes, and polymorphisms of the β subunit of the IL-18 receptor (IL-18rap) have emerged in genome-wide association studies as being associated with type 1 diabetes.

Studies show that lifestyle modification has the ability to modify IL-18 levels, with exercise interventions reducing plasma IL-18 levels in either low-level or extreme exercise conditions, including in patients with type 2 diabetes. Lifestyle intervention studies resulting in weight loss in obese individuals report reductions in IL-18 levels, as do studies of gastric weight-reducing surgery.

Metabolic studies show that acute hyperglycaemia results in marked elevations of circulating IL-18, and that IL-18 levels are acutely modulated by diet composition with a high-fat diet increasing, and high-fibre diet decreasing, IL-18 levels. Thus, IL-18 levels appear to be moderated by diet in the same direction as risk of cardiovascular disease and diabetes, i.e. a high fat diet has been linked with the development of cardiovascular disease and diabetes, and increases IL-18, while a high fibre diet has been linked with protection from cardiovascular disease and diabetes, and reduces IL-18.

One hypothesis is that IL-18 is in fact not a causative agent as regards the associations with glucose metabolism and insulin sensitivity described above, but acts as a compensatory mechanism and many studies could be viewed as lending support to this hypothesis. IL-18 enhances glucose uptake in adipocytes, decreases hepatic gluconeogenesis and prolongs the hypoglycaemia induced during an insulin tolerance test and thus, for example, elevations of IL-18 during hyperglycaemia or elevated IL-18 associated with higher blood glucose and insulin levels or poor insulin sensitivity could be viewed as a mechanism by which the body attempts to reduce blood glucose. Similarly, reports of elevated IL-18 in obese individuals coupled with animal data suggesting IL-18 can act centrally to decrease food intake (discussed further below) indicate that an elevation of IL-18 in obesity could play a compensatory role, much akin to the manner in which leptin, also an
anorectic cytokine, is elevated in obesity and appears to be associated with 'leptin resistance'. In a similar fashion a degree of 'IL-18 resistance' was reported in a study comparing obese vs. normal weight individuals, where peripheral blood mononuclear cells from obese individuals were shown to exhibit a reduced production of interferon-γ (IFN-γ) in response to IL-18 stimulation. On the other hand, the involvement of IL-18 in activating the NF-κB pathway, which has been shown to be involved in the pathogenesis of insulin resistance and diabetes (as discussed in the Introduction, Chapter 1), suggests that higher levels of IL-18 would in fact be a causative agent in the development of insulin resistance.

A key study which led to the development of experiments into the role of IL-18 included in this thesis is that by Netea et al., which was the first to report an obese phenotype of IL-18 knockout mice, supported a year later by similar findings from another laboratory. Together these studies show that IL-18 knockout animals display a phenotype of overeating leading to obesity and its associated comorbidities including glucose intolerance. In these animals, delivery of IL-18 into the brain reduced appetite to normal levels, an effect also observed in 'wild type' mice with normal IL-18 levels. These studies thus unveiled a previously unknown neuroendocrine effect of IL-18 on energy homeostasis, mediated by unknown mechanisms in the brain.

Thus, on the basis of the evidence linking IL-18 with the risk and pathogenesis of cardiovascular disease and diabetes, observations surrounding its elevation in obesity and modification by lifestyle intervention (discussed further in Chapter 4), its role as an activator of the NF-κB signalling pathway implicated in dysglycaemia and insulin resistance, and evidence from animal studies for a role in appetite and glucose homeostasis, another avenue of investigation in this thesis was to investigate further the neuroendocrine effects of IL-18, with a view to aid in understanding the mechanisms by which it reduces appetite and affects glucose metabolism, and thus contribute further to the emerging evidence of a role of inflammation in the pathogenesis of diabetes and insulin resistance. These works form Section 3 of this thesis.

Although obesity has often been referred to as a state of leptin resistance, there is little consensus as regards to how this term is used or such resistance is measured. The widespread, but ill-defined, use of the phrase 'leptin resistance' was such that it was the topic of a recent workshop convened by the US National Institutes of Health aimed at deriving a clinically useful consensus in order to encourage clearer application of the term in the future.
1.8 Patterns and dynamics of cytokine release

One aspect of cytokine biology which confounds efforts at understanding the link between circulating cytokines and health outcomes is the nature of cytokine release. Cytokines are usually activated and released upon some form of inflammatory stimulus, such as physical tissue injury, viral or bacterial infection, or other forms of tissue injury such as exposure to ultraviolet light. In some cases such inflammatory reactions can remain localised in a particular tissue, while in others alterations in circulating cytokine levels can be observed. For example, after an acute illness, following myocardial infarction, exposure to air pollution, or an artificial inflammatory stimulus in a research setting such as injection of bacterial lipopolysaccharide, levels of circulating cytokines can be seen to follow increase and subsequently decline, with time courses dependent on the type and extent of inflammatory event and inter-individual variation. Interleukin-6, for example, is known as a ‘myokine’ – a cytokine released from muscle tissue during physical exertion and thus circulating levels increase dramatically post-exercise.

As such, measuring circulating cytokines at one time point, as is commonly done in studies involving humans (for example epidemiological studies, or dietary intervention studies), may not provide an accurate reflection of the more complex biology of cytokine action. Nevertheless, in terms of research into the role of inflammation in obesity and type 2 diabetes, even in the absence of an acute inflammatory stimulus elevations of cytokines can be observed, which in itself, is the basis for these elevations being named ‘chronic sub-clinical’ inflammation. Given that the focus of this thesis is chronic inflammation in the absence of an acute inflammatory stimulus, and the participant burden which would be required for a more intensive analysis of cytokine dynamics, in the LOADD study described in Section 2 only single measures of circulating cytokines have been made. Nevertheless, such measures should be interpreted keeping in mind the potential limitations of examining one time point.

In addition, in Section 3, which focuses on the neuroendocrine role of IL-18, this cytokine is constitutively present in the circulation, as opposed to the more sporadic release of many other inflammatory markers. Furthermore, and as detailed in Chapter 7, IL-18 production in the medial habenula is constitutive and can be observed in the absence of any inflammatory stimulus. Data from knockout animals with an absence of IL-18 suggest that this constitutive presence plays a role in body weight regulation, and it is this constitutive aspect of IL-18 biology which is the focus of the works in Section 3, as opposed to the typical dynamic of cytokine induction followed by remission observed with acute inflammatory stimuli.
Section 2:

The effect of dietary intervention on inflammatory markers and adipokines in type 2 diabetes: an analysis in the Lifestyle Over and Above Drugs in Diabetes (LOADD) study
Chapter 2. Overview and methods of the LOADD study

2.1 The LOADD study: rationale

In contrast to the studies discussed in the Introduction examining the effect of glucose-lowering agents on cardiovascular endpoints, there have been no studies conducted in which the effect of dietary or lifestyle modification in patients with type 2 diabetes has been examined for potential benefits on clinical endpoints of macrovascular disease. While the evidence is not conclusive of lowering of blood glucose per se via medication-driven strategies for the prevention of macrovascular disease in type 2 diabetes, there is a sound body of evidence that lifestyle modification and dietary intervention can improve surrogate markers of diabetes and cardiovascular risk in those with diabetes, to the extent that evidence-based nutritional guidelines for the treatment of diabetes have been released by a number of authorities.\(^1\) The Diabetes and Nutrition Study Group of the European Association for the Study of Diabetes (EASD) released guidelines in 2004 which were derived principally from studies which included outcomes of pre-determined surrogate markers of diabetes and cardiovascular risk (blood pressure, lipoprotein profile, glycaemia, insulin sensitivity, body composition and renal function).\(^2\) Many of the nutritional studies involving people with type 2 diabetes upon which these guidelines were based were conducted prior to the widespread use of agents such as statins and ACE inhibitors which are recommended as part of a modern diabetes treatment regimen. The New Zealand Guidelines Group (NZGG)\(^1\) for example recommends patients with type 2 diabetes be treated with:

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\(^1\) Whether the improvement in clinical outcomes derives from an improvement in glycaemic control itself, or whether lifestyle modification induces improvement in other metabolic variables (which are associated with changes in glycaemic control) which themselves influence the pathogenesis of micro- and macrovascular diabetes complications is not known; the relative contributions of the glucose lowering vs. other effects of dietary interventions (e.g. changes in macronutrient composition or nutrient quality) cannot be easily disentangled. The scientific difficulty of ascribing cause and effect of a dietary intervention to an endpoint via intermediate markers is discussed further in Chapter 4 while addressing the potential benefit of the LOADD dietary intervention on clinical endpoints.
- aspirin, a beta-blocker, statin and ACE (angiotensin converting enzyme) inhibitor after a previous cardiovascular event (irrespective of blood pressure or lipid levels)
- and/or ACE inhibitor, A2 (angiotensin 2) receptor blocker, aspirin and lipid-modifying agent in the event of evidence of renal disease (including microalbuminuria and irrespective of baseline lipid levels)
- and/or fibrates in the event of high triglycerides and low HDL

Thus, the current clinical management of those with type 2 diabetes typically involves not only agents for the control of glycaemia, but also renoprotective, cardioprotective and lipid-modifying agents. This presents a situation in which recommendations for the nutritional management of diabetes were based upon studies in which participants were often taking a much simpler range of medications than would now be regarded as acceptable diabetes management. Researchers at the Edgar National Centre for Diabetes Research therefore posed the question as to whether implementation of these evidence-based guidelines would be able to further improve cardiovascular risk and glycaemic control in diabetes patients treated according to guidelines such as those above. Although some studies had conducted comparisons of the relative, or additive, effects of statins and dietary intervention to lower blood cholesterol levels following the introduction of statins, similar studies did not exist for the comparison of the relative or additive effects of dietary intervention and the currently recommended range of diabetes medications on diabetes complications.

In addition, at a local level, data collected by the Otago Diabetes Project also showed that in spite of an increasing use of recommended medications, glycaemic control and body weight of enrolled patients (estimated to be approximately 70% of those with diabetes in the Otago region) were worsening, suggesting that lifestyle measures were either not being encouraged or that compliance was poor.

These diabetes management guidelines have been updated at the time of writing, however, the guidelines cited above were those current during the planning and conduct of the LOADD study. Edgar National Centre for Diabetes Research – based at the University of Otago, one of the host institutions for this PhD thesis; now known as the Edgar National Centre for Diabetes and Obesity Research.

Otago is a region in the South Island of New Zealand, where the Edgar National Centre for Diabetes Research is based. Staff at the centre were involved in the running of the Otago Diabetes Project which has since ceased.
As noted above, there have also been no studies which have examined the potential for dietary modification to reduce macro- or microvascular events in persons with type 2 diabetes. The Steno-2 study comes close to examining this question; however, while the Steno-2 study involved lifestyle and dietary modification in the intervention arm, rates of medication use also differed between treatment groups such that a more intensive medication regimen was used in the intervention group. The currently recommended range of medications for diabetes listed above by the NZGG is similar in many respects to those used in the intervention group in the Steno-2 study, with the control arm in that study receiving what would be considered a sub-standard level of care according to NZGG guidelines. Thus, the extent to which the observed benefit in macrovascular endpoints in the Steno-2 study is due to medication or lifestyle effects cannot be easily determined.

From these multiple concerns the Lifestyle Over and Above Drugs in Diabetes (LOADD) study was initiated. The aim of this study was to examine the extent to which implementation of the nutritional guidelines recommended by the EASD could improve cardiovascular risk indicators in people with type 2 diabetes. All participants would be treated with the full gamut of medications as recommended by national treatment guidelines, and would be derived from the same population that was assessed by the Otago Diabetes Project, the results of which suggested poor adherence to lifestyle and nutrition advice. Those randomised to the intervention group would receive intensive dietary advice as well as initiating an optimized drug regimen, whereas those in the control group would not be given special nutrition-related advice.

While ultimately clinical endpoints of microvascular and macrovascular complications are preferred outcomes for measuring the effectiveness of an intervention aimed at improving diabetes control (as has been particularly pertinent in recent history regarding evaluating the effectiveness of diabetes medications), this would obviously require a substantial time and cost investment. The LOADD study was designed to examine outcomes based on surrogate measures in the first instance. This could possibly be followed by a larger study based on clinical endpoints in the event of positive findings in the initial study on surrogate measures. The primary endpoint in the LOADD study was glycaemic control as measured by HbA1c (glycaemic control having often been regarded as the principal factor of interest in the management of type 2 diabetes). However, this study provided an opportunity to examine the effect of the LOADD study dietary intervention on a range of additional outcome measures including adipokines and inflammatory markers, the study of which forms part of the work contained in this thesis.
2.2 Inflammatory markers and adipokines of interest:

There is a vast array of inflammatory markers and adipokines which could potentially be of relevance to the pathogenesis of type 2 diabetes and its complications. However, for practical and budgetary reasons a selection of analytes of interest was made. These were: interleukin-1 beta (IL-1β), interleukin-1 receptor antagonist (IL-1ra), interleukins 6, 10, and 18 (IL-6, IL-10, IL-18), tumour necrosis factor (TNFα), the soluble TNFα receptors sTNF-R1 and sTNF-R2, neopterin, and leptin. A brief summary of the reasons why these were chosen is given below.

i. Interleukin-18 (IL-18)

The study of IL-18 was the basis of the animal work undertaken as a major part of this thesis, and the scientific basis for interest in the cytokine in relation to type 2 diabetes and metabolic disease is described in the introduction of this thesis (Section 1.6.1) and Chapter 5. In brief, a number of studies suggest IL-18 plays an active role in the development of cardiovascular disease (discussed in Chapter 5) and a meta-analysis of prospective studies found elevated baseline IL-18 was associated with an increased risk of developing coronary heart disease, even after adjustment for traditional cardiovascular risk factors. In addition, two prospective cohort studies have shown elevated IL-18 levels to be predictive of the development of type 2 diabetes, suggesting a role for this cytokine in diabetes pathogenesis.

ii. Neopterin

Neopterin is released from macrophages in response to interferon-gamma (IFN-γ), which is in turn released in response to IL-18 (initially named IFN-γ inducing factor). A number of studies have shown neopterin levels to be predictive of cardiovascular events, cardiovascular mortality and all-cause mortality, including in individuals with type 2 diabetes. Serum levels correlate with BMI, and are elevated in stress and depression. Neopterin levels have also been shown to relate to plaque morphology, and to correlate with the extent and severity of coronary artery disease, and carotid intimal medial thickness. In addition, levels are predictive of left ventricular remodelling in myocardial infarction survivors and of low left ventricular ejection fraction. Human endothelial cells have been shown to release tissue factor, ICAM-1 and VCAM-1 upon exposure to neopterin, by a mechanism which involves NF-κB activity. Given that IL-18 induces IFN-γ, and that in turn neopterin is produced in response to IFN-γ, it is not surprising that administration of recombinant IL-18 was observed in primates to increase circulating neopterin.
levels\textsuperscript{547,548}, such that IL-18 and neopterin can be regarded as different points in the same inflammatory pathway.

\textbf{iii. IL-1β and IL-1ra}

IL-1β is a pro-inflammatory cytokine which stimulates NF-κB activity, and there are many similarities in the processing and signalling pathways activated by IL-18 and IL-1β, and in phenotypes resulting from their manipulation. Variants in the IL-1 gene have been shown to be associated with fat mass in men\textsuperscript{549}, obesity and the metabolic syndrome in persons with coronary heart disease\textsuperscript{550} and diabetes and blood glucose homeostasis in men and women of European descent\textsuperscript{204}. In animal studies, knockout of the interleukin-1 receptor has been shown to protect against high-fat diet induced insulin resistance\textsuperscript{551} and increased blood pressure\textsuperscript{552}, and administration of an IL-1β antibody improves insulin resistance in mice on a high fat diet\textsuperscript{553,554}. IL-1β is produced from the immune system inflammasome and inflammasome activation and IL-1β release has been identified as a key component of the development of diet-induced insulin resistance in animal studies\textsuperscript{555}.

IL-1ra acts as a natural circulating antagonist able to bind the interleukin-1 receptor but not induce signal transduction. The use of IL-1ra as a potential diabetes therapy has also been examined in randomised controlled trials in humans with type 2 diabetes, and has been shown to be a potential therapy with improvements in glycaemia\textsuperscript{281,282}, a finding also observed in animal models\textsuperscript{556}. Elevation of IL-1ra has been shown to be a predictor of future development of diabetes in prospective cohort studies\textsuperscript{190,191,557}, thought to reflect an underlying pathogenic process relating to changes in inflammatory signalling early in the pre-clinical stage of disease.

\textbf{iv. Interleukin-6 (IL-6)}

Interleukin-6 is a pro-inflammatory cytokine which is released from a range of tissues and cell types including adipocytes, skeletal muscle and macrophages\textsuperscript{558}. It is the most immediate upstream regulator of circulating CRP levels, as IL-6 action on hepatocytes induces the release of CRP\textsuperscript{559}. Studies which have compared the predictive power of IL-6 and CRP on clinical outcomes such as cardiovascular disease have often found IL-6 to be a more powerful predictor than CRP\textsuperscript{184}. IL-6 has been also named a ‘myokine’ due to large amounts being released from skeletal muscle following exercise\textsuperscript{560,561}. In keeping with other pro-inflammatory cytokines, IL-6 levels are chronically elevated in those with obesity and diabetes\textsuperscript{399,562}. Up to 30% of circulating IL-6 in humans may be produced by adipose tissue\textsuperscript{563} and circulating IL-6 levels can be reduced by weight loss\textsuperscript{284,399,400}. IL-6
has also been shown to induce hepatic insulin resistance, a key pathogenic feature of type 2 diabetes.

v. Interleukin-10 (IL-10)

Interleukin-10 is an anti-inflammatory cytokine which suppresses the release of pro-inflammatory cytokines from macrophages. Thus, not surprisingly, given the relationship between inflammation and pathways of insulin resistance, this anti-inflammatory cytokine typically shows associations with insulin sensitivity which are inverse to those of pro-inflammatory cytokines. IL-10 levels have been shown to be associated with insulin sensitivity determined by clamp, and just as a number of studies have shown a reduction in pro-inflammatory cytokines with weight loss, a combined medication and diet regimen in obese individuals was shown to increase IL-10 levels. The ratio of IL-18 to IL-10 has been documented as being predictive of coronary events, and IL-10 is associated with the metabolic syndrome and type 2 diabetes. Variations in the IL-10 gene have been shown to associate with obesity and insulin resistance, although not diabetes, in people of European descent and with type 2 diabetes in people of Asian descent. In animal studies transgenic overexpression of IL-10 in skeletal muscle was shown to prevent high-fat diet induced insulin resistance, inhibition of IL-10 worsened hepatic insulin resistance induced by a high-fat diet, and treatment with IL-10 was found to prevent the insulin resistance induced by lipids or IL-6.

vi. TNFα and its soluble receptors

TNFα is a pro-inflammatory cytokine which activates the NF-κB signalling pathway and also interferes with insulin signalling pathways by inducing serine phosphorylation of the insulin receptor substrate 1 (IRS-1). TNFα exerts its effects by the membrane bound receptors TNF-R1 and TNF-R2. Binding of TNFα induces shedding of the extracellular portion of the receptor, forming the soluble receptors sTNF-R1 and sTNF-R2. These soluble receptors are thought to modulate TNFα activity by acting as a buffer system to bind TNFα and prevent activation of membrane-bound receptors, and also as a means of prolonging TNFα activity by binding TNFα (preventing degradation) and later release. As such, it has been suggested that levels of the soluble TNFα receptors may be a more accurate marker of TNFα activity than the ligand itself. In the in vivo situation in humans, the use of monoclonal antibodies against TNFα in the treatment of rheumatoid arthritis has been shown to be associated with an improvement in insulin sensitivity, and a case report details a sudden deterioration of glycaemia upon termination of TNFα antibody treatment. There is also evidence that pulsatility of TNFα determines the responsiveness of
TNFα-induced NF-κB activation, and thus chronically elevated TNFα in obesity may exert differing effects on NF-κB activity than TNFα activity in healthy persons 584. TNFα and its receptors play a role in heart disease 585, including myocardial infarction 586 and heart failure 587,588 with evidence for opposing effects of the TNFα receptor types on heart failure pathogenesis 587. TNFα-induced serine phosphorylation of IRS-1 (which reduces downstream insulin signalling) has also been shown to be inhibited by salicylic acid 589, thus implicating this as a possible mechanism involved in salicylate-induced improvements in glycaemic control.

vii. Leptin

Leptin is often referred to as the archetypal adipokine, as it was the first cytokine released from adipose tissue to be discovered 590. Leptin levels are elevated in obesity, which appears to be a state of pathogenic leptin resistance 591, although evidence exists that while central (brain) responsiveness is impaired in obesity, leptin signalling in the periphery may be spared 592. Leptin shares many downstream signalling mechanisms with insulin and various studies have shown that central leptin signalling modulates peripheral insulin sensitivity 593–595. In addition, rodents made insulin-deficient with streptozotocin or through autoimmune processes have been shown to be viably maintained with normal glucose homeostasis with the administration of leptin (even in the absence of insulin) 596,597, highlighting the powerful effects of this adipokine on glycaemic control. High leptin levels have been proposed to exert adverse cardiovascular effects 598,599 and thus may contribute to the excess cardiovascular morbidity observed in obesity, although this relationship may be confounded by other processes related to adiposity 600.

* although obesity has often been referred to as a state of leptin resistance, there is little consensus regarding how this term is used or how such resistance is measured. The widespread, but ill-defined, use of the phrase 'leptin resistance' was such that it was the topic of a recent workshop convened by the US National Institutes of Health aimed at deriving a clinically useful consensus in order to encourage clearer application of the term 504.
2.3 Overview of the LOADD study:

Participants, inclusion and exclusion criteria:

The Lifestyle Over and Above Drugs in Diabetes study was a randomised controlled trial conducted by the Edgar National Centre for Diabetes Research, Dunedin, New Zealand. The study enrolled participants with type 2 diabetes within 1 to 5 years of diagnosis who were treated with oral hypoglycaemic agents. Inclusion criteria stipulated that participants should be less than 70 years of age, have unsatisfactory glycaemic control (defined as an HbA1c of ≥ 7 %) and in addition have two of the following three cardiovascular risk factors: overweight or obesity, hypertension or dyslipidaemia. Hypertension was defined as the use of antihypertensive medications or blood pressure ≥ 140/90 mmHg with or without antihypertensive medication. Dyslipidaemia was defined as either a total cholesterol level of greater than 5.2 mmol/l, LDL cholesterol > 3.5 mmol/l, triglycerides > 2.0 mmol/l, HDL cholesterol < 1.0 mmol/l or a combination of the above, or the use of lipid modifying agents. Overweight was defined as a BMI ≥ 25 kg/m². The baseline characteristics of participants randomised to control and intervention groups is shown in Appendix 1, Table 1.

Pre-randomisation period and randomised treatment groups:

The study commenced with a pre-randomisation period in which participants saw a study physician who ensured medication regimens were in accordance with the NZGG recommendations. Medication regimens were altered where necessary and participants saw the study physician on multiple occasions if needed during this run-in phase. Once it was deemed that participants were receiving an optimal level of treatment according to NZGG recommendations they were then randomised to either an intensive dietary treatment arm, or a control arm in which participants continued with their usual standard of care provided by their General Practitioner and/or Diabetes Specialist. Participants in the usual care group were therefore in theory receiving the same level of diabetes care as afforded to all individuals with diabetes in New Zealand, though arguably the level of care was somewhat higher due to contact with the study physician and medication review prior to randomisation. The intensive dietary treatment arm consisted of a six month dietary intervention supervised by the study dietitian, both in one-on-one and group visits. Dietary advice was tailored to assist participants to follow a diet in line with recommendations in the evidence-based dietary guidelines for the treatment of diabetes issued by the Diabetes and Nutrition Study Group of the European Association for the Study of Diabetes, but also took into account individual preference.
Dietary intake:

Dietary intake was assessed by the use of three-day weighed diet records collected by participants at baseline and six months. Diet records were used to calculate total energy, macronutrients and fat content, and fibre intake, as shown in Appendix 1, Table 2.

Study visits and blood sampling:

Participants were invited for two baseline visits (titled S0 and S1) for blood collection and anthropometric measurements. An average of the blood tests and clinical results for both S0 and S1 visits was taken as the baseline level for measures such as blood lipid levels, HbA1c, blood pressure, and anthropometric measures. Six month end-of-study measures were taken in a similar manner, with repeat measures taken one week apart (labelled S10 and S11). For the purposes of the current analysis of inflammatory markers and adipokines, blood samples taken at S0 (baseline) and S10 (6-months) were used, as the use of repeat measures would have exceeded the available budget.

Power studies, endpoints and ethical approval:

The main endpoint for the study was glycaemic control, and the study was designed to have 80% power to detect a 0.5% difference in HbA1c between treatment arms at a 5% level of significance. A number of additional measures of interest were included as secondary outcomes. These were: anthropometric variables and markers of diabetes control and cardiovascular risk, an analysis of which has been published 601 (note that this is the work of other researchers - the author of the current thesis did not contribute to the collection or analysis of those variables); and a selection of inflammatory markers and adipokines, which were measured from stored plasma samples and analysed as part of the current thesis. The study was approved by the Lower South Regional Ethics Committee of New Zealand (project key LRS/05/07/026), and registered prior to commencing at www.clinicaltrials.gov under the accession number NCT00124553.
2.4 Summary of previously published LOADD study findings:

A flow diagram of participants through the LOADD study is shown below in Figure 1, as published previously \(^6\). A total of 94 participants completed the 6-month study (n = 46 for the intervention group, n = 48 control group), which was completed through 2006 to 2009. One of the intervention participants was subsequently diagnosed with slow onset type 1 diabetes and was excluded from further analysis.

An analysis of the clinical, anthropometric, biochemical and dietary data from the LOADD study has already been made and published \(^6\). Although that work does not form part of the current thesis, some of these measures are included in analyses here, or are referred to during interpretation of the present analyses. Therefore, by way of explanation, the collection of these variables and the results presented in the published work are summarized below in order to assist in interpreting the findings presented here.

Collection of clinical, anthropometric, biochemical and dietary data:

Briefly, the methods used for the collection and measurement of these data were as follows: plasma total cholesterol, HDL cholesterol, and triglycerides were measured using enzymatic test kits (Roche Diagnostics, USA) on a Cobas Mira Plus autoanalyser (Roche Diagnostics, USA). Low-density-lipoprotein cholesterol was subsequently calculated using the Friedewald formula. Fasting glucose was measured using the hexokinase method using a commercially available kit (Roche Diagnostics, USA) and HbA1c was measured enzymatically (Roche Diagnostics, USA). Uric acid was measured using the Unimate 5 enzymatic colorimetric test (Roche Diagnostics, USA). Anthropometric and blood pressure measurements were made by trained nurses, with anthropometric measures made in duplicate and blood pressure measurements in triplicate. Dietary intake was assessed by 3-day weighed diet records.

Results: dietary intake:

Dietary intakes of control and intervention groups are shown in Appendix 1, Table 2. In brief, at the end of the 6-month study period participants in the intensive dietary intervention group were consuming a diet significantly higher in protein (difference (95 % CI) in percent energy intake adjusted for age, sex and baseline measures: 1.6 (0.04, 3.1) %), significantly lower in saturated fat content (-1.9 (-3.3, -0.6) %), with a trend towards a higher fibre intake, of borderline statistical significance (3.0 (-0.2, 6.1) g). No other measures of dietary composition differed between groups.
Results: glycaemic control and surrogate markers of cardiovascular risk:

The intensive dietary intervention resulted in statistically significant reductions in HbA1c (difference (95 % CI) adjusted for age, sex and baseline measures: - 0.4 (- 0.1, - 0.7) %), and measures of anthropometry in the intervention group compared to control (weight: - 1.3 (- 0.1, - 2.4) kg; waist circumference: - 1.6 (- 0.5, - 2.7) cm; BMI: - 0.5 (- 0.1, - 0.9) kg/m²).

No statistically significant changes in other markers of glycaemic control and surrogate measures of cardiovascular risk were observed, including fasting glucose, blood pressure, lipid profile, triglycerides, uric acid, and urinary albumin to creatinine ratio.
Figure 1: LOADD study participant flow chart, adapted from Coppell et al. 601.

165 assessed for eligibility

- 43 not meeting inclusion criteria

122 had clinical assessment

- 17 excluded:
  - 13 HbA1c ≤ 7%
  - 1 diet only treatment
  - 1 too busy
  - 2 unspecified reasons

105 eligible

- 1 withdrew

104 randomised

- 6 discontinued:
  - 2 withdrew
  - 2 lost to follow-up
  - 1 moved out of area
  - 1 admitted to hospital

- 52 intensive dietary intervention
- 52 usual care

- 4 discontinued:
  - 1 withdrew
  - 1 died
  - 1 loss of family member
  - 1 pregnancy

46 completed

(1 excluded from analysis due to misdiagnosis)

48 completed
2.5 The current work: measurement of inflammatory markers and adipokines:

Plasma samples from LOADD study participants were stored at -80 °C until processing. Two aliquots of approximately 300 µl each were initially set aside for analysis of inflammatory markers and adipokines. IL-18 was initially measured by ELISA from Bender MedSystems (Austria, catalogue number BMS267/2), however the results were found to have an unacceptably high inter-assay coefficient of variation (data not shown). IL-18 measurement was subsequently redone with an ELISA kit from another manufacturer (MBL, Japan). This kit has been used in a number of other publications (e.g. see 426, 434, 437, 460, 461, 499, 602–604). Leptin and neopterin were measured by 125I-radioimmunoassay (Leptin: Millipore, MA, USA, catalogue number HL81K; Neopterin: BRAHMS Diagnostica, Germany, B-R-A-H-M-S Neopterin RIA). The human IL-18BP gene encodes four different isoforms of IL-18BP, labelled ‘a’ through ‘d’. IL-18BPa and IL-18BPc appear to have the greatest ability to inhibit IL-18, with IL-18BPa having a much greater affinity. Much of the research into the role of IL-18BP has focused on the IL-18BPa isoform (e.g. 448, 454, 606–611), including the generation of transgenic mice which overexpress the IL-18BPa isoform 612, 613, and therefore IL-18BP was measured using a commercial ELISA designed to bind IL-18BPa (RayBiotech, Inc., GA, USA, catalogue number ELH-IL-18 BPA-001). The remaining cytokines were measured in a Lumenix™ bead array multiplex system with multiplex beads and plates from Millipore (MA, USA) which were read on a Bio-Plex suspension array instrument (Bio-Rad Laboratories, CA, USA). Multiplex arrays were performed with two separate plates: one for analysis of IL-1β, IL-1ra, IL-6, IL-10 and TNFα (catalogue number MPXHCYTO-60K), and another for analysis of sTNF receptors (sTNF-R1 and sTNF-R2, catalogue number HSCR-32K). Because of the practical issues involved in conducting a number of different measurements on frozen aliquots, and to ensure consistent freeze-thaw cycles across samples, a schedule was set up in which the two aliquots were thawed for use in assays and refrozen as follows:

aliquot 1: leptin RIA, neopterin RIA, sTNF receptor (sTNF-R1, sTNF-R2) multiplex

aliquot 2: cytokine multiplex (IL-1β, IL-1ra, IL-6, IL-10, TNFα), initial failed IL-18 ELISA (BenderMedSystems) (note - results not included in this thesis), IL-18BP ELISA

As noted above, the IL-18 ELISA was subsequently repeated using kits from MBL (Japan). This was performed on a separate plasma sample which had previously been through one freeze-thaw cycle for the measurement of C-reactive protein from the same clinic visit.
Analyses were conducted at the Department of Microbiology (for Luminex™ bead multiplex assays), or Lipid and Diabetes Laboratory, Department of Human Nutrition (all other assays), both within the University of Otago, Dunedin, New Zealand. Assay plates were loaded to have a balanced number of samples from control and intervention groups to reduce any systematic error introduced from interassay variability. In addition, the repeat IL-18 ELISA assay (MBL) was also balanced to have the same number of baseline and 6-month follow-up samples for each randomisation group. Assays were conducted according to manufacturer’s instructions and where instructions allowed optional overnight incubations (Millipore multiplex plates, RayBiotech IL-18BPa ELISA), this was done for greater assay sensitivity. Participant samples were analysed in duplicate in RIAs and ELISAs, and with single samples in multiplex assays. Duplicate samples from RIAs with a coefficient of variation of 20 % or greater were excluded from further analysis, as were samples from ELISAs where raw absorbance readings of duplicates differed from their mean by 20 % or more. One participant in the control group was found to have extremely high levels of neopterin, sTNF-R1 and sTNF-R2 both at baseline and follow-up visits, for unknown reasons, and these values were excluded from further analysis (baseline: neopterin 155.6 nmol/l, sTNF-R1 16,624.0 pg/ml, sTNF-R2 21,594.6 pg/ml; 6-months: neopterin 259.16 nmol/l, sTNF-R1 14,138.2 pg/ml, sTNF-R2 21,790.1 pg/ml).

2.5.1 Intra- and inter-assay variability:

Pooled plasma samples, which consisted of plasma left over from other unrelated analyses conducted at the Department of Nutrition Lipid Laboratory, were added as unknowns in the assays in order to aid in the calculation intra- and inter-assay coefficients of variation (CVs), shown in Tables 2a and 2b. A number of assays also included internal controls provided by the manufacturer and thus where possible CVs were calculated using pooled plasma samples and control(s) as CV calculations can differ across the range of the standard curve. Using samples at various points in the curve provides a more accurate measure of overall variability. Controls from different plate kits had the same manufacturing lot number and thus could be included in inter-assay CV calculations. In some assays pooled plasma samples were either accidentally omitted (Leptin RIA), or had undetectable levels of analytes in some or all assay plates (IL-18 BPa ELISA, Millipore cytokine multiplex – IL-1β, IL-1ra, IL-6, IL-10, TNFα). Thus, for the Millipore cytokine multiplex internal control samples were used as the basis for CV calculation. To test the validity of calculating CV values on the basis of internal control samples, CV calculations for the other multiplex performed here (Millipore soluble cytokine receptor multiplex – sTNF-R1 and sTNF-R2) were made on the basis of both pooled plasma samples and controls as a comparison, shown in Table 2a. Both methods gave comparable results. The IL-18 BPa ELISA used, manufactured by RayBiotech Ltd, lacked internal control samples, and in addition
pooled plasma samples were below the limit of detection (for reasons unknown). This excluded the possibility of evaluating coefficients of variation based on traditional methods. Thus, alternative methods to calculate assay CV values were investigated, and the same calculations made with the other ELISA used (IL-18, MBL) as a comparison to gauge the validity of these different methods, which were: (i) using the variation in absorbance readings of the standards used to create the standard curve, and (ii) the intra-assay average CV values calculated from duplicates of all unknown samples (participant samples - IL-18 and IL-18 BP ELISAs; and plasma pools - IL-18 ELISA). Comparisons are shown in Table 2b. For the IL-18 ELISA assay, intra-assay CV values calculated with these alternative methods were lower, and the inter-assay CV value marginally higher, than those calculated using plasma pools. This suggests that if calculating CV values of the IL-18 BPa ELISA by using pooled plasma samples had been possible, it would likely give values somewhat different than those shown in Table 2b by these two alternative methods, although would likely remain within an acceptable range of variance. Limits of detection for ELISAs are stated by the manufacturers as 12.5 pg/ml for MBL IL-18 ELISA, and < 20 pg/ml for Raybiotech Ltd. IL-18 BPa ELISA. Limits of detection for the remaining assays are shown in Table 2a.
2.6 Statistical analysis:

Statistical analysis was performed under the direction of the study statistician (Assoc Prof S Williams, University of Otago), using Stata software (Stata Corporation, Texas, USA) or GraphPad Prism 5.0d for Mac OS X (GraphPad Software, Inc., CA, USA).

Effect of dietary intervention:

The effect of the intervention was assessed by multiple regression adjusting for baseline measures. As the majority of analytes showed skewed distribution, measures were log-transformed before analysis. Differences are represented as the ratio of difference at 6-month follow-up of the intervention group over the difference at 6-month follow-up of the control group, using log-transformed values of each variable. For some variables, further analyses were conducted with additional adjustment for changes in BMI and HbA1c, as indicated in the text. The effect of dietary intervention on levels of inflammatory markers and adipokines was corrected for multiple testing with Bonferroni adjustment. The relationship between changes in neopterin and IL-18 levels, or between these cytokines and changes in measures of dietary composition, was performed using linear regression in GraphPad Prism 5.0d for Mac OS X.

Correlation analysis:

In order to examine the relationships between the different adipokines and inflammatory markers, as well as with other surrogate markers of cardiovascular risk, pairwise correlation coefficients were generated using baseline measures of participants from both intervention and control groups (using non-log transformed measures) with Stata. As leptin is highly correlated with measures of adiposity partial correlation coefficients corrected for BMI were generated. No corrections were made for multiple testing of baseline correlations.

For variables showing significant pairwise correlation coefficients, further analyses and visualisation were made by examining scatter plots with the use of GraphPad Prism. Given the large number of pairwise correlations generated, and that outlying points can generate false positive associations, tests were made to examine the robustness of these associations by excluding possible outliers from scatter plots (Appendix 1, Figure 2). On the one hand, the exclusion of potential outliers can highlight whether an association is driven by one or a few select points, on the other, excluding variables without an a priori condition can result in biased selection of data points. Nevertheless, these analyses were performed to check for false positive associations driven by outlying data points. No attempt was made to perform a similar analysis with non-significant pairwise correlations to
check for false negative associations. Note that due the differing methods GraphPad Prism and Stata software use to generate linear regression of scatter plots, and pairwise correlation coefficients, respectively, the r and p values differ slightly for the same associations between variables.
Table 2a: Measured intra- and inter-assay coefficients of variation (CV) for RIAs and multiplexes, and limits of detection as stated by the manufacturer.

<table>
<thead>
<tr>
<th>Assay &amp; method (Company)</th>
<th>Limit of detection</th>
<th>Intra-assay CV (%)</th>
<th>Inter-assay CV (%)</th>
<th>Method of CV calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neopterin RIA (BRAHMS Diagnostica)</td>
<td>n.s.</td>
<td>7.8</td>
<td>7.8</td>
<td>pooled plasma and control samples</td>
</tr>
<tr>
<td>Leptin RIA (Millipore)</td>
<td>0.5 ng/ml</td>
<td>2.9</td>
<td>10.7</td>
<td>control samples</td>
</tr>
<tr>
<td>Multiplex – cytokine 5-plex (Millipore)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.4 pg/ml</td>
<td>8.0</td>
<td>31.1</td>
<td>control samples</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>2.9 pg/ml</td>
<td>10.7</td>
<td>26.0</td>
<td>control samples</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.3 pg/ml</td>
<td>6.5</td>
<td>33.1</td>
<td>control samples</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.3 pg/ml</td>
<td>9.9</td>
<td>32.9</td>
<td>control samples</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.1 pg/ml</td>
<td>7.7</td>
<td>28.0</td>
<td>control samples</td>
</tr>
<tr>
<td>Multiplex – soluble TNFα receptors (Millipore)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sTNF-R1</td>
<td>9.6 pg/ml</td>
<td>9.6</td>
<td>13.4</td>
<td>pooled plasma samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.0</td>
<td>9.3</td>
<td>control samples</td>
</tr>
<tr>
<td>sTNF-R2</td>
<td>16.9 pg/ml</td>
<td>5.5</td>
<td>6.4</td>
<td>pooled plasma samples</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.7</td>
<td>8.5</td>
<td>control samples</td>
</tr>
</tbody>
</table>

n.s - not stated. Lowest standard is 3.0 nmol/l.
Table 2b: Calculated intra- and inter-assay coefficients of variation (CV) for ELISAs, showing the comparison of different methods for IL-18 BPa and IL-18 ELISAs.

<table>
<thead>
<tr>
<th>Method of CV calculation</th>
<th>IL-18 ELISA</th>
<th></th>
<th>IL-18 BPa ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-assay CV (%)</td>
<td>Inter-assay CV (%)</td>
<td>Intra-assay CV (%)</td>
</tr>
<tr>
<td>pooled plasma samples</td>
<td>8.0</td>
<td>9.9</td>
<td>*</td>
</tr>
<tr>
<td>average variance of absorbance of</td>
<td>3.1</td>
<td>10.9</td>
<td>6.7</td>
</tr>
<tr>
<td>standard curve points</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>average variance of absorbance of all</td>
<td>2.2</td>
<td>-</td>
<td>13.4</td>
</tr>
<tr>
<td>unknown samples</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Assay lacks internal control samples and pooled plasma samples were below the limit of detection, such that CV values cannot be calculated with this method.
Chapter 3: Results

3.1 Effect of dietary intervention on inflammatory markers and adipokines

The effect of the 6-month LOADD study dietary intervention on levels of adipokines and inflammatory markers is shown in Table 3, with group differences presented in graphical format in Figure 2. Because of the skewed distribution of the data, values for each measure were log-transformed and effect sizes calculated as the ratio between groups of change from baseline.

The intervention resulted in significant reductions in IL-18 and neopterin levels relative to control, with difference ratios (95% CI) of 0.91 (0.83, 0.99) and 0.87 (0.77, 0.98), respectively. No significant changes were observed in other measured inflammatory markers and adipokines. Bonferroni adjustment for multiple testing rendered these differences non-significant.

Given that the intervention produced significant reductions in anthropometric measures (weight: 1.3 (0.1, 2.4) kg, waist circumference: 1.6 (0.5, 2.7) cm, BMI: 0.5 (0.1, 0.9) kg/m$^2$) and HbA1c (0.4 (0.1, 0.7) %) (data from $^{601}$), the relationship between these changes and those seen here in IL-18 and neopterin was further investigated by examining the correlation between change in BMI, HbA1c, IL-18 and neopterin across all participants (Table 4 and Appendix 1, Table 3). Changes in HbA1c were correlated with change in BMI ($r = 0.30$, $p = 0.003$), however, there was no association between changes in IL-18 or neopterin and change in BMI or HbA1c. In contrast, changes in IL-18 were correlated with changes in neopterin, with correlation of a similar magnitude to that observed between HbA1c and BMI ($r = 0.299$, $p = 0.009$). A scatter plot of change in neopterin levels vs. change in IL-18 levels for all participants revealed a selection of data points which could lend undue weight to this correlation (Figure 3, Panel A). Sequential removal of two participants with large increases in neopterin values reduced this association to $r = 0.225$ ($p = 0.053$, $n = 74$; Figure 3, Panel B).

Another method of examining the relationship between changes in multiple variables arising from the intervention is to perform multiple regression with adjustment for the additional variables as...
confounders. Intervention effect sizes were therefore calculated for changes in IL-18 and neopterin with additional adjustment for changes in BMI and/or HbA1c, as shown in Table 5. Effect sizes for change in IL-18 and neopterin were minimally altered by further adjustment for BMI or HbA1c, with effect sizes either marginally larger (after adjustment for change in BMI) or smaller (after adjustment for HbA1c) for the effect of the intervention on IL-18 levels, or slightly larger after adjustments for the effect of the intervention on neopterin levels.

The association of changes in neopterin and IL-18 levels with dietary components was further analysed in Appendix 1, Figures 1 and 2. Changes in neopterin levels (henceforth Δ neopterin) showed a significant correlation with changes in saturated fat intake (Δ SFA: Appendix 1, Figure 1, Panel D; $r = 0.29$, $p = 0.03$). Examining changes in intervention vs. control participants separately showed that this was entirely driven by an association between Δ neopterin and Δ SFA in intervention participants ($r = 0.43$, $p = 0.01$; Appendix 1, Figure 1, Panel F) which was not observed in control participants ($r = 0.01$, $p = 0.94$; Appendix 1, Figure 1, Panel E). No other significant associations were observed between dietary variables and changes in neopterin or IL-18 levels.
Table 3: Comparison of effect sizes of control and dietary interventions on adipokines and inflammatory markers. Differences are depicted in graphical format in Figure 2.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Intervention</th>
<th></th>
<th>Control</th>
<th></th>
<th>Difference * (95 % CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>median (IQR)</td>
<td>n</td>
<td>median (IQR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>leptin (ng/ml)</td>
<td>36</td>
<td>36.4 (28.8, 48.0)</td>
<td>36</td>
<td>37.4 (20.9, 54.5)</td>
<td>1.10 (0.80, 1.50)</td>
<td>0.56</td>
</tr>
<tr>
<td>neopterin(nmol/l)</td>
<td>39</td>
<td>5.36 (4.55, 6.09)</td>
<td>39</td>
<td>5.04 (4.42, 5.47)</td>
<td>0.87 (0.77, 0.98)</td>
<td>0.03</td>
</tr>
<tr>
<td>sTNF-R1 (pg/ml)</td>
<td>40</td>
<td>836 (615, 1019)</td>
<td>40</td>
<td>901 (671, 1127)</td>
<td>1.00 (0.89, 1.11)</td>
<td>0.94</td>
</tr>
<tr>
<td>sTNF-R2 (pg/ml)</td>
<td>40</td>
<td>2727 (2413, 3300)</td>
<td>40</td>
<td>2919 (2418, 3254)</td>
<td>1.10 (0.87, 1.40)</td>
<td>0.42</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>12</td>
<td>1.60 (0.52, 4.16)</td>
<td>12</td>
<td>0.51 (0.20, 4.03)</td>
<td>0.56 (0.11, 2.74)</td>
<td>0.45</td>
</tr>
<tr>
<td>IL-1ra (pg/ml)</td>
<td>10</td>
<td>12.7 (7.4, 55.2)</td>
<td>10</td>
<td>41.3 (3.8, 102.5)</td>
<td>1.20 (0.45, 3.15)</td>
<td>0.70</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>29</td>
<td>4.94 (1.43, 9.76)</td>
<td>29</td>
<td>5.61 (1.88, 23.21)</td>
<td>1.12 (0.51, 2.44)</td>
<td>0.78</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>20</td>
<td>3.08 (1.74, 6.61)</td>
<td>20</td>
<td>2.42 (1.60, 8.63)</td>
<td>0.57 (0.30, 1.10)</td>
<td>0.09</td>
</tr>
<tr>
<td>TNFα (pg/ml)</td>
<td>44</td>
<td>9.7 (6.7, 16.3)</td>
<td>44</td>
<td>14.0 (10.2, 21.8)</td>
<td>1.00 (0.79, 1.27)</td>
<td>1.00</td>
</tr>
<tr>
<td>IL-18 (pg/ml)</td>
<td>45</td>
<td>506 (430, 666)</td>
<td>45</td>
<td>449 (354, 553)</td>
<td>0.91 (0.83, 0.99)</td>
<td>0.04</td>
</tr>
<tr>
<td>IL-18 BP (pg/ml)</td>
<td>32</td>
<td>455 (140, 857)</td>
<td>32</td>
<td>362 (163, 768)</td>
<td>0.82 (0.53, 1.26)</td>
<td>0.36</td>
</tr>
</tbody>
</table>

* analyses adjusted for baseline but no other variables. Difference represents the ratio of change in intervention group to change in control group.
Figure 2: Forest plot of LOADD dietary intervention effect sizes on adipokines and inflammatory markers. x-axis represents the effect size as shown in Table 3, being the ratio of the change from baseline in the intervention group over the change from baseline in the control group.
Table 4: Pairwise correlation coefficients of the change in BMI, HbA1c, IL-18 and neopterin for participants from both control and intervention groups.

<table>
<thead>
<tr>
<th></th>
<th>Δ BMI</th>
<th>Δ HbA1c</th>
<th>Δ IL-18</th>
<th>Δ neopterin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ HbA1c</td>
<td>0.30 **</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Δ IL-18</td>
<td>-0.04</td>
<td>0.14</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Δ neopterin</td>
<td>-0.11</td>
<td>-0.02</td>
<td>0.30 **</td>
<td>-</td>
</tr>
</tbody>
</table>

* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001

Figure 3: Scatter plots of change in IL-18 levels (x axis) versus change in neopterin levels (y axis) across all participants with linear regression correlations shown with a red dashed line. **A**: r = 0.274 (p = 0.017, n = 76) *; **B**: exclusion of two uppermost Δ neopterin values (circled in red in A), r = 0.225 (p = 0.053, n = 74).

* note there are slight differences in the r and p values shown here versus those in Table 4 and Appendix 1, Table 4, due to differences in the software used (Stata pairwise correlation function in Table 4 and Appendix 1, Table 4; GraphPad Prism regression analysis in Figure 3).
Table 5: Intervention effect sizes for IL-18 and neopterin with additional adjustment for changes in HbA1c and BMI.

<table>
<thead>
<tr>
<th>Multiple regression model</th>
<th>IL-18 (pg/ml)</th>
<th>neopterin(nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Difference (95 % CI)</td>
<td>p-value</td>
</tr>
<tr>
<td>Base model *</td>
<td>0.91 (0.83, 0.99)</td>
<td>0.04</td>
</tr>
<tr>
<td>+ adjustment for change in BMI</td>
<td>0.90 (0.82, 0.99)</td>
<td>0.03</td>
</tr>
<tr>
<td>+ adjustment for change in HbA1c</td>
<td>0.92 (0.83, 1.01)</td>
<td>0.07</td>
</tr>
<tr>
<td>+ adjustment for changes in both BMI &amp; HbA1c</td>
<td>0.91 (0.83, 1.00)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* multiple regression adjusted for baseline measures, expressed as the ratio of change in intervention group over change in control group. As shown in Table 3 above.
3.2 Correlation of inflammatory markers and adipokines with anthropometric variables and glycaemic control

In order to examine the relationship of the selected inflammatory markers and adipokines with other clinical and anthropometric measures of cardiovascular risk, the baseline (pre-intervention) measures from both control and intervention arms were pooled. Distribution of measures are shown in scatter graphs in Appendix 1, Figure 3. Pairwise correlation coefficients are shown in Table 6 and Appendix 1, Table 4 for relationships among adipokines and inflammatory markers and with other clinical variables. In order to account for the potential confounding effect of outlying data points, those variables with significant correlations were plotted in scatter graphs (Appendix 1, Figures 4 through 9) to check for outlying data points and reassess correlations excluding these points.

Leptin was significantly correlated with waist circumference, body weight and BMI, and was also correlated with measures of glycaemia and various lipid measures. Leptin was also lower in men and current smokers. Scatter graphs of these relationships did not reveal any outlying data points to skew these correlations (Appendix 1, Figures 4 – 9). To further investigate the correlation of leptin with measures of glycaemia and blood lipids, partial correlation coefficients were generated for leptin corrected for BMI as a measure of adiposity (Table 7). These showed that the association of leptin with measures of glycaemia appeared to be mediated by adiposity, as the correlations of leptin with glucose or HbA1c were no longer statistically significant after taking into account the association with BMI. In contrast, the partial correlation of leptin with total cholesterol remained statistically significant (p = 0.015) and of similar magnitude after accounting for adiposity (r = 0.29 for pairwise correlation, r = 0.27 for partial correlation adjusted for BMI).

IL-18 was significantly associated with measures of anthropometry, with associations with waist circumference (r = 0.31, p = 0.002) and weight (r = 0.28, p = 0.007) comparable to those seen with leptin (with values of r = 0.30, p = 0.006 for waist circumference and r = 0.27, p = 0.013 for weight; see Appendix 1, Table 4 for full pairwise correlation data). These associations appeared robust in scatter graphs with no outlying data points (Appendix 1, Figures 4 – 9). Other associations reaching a high level of significance (p ≤ 0.01) include lower levels of sTNF-R2 with increasing fasting glucose, and lower TNFα levels with increasing age. Associations of smoking with the proinflammatory cytokines TNFα and IL-1β appeared to be driven by outlying data points (Appendix 1, Figure 5). Neopterin was significantly correlated with age and diabetes duration. While these associations would also be expected, since diabetes duration also correlates with age, the association of neopterin...
levels with diabetes duration appeared to be unduly influenced by one outlying data point (Appendix 1, Figure 8, Panels C and C’).

In correlations amongst adipokines and inflammatory markers, levels of the soluble TNFα receptors were highly correlated (Appendix 1, Figure 9, Panel G). A number of correlations amongst inflammatory markers and adipokines appeared to have outlying data points, and correlations were even stronger after their exclusion, including the associations of the soluble TNFα receptors and neopterin, the anti-inflammatory cytokines IL-10 and IL-1ra, IL-6 and TNFα, and IL-10 and TNFα. The association of IL-1ra with neopterin, however, appeared to be influenced by select outliers and was no longer significant after their exclusion (Appendix 1, Figure 9, Panels A and A’).
Table 6: Pearson correlation coefficients of adipokines and inflammatory markers with anthropometric, clinical and biochemical measures at baseline. Full data including number of observations and p-values for each correlation are shown in Appendix 1, Table 2.

|                            | leptin (ng/ml) | neopterin (nmol/l) | TNF (pg/ml) | sTNF-R1 (pg/ml) | sTNF-R2 (pg/ml) | IL-1β (pg/ml) | IL-1ra (pg/ml) | IL-6 (pg/ml) | IL-10 (pg/ml) | IL-18 (pg/ml) | IL-18 BP (pg/ml) |
|-----------------------------|----------------|--------------------|-------------|-----------------|----------------|---------------|---------------|--------------|---------------|---------------|----------------|----------------|
| **Glycaemia:**              |                |                    |             |                 |                 |               |               |              |               |               |                 |               |
| glucose (mmol/l)            | 0.25 *         | -0.03              | -0.04       | -0.07           | -0.27 **        | -0.04         | -0.09         | -0.10        | -0.12         | -0.04         | 0.09            |
| HbA1c (%)                   | 0.30 **        | 0.05               | 0.03        | -0.05           | -0.16           | 0.01          | 0.03          | -0.10        | -0.05         | 0.13          | 0.11            |
| diabetes duration (yrs)     | 0.14           | 0.22 * †           | -0.08       | 0.03            | 0.13            | -0.12         | 0.26          | -0.19        | -0.06         | 0.14          | 0.07            |
| **Anthropometry:**          |                |                    |             |                 |                 |               |               |              |               |               |                 |               |
| waist (cm)                  | 0.30 **        | 0.14               | 0.10        | 0.22 *          | 0.12            | -0.05         | -0.04         | -0.11        | -0.07         | 0.31 **       | 0.12            |
| weight (kg)                 | 0.27 *         | 0.06               | 0.18        | 0.14            | 0.04            | -0.02         | -0.12         | -0.15        | -0.01         | 0.28 **       | 0.13            |
| BMI (kg/m²)                 | 0.48 ***       | 0.13               | 0.14        | 0.17            | 0.08            | 0.05          | -0.14         | -0.10        | -0.11         | 0.26 *        | 0.10            |
| **Biochemistry:**           |                |                    |             |                 |                 |               |               |              |               |               |                 |               |
| total chol. (mmol/l)        | 0.29 **        | -0.14              | 0.03        | 0.00            | -0.06           | 0.16          | 0.16          | -0.01        | 0.03          | -0.08         | 0.00            |
| HDL (mmol/l)                | 0.24 *         | -0.01              | -0.17       | -0.04           | 0.01            | 0.17          | 0.30          | 0.19         | 0.29 * †      | -0.23 *       | 0.00            |
| LDL (mmol/l)                | 0.26 *         | -0.16              | 0.00        | -0.06           | -0.07           | 0.21          | 0.10          | -0.03        | 0.05          | -0.15         | 0.00            |
| TAG (mmol/l)                | 0.00           | 0.01               | 0.20        | 0.17            | -0.01           | -0.16         | -0.01         | -0.10        | -0.24 * †     | -0.03         | 0.00            |
| uric acid (µmol/l)          | -0.04          | 0.02               | -0.04       | 0.07            | 0.19            | -0.16         | -0.12         | -0.17        | -0.06         | 0.14          | -0.03           |
| **Blood pressure:**         |                |                    |             |                 |                 |               |               |              |               |               |                 |               |
| Systolic (mmHg)             | -0.09          | 0.09               | -0.18       | 0.06            | 0.09            | 0.09          | 0.16          | -0.09        | -0.07         | 0.03          | 0.19            |
| Diastolic (mmHg)            | -0.14          | -0.04              | -0.08       | -0.15           | -0.18           | 0.20          | 0.07          | -0.07        | 0.05          | 0.03          | 0.23 *          |
### Table 6 cont:

<table>
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<tr>
<th></th>
<th>leptin (ng/ml)</th>
<th>neopterin (nmol/l)</th>
<th>TNFα (pg/ml)</th>
<th>sTNF-R1 (pg/ml)</th>
<th>sTNF-R2 (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
<th>IL-1ra (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>IL-18 (pg/ml)</th>
<th>IL-18 BP (pg/ml)</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>age (yrs)</td>
<td>-0.04</td>
<td>0.32 **</td>
<td>-0.28 **</td>
<td>0.22 *</td>
<td>0.24 *</td>
<td>-0.25 * †</td>
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<td>0.01</td>
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<tr>
<td>male</td>
<td>-0.38 ***</td>
<td>-0.02</td>
<td>0.10</td>
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<td>-0.10</td>
<td>0.16</td>
<td>-0.19</td>
<td>0.16</td>
<td>0.18</td>
<td>0.09</td>
</tr>
<tr>
<td>current smoker</td>
<td>-0.24 *</td>
<td>0.07</td>
<td>0.30 ** †</td>
<td>0.11</td>
<td>0.22 *</td>
<td>0.38 ** †</td>
<td>-0.15</td>
<td>-0.01</td>
<td>-0.10</td>
<td>0.05</td>
<td>-0.02</td>
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#### Adipokines and inflammatory markers:

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<tr>
<th></th>
<th>leptin (ng/ml)</th>
<th>neopterin (nmol/l)</th>
<th>TNFα (pg/ml)</th>
<th>sTNF-R1 (pg/ml)</th>
<th>sTNF-R2 (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
<th>IL-1ra (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>IL-18 (pg/ml)</th>
<th>IL-18 BP (pg/ml)</th>
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</thead>
<tbody>
<tr>
<td>leptin (ng/ml)</td>
<td>-</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>neopterin (nmol/l)</td>
<td>0.02</td>
<td>-</td>
<td></td>
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<td>TNFα (pg/ml)</td>
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<td>-0.06</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>sTNF-R1 (pg/ml)</td>
<td>0.16</td>
<td>0.22 *</td>
<td>0.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>sTNF-R2 (pg/ml)</td>
<td>0.12</td>
<td>0.25 *</td>
<td>-0.09</td>
<td>0.53 ***</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>-0.01</td>
<td>0.00</td>
<td>0.13</td>
<td>-0.03</td>
<td>-0.16</td>
<td>-</td>
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<tr>
<td>IL-1ra (pg/ml)</td>
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<td>0.50 ** †</td>
<td>-0.06</td>
<td>0.01</td>
<td>-0.10</td>
<td>-0.04</td>
<td>-</td>
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<tr>
<td>IL-6 (pg/ml)</td>
<td>-0.05</td>
<td>0.00</td>
<td>0.28 *</td>
<td>0.11</td>
<td>0.03</td>
<td>0.05</td>
<td>0.12</td>
<td>-</td>
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<td>IL-10 (pg/ml)</td>
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<td>-0.01</td>
<td>-0.14</td>
<td>0.01</td>
<td>0.58 ***</td>
<td>0.08</td>
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<td>IL-18 (pg/ml)</td>
<td>0.03</td>
<td>0.18</td>
<td>0.18</td>
<td>0.15</td>
<td>0.07</td>
<td>0.30</td>
<td>-0.11</td>
<td>0.16</td>
<td>0.05</td>
<td>-</td>
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</tr>
<tr>
<td>IL-18 BP (pg/ml)</td>
<td>0.00</td>
<td>0.17</td>
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<td>0.07</td>
<td>-0.06</td>
<td>0.11</td>
<td>-0.05</td>
<td>0.24</td>
<td>0.12</td>
<td>-</td>
</tr>
</tbody>
</table>

* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001

† Association was not robust to exclusion of outlying data points as shown in Appendix 1, Figures 4–9.
Table 7: Partial correlation coefficients for leptin when adjusting for BMI.

<table>
<thead>
<tr>
<th>Variable X</th>
<th>Pairwise correlation (as shown in Table 1)</th>
<th>p-value</th>
<th>Variable X partial correlation adjusting for BMI</th>
<th>p-value</th>
<th>BMI partial correlation adjusting for variable X</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose (mmol/l)</td>
<td>0.25</td>
<td>0.024</td>
<td>0.14</td>
<td>0.207</td>
<td>0.44</td>
<td>0.000</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>0.30</td>
<td>0.006</td>
<td>0.15</td>
<td>0.185</td>
<td>0.42</td>
<td>0.000</td>
</tr>
<tr>
<td>total chol. (mmol/l)</td>
<td>0.29</td>
<td>0.008</td>
<td>0.27</td>
<td>0.015</td>
<td>0.47</td>
<td>0.000</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.48</td>
<td>0.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</table>
Chapter 4: Discussion

4.1 Discussion of LOADD study findings

This study examined the effects of a randomised controlled trial of dietary intervention in patients with type 2 diabetes on a range of adipokines and inflammatory markers. Analyses included an examination of the associations between these adipokines and inflammatory markers and other markers of cardiovascular risk, and an assessment of the effect of dietary intervention on circulating levels of these analytes. The key finding from this study was that the 6-month dietary intervention in the LOADD study produced significant reductions in circulating IL-18 and neopterin levels. Adjustment of the results shown in Table 3 for multiple testing reduces the required p value to 0.0047, and thus none of these results are significant after Bonferroni correction. There is, however, debate about the utility of correction for multiple testing. In particular, the Bonferroni test assumes all measures are independent. However, substantial interaction would be expected amongst inflammatory markers due to their biological roles and often production and release from the same cells, and this is suggested by the analyses of correlation of inflammatory markers and adipokines at baseline (Table 6 and Appendix 1, Figures 4 – 9). Under these circumstances, Bonferroni correction will substantially reduce the available statistical power to detect differences between groups; in other words, correction for multiple testing would reduce the chance of a type I error, but is likely to increase the chance of a type II error. In the of the results here, it is perhaps best concluded that significant reductions in neopterin and IL-18 were observed in the intervention group, but that it is possible these reflect a type I error.

There have been no previous studies where the effect of a dietary intervention on inflammatory markers has been assessed in participants with type 2 diabetes. A number of other publications in persons with obesity have shown that weight loss can reduce circulating IL-18 levels, including studies of liposuction, bariatric surgery, and diet and/or exercise interventions. In this regard, the results of the LOADD study in people with type 2 diabetes are in accordance with the findings of previous lifestyle studies in persons with obesity. However, analyses
of the correlation of change in IL-18 or neopterin with the change in BMI or HbA1c, or further adjustment of regression models for changes in BMI or HbA1c, suggest that the reductions in IL-18 and neopterin were largely independent of the observed reductions in BMI and HbA1c which also resulted from the intervention, and thus arise from other factors relating to the LOADD study intervention aside from weight loss. Given that previous metabolic studies by Esposito et al. have shown that IL-18 increases after a high fat meal and decreases after a high fibre meal, and that the dietary intervention in the LOADD study resulted in a significant increase in protein intake, significant reduction in saturated fat intake, and an increase in dietary fibre intake of borderline significance, it seemed plausible that the reduction in IL-18 could thus be reflective of changes in dietary composition. However, a post-hoc analysis of the correlation of changes in neopterin and IL-18 levels with various aspects of diet composition largely failed to show any correlations between these variables. The only exception was a significant association of changes in saturated fat intake with changes in circulating neopterin levels. This association was only observed amongst participants in the intervention group. It is not clear how this effect could be biologically plausible; if neopterin levels were regulated by saturated fatty acid intakes then changes in intake levels in control group participants would also be expected to show the same relationship, unless somehow the intervention participants were directed towards, for example, a different profile of saturated fatty acid composition and specific fatty acids exhibited differing effects on neopterin release, or the changes in saturated fatty acid intake were correlated with intake of a specific dietary variable which influences neopterin levels. Given the post-hoc nature of this assessment, it is possible that this is a spurious result. It should be noted, however, that the lack of association between changes in IL-18 and neopterin and other measures of dietary composition does not necessarily rule out an effect of diet composition on these variables; even weighed diet records, as used here in the LOADD study, are prone to error and based on self-reported intake, and an effect could exist which we are unable to detect with the LOADD study data (i.e. a type II error).

If the lack of association between changes in IL-18 and neopterin, and changes in anthropometric, glycaemic, or dietary variables is taken as an accurate finding, this then leaves open the question as to what mediates these changes in cytokine levels in the LOADD study. One hypothesis relates to the role of gut bacteria in generating inflammatory cytokines. A number of studies have shown a role for IL-18 in the pathogenesis of intestinal inflammatory disorders, and there is recent interest in the role of gut microbiota in mediating obesity-associated metabolic changes. A recent study by Maslanik et al. in rats showed that stress-induced changes in circulating IL-18 can be blunted by oral administration of antibiotics, and this raises the possibility that diet-induced
alterations in gut microbiota could be one mechanism underlying the reduction in IL-18 observed here. This could also potentially form a mechanism by which changes in diet composition acutely regulate circulating IL-18 levels, as reported by Esposito et al.\textsuperscript{501} (discussed above). Other possible explanatory mechanisms exist, such as the role of IL-18 in the hypothalamic-pituitary-adrenal stress axis\textsuperscript{440}. A number of studies in animals have shown that acute stress increases circulating or saliva IL-18 levels\textsuperscript{626–632}, and while the results of Maslanik et al. suggest that this may involve gut microbiota in part, their results also reinforce the influence of psychological stress on circulating IL-18 levels. While the LOADD study was designed as a dietary intervention, it is important to recognize the unplanned ‘non-intervention’ effects of involvement in a research project. Whether the increased interpersonal contact with a dietitian and discussion of diet and diabetes-related problems by intervention group participants had psychological effects which influenced the stress axis is unknown.

A significant reduction in neopterin was also observed in the current study. Given that: (i) IL-18 induces neopterin release from macrophages (via induction of IFN-γ\textsuperscript{530}), (ii) levels of neopterin are increased in monkeys after an acute injection of IL-18\textsuperscript{547,548}, it seemed likely that the observed reductions in neopterin could occur downstream of the reductions in IL-18. Correlation analyses showed that changes in neopterin levels in the LOADD study correlated with changes in IL-18 ($r = 0.299$, $p = 0.009$, Table 4), however, the significance of this relationship was sensitive to the exclusion of a few data points with large delta values (Figure 3). Thus, it appears possible that the reduction in neopterin may be dependent in part on a reduction in circulating IL-18 levels, although there are clearly other factors which have a greater contribution to changes in neopterin levels.

Given that the LOADD dietary intervention resulted in significant changes in anthropometric measures, and that leptin levels at baseline correlated with these anthropometric measures, it was somewhat surprising that these anthropometric changes were not paralleled by a change in circulating leptin. While changes in BMI, waist circumference and weight were statistically significant, the effect sizes of 0.5 kg/m$^2$, 1.6 cm, and 1.3 kg represent changes of 1.4 %, 1.4 %, and 1.3 %, respectively, relative to the mean baseline values in the intervention group. Thus, it is possible that the effect sizes were simply not sufficient to detect a change in circulating leptin levels.

In addition to examining the effect of the LOADD study dietary intervention on selected inflammatory markers and adipokines, analyses were conducted here to examine the association of these variables with glycaemic control and surrogate markers of cardiovascular risk. These analyses
showed a number of expected associations, such as lower leptin levels in males and non-smokers and an association with anthropometric measures, a correlation between of soluble TNFα receptors and neopterin, and associations of IL-18 with measures of anthropometry. Interestingly, the correlation of IL-18 with baseline body weight and anthropometric measures was of a similar magnitude to those seen between leptin and anthropometric measures (IL-18 correlations $r = 0.31$, $p = 0.002$ for waist circumference and $r = 0.28$, $p = 0.007$ for weight; leptin correlations $r = 0.30$, $p = 0.006$ for waist circumference and $r = 0.27$, $p = 0.013$ for weight; see Appendix 1, Table 4 for full pairwise correlation data). The correlation of IL-18 with measures of anthropometry has been previously noted in other studies, although not all have observed this relationship. Whether or not IL-18 is a true ‘adipokine’ and actually released from adipose cells is controversial, however, this argument is largely academic as while IL-18 may not be produced by adipose cells, it is produced from adipose tissue with production from non-adipose stromal cells and studies have reported higher adipose tissue IL-18 expression production in obesity in humans and animals. The association of IL-18 with anthropometric measures at baseline presents a scenario which, at face value, appears at odds with the finding that IL-18 levels were reduced by the dietary intervention but were not associated with changes in BMI. It is not known how such an effect arises and presents an interesting topic of enquiry for further research.

A few previous studies have found a positive association of neopterin with age, and an association of neopterin with the soluble TNFα receptors has also been found in a number of studies (reviewed in). Studies have shown higher levels of neopterin in patients with diabetes than in those without or conversely, a higher proportion of participants with diabetes across increasing quarters of neopterin. One study in patients with type 1 diabetes noted a trend towards a significant association of neopterin with diabetes duration ($r = 0.205$; $p < 0.0539$), and other studies in patients with type 2 diabetes have shown increased levels in patients with nephropathy and microalbuminurie. An association of neopterin with diabetes duration could therefore be indicative of an underlying pathogenic process, such as the development of renal disease or atherosclerosis. However, given age and duration of diabetes are inevitably correlated, the observed positive association of neopterin with duration of diabetes could arise from confounding by age; indeed in the aforementioned study which showed higher neopterin levels in patients with microalbuminurie, these patients were significantly older than the comparison group without. In the results presented here, neopterin was significantly positively associated with age, and yet the association with diabetes duration appeared to be influenced by one data point. Clearly, one of the
difficulties in examining factors which show a correlation with age is delineating whether the association is reflective of an underlying pathogenic process such as atherosclerosis, or part of a normal, healthy, alteration associated with aging. Forming a distinction between the two would require a large amount of scientific study, and depend to large extent on the definitions used to differentiate between 'normal' aging and a pathogenic process associated with aging. However, at least in the case of neopterin, there is sufficient evidence of an association or even active role in the development of cardiovascular disease \(^{531-536,540,541,543,545,546,646-658}\) to suggest that the association with age is unlikely to reflect a process of 'healthy' aging, and probably arises due to the association with subclinical or clinical atherosclerosis.

Leptin levels correlated with anthropometric measures of adiposity, as expected based on its production from adipose tissue, as well as associations with glycaemia and cholesterol. While the association of leptin with glycaemia appeared to be explained by differences in adiposity, the association with cholesterol was not affected by controlling for BMI. A number of studies have shown direct effects of leptin on aspects of lipid metabolism, such as changes in fat oxidation \(^{659,660}\) and changes in lipolysis \(^{661}\). However, changes in lipid metabolism seen in people with congenital leptin deficiency, or leptin-deficient rodents are confounded by changes in body weight with leptin treatment, and in addition, these subjects are leptin-sensitive, in contrast to the leptin resistance associated with excess adiposity which would be expected in the participants of the LOADD study. While much research has been conducted into the blunted effects of leptin on appetite, energy expenditure and glucose metabolism with obesity (so-called 'leptin resistance'), whether the effects of leptin on lipid metabolism are also affected is less clear \(^{662}\). A study in diet-induced obese mice showed that leptin reduces liver triglyceride content in livers from normal-weight but not diet-induced obese animals, supporting the notion of a more global leptin resistance which also blunts the effects of leptin on lipid metabolism. In contrast, a study involving acute disruption of leptin signalling in normal weight mice showed no effects on circulating lipids \(^{663}\). Given a number of studies in humans showing that leptin therapy can reduce circulating lipid levels in leptin deficiency\(^{664,665}\) and lipodystrophy \(^{666-668}\), the positive association of leptin levels with cholesterol seen in the LOADD study participants could be taken as evidence of resistance to the effects of leptin on lipid metabolism, as based on the aforementioned works higher circulating leptin levels would be expected to result in lower circulating lipid levels. Of note, a recent randomised controlled trial of leptin therapy in obese patients with type 2 diabetes enrolled subjects with a similar mean BMI (33.2 ± 3.8 kg/m\(^2\)), age (53.3 ± 11.4 yrs), and leptin levels (38.0 ± 6.4 ng/ml placebo group, 35.2 ± 3.5 ng/ml leptin treatment group at baseline) as the LOADD study participants. The authors of that study
found no effect of leptin treatment on a variety of metabolic parameters, consistent with a diagnosis of leptin resistance, which supports the notion that the LOADD study participants were also likely to have been leptin resistant.

This study had a number of weaknesses. Firstly, it is acknowledged that a number of analyses have been conducted here and there is a high probability of a type I error producing spurious 'positive results'. Indeed, for any randomised controlled trial, analysis of 20 variables would be expected to produce one false positive result by chance at the 5 % level of significance. The key analysis included here, of examining the effect of the LOADD study dietary intervention on circulating levels of a selection of inflammatory markers, was however, planned a priori, and the finding of significant reductions in both IL-18 and neopterin, which form part of the same inflammatory signalling pathway, possesses an additional biological plausibility suggestive of being a 'true' positive result. The results of multiple pairwise correlation comparisons, involving correlations between a large number of variables, are best viewed with some caution as these could similarly be subject to a type I error. However, the use of scatter graphs and exclusion of outlying data points shown in Appendix 1 provides some measure of the robustness of the reported associations.

Another weakness of the study is that for a number of the inflammatory markers and adipokines measured (IL-1β, IL-1ra, IL-6, IL-10 and IL-18 BP) samples were outside the range of measurement or excluded on the basis of large coefficients of variation, resulting in a number of missing values. This is particularly evident in the measures of IL-1β, IL-1ra, IL-6, and IL-10, which were all measured as part of the same multiplex (in addition to TNFα, for which more reliable measures were obtained, suggesting there was not simply a more global problem with this multiplex assay). The principal analysis examining the effect of the dietary intervention was also limited to participants with values for each analyte at baseline and 6-month end of study visits. The resulting modest sample gave rise to wide confidence intervals and hence greatly reduced statistical power to detect any alterations arising from the intervention. A number of studies have been published which have aimed to systematically investigate the use of multiplex technology versus ELISAs, or assess the accuracy of different multiplex panels for the measurement of cytokines. These studies paint a picture generally in agreement that some problems exist in the use of multiplex technologies, such as the presence of heterophilic antibodies (i.e. antibodies for the detection of different analytes interacting with each other during the assay), and that the overall agreement of absolute values can differ widely between multiplex technologies and ELISAs, depending on the analytes in question, and this can subsequently interfere with cross-study comparisons. However, despite these
differences multiplex assays have generally been confirmed as providing the ability to provide discrimination between relative levels across samples, and thus, while the absolute levels detected may differ from those seen by ELISA assays, they are still capable of being used for a number of the same comparisons offered by ELISA or RIA methods, such as ranking samples and providing correlations, and for assessing relative changes across time. Both Martos-Moreno et al. 672 and Khan et al. 680 have compared changes in cytokine levels across time (Martos-Moreno et al. in a weight loss intervention in obese children, and Khan et al. following an acute inflammatory challenge) and found changes in cytokine concentrations measured by multiplex comparable to those measured with ELISA or RIA in the same study. In their comparison across multiple laboratories, Breen et al. concluded that "in the context of smaller studies in a single laboratory or when all samples have been collected and can be run at a single time and place, multiplex kits may be useful and offer the benefit of greatly reduced sample volumes" 670. Given that the analyses conducted here in the LOADD study conform to these criteria and present a comparison of relative changes with a dietary intervention, or correlation with other cardiovascular risk variables based on ranking, the use of the multiplex values of IL-1β, IL-1ra, IL-6 and IL-10 in this analysis seems justified. However, given the number of samples outside of the detectable range, results should be interpreted with caution as the presence of a systematic difference between samples within or outside of the detectable range cannot be excluded. In addition, current consensus appears to suggest the comparison of absolute levels across studies should also be made with caution as differences in antibody pairs and laboratory procedures can give highly differing levels for some analytes.

In conclusion, the LOADD study dietary intervention resulted in significant reductions in circulating levels of IL-18 and neopterin. IL-18 has been associated with the development of cardiovascular disease, including a meta-analysis of prospective studies showing a relative risk (95 % CI) of developing of coronary heart disease of 1.34 (1.17, 1.53) after adjustment for age, gender, classical CHD risk factors, and inflammatory markers. Similarly, neopterin levels have been evaluated as a risk marker in numerous studies and associated with the development of a variety of cardiovascular events 532,535,540,541,543,545,602,646–654, including an association with ischaemic heart disease in people with type 2 diabetes 536. Although evidence exists for both of these factors for an association with, or even active role in, the development of cardiovascular disease, there are no trials demonstrating that a reduction in either of these markers results in a reduced rate of clinical endpoints. As such, the change in inflammatory markers seen in the current study appears to move in a favourable direction, although only a larger, longer trial of the LOADD dietary intervention with follow-up for clinical events would provide sufficient evidence of an effect on risk of
cardiovascular disease or diabetes complications. Subsequently delineating whether such a reduction in risk derives from changes in inflammatory markers would be subject to numerous limitations; interpreting cause-and-effect relationships and future expectations of benefit with lifestyle intervention studies is discussed below.
4.2 Interpreting the effects of the LOADD study dietary intervention; theoretical considerations of establishing cause and effect in studies of metabolic disease

One of the main driving ideas behind the research in this thesis was that sub-clinical inflammation associated with type 2 diabetes could be a causative factor in the high rates of heart disease in this population, if not even a cause of type 2 diabetes and insulin resistance itself. The question was posed as to whether dietary intervention in people with type 2 diabetes, who are treated according to current guidelines, could alleviate sub-clinical inflammation as assessed by a panel of inflammatory markers. The study in which this question was examined, the LOADD study, was itself designed principally as a pilot study, to assess the effects of dietary intervention on a range of markers of cardiovascular risk and diabetes control, with the intention of following this study with a larger trial with clinical endpoints; as recent trials in diabetes have underscored, these are the outcomes truly of interest in the treatment of diabetes.

In the event then that a hypothetical future study were to be conducted and show a reduction, for example, of diabetes complications (whatever endpoint that may include) with the LOADD study dietary intervention in people with type 2 diabetes, the question many would ask is how is this effect is achieved? Most likely, people would look to changes in physiological variables such as blood pressure or surrogate markers of cardiovascular risk such as lipid profile or inflammatory markers, and put forward the idea that the hypothetical risk reduction could be mediated by changes in such factors. In scenarios such as these, people often look to ‘basic research’ in order to try and understand the physiological processes taking place and deduce how they could impact on disease progression or rates of diabetes complications. In the main analysis of the LOADD study, statistically significant reductions in HbA1c, waist circumference, weight, and BMI were seen, without any changes in the conventional cardiovascular risk factors of lipid measures, blood pressure, or kidney function as measured by urinary albumin:creatinine ratio. The analysis included here also showed reductions in the inflammatory markers of neopterin and IL-18. Clearly, there are also a large number of other possible inflammatory markers not considered here, as well as markers reflective of other pathological processes currently being investigated for active roles in the development of diabetes complications, such as ectopic lipid deposition, endoplasmic reticulum stress, or levels of reactive oxygen species. Any hypothetical reduction in diabetes complications could also be derived from as yet unknown mechanisms or biological functions. Indeed, the rate of scientific progress in
the understanding of metabolism is presently giving rise to an increasing number of potential mediators including endocrine effects of bone 681, increasing numbers of adipokines 682, and differing levels of metabolites 683,684 and "lipokines" 685 in people with type 2 diabetes. In addition, other studies highlight the role of factors carried by one’s diet but not conventionally thought of as ‘dietary’, such as cooking method 686, or co-ingested factors including pesticide residues 687 or plastics such as bisphenol A 688,689. Therefore, were the LOADD study dietary intervention to be shown in the future to have any effects on diabetes complications, ascribing causal mechanisms to changes in any of these biomarkers, whether they be ‘classical cardiovascular risk factors’, or newer ‘novel’ risk factors, or delineating the relative contributions of changes in multiple biomarkers to a reduction in disease risk would be incredibly difficult to put into practice. Any such conclusions would also be theoretically marred by the possibility that other unmeasured variables mediate changes in risk (in effect, the biological equivalent of the ‘unknown/unmeasured confounder’ in epidemiological terms).

With the multitude of biological processes and measurable chemical entities in the human body, ascribing cause-and-effect to changes in a variable such as a blood-borne analyte quickly leads one into a quagmire fraught with the difficulties of discerning association from causation. This is the quagmire into which research into the role of glucose as a cardiovascular risk factor, discussed in the Introduction, has marched head first by attempting to hail glucose reduction as the curative factor of the diverse interventions employed. The key factor necessary to bear in mind is that descriptions of cause-and-effect must hold the intervention in question as the ‘cause’, which in the case of the glucose-lowering studies discussed earlier are the medication regimens employed (as opposed to their effect; a reduction in glycaemia). Discerning the possible biological mediators which lie in between the administered ‘cause’ and measured ‘effect’ is another scientific venture altogether, whether the intervention be lifestyle modification, as used here, or drug therapy as in the glucose-lowering trials discussed in the Introduction. Thus, were the LOADD study dietary intervention to be shown to influence rates of diabetes complications, the dietary intervention would be the cause (including its associated ‘non-dietary’ components including, but not limited to, the psychological effects of visiting a study dietitian, or inadvertent alterations in the intake of pesticide or plastics residues); we could posit theories regarding reductions in glycaemia, adiposity, IL-18 or neopterin mediating risk reductions (assuming that future hypothetical interventions would show the same effects on surrogate markers as measured here), however, these would remain purely theoretical postulations.
Section 3:

Animal studies into the biological role of IL-18
Chapter 5: Introduction

5.1 CNS control of appetite and blood glucose

The idea that the CNS may exert a substantial effect on peripheral glucose homeostasis is not at all new, and Claude Bernard, the ‘father of modern physiology’ suggested this to be so as far back as 1849. More recently the career trajectory of Robert Sherwin, a physician-scientist with an interest in hypoglycaemia, is one which began in the 1970’s with investigations into the peripheral regulation of blood glucose and progressed to focus increasingly on CNS mechanisms as the key events involved in sensing and responding to hypoglycaemia.

By the early twentieth century it had been known for some time that tumors or injuries of the hypothalamic region could produce a cluster of symptoms including adiposity, fever, polyuria and reproductive disorders, and a few experimental studies of this era showed that lesions of the hypothalamus could produce obesity. However, it was not until the development of stereotaxic equipment in the 1930’s by Horsley-Clarke, which allowed for precise, reproducible lesions of the brain, that research into the role of the hypothalamus in the control of appetite and blood glucose began in earnest.

Researchers quickly realised that the bilateral ventromedial hypothalamic nuclei play an important role in weight maintenance, and were labelled a ‘satiety centre’, as lesioning of these nuclei would produce weight gain. It was also established that this was not due to food intake alone, as pair-feeding of lesioned animals to the same amount of food consumed by non-lesioned controls did not completely impede weight gain. The neighbouring lateral hypothalamic nuclei were soon after documented as producing the opposite effect and became known as a ‘feeding centre’. At the same time as these studies, dramatic effects of the brain on blood glucose control were also uncovered, including effects of the pituitary and ventromedial hypothalamic nuclei on blood glucose homeostasis, and the role of descending signals from the vagus nerve.

Further works into the control of food intake over the following decades focused on results obtained from ‘parabiosis’ experiments where rodents were surgically joined to allow subcutaneous transport.
of extracellular and blood-borne proteins from one animal to another. In combination with the serendipitous occurrence of spontaneous mutations resulting in heritable obesity in strains of mice at the Jackson Laboratories, various parabiosis experiments involving these mutant strains or overfeeding of normal rodents, lesioning of the ventromedial hypothalamus or electrical stimulation of the lateral hypothalamus, suggested that “the hypothalamus may respond to body fat content by way of a slowly turned over blood-borne factor” (from Hervey et al., 1977). Later, painstaking positional cloning experiments led to the discovery of leptin, for which Douglas Coleman and Jeffrey Friedman – key figures in the parabiosis and cloning works, respectively – were awarded the 2010 Albert Lasker Basic Medical Research Award. A wealth of research into the role of leptin and hypothalamic feeding pathways has since followed; while the twentieth century marked a slow but sure development of knowledge regarding the role of the hypothalamus in the control of appetite and blood glucose homeostasis, the final years of the twentieth century and initial decade of the twenty-first have seen the rate of progress greatly accelerated by modern molecular biology and genetic engineering techniques, as well as a heightened scientific interest in elucidating the mechanisms by which metabolism is regulated. Recent works in particular show that the actions of leptin and other metabolic signals in the hypothalamus influence peripheral insulin sensitivity and glucose uptake, and that the hypothalamic effects of nutrients also influence food intake, metabolism and hepatic glucose production. Leptin administration into the hypothalamus has even been shown to reverse the glycaemic abnormalities of rodents with type 1 diabetes, apparently by a mechanism involving increased peripheral glucose uptake and depressed hepatic glucose output. These findings reinforce the discovery first elucidated by Claude Bernard; that the brain plays a powerful role in the regulation of peripheral glucose levels. In today’s modern setting of diabetes research, recent reviews discuss the CNS as having potentially overlooked powerful effects on peripheral insulin action and glucose homeostasis which could be of clinical relevance to the pathogenesis and treatment of diabetes.

This experiment is similar in many regards to findings approximately 80 years earlier, in which pancreatectomized dogs were kept alive for months by removal of the pituitary. These studies underscore the key role of CNS mechanisms in controlling glucose homeostasis; even, as is the case in these works, in the face of a complete absence of insulin (in both studies) and glucagon (in pancreatectomized dogs).
5.2 Architecture of the hypothalamus

Given the relatively short time frame of recent intense research into the functioning of the hypothalamus and mechanisms of energy homeostasis, it is not surprising that there remain many unanswered questions with regard to precisely how different metabolic signals influence the hypothalamus, and how signalling mechanisms involved in energy homeostasis interact in the living animal. Nevertheless, there is general consensus on some basic aspects of hypothalamic function and architecture. The arcuate nucleus at the base of the hypothalamus has been a focal point of research, and it appears that it forms a unique nucleus in the mammalian brain; evolved to be capable of detecting signals in the peripheral circulation or cerebrospinal fluid in the 3rd ventricle, which could be achieved via a more permeable blood-brain barrier and/or direct projections outside of the blood-brain barrier. Although these mechanisms remain somewhat controversial, it is generally agreed that the arcuate is particularly responsive to changes in circulating metabolic signals. For example, leptin receptors are found in high density in the arcuate nucleus, which respond to physiological levels of circulating leptin (with other hypothalamic nuclei responding at greater concentrations) and a loss of response to leptin in the arcuate nucleus is a key feature of diet-induced obesity. Leptin signalling in the arcuate also appears to play a role in synaptic development and plasticity, thus demonstrating that metabolic signals have a role beyond the early signalling events which are typically monitored as outcomes in most research experiments. The arcuate nucleus contains two populations of neurons that have been particularly well-studied as targets of leptin action, one which expresses proopiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART), activation of which causes a decrease in food intake, and another expressing agouti-related peptide (AgRP) and neuropeptide Y (NPY), activation of which increases food intake. Another key population of neurons is those expressing the melanocortin receptors (MCRs), which are particularly dense in the paraventricular nucleus of the hypothalamus (PVN), and which receive input from POMC neurons in the arcuate nucleus. The key role of MCR expressing neurons in energy homeostasis has been underscored by a number of studies and their relevance in human physiology is evident in studies showing that mutations of the melanocortin receptors are the most prevalent known genetic cause of obesity in people.

Figure 4 shows a basic schematic diagram of the location of these nuclei and neuronal populations in the hypothalamus. While the existing knowledge of hypothalamic function is more complex than that shown in the diagram, and will no doubt become increasingly more so as further research is
Figure 4: Nissl-stained coronal rat brain section, showing the position of hypothalamic nuclei. Paraventricular (PVN), ventromedial (VMH), and arcuate (ARC) hypothalamic nuclei are labelled. The arcuate nucleus contains two neuronal populations, expressing POMC/CART and NPY/AgRP, which are involved in energy homeostasis and metabolism. Although found throughout the hypothalamus, the paraventricular nucleus contains a particularly high concentration of cells expressing melanocortin receptors, which are also important regulators of food intake and metabolism. POMC-expressing cells in the arcuate nucleus project to the paraventricular nucleus, and much of their effects on food intake and metabolism may arise from downstream effects on melanocortin receptor-expressing cells. Image adapted from http://brainmaps.org.
conducted, the components of this model and their importance in energy homeostasis stand up well to the evidence presented by the existing literature.

Of note, the bulk of the characterisation of these metabolic pathways has been conducted in rodents, with some work in other species (particularly sheep) 745-751. Thus, to what extent the current knowledge of these metabolic signalling pathways also holds true in humans is not known. Similarities in findings across mammalian species (for example sheep and rodents), the phenotypes observed in humans with genetic mutations of melanocortin receptors or leptin signalling (which largely agree with expected effects based on animal studies), and genetic homology between humans and rodents in genes encoding many of these metabolic factors, suggest that the basic system of metabolic control by the hypothalamus also operates in a similar fashion in humans. However, differences obviously exist, such as the processing of POMC transcripts 743, and the precise role of any uniquely human traits will be incredibly difficult to ascertain.
5.3 Inflammation in the hypothalamus

Just as there are links between inflammatory markers in the periphery and insulin resistance (as discussed in the Introduction), a number of works have also shown that activation of inflammatory pathways in the brain are linked to central responses to leptin and insulin. Activation of NF-κB and transcription of pro-inflammatory cytokines is upregulated in the hypothalamus upon consumption of a high fat diet\(^{300,419,752}\), which appears to involve the inflammatory pathways of TLR-4 (toll-like receptor 4)\(^4\) and MyD88 (myeloid differentiation primary response gene 88)\(^{758}\) signalling.

Increased NF-κB signalling in the hypothalamus reduces responses to the hormones insulin\(^{300,752}\) and leptin\(^{300}\), and also appears to contribute to diet-induced weight gain, as manipulations to reduce hypothalamic inflammation also decrease weight gain on a high fat diet\(^{300,752,758}\). Leptin-induced changes in appetite, or anorectic sickness behaviour induced by common inflammatory signals, have also been implicated to involve activation of the NF-κB signalling pathway in the hypothalamus\(^{759}\), and NF-κB activation induces hypothalamic suppressor of cytokine signalling-3 (SOCS-3), a negative regulator of leptin signalling\(^{300}\), thus appearing to form a classical negative feedback loop (in much the same way as leptin-induced STAT-3 signalling). Other studies show that the beneficial effects of exercise on insulin sensitivity may involve, at least in part, cytokine effects on hypothalamic inflammatory signalling\(^{760}\). In addition, a number of proinflammatory cytokines are known to influence appetite (although it has not been demonstrated that their mechanisms of action all involve activation of hypothalamic inflammatory pathways), and their elevation is believed to underlie cancer cachexia\(^{761}\).

\(^{4}\) There is some evidence that saturated fatty acids directly activate TLR-4 receptors\(^{421}\) although this has been contested\(^{753}\). In vivo it appears that TLR-4 receptors do play a role in diet-induced obesity as mice with a gene ‘knock-out’ or manipulation of TLR-4 are resistant to diet-induced obesity\(^{419}\), and the in vivo situation could involve other, indirect, means of TLR-4 activation such as the generation of ‘endotoxemia’ by gut microbiota in response to a high fat or hypercaloric diet\(^{301,754–757}\).
5.4 Key questions regarding the biological actions of interleukin-18: how are the central effects of IL-18 mediated?

What is the expression and distribution of the IL-18 receptor in the brain?

The studies of Netea *et al.* 503 and Zorrilla *et al.* 506, which showed that intracerebroventricular injections of IL-18 could act centrally to reduce food intake, present a 'black box' scenario where IL-18 acts within the brain but the mechanism by which it produces a reduction in appetite is unknown. Neither of these studies offer hypotheses or putative mechanisms by which these effects could be mediated. Given the evidence linking the hypothalamus with appetite, an obvious hypothesis was that IL-18 would act on neuronal populations in the hypothalamus known to control food intake. In addition, this line of reasoning is supported by similarities between manipulation of IL-18 and manipulation of its related cytokine family member IL-1: mice lacking IL-1 receptors display a phenotype remarkably similar to that reported for mice lacking IL-18 receptors, with mature-onset obesity leading to impairment of glucose homeostasis (IL-18 receptor knockouts: 503; IL-1 receptor knockouts: 762); IL-1 receptor antagonist (an endogenous antagonist of IL-1) knockout mice have also been characterised and show protection from diet-induced obesity and a lean phenotype 763; and two papers by the same group report the expression of IL-1 receptors on proopiomelanocortin (POMC) and agouti-related peptide (AgRP) expressing neurons in the hypothalamus 764,765. Therefore, it was reasoned that given similarities in phenotypes resulting from manipulations of IL-1 and IL-18 genes, and in the signalling pathways of these cytokines 423,766, that IL-18 receptors may also be expressed on POMC and AgRP neurons in the hypothalamus.

Could IL-18 contribute to hypothalamic inflammation?

Given the key role of the hypothalamus in influencing energy homeostasis and glucose control, links between inflammation and insulin resistance, studies suggesting a link between IL-18 and measures of insulin resistance and development of diabetes, and the fact that IL-18 is a pro-inflammatory cytokine which acts through NF-κB signalling, it was hypothesized that IL-18 could act in the hypothalamus to influence food intake and glucose homeostasis via NF-κB activation.
5.5 Hypotheses:

Two hypotheses arose from interpretations of the current literature on the role of IL-18:

(i) The brain as a target of peripheral IL-18

One key hypothesis or model of IL-18 action in this thesis is that elevated circulating IL-18, as seen in obesity and diabetes, could drive NF-κB signaling in the hypothalamus, which has been linked to hypothalamic responsiveness to insulin and leptin. In this scenario, circulating IL-18 could influence appetite by interacting with hypothalamic pathways which govern energy homeostasis. Thus, experiments were planned in this thesis to examine the distribution of the IL-18 receptor in the rodent brain by in situ hybridisation and polymerase chain reaction (PCR) experiments.

(ii) Brain-derived IL-18 as a mediator of the metabolic effects of IL-18

Another hypothesis, is that IL-18 production within the brain itself could influence metabolic pathways involved in appetite. Such a possibility could explain observations from animal experiments showing modulation of appetite by IL-18 gene knockout or intracerebroventricular IL-18 administration.

Note that these two hypotheses are not necessarily mutually exclusive; a scenario could exist whereby both peripheral and brain-derived IL-18 jointly influence metabolism. Nevertheless, the chapters in this section of the thesis are organised around investigations into these two hypotheses.
Chapter 6: General laboratory methods

6.1 Animals

Animals were obtained from the Hercus Taieri Resource Unit, Dunedin, New Zealand and housed under Specific Pathogen Free (SPF) conditions until use in the current experiments. Animals used were either adult male Sprague-Dawley rats or adult male CJ57BL/6 mice. All procedures were performed with ethical approval from the Animal Welfare Office, University of Otago (approval numbers 58/07 and 64/08).

6.2 Reverse transcription polymerase chain reaction (RT-PCR)

Tissue collection of whole hypothalamus, liver, spleen and adrenal samples:

Samples of liver, spleen, adrenal and hypothalamus were collected by gross dissection after sacrificing animals by decapitation. Samples were wrapped in tin foil and placed on dry ice until frozen, and then transferred to a freezer at -80 °C as soon as possible. For extraction of hypothalamic tissue, brains were placed on tin foil with the inferior surface facing upwards, and the hypothalamus grossly dissected with a razor blade. For liver, spleen and adrenal samples, segments of tissue small enough to be dissolved in 1 mL of Trizol were dissected.

Collection of brain micropunches:

Brains were collected following decapitation as above. Tissue was kept at -80 °C until needed. Brains were transferred to a cryostat set to -10 °C and mounted onto sectioning chucks using Tissue-Tek® (Sakura Finetek USA, OCT Compound 4583). Coronal sections were cut at a thickness of 300 μm and mounted onto glass slides, which were placed onto a steel block kept cool with dry ice and viewed under dissecting microscope. Micropunches through nuclei of interest were collected by punching circular holes using modified 21-gauge hypodermic needles 500 μm in diameter.

For collection of ventromedial hypothalamic and paraventricular nuclei, two punches per section were made either side of the third ventricle. Collection of arcuate nuclei was done by a single punch, centred on the middle of the third ventricle at its lowest point. Two micropunches per section were...
collected through the habenula, interpeduncular nucleus and ventral tegmental area. The rat brain atlas of Paxinos and Watson was used as a guide. Appendix 3, Figures 1 through 4 show images of micropunch collection from the various nuclei for experiments described in Chapter 7, Section 7.5 and Chapter 8, Section 8.5.

RNA extraction:

Collected tissue samples or brain micropunches were placed into 1.7 ml pre-sterilized microcentrifuge tubes (Axygen, CA, USA; product number: MCT-175-C-S) containing Trizol reagent (Invitrogen, USA, SKU# 15596-026). Spleen, liver, adrenal and whole hypothalamus samples were digested in 1 ml Trizol, and micropunches in 800 μl of Trizol. Samples were homogenised by using a plunger which had been treated with RNase Zap (Ambion, USA, Catalogue #AM9780) according to manufacturers instructions, and samples were subsequently passed through a sterile 26 gauge needle to further break up tissue. Following homogenisation, samples were sonicated for approximately 10 minutes and 10 μg of glycogen added. Samples were incubated at room temperature for 5 minutes and at this stage some samples were optionally frozen at -80 °C for later RNA extraction. For RNA extraction, 0.2 ml of chloroform solution was added per 1 ml of Trizol and tubes shaken for approximately 15 seconds, then incubated at room temperature for a further 3 minutes. Samples were then spun in a refrigerated centrifuge set at 4 °C for 15 minutes at 12,000 × g to produce a clear upper phase containing RNA. This clear phase (between 300 - 600 μl) was transferred to fresh tubes using sterile pipette tips, and isopropyl alcohol added at a quantity of 0.5 ml for every 1 ml of Trizol originally used for extraction. Tubes were vortexed and incubated at room temperature for 10 minutes, after which tubes were spun in a centrifuge for 10 minutes at 12,000 × g at a temperature of 4 °C, leaving a pellet of RNA at the bottom of the tube. The supernatant was discarded and 1 ml of 75 % ethanol added. Ethanol solution was diluted from 100 % Analar grade ethanol using DEPC-treated water. The RNA pellet was washed by vortexing and spun in a centrifuge at 7,500 × g for 5 minutes at a temperature of 4 °C. The resulting supernatant was poured off and any remaining excess supernatant removed by pipette. Samples were left in open tubes at room temperature for approximately 10 minutes to further air dry the RNA pellet, after which 20 μl of DEPC-treated water was added and tubes incubated for 10 minutes at 60 °C with occasional vortexing to resuspend the collected RNA. Samples were either used for reverse transcription immediately or were kept frozen at -80 °C for reverse transcription at a later time.
Measurement of DNA and RNA quantity and quality:

Quantity and quality of DNA and RNA was measured using a Nanodrop spectrophotometer (Nanodrop® ND-1000 Spectrophotometer), and assessed via the ratio of absorptions at wavelengths of 260 nm to 230 nm (260/230 ratio), and 260 nm to 280 nm (260/280 ratio).

Reverse transcription:

Reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen, Catalogue number 18080-044). Tubes were prepared with up to 11 μl of RNA solution, 50 ng of random hexamers, 1 nmol of dNTP, and where necessary made up to 13 μl of total solution with DEPC-treated water. The amount of RNA added was calculated using previous measurements obtained from spectrophotometer analysis. Tubes were incubated for 5 minutes at 65 °C and then placed on ice. To each tube 7 μl of a second solution was added which contained 4 μl of 5 × first strand buffer, 0.1 ng of DDT, 40 U of RNase OUT, and either 200 U of Superscript III enzyme for reverse transcription positive (RT+) samples, or an equal volume (1 μl) of DEPC-treated water for reverse transcription negative (RT-) samples. Samples were placed in a thermocycler machine and incubated for 5 minutes at a temperature of 25 °C, followed by 50 °C for 50 minutes and 70 °C for 15 minutes. Following reverse transcription samples were stored at -20 °C until further use. Calculation of approximate cDNA concentrations following reverse transcription were made based on the amount of RNA added and assumed a complete 100 % conversion of RNA to cDNA.

Primer design for polymerase chain reactions:

Primers were designed against target gene sequences using either Invitrogen’s OligoPerfect™ Designer or Primer3 online software systems, by targeting desired segments of the reference gene sequences, or using the National Center for Biotechnology Information PrimerBLAST (Basic Local Alignment Search Tool) after this was released. Gene sequences were accessed from the National Center for Biotechnology Information online Genbank database, where available, or otherwise from the Ensembl Genome Browser. Primers were checked for possible amplification of non-target genes by the use of the online BLAST or PrimerBLAST tools from the National Center for Biotechnology Information, and checked for the probability of primer dimers using the online software Netprimer.

Polymerase chain reaction (PCR):

Polymerase chain reaction was carried out using either 1.1 × or 2 × Reddymix™ PCR Master Mix (Thermo Scientific, USA, product numbers AB-0575/LD/A and AB-0575/DC/LD/A respectively)
which incorporates loading buffers and dyes to enable direct loading of PCR products onto electrophoresis gels. Reactions were carried out in either 25 μl or 50 μl total reaction volumes, which incorporated: the appropriate amount of Reddymix as per manufacturer’s instructions; cDNA, added with the aim of achieving a concentration of 1-4 ng of cDNA per μl of reaction mix up to a maximum of 4 μl/50 μl reaction (or 2 μl/25 μl reaction), on the assumptions that all RNA as measured by spectrophotometer (above) was converted to cDNA during reverse transcription, and that no cDNA was subsequently degraded. Forward and reverse primers were added at 20 pmol each, with the exception of amplification of rat IL-18r1 using the primers of Andre et al. when 50 pmol of primers was added (as per reported methodology of Andre et al. ). Primers and target sequences of desired transcripts are given later in each chapter. Reactions were carried out in appropriate thermocycler 200 μl tubes (either Abgene product number ABGAB-0337 from Thermo Scientific, USA or Axygen, USA, product number PCR-02D-C) and using any of three thermocycler machines:

- Minicycler™, MJ Research Inc, Watertown, MA, USA, Model PTC-150HB
- Biometra TProfessional Basic, Biometra, Germany, order number 070-701
- Corbett Research Thermocycler, Corbett Research, USA, Model CG1-96

Unless stated otherwise, PCR settings were an initial denaturing step of 3 minutes at 94 °C, followed by 35 cycles of a denaturing step of 94 °C for 45 s, an annealing step at 60 °C for 45 s, and an extension step of 72 °C for 60 s, followed by a final extension step of 72 °C for 5 minutes.

**Nested and semi-nested PCR:**

Nest and semi-nested PCR reactions were carried out as above, with first round PCR products diluted 1:200 in DEPC-treated water, and a 1 μl volume of the resulting diluted PCR product used per 50 μl total reaction as the cDNA template in the second round of amplification.

### 6.3 Gel electrophoresis: visualization of PCR products

Products of polymerase chain reactions were loaded onto 1.5 % agarose gels, prepared with Ultrapure™ agarose (Invitrogen, SKU #16500500) and either Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE) buffers, and ethidium bromide at a concentration of 0.3 μg per ml of buffer. Samples were run with a 100 bp DNA ladder (Invitrogen, SKU #15628-019), at a fixed voltage ranging from 70 V to 120 V depending on the size of the gel used, and for a variable length of time until sufficient separation of DNA bands was observed with an ultraviolet gel viewer. Gels were viewed on a viewing platform lit with ultraviolet bulbs, and photographed with a camera with Polaroid film 667 (Polaroid Corporation UK), or using a digital camera connected to a computer.
Polaroid photographs were scanned into digital images for archiving and use in the current thesis document. Images were colour inverted to give black bands on a white background for clearer print reproduction. For some gel images, brightness and contrast were adjusted to give clearer definition of bands, but no other digital manipulation of images was performed.

6.4 Product sequencing: verification of amplification of desired gene products

Products were sequenced either at the Allan Wilson Centre for Molecular Ecology and Evolution (AWCMEE, Massey University, New Zealand) or the Genetic Analysis Services at the Department of Anatomy, University of Otago, New Zealand. PCR products were first purified by column purification, using QIAquick® Column Extraction kit (Cat No 28104, QIAGEN) according to manufacturer’s instructions.

6.5 In situ hybridisation methods

Preparation of tissue sections for in situ hybridisation procedure:

Whole brains were collected from animals following decapitation and stored at -80 °C until use. Sections through rat or mouse brain were cut in a cryostat set between -20 °C and -16 °C at a thickness of 30 μm in either coronal or sagittal orientations. Sections of liver and spleen collected from the same animals were used as positive control tissues, and placed on cryostat chucks at an orientation which provided a reasonable ease of mounting and sectioning. Sections were collected onto either Superfrost®Plus microscope slides (Biolab New Zealand, Cat No. LBS4951) or ATS-coated glass slides prepared in house. Slides were stored at -80 °C until used in in situ hybridisation procedures.

Generation of hybridisation probe cDNA templates:

RT-PCR was used to amplify targeted transcripts using the primers shown in Table 8, using cDNA reverse transcribed from total RNA extracted from tissues of the respective species. PCR products were sequenced to ensure amplification of the desired sequences. New primers were then made with either ‘SP6’ (AATTAGGTTGACACTATAAATAG) or ‘T7’ (TAATACGACTCAGCTAAGGGAGA) promoter nucleotide sequences added to the original primers, both placed at the 5’ end of ‘forward’ and ‘reverse’ primers, respectively, in order to generate anti-sense probes from a T7 promoter, and sense probes from an SP6 promoter. Templates for use in in situ experiments were made by performing PCR amplification using these primers with either RT product or diluted PCR product from previous
Table 8: Primer sequences for use in generating *in situ* hybridisation probes for use in rat and mouse tissues

<table>
<thead>
<tr>
<th>Gene product of interest</th>
<th>Primer</th>
<th>Primer sequence</th>
<th>Designed to bind †</th>
<th>Expected probe length ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rat:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-18</td>
<td>F</td>
<td>TGGCTGTGACCCCTATCTGTG</td>
<td>NM_019165.1, binding nt 287 - 543</td>
<td>256 bp</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>ATCCCAATTTCATCCATCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mouse:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-18 rap</td>
<td>F</td>
<td>ACTGCTCCATTCATTGTCG</td>
<td>NM_010553.2, binding nt 941 - 1195</td>
<td>254 bp</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CAGAATTTCCGGCTTCAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-18r1</td>
<td>F</td>
<td>TGATATCCAGGCCATGTCT</td>
<td>NM_008365.1, binding nt 1331 - 1461</td>
<td>130 bp</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CCGCTATGCGCCTATAGAAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F - forward primer; R - reverse primer

† reference codes are for the NCBI Genbank database 771,776

‡ Excludes SP6 and T7 promoter nucleotide sequences
reactions which utilised the original primers without SP6/T7 promoter extensions as a reaction template. Subsequently, DNA templates were sequenced at the Allan Wilson Centre for Molecular Ecology and Evolution or Genetic Analysis Services, Department of Anatomy to ensure appropriate amplification of desired gene sequences prior to use in \textit{in situ} hybridisation experiments.

\textbf{Generation of hybridisation probes:}

Radiolabelled hybridisation probes were generated using a Promega Riboprobe\textsuperscript{®} Combination System SP6/T7 RNA Polymerase kit (Promega, USA, product number P1460) and Easytides\textsuperscript{®} S\textsuperscript{35}-labelled UTP (Perkin Elmer, Boston, MA, USA, product number NEG739H001MC). Probes were made by drying 9 μl of Easytides S\textsuperscript{35}-labelled UTP in a 1.5 mL screw top centrifuge tube (Axygen Scientific, USA, product number SCT-150-C-S) in a vacuum centrifuge (ThermoSavant DNA120 SpeedVac\textsuperscript{®}) until very little solution remained. The volume of S\textsuperscript{35}-labelled UTP was adjusted as deemed necessary depending on the age of the stock and how far through its half-life the S\textsuperscript{35}-labelled UTP had progressed, using 9 μl for a fresh stock and greater amounts for older radiolabelled UTP supplies. Subsequently 1 μl of 100 nmol of DTT, 1.25 nmol each of ATP, GTP and CTP nucleotides, approximately 200 ng of cDNA hybridisation template, and DEPC-treated water were added to give a final volume of 8.5 μl. Tubes were vortexed to resuspend radiolabelled UTP nucleotides and then 20 U of RNaseIn Ribonuclease inhibitor and 10 - 20 U of T7 or SP6 RNA polymerase added. Tubes were incubated for one and a half hours at 37 °C in a dry bath, after which RQ1 DNase was added to a concentration of 1 U per microgram of template cDNA and tubes incubated for a further 15 minutes. Reactions were terminated with the addition of 1 μl of 0.2 M EDTA. Samples were diluted to 50 μl total volume with TE buffer and duplicate measures of 1 μl of solution set aside to determine the total radioactivity present.

Unincorporated nucleotides were removed using Roche mini Quick Spin columns (Roche Applied Science, catalogue number 11 814 427 001) according to manufacturer’s instructions and duplicate 1 μl measures removed for measuring of incorporated radioactivity.

Radioactivity was measured using 4 mL of scintillation fluid (Biodegradable Counting Scintillant, Product code NBCS104, Amersham) in 5 mL scintillation tubes, and counted using a 1450 microbeta Trilux system (Wallac) running Microbeta Windows Workstation software. Probes were used for subsequent \textit{in situ} hybridisation if they showed at least 25 % incorporation of radiolabelled UTP.
Following removal of unincorporated nucleotides, samples were made up to 100 μl with TE buffer and 5 μl removed for visualising the probe on an electrophoresis gel. To the remaining solution 10 μl of 3 M sodium acetate was added, followed by 250 μl of cold 100 % Analar grade ethanol. Tubes were then placed in a -80 °C freezer for at least 30 minutes prior to use.

**Gel electrophoresis of radiolabelled probes:**

Radiolabelled probes were run on an RNA-denaturing 1.5 % agarose gel. Prior to adding probe samples to the gel wells, to the 5 μl of the transcription solution (removed in steps above), 15 μl of sample buffer was added, containing deionised formamide, formaldehyde and MOPS buffer and heated for 10 minutes at 65 °C. Samples were then loaded with loading buffer and run with a DNA ladder to facilitate evaluation of band size. Following electrophoresis, gels were photographed under UV light and then loaded into light-proof cassettes (Hypercassette™, Amersham Life Science, UK) and autoradiograph film (Kodak Medical X-ray film, supplied by Radiographic Supplies Ltd, Christchurch, NZ) loaded into the cassettes in a dark room. Cassettes were left at 4 °C overnight and developed the following day. Autoradiographs were developed as described below and lined up against photos of the electrophoresis gel to estimate the size of bands visible on the autoradiograph film, in order to verify that they were in accordance with the expected length of RNA probes.

*In situ* hybridisation procedure:

Slides were thawed at 55 °C for 5 minutes in an oven and loaded into slide racks. Racks were placed in 4 % paraformaldehyde solution and left for 20 minutes in a fridge at 4 °C. Slides were washed in 0.5 × SSC buffer for 5 minutes and placed into RNase buffer containing 2 μg/ml of proteinase K for 10 minutes for membrane permeabilisation, after which slides were washed again in 0.5 × SSC buffer for 10 minutes with agitation. Slides were then placed in a 1 × TEA solution for 3 minutes with agitation followed by 1 × TEA solution with 0.25 % acetic anhydride for 10 minutes with agitation to decrease non-specific binding. Slides were subsequently washed twice in 2 × SSC solution for 2 minutes per wash. Slides were dried using Kimwipe tissues (Kimberly Clark Professional, NSW) and placed elevated on Perspex bars in an air-tight box which was kept humid by lining the bottom of the box with paper towels which had been moistened with buffer containing SSC buffer, formamide and DEPC-treated water. Sections were covered with a hybridisation buffer solution containing DTT, NaCl, Tris buffer, EDTA, Denhardt’s solution, dextran sulphate and formamide. Slides were then incubated in an oven at 42 °C for 3 hours for pre-hybridisation. Afterwards, 20 μl of a hybridisation mix containing the radiolabelled probe (2 μl), tRNA (1 μl) and buffer (17 μl) as listed
above was added to each section. Slides were covered with glass coverslips and incubated at 55 °C overnight for hybridisation of radiolabelled probes to target mRNA.

Following hybridisation, slides were loaded into slide racks and washed twice in 2 × SSC solution containing 10 mM β-mercaptoethanol and 1 mM EDTA for 10 minutes each, and then placed in RNase A solution containing 20 μg/l of RNase A for 30 minutes to remove any remaining unbound RNA. Slides were again washed twice in 2 × SSC solution containing 10 mM β-mercaptoethanol and 1 mM EDTA for 10 minutes each, and slide racks were then placed in a 1 L container of 0.1 × SSC solution and incubated for an hour at 55 °C as a high stringency wash, after which the incubation solution was replaced with fresh 0.1 × SSC solution and left for a further hour. Slides were washed twice in 0.5 × SSC for 10 minutes each.

Visualisation of binding using autoradiographs:

Following the in situ hybridisation procedure, sections were dried by submersion in a graded alcohol series by placing slides for 3 minutes each in solutions of 50 % ethanol with 0.3 M NH₄, 70 % ethanol with 0.3 M NH₄, 95 % ethanol, and 100 % ethanol. Any remaining alcohol solution was dried off by placing slides on a bench in front of a fan. Slides were placed into light-proof cassettes and radiographic film (Kodak Medical X-ray film, supplied by Radiographic Supplies Ltd, Christchurch, NZ) placed over slides in a dark room. Cassettes were shut and additionally wrapped in black polyurethane if deemed necessary to inhibit the leakage of light onto radiographic film. Cassettes were placed in a 4 °C fridge and left to expose for 1 - 3 weeks prior to developing.

Developing autoradiographs:

Films were developed in a dark room using either an automated developer machine (Ecomax X-ray film processor, Protec Medical Systems, Oberstenfeld, Germany), or manually by immersion for 2 minutes in developer (Kodak D19 developer, Eastman Kodak, USA), rinsing for 30 seconds in running cold water, and 1 minute in fixative (Ilford Hypam fixer, Ilford Photo, England). Films were scanned into digital images for archiving and inclusion in this thesis document.

6.6 Immunohistochemistry

Sample preparation:

Adult male Sprague-Dawley rats were anaesthetized by interperitoneal injection of sodium pentobarbitone (Pentobarb 300, Provet NZ Ltd, Auckland, NZ) combined with sodium heparin
(Multiparin, CP Pharmaceuticals Ltd, UK) to aid in blood letting during perfusion. Rats were perfused by transcardiac perfusion, initially with sterile saline containing 500 IU/L of heparin, followed by 4 % paraformaldehyde. Brains were removed, along with spleens for use as a positive control, and were post-fixed in 4 % paraformaldehyde overnight at 4 °C and then transferred to sucrose infiltration solution. After 24 hours infiltration solution was refreshed and tissues left until samples had sunk (typically another 24 to 48 hours), and were then either sectioned immediately or transferred to a -80 °C freezer until sectioning. Tissues were sectioned by microtome, with the brains of three animals cut into different planes: coronal, sagittal or coronal oblique. The coronal oblique plane was oriented with the ventral portion of the brain more posterior than the dorsal, with the aim of sectioning along the length of the fasciculus retroflexus, as previous studies had shown IL-18-positive fibres running within this fasciculus \(^777\). The approximate plane of sectioning is shown in Figure 5. Brains were cut into 30 µm sections in the coronal and coronal oblique planes, and 40 µm sections in the sagittal plane. Spleen sections used as positive controls in IL-18 immunohistochemistry were cut into 30 µm sections. All sections were placed in cryoprotectant solution and stored at -20 °C until processing for immunohistochemistry. Cryoprotectant solution consisted of 0.05 M phosphate buffer with sodium chloride at physiological concentration, 30 % ethylene glycol, 30 % weight by volume sucrose, and 1 % weight by volume polyvinylpyrrolidone.

**Immunohistochemistry procedure:**

Tris-buffered saline (TBS) with 0.1 % triton was used as the wash buffer throughout, and immunohistochemistry was performed with free-floating sections in incubation wells. Sections were washed three times for 10 minutes each in wash buffer to remove cryoprotectant, and then placed in distilled water with 30 % hydrogen peroxidase and 1 % sodium hydroxide for 20 minutes to deplete endogenous peroxidase activity. Sections were washed for 5 minutes in wash buffer and then endogenous biotin blocked using a method adapted from Wood and Warnke \(^778\) to reduce labelling of endogenous biotin in the rat brain \(^779\) by incubation in 0.01 % avidin solution (Invitrogen, catalogue number 43-4401) followed by 0.001 % biotin solution (Invitrogen, catalogue number B-1595) for 15 minutes each, followed by 5 minute washes. For IL-18 immunohistochemistry described in Chapter 7, a mild antigen retrieval step was used to enhance staining in fasciculus retroflexus fibres by incubation in 0.03 % SDS (sodium dodecyl sulphate) for 10 minutes followed by a 5 minute wash in wash buffer. Non-specific antibody binding was blocked by incubating sections in 4 % serum from the appropriate species in TBS for 1 to 2 hours at room temperature. Since some of the secondary antibodies used exhibit cross-reactivity with bovine IgG, the use of bovine serum albumin was
avoided in all experiments. Primary and secondary antibodies, concentrations, and controls are as described in each chapter. Following primary antibody incubation, sections were washed three times for 10 minutes each in wash buffer and then incubated in biotinylated secondary antibody (as indicated). Sections were developed using an avidin-biotin complex (ABC)/3,3’-diaminobenzidine (DAB) reaction, both performed according to manufacturer’s instructions (both from Vectorlabs: VECTASTAIN ABC standard kit, PK-4000; DAB Peroxidase Substrate Kit, SK-4100). Nickel was added to the DAB reaction to produce a black precipitate. Following DAB development, sections were refrigerated in TBS until mounting on slides. Glass gelatin-coated slides were used for mounting sections, which were then passed through an alcohol dehydrating series, incubated in xylene and coverslipped in DPX (dibutyl phthalate xylene) microscopy medium.

Images of sections included here were obtained by photographing with a digital camera attached to an Olympus AX70 light microscope. Each image was white-balanced against a blank section of the slide. Due to uneven background levels across different sections and magnifications, images were later processed through Adobe Photoshop CS5, adjusting the input levels for each image until background portions of each image appeared white.
Chapter 7: Brain-derived IL-18 as a mediator of the metabolic effects of IL-18

7.1 Overview

One potential explanation for both the phenotype of IL-18 knockout animals, and observations of intracerebroventricular IL-18 administration reducing appetite reported by Netea et al. and Zorrilla et al., is that there is production of IL-18 within the brain which acts to modulate appetite.

A few studies have shown increases in IL-18 in the brain in various models of brain injury, where IL-18 seems to fulfill a role typically expected of pro-inflammatory cytokines, which are often upregulated following an acute injury. In other studies examining constitutive presence of IL-18 under more physiological conditions, IL-18 has been reported in a number of brain areas: the first study to clone rat IL-18 cDNA reported its expression in the cerebellum, hippocampus, hypothalamus, striatum and cortex; other works have shown amplification of IL-18 mRNA by PCR in various brain regions, such as the hypothalamus, amygdala, pituitary, and cerebellum. Studies using cell cultures have shown expression of IL-18 in microglia and astrocytes, which has also been observed in vivo, and IL-18 knockout mice have been shown to exhibit reduced microglial activation following a neurotoxic insult or acute short-term stress, demonstrating a role for IL-18 in microglial function. A study by Prinz and Hanisch shows reducing brain IL-18 content with age in mice with little evidence of brain IL-18 in adult mice, which is in agreement with data from the Allen Brain Atlas showing no detectable expression of the IL-18 gene in the adult mouse. On the other hand, the Eurexpress project, which examines gene expression across the entire developing embryo, shows no evidence of IL-18 brain expression at 14.5 days post-conception. Of particular importance to the current thesis is one previous report from 2002 in which Sugama et al. conducted in situ hybridisation and immunohistochemistry for IL-18 in the Sprague-Dawley rat. They reported that IL-18 is produced within the medial habenula and ependymal cells lining the 3rd ventricle. Although the reproduction of immunohistochemistry

- 109 -
images in that paper is somewhat poor, they present evidence from dark field images of immunohistochemistry experiments showing that IL-18 is transported down fibres within the fasciculus retroflexus, a major fibre tract which connects the habenula (medial and lateral), stria terminalis, interpeduncular nucleus and ventral tegmental area and forms part of the dorsal diencephalic conduction system. This was the first paper to describe such a selective distribution of IL-18 in the brain, and no other studies have been published since describing similar findings.

Given the conflicting evidence presented by the above studies, we sought to characterize the expression of IL-18 mRNA and its translation into protein in the adult rat, through in situ hybridisation with radioactive probes and immunohistochemistry, respectively, in order to examine whether central expression of IL-18 could play a role in the phenotype of IL-18 knockout animals and associations of IL-18 with metabolic diseases.

7.2 Methods

7.2.1 In situ hybridisation

In situ hybridisation for expression of the IL-18 gene was performed in adult male Sprague-Dawley rat tissue, with methods and probes as described in Methods section, Chapter 6, Section 6.3. Radiographic film was left to develop for 2 weeks.

7.2.2 Immunohistochemistry

Immunohistochemistry was performed as described in the General Laboratory Methods, Chapter 6. Anti-IL-18 primary antibody (Santa Cruz, sc-6179) was added to 4 % normal horse serum diluted in TBS with 0.1 % triton at a ratio of 1:1000 and incubated at 4 °C for approximately 36 hours. Secondary antibody (Vectorlabs horse anti-goat IgG, BA-9500) was diluted 1:1000 in blocking solution and incubated for one hour at room temperature. Sections from the same brains were run through three negative control procedures at the same time, where either: the primary antibody was omitted, the primary antibody was replaced with non-immune IgG from the same species (goat IgG, Vector Labs, I-5000), or the primary antibody was pre-incubated during the blocking step in 500 µl of TBS to which a 5-fold concentration of the immunizing antigen was added (pre-absorption control; antigen from Santa Cruz, sc-6179P).
7.2.3 IL-18 and IL-18s RT-PCR

Appendix 2, Section 2.1 describes the design and testing of primers to amplify the full-length IL-18 and IL-18s transcripts. Primers were designed to bind to either the 57 bp segment present only in the full-length IL-18 transcript, or to span the nucleotides either side of this segment to amplify the IL-18s transcript, shown in Appendix 2, Table 1. Primers were tested as shown in Appendix 2 on spleen and adrenal tissue. Micropunches of the habenula were made as described in General Laboratory Methods, Chapter 6; images of sections before and after punching the habenula nuclei are shown in Appendix 3, Figures 1 and 2. RNA extraction and reverse transcription were performed as detailed in Chapter 6, and PCR reactions carried out as described in Appendix 2, Section 2.1.

7.3 In situ hybridisation to examine IL-18 mRNA expression

Autoradiographs from in situ hybridisation experiments are shown in Figure 6. No signal was noted in the liver, which had previously proved a good positive control during PCR amplification of IL-18 mRNA (not shown). Brain sections showed no signal in sections incubated with sense probes, and a specific strong signal in the habenula with anti-sense probes, as had been reported previously by others. A lighter signal was also observed more anteriorly in the brain in a region within the triangular septal nucleus.
Figure 6: In situ hybridisation for expression of the IL-18 gene in the rat brain. Left and middle panels show autoradiograph images of sections incubated with antisense and sense probes, as indicated. To the right are shown Nissl-stained coronal sections of the rat brain with features of interest labelled (adapted from http://brainmaps.org). Note focal expression is seen in the medial habenula but is absent in other brain areas, with the exception of some evidence of radiolabelling in the triangular septal nucleus.
7.4 Immunohistochemistry to examine the distribution of IL-18 protein

Immunohistochemistry experiments for IL-18 produced strongly specific staining in the habenula, principally in the dorsal region of the medial habenula bordering the dorsal 3rd ventricle, but some IL-18 positive cells were also present more laterally in the medial habenula (Figure 7, Panels A - D). Sections where the primary antibody had been pre-absorbed with a 5-fold excess of antigen showed light background staining as well as brown staining along the fibres of the stria terminalis, which runs along the dorsal edge of the habenula (Panel E); this non-specific signal was also present in sections incubated with primary antibody, where a similar brown pattern of staining is visible superior to the darker stained IL-18 positive cell bodies (Panels A and B). Sections through the triangular septal nucleus, which gave in situ hybridisation results suggestive of IL-18 expression (above, Figure 6), showed no signs of positive staining (not shown). Examination of the distribution of IL-18 positive cells in sagittal (Figure 8) and coronal (Figure 7) planes reveals that anteriorly, IL-18 positive cells begin to appear interspersed with afferent fibres from the stria terminalis. Cell numbers increase more posteriorly in the habenula as the stria terminalis fibres terminate, with the heaviest immunolabelling found in the dorsal medial habenula. Throughout the habenula, IL-18 positive cells are concentrated around the most medial aspect of the habenula, lining the dorsal 3rd ventricle.

Fibres within the fasciculus retroflexus also stained positive for the presence of IL-18. These could be seen in coronal (Figure 7), and sagittal planes (Figure 8). The fasciculus retroflexus projects to the interpeduncular nucleus, and postively-stained fibres could be seen throughout the fasciculus, terminating in a densely stained projection field in the interpeduncular nucleus (Figure 8, Panel C).

Staining of ependymal cells was noted (Figure 9), which was also observed by Sugama et al. when conducting immunohistochemistry for IL-18 in the Sprague-Dawley rat. The positive staining observed during incubation with anti-IL-18 primary antibody was, however, also observed when sections were incubated with anti-IL-18 antibody which had been preabsorbed with a 5-fold excess of antigen (Figure 9, Panel B); the intensity of staining was lighter in these negative control sections, but still present. Given that staining of ependymal cells was visible with antibody preabsorption, and that the preabsorption procedure used successfully blocked antibody binding to the more numerous habenula IL-18-containing cells (Figure 8, Panel E), the staining seen in ependymal cells with anti-IL-18 antibody appears non-specific.
Figure 7 (following pages): Immunohistochemistry for IL-18 in the rat brain showing coronal sections focused on the habenula and surrounding brain regions. A - D show sections incubated with primary antibody demonstrating IL-18 immunostaining anteriorly (A) through posteriorly (D) in the medial habenula. E shows a section at the level of that shown in Panel B where primary antibody was pre-absorbed with antigen prior to incubation. Clear presence of IL-18 immunoreactive cells are seen in the medial habenula, principally in the dorsomedial portion. Fibres exiting the medial habenula can be seen (as indicated by arrows) joining the emerging fasciculus retroflexus more ventrolaterally. Scale bars = 500 µm.
Figure 8 (following page): Sagittal view of IL-18 staining in the rat habenula. **A:** IL-18 positive cells in the medial habenula are interspersed among afferent stria terminalis fibres. **B:** More laterally, IL-18-containing fibres can be seen in the emerging fasciculus retroflexus. **C:** Terminating fasciculus retroflexus fibres entering the interpeduncular nucleus, which stains densely for IL-18. Scale bars = 500 µm.
Figure 9: Staining of ependymal cells surrounding the 3rd ventricle during immunohistochemistry for IL-18. Ependymal cells stain positively when incubated with anti-IL-18 antibody (A), but also with preabsorbed antibody (B). Scale bars = 200 µm.
7.5 Examining expression of an IL-18 splice variant in the rat habenula

Previous studies have reported the existence of a truncated IL-18 splice variant, IL-18s in the rat and mouse. In the study showing its existence in the mouse, expression in brain homogenates was seen by RT-PCR. In the study by Conti et al., which discovered the IL-18s transcript in the rat, the existence of the IL-18s transcript was detected in the adrenal gland by RT-PCR. In that study, expression of IL-18 mRNA in the adrenal gland was also observed to increase after cold stress, or treatment with the antihypertensive reserpine. Similarly, Sugama et al. have also shown in mice that immersion and immobilisation stress causes an upregulation of IL-18 mRNA and protein in the adrenal gland, and adrenalectomy experiments showed that the adrenal gland was the source of stress-induced elevated circulating IL-18. In a separate study, Sugama et al. also showed that restraint stress in rats induces an upregulation of IL-18 in the habenula. Given the similar upregulation of IL-18 transcription and translation in the adrenal gland and habenula in response to stress, the presence of an IL-18 splice variant reported in the adrenal gland, and detected in brain homogenates in the mouse, experiments were undertaken to determine if this splice variant is similarly present in the rat habenula. Previous in situ hybridisation and immunohistochemistry experiments reported above had shown the habenula to be the only site of brain IL-18 production.

Results of RT-PCR experiments utilising habenula samples are shown in Figure 10. As observed previously with adrenal RT samples (Appendix 2, Figure 2), the primer combination designed to amplify the IL-18s transcript also showed amplification of the full length IL-18 transcript. The IL-18s transcript was present spleen, included as a positive control, and the three habenula micropunch samples showed the presence of the IL-18s transcript, which was however much lighter in samples 2 and 3 than sample 1.

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"only site of brain IL-18 production" – these experiments show that the overwhelming amount of radioactive signal from IL-18 mRNA in situ hybridisation experiments arises from the habenula, and likewise immunohistochemistry experiments show specific staining for the IL-18 protein in the habenula. These findings do not necessarily definitively rule out IL-18 production in other areas, particularly for example during other disease conditions, as IL-18 production in other areas has been observed during ischaemic brain injury. They do, however, show that in a typical healthy male Sprague-Dawley rat the habenula is the only substantial source of IL-18 production.

- 120 -
Figure 10: RT-PCR for IL-18 splice variants in the rat habenula. Primer combinations were used, as indicated, to amplify IL-18 and IL-18s transcripts in habenula micropunches. Numbers above lanes represent samples from different animals. spl - spleen (control); H20 - water only sample without RT product (negative control, in RT- panel only). RT+ samples are shown on the image above and RT- samples below as indicated; RT+ and RT- samples were run on the same gel. Expected product sizes - IL-18: 352 bp, IL-18s: 182 bp (239 bp for amplification of full-length IL-18), full IL-18 only: 231 bp.
7.6 Discussion:

Results from both the *in situ* hybridisation and immunohistochemistry experiments included here revealed an intense focus of IL-18 expression in the brain in the medial habenula. Some signal for the presence of IL-18 mRNA in the triangular septal nucleus was seen in the *in situ* hybridisation experiment (Figure 6). This, however, was not seen at the protein level in immunohistochemistry experiments, although given only a few sections collected at the level of the triangular septal nucleus were included in the experiment, the expression of IL-18 protein in this nucleus cannot be ruled out from these results. While IL-18 mRNA had previously been amplified from liver samples during primer design and testing, liver sections included as positive control sections in the IL-18 *in situ* hybridisation experiments showed no discernable signal of IL-18 expression. There could be a number of reasons for this observations, such as that there were problems with the liver sections included as a positive control in this assay, that liver samples previously used during RT-PCR were from an animal exposed to an unrecognized inflammatory stimulus, or that the *in situ* hybridisation sensitivity was too low to detect liver IL-18 mRNA expression.

Other works in the literature have also detected IL-18 expression by qPCR and transcriptome sequencing in brain regions other than the habenula, such as the hypothalamus and medial prefrontal cortex (Gregg *et al.*). One must bear in mind that PCR is a powerful technique allowing amplification of even very low levels of gene expression, and tissue samples collected in that study, and here for RT-PCR, used fresh tissue samples and thus also contain mRNA present in circulating blood-borne cells such as lymphocytes. Additionally, in the work of Gregg *et al.*, IL-18 expression is reported in the medial prefrontal cortex and hypothalamus based on qPCR and Illumina and Sequenom transcriptome sequencing experiments. However, while the data are presented as reads (Illumina and Sequenom transcriptome sequencing) and IL-18 transcript levels normalised to GAPDH expression (qPCR), the relative amounts compared to other transcripts (such as the control transcript GAPDH) is not clear and it is entirely possible from those experiments that the actual levels of IL-18 detected were low. This certainly appears to be the case from experience with a pilot RT-PCR conducted as part of this thesis (described below in Chapter 8, Section 8.1). In these hypothalamic micropunch samples, while IL-18 was detectable, the resulting bands visible in gel electrophoresis of PCR products were very faint. Brain tissue used here for *in situ* hybridisation was also collected fresh, and in sections of this tissue the overwhelming bulk of radioactive hybridisation signal was seen in the habenula, with very little signal present in other brain areas (with the
exception of some sections showing a positive signal in the triangular septal nucleus). In addition, the results of both \textit{in situ} hybridisation and immunohistochemistry experiments here present a consistent finding of IL-18 expression almost exclusively in the medial habenula. Together, these findings suggest that while it is possible to detect IL-18 transcripts in other brain regions, the only sizeable source of IL-18 in the brain of a healthy rat is the habenula. Other studies examining disease states have shown IL-18 expression in the brain in other regions, with Hedtjärn \textit{et al.}, for example, showing clear presence of IL-18 by immunohistochemistry in other brain regions following hypoxic-ischaemic brain injury \cite{Hedtjärn2008}. In that study IL-18 appears to be playing a role regarded as typical for pro-inflammatory cytokines – that of being upregulated by injury or infection – whilst the strong constitutive expression present in the habenula in healthy rodents shown here appears to be fulfilling an entirely distinct function.

The above experiments also show that the habenula expresses the IL-18s splice variant. The role this splice variant plays in IL-18 signalling is largely unknown, and indeed the only study which examines the functional role of this variant is a report by Yang \textit{et al.} who examine its ability to induce IFN-γ in mouse splenocytes \cite{Yang2006}, showing that IL-18s appears to amplify the production of IFN-γ induced by IL-18, however only does so at low IL-18 levels.

The implications of, and further questions which arise from, these findings and how they relate to the hypothesis of brain-derived IL-18 playing a role in the previously observed metabolic effects of IL-18 are discussed further in the final discussion for Section 2, Chapter 10, which also examines previous research into the function of the medial habenula.
Chapter 8: Central expression of IL-18 receptors

8.1 Overview

At the time of beginning this thesis there was little information available regarding the central distribution of the IL-18 receptor. A few studies had reported amplification of transcripts of the IL-18 receptor subunits in the rodent brain, showing expression across a number of brain regions including the cortex, striatum, hippocampus and hypothalamus. One of the hypotheses proposed for the associations of IL-18 with metabolic disorders and its effect on appetite in rodents was that circulating IL-18 could act in a neuroendocrine manner by activating pro-inflammatory signals within the hypothalamus.

Experiments were therefore planned to examine the distribution of the IL-18 receptor subunits, IL-18r1 and IL-18rap, in the rodent brain by in situ hybridisation, as (i) this had not been published previously, and (ii) in situ hybridisation images would enable visualisation of specific regions of expression in the hypothalamus, which could guide further experiments examining expression in distinct cell types such as POMC, NPY, or melanocortin-receptor expressing neurons which are largely demarcated into different hypothalamic nuclei.
8.2 Methods

8.2.1 Pilot examination of the expression of components of the IL-18 signalling system in the rat hypothalamus

Primers were designed to amplify the IL-18, IL-18 BP, IL-18r1, IL-18rap, and Myd88 transcripts in the rat, as shown in Table 9. Primers for the detection of IL-18 spanned a 57 bp segment missing in the IL-18s transcript described above in Chapter 7, and thus could theoretically amplify both transcripts. Design and testing of primers for the amplification of IL-18r1 and IL-18rap splice variants are described in Appendix 2, Sections 2.2 and 2.3, respectively.

8.2.2 In situ hybridisation

In situ hybridisation for IL-18r1 and IL-18rap was performed as described in the General Laboratory Methods chapter, Chapter 6, Section 6.5. Probes for the detection of IL-18r1 in the mouse were designed to cross the portion of the IL-18r1 mRNA transcript which encodes the transmembrane region of IL-18r1 protein, upstream of the site of divergence of a reported splice variant (IL-18r1 type II) with a truncated intracellular portion, and would therefore be expected to bind mRNA transcripts for both splice variants. Probes for the detection of IL-18rap in mice spanned a region downstream of a reported IL-18rap splice variant which includes an unspliced intron, and would also be expected to detect both transcripts. Hybridisation experiments were performed on brain tissue from adult male C57BL/6 mice, with spleen sections included as a positive control.

8.2.3 Examination of IL-18 receptor splice variants across various nuclei of the rat brain

Primers were designed to amplify splice variants of the IL-18r1 and IL-18rap transcripts, using the primers and PCR conditions described in Tables 10 and 11. Primer positions are depicted in schematic diagrams in Figures 11 and 12. Prior to conducting the experiment, methodology was established to amplify different splice variants of the IL-18r1 and IL-18rap transcripts in the rat, particularly as the IL-18r1 type II transcript had not been described previously in the rat, and is described in Appendix 2. Following primer testing, micropunches of brain nuclei and spleen tissue samples were collected and processed through Trizol-based RNA extraction as described in the Methods section, Chapter 6, Section 6.2. Appendix 3, Figures 1 through 4 show images of micropunch collection from the various nuclei.
Table 9: Primer sequences for amplification of IL-18 system transcripts in rat hypothalamic micropunch samples, shown in Figure 13.

<table>
<thead>
<tr>
<th>Gene product of interest</th>
<th>Primer</th>
<th>Primer sequence</th>
<th>Designed to bind †</th>
<th>Expected product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-18</td>
<td>F</td>
<td>TGGCTGTGACCCTATCTGTG</td>
<td>NM_019165, binding 287 – 306</td>
<td>256 bp</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>ATCCCCATTTTCATCCTTCC</td>
<td>NM_019165, binding 524 – 543</td>
<td></td>
</tr>
<tr>
<td>IL-18 BP</td>
<td>F</td>
<td>GCTCCTTCATTGAGCACCTC</td>
<td>NM_053374.1, binding 306 – 325</td>
<td>189 bp</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TGGGCCAGAATGACATGATA</td>
<td>NM_053374.1, binding 476 – 495</td>
<td></td>
</tr>
<tr>
<td>IL-18r1</td>
<td>F</td>
<td>CCAACGAAGAAGCCACAGACA</td>
<td>NM_001106905.2, binding 1269 – 1289</td>
<td>259 bp</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CTCAGGATGACACTCTCTCAG</td>
<td>NM_001106905.2, binding 1508 – 1528</td>
<td></td>
</tr>
<tr>
<td>IL-18 rap</td>
<td>F</td>
<td>ACGTCCAGTGGTACATGCAA</td>
<td>NM_184047, binding 245 – 264 / ENRSRNOT00000020260, binding 245 – 264</td>
<td>454 bp</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GACGGTCCAGGAAGTCACAT</td>
<td>ENRSRNOT00000020260, binding 680 – 699</td>
<td></td>
</tr>
<tr>
<td>Myd88</td>
<td>F</td>
<td>GAGATCCCGAGTTTGGAGAC</td>
<td>NM_198130.1, binding 260 – 279</td>
<td>191 bp</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CTGTTTCTGCTGGTGCGTA</td>
<td>NM_198130.1, binding 432 – 451</td>
<td></td>
</tr>
</tbody>
</table>

F – forward primer

R – reverse primer

† reference codes are for either NCBI Genbank database (for sequences beginning in 'NM') \( ^{771} \) or Ensembl gene database (for sequences beginning in 'EN') \( ^{772} \)
Table 10: Primers used for amplifying IL-18 receptor transcripts in brain micropunches.

<table>
<thead>
<tr>
<th>Transcript(s)</th>
<th>Primer pair</th>
<th>Primer sequences</th>
<th>Primers designed to bind</th>
<th>Expected product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total IL-18r1</td>
<td>Forward A</td>
<td>ATCCTAGCTATGGAGAGCTG</td>
<td>ENSRNOT00000020194, 804 - 823; IL18r1 reference sequence within exon 4</td>
<td>196 bp</td>
</tr>
<tr>
<td></td>
<td>Total IL18r1 reverse</td>
<td>ACTGGTCCCCTTCAATAACCGT</td>
<td>ENSRNOT00000020194, 980 - 1000; IL18r1 reference sequence crossing exons 5-6.</td>
<td></td>
</tr>
<tr>
<td>Total IL-18r1 Primers from Wheeler et al.</td>
<td>Forward</td>
<td>TGCATGAAGACAGGAACGAG</td>
<td>ENSRNOT00000020194, 1149 - 1168; IL18r1 reference sequence within exon 7</td>
<td>237 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTGCCACAGAGGTGAGAACA</td>
<td>ENSRNOT00000020194, 1366 - 1385; IL18r1 reference sequence within exon 9</td>
<td></td>
</tr>
<tr>
<td>IL-18r1 type I</td>
<td>Forward C</td>
<td>CCAACGAAGAAGCCACAGACA</td>
<td>ENSRNOT00000020194, 1269 - 1289; IL18r1 reference sequence within exon 8</td>
<td>360 bp</td>
</tr>
<tr>
<td></td>
<td>IL18r1 type I reverse</td>
<td>CAGGCACCACATCTCTTTCA</td>
<td>ENSRNOT00000020194, 1609 - 1628; IL18r1 reference sequence within exon 10</td>
<td></td>
</tr>
<tr>
<td>IL-18r1 type II, 1st round PCR</td>
<td>Forward B</td>
<td>GCAAACTGACATGCTTCAAAAA</td>
<td>1188 - 1192/1194 - 1207; IL18r1 reference sequence within exon 8*</td>
<td>544 bp</td>
</tr>
<tr>
<td></td>
<td>IL18r1 type II reverse</td>
<td>AGCACGGGACATGTGAGGAGA</td>
<td>ENSRNOG00000000152027, 19589 - 19609; IL18r1 reference sequence, intron between exons 9 and 10.</td>
<td></td>
</tr>
<tr>
<td>IL-18r1 type II, 2nd round PCR §</td>
<td>Forward C</td>
<td>CCAACGAAGAAGCCACAGACA</td>
<td>ENSRNOT00000020194, 1269 - 1289; IL18r1 reference sequence within exon 8</td>
<td>463 bp</td>
</tr>
<tr>
<td></td>
<td>IL18r1 type II reverse</td>
<td>AGCACGGGACATGTGAGGAGA</td>
<td>ENSRNOG00000000152027, 19589 - 19609; IL18r1 reference sequence, intron between exons 9 and 10.</td>
<td></td>
</tr>
<tr>
<td>Table 10 cont:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>IL-18rap transcripts</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-18rap</strong>&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>sIL-18rap</strong>&lt;sup&gt;§&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Positive control transcript</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-actin</strong>&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

<sup>*</sup> Primer was originally designed against mouse IL-18r1 reference sequence and thus contains a one base pair mismatch, underlined in the primer sequence.

<sup>†</sup> Primer pair can theoretically also amplify sIL-18rap transcript which includes a transcribed intron between the forward and reverse primer. The sIL18rap transcript amplified would be 543 bp, although in practice this transcript is not observed and is revealed in a second round of semi-nested PCR.

<sup>§</sup> Semi-nested PCRs. For detection of IL-18r1 type II, the PCR product from the 1<sup>st</sup> round primer combination was diluted and used as the template for amplification with the 2<sup>nd</sup> round primer pair. Likewise for IL-18rap, the PCR product for IL-18rap was diluted DEPC-treated water and this used as the template for a second round of semi-nested PCR utilising the primer pair stated.
Table 1: PCR settings for amplifying IL-18 and receptor transcripts from brain micropunches

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Initial Denaturing step</th>
<th>Cycles</th>
<th>Denaturing</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>94˚C for 3 min</td>
<td>35</td>
<td>94˚C for 30 s</td>
<td>58˚C for 30 s</td>
<td>72˚C for 45 s</td>
<td>72˚C for 5 min</td>
</tr>
<tr>
<td>Total IL18r1</td>
<td>94˚C for 3 min</td>
<td>35</td>
<td>94˚C for 45 s</td>
<td>54˚C for 45 s</td>
<td>72˚C for 60 s</td>
<td>72˚C for 5 min</td>
</tr>
<tr>
<td>Total IL18r1, Wheeler et al. primers</td>
<td>94˚C for 3 min</td>
<td>35</td>
<td>94˚C for 45 s</td>
<td>57˚C for 45 s</td>
<td>72˚C for 60 s</td>
<td>72˚C for 5 min</td>
</tr>
<tr>
<td>IL18r1 type I</td>
<td>94˚C for 3 min</td>
<td>35</td>
<td>94˚C for 45 s</td>
<td>55˚C for 45 s</td>
<td>72˚C for 60 s</td>
<td>72˚C for 5 min</td>
</tr>
<tr>
<td>IL18r1 type II 1st round</td>
<td>94˚C for 3 min</td>
<td>35</td>
<td>94˚C for 45 s</td>
<td>55˚C for 45 s</td>
<td>72˚C for 60 s</td>
<td>72˚C for 5 min</td>
</tr>
<tr>
<td>IL18r1 type II 2nd round *</td>
<td>95˚C for 3 min</td>
<td>35</td>
<td>95˚C for 30 s</td>
<td>62˚C for 30 s**</td>
<td>72˚C for 45 s</td>
<td>72˚C for 5 min</td>
</tr>
<tr>
<td>IL-18rap</td>
<td>94˚C for 3 min</td>
<td>35</td>
<td>94˚C for 45 s</td>
<td>54˚C for 45 s</td>
<td>72˚C for 60 s</td>
<td>72˚C for 5 min</td>
</tr>
<tr>
<td>sIL-18rap †</td>
<td>94˚C for 3 min</td>
<td>35</td>
<td>94˚C for 30 s</td>
<td>57˚C for 30 s</td>
<td>72˚C for 45 s</td>
<td>72˚C for 5 min</td>
</tr>
</tbody>
</table>

* using as template the diluted product from IL18r1 type IIa first round, diluted 1:200 with DEPC-treated water.

** touchdown step dropping 0.2 ˚C per cycle

† using as template the diluted product from IL18rap, diluted 1:200 with DEPC-treated water.
Figure 11: Schematic diagram of positions of primers used to amplify IL-18r1 splice variants. Depicted is the IL-18r1 type I reference transcript. Exons are depicted as boxes, with open boxes representing untranslated regions. Introns are shown as lines. Primer positions are indicated, with forward primers above the sequence and reverse primers below. Primers correspond to those listed in Table 10. Also shown is the position of the intron insert present in IL-18r1 type II transcript. In the mouse, the type II transcript includes an intron sequence located between exons 9 and 10, but not the remaining downstream exons. Assuming the same occurs for the type II transcript in the rat, reverse primers placed downstream of this segment (such as ‘IL-18 type I reverse’) will not amplify the type II transcript. Primers upstream would be expected to amplify both.
Figure 12: Schematic diagram of positions of primers used to amplify IL-18rap splice variants. Depicted above is the IL-18rap reference transcript (ENSRNOT00000020260). Exons are depicted as boxes, introns as lines. Shown as a grey box is the intron which is inserted to form the sIL-18rap transcript. Primer positions are indicated, with forward primers above the sequence and reverse primers below. Primers correspond to those listed in Table 10. Also shown is the Genbank entry NM_184047.1 and the region this covers of the sIL-18rap transcript. The IL-18rap transcript was amplified by the primers indicated on the sequence above. For amplification of the sIL-18rap transcript, a semi-nested PCR was performed where the 2nd PCR used a primer within the intron insert, as shown on the sequence below.
8.3 Pilot examination of the expression of components of the IL-18 signalling system in the rat hypothalamus

Prior to conducting experiments involving \textit{in situ} hybridisation to examine receptor distribution in the hypothalamus, a preliminary pilot experiment was conducted to ensure that components of the IL-18 signalling system could be detected by RT-PCR. Hypothalamic micropunches were performed as described in the Methods section using brain tissue extracted from an adult male Sprague-Dawley rat. Primer combinations were used to amplify subunits of the IL-18 receptor (IL-18r1 and IL-18rap), as well as IL-18 itself, IL-18 BP (the natural antagonist of IL-18), and Myd88 which is involved in downstream signalling of the IL-18 receptor. Gel electrophoresis of PCR products is shown in Figure 13.

Very low levels of expression of IL-18 were observed in micropunches of the ventromedial and paraventricular hypothalamic nuclei. IL-18 BP expression was observed in all nuclei, as was the intracellular signalling component Myd88 (which is also involved in signal transduction from the interleukin-1 receptor). Expression of IL-18 receptor subunits IL-18r1 and IL-18rap was detected in micropunches of hypothalamic nuclei. The primer combination used to amplify IL-18r1 produced a band of the expected size in the RT- sample of the hypothalamus homogenate. A subsequent PCR using the same sample gave a clear RT- lane and band of the expected size in the RT+ lane (not shown), and it was concluded that the whole hypothalamus homogenate IL-18r1 RT- tube used here was contaminated in this PCR run only.

Although not a quantitative PCR experiment, approximately 200 ng of cDNA was added to each reaction tube and results suggest expression could be higher in the arcuate and ventromedial hypothalamic nuclei than in the paraventricular nucleus. It was noted that both of the primer combinations used to amplify IL-18r1 and IL-18rap produced clear bands in the whole hypothalamus and paraventricular nucleus samples, but extra bands in the arcuate and ventromedial hypothalamus samples. It is not known why this occurred, as if these bands were to represent, for example, additional splice variants or non-specific binding, it would be expected that this would also be present in the whole hypothalamus homogenates as these include both the arcuate and ventromedial hypothalamic nuclei. Regarding the additional bands present in the arcuate and ventromedial hypothalamus IL-18rap lanes, the sIL-18rap splice variant includes an unspliced intron and therefore would in fact be heavier than the product for IL-18rap, not lighter as is the case for the additional
bands present. Heavier bands present in the IL-18 BP and Myd88 samples are the expected size for amplification of the target DNA band, as these RT samples were not DNase treated.
Figure 13: Expression of IL-18 signalling components in rat hypothalamic micropunches. Amplified transcripts are indicated above gel segments. Samples are as follows: arc – arcuate nucleus; pvn – paraventricular nucleus; vmh – ventromedial hypothalamus; hyp – whole hypothalamus homogenate. Hypothalamic micropunch samples (arc, pvn, vmh) shown are RT+, run alongside whole hypothalamus RT+ (hyp +) and RT- (hyp -) control samples as indicated.

8.4 Examination of central expression of IL-18 receptor subunits using in situ hybridisation

Following the detection of mRNA encoding IL-18 receptor subunits in the RT-PCR pilot experiment above, in situ hybridisation was performed to examine the distribution of expression of the IL-18 receptor genes in the mouse brain.

8.4.1 Interleukin-18 receptor alpha subunit (IL-18Rα/IL-18r1)

Radiographic film was left opposed to sections for 16 days prior to development for hybridisation with IL-18r1 probes (Figure 14). In situ hybridisation for the interleukin-18 receptor alpha subunit produced clear pockets of staining in the spleen, which was observed with antisense but not sense probes. Brain sections, however, produced no specific staining in either coronal or sagittal sections.

8.4.2 Interleukin-18 receptor beta subunit (IL-18Rβ/IL-18rap)

In situ hybridisation using probes for IL-18rap resulted in little visible signal in an autoradiograph developed after one week of exposure. A subsequent film exposed for 25 days resulted in visible foci of staining in spleen sections (Figure 15), with a similar staining pattern in the spleen to that seen in in situ hybridisation experiments for mouse IL-18r1 (compare Figure 14), with pockets of expression visible. However, no specific staining was seen in brain sections, as was the case in in situ hybridisation experiments for IL-18r1.
**Figure 14:** In situ hybridisation for IL-18r1 expression in the mouse brain. Sections were incubated with sense and antisense probes for the IL-18r1 transcript and left exposed for 16 days. Middle row shows coronal (top) or sagittal (middle row) brain and spleen (bottom row) sections incubated with antisense probes. Corresponding positions in the mouse brain are shown to the left with Nissl-stained coronal sections (adapted from http://brainmaps.org). Right hand column shows sagittal brain and spleen sections incubated with sense probes.

**Figure 15:** In situ hybridisation for IL-18rap expression in the mouse brain. Middle and right hand columns show mouse brain and spleen sections incubated with anti-sense probe for IL-18rap and left exposed for 25 days against radiographic film. Left-hand column shows corresponding Nissl-stained coronal and sagittal sections with major features indicated (adapted from http://brainmaps.org). Note the spleen section included as a positive control, which shows foci of expression corresponding to the white pulp.
8.5 Examination of expression of IL-18 receptor splice variants by RT-PCR in rat brain micropunches

The results from the in situ hybridisation experiments presented above in Section 8.4, suggest that in fact there is very little or no detectable IL-18 receptor expression in the brain. Given these experiments were preceded by a pilot study examining IL-18 receptor expression by RT-PCR (Section 8.3), which did demonstrate amplification of IL-18 receptor transcripts, these results presented seemingly contradicting results. A further experiment was planned to repeat that pilot experiment in more animals with a revised experimental plan. Different brain nuclei were chosen to reflect the differing hypotheses of possible IL-18 involvement in eating behaviour; hypothalamic micropunches were collected through the arcuate, ventromedial and paraventricular hypothalamic nuclei, on the hypothesis that circulating IL-18 could influence signalling in these areas. Given that habenula-derived projections were shown to terminate in the interpeduncular nucleus, micropunches through the interpeduncular nucleus and neighbouring ventral tegmental area were made. In addition, additional primer sets and amplification strategies were designed to amplify different portions of the IL-18r1 and IL-18rap mRNA transcripts, including primers to specifically amplify different splice variants for IL-18r1 (IL-18r1 type I and type II described in the mouse in \(^{804}\), and in the rat as described in Appendix 2) and IL-18rap (IL-18rap and sIL-18rap, described in the rat in \(^{775}\)).

Photographs of agarose gels following electrophoresis for each PCR reaction are collated in Figures 16 and 17. It was apparent that bands of the expected product size were present in RT- samples for β-actin, as well as the presence of products of expected size in some RT- samples for amplification of IL-18r1, showing the presence of contamination. Bands were also present in RT- samples during semi-nested PCR for the amplification of IL-18r1 type II, however, these were not of the expected product size (approx 180 bp vs. 463 bp) and thus likely represent non-specific amplification of unknown sequences; similar non-specific bands were also present in some RT+ samples.
Figure 16 (following pages): RT-PCR for the detection of IL-18r1 transcripts in rat brain micropunches.
Various primer combinations were used, as indicated, to amplify IL-18r1 transcripts or β-actin. arc - arcuate nucleus; pvn - paraventricular nucleus; vmh - ventromedial hypothalamus; ipn - interpeduncular nucleus; vta - ventral tegmental area; spl - spleen (control); H20 - water only sample without RT product (negative control).
Panel A - RT+ samples; Panel B - RT- samples. The darker band present toward the centre of the 100 bp ladder is 600 bp.
A

Total IL-18r1: 196 bp

Total IL-18r1: 237 bp
Wheeler et al.

IL-18r1 type I: 360 bp

IL-18r1 type II:
1st round PCR, 544 bp

IL-18r1 type II:
2nd round PCR, 463 bp

β-actin: 337 bp
Figure 17 (following pages): RT-PCR for the detection of IL-18rap transcripts in rat brain micropunches. Primer combinations were used, as indicated, to amplify IL-18rap, sIL-18rap transcripts or β-actin. arc - arcuate nucleus; pvn - paraventricular nucleus; vmh - ventromedial hypothalamus; ipn - interpeduncular nucleus; vta - ventral tegmental area; spl - spleen (control); H2O - water only sample without RT product (negative control).

Panel A - RT+ samples; Panel B - RT- samples.
<table>
<thead>
<tr>
<th>100 bp</th>
<th>Rat 1</th>
<th>Rat 2</th>
<th>Rat 3</th>
</tr>
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<tr>
<td>arc</td>
<td>pvn</td>
<td>vmh</td>
<td>ipn</td>
</tr>
<tr>
<td>IL18rap: 454 bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sIL18rap: 386 bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd round PCR</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>β-actin: 337 bp</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
8.6 Discussion:

Despite an initial pilot experiment showing amplification of IL-18 receptor subunits in the rat hypothalamus, subsequent \textit{in situ} hybridisation in the mouse brain showed no signals suggesting central expression of the genes which encode the IL-18 receptor. A further RT-PCR experiment which was conducted across a wider range of brain regions showed a low level of expression across all brain regions sampled. The results of the RT-PCR experiments here are reminiscent of those published by Andre \textit{et al.} who published RT-PCR experiments which resulted in bands of similar intensity across samples from different regions of the rat brain \cite{Andre2003}. In those experiments, and those performed here, interpretation of the relative amounts of each transcript type and across different brain areas is complicated by the fact that the amplifications performed were not done by qPCR; thus, different intensities of bands of different transcripts could represent differing amplification efficiencies of the various primer combinations. Likewise, differing intensities of bands across different brain regions could reflect differing amounts of starting material (added cDNA per reaction tube); the intensity of \(\beta\)-actin amplification products from samples from Rat 3 appear lighter than those from Rats 1 and 2 in the latter experiment included here, and could therefore support the interpretation that less RNA was reverse transcribed from these samples.

Bearing these limitations in mind, and notwithstanding the limitations imposed by contamination of RT- samples, these experiments and previously published works \cite{Andre2003,Alboni2004} generally show that IL-18r1 and IL-18rap transcripts can be amplified from samples of various brain regions in the mouse or rat brain. Regarding the expression of the IL-18r1 type II splice variant, this was only detectable in brain micropunches by a second round of semi-nested PCR in the present study. In contrast, Alboni \textit{et al.} found that this transcript could be reliably amplified by a single round PCR strategy in the mouse \cite{Alboni2004}. Whether these differences relate to a species difference between the mouse and rat, or differences in amplification efficiencies of the primers used, is unknown. From the results here it could be concluded that (i) given semi-nested PCR is an incredibly powerful technique able to amplify minute levels of gene expression, and (ii) positive bands are only seen in approximately half of the micropunch samples, with no particular clear pattern across different brain regions, it appears that this variant is probably not expressed at sufficient levels to be physiologically relevant in the rat brain, at least under the physiological conditions of the animals used here.
The IL-18rap transcript, which encodes the receptor subunit necessary for signal transduction, was also detectable, albeit produced bands of much lesser intensity than that seen in the positive control tissue of spleen. Three out of three arcuate nucleus samples showed IL-18rap expression, as did three of three interpeduncular nucleus samples. Other brain regions showed less consistent results, with only one ventral tegmental area micropunch showing evidence of expression. Given that in general many bands produced on electrophoresis were faint, and the inherent weakness noted above of comparing transcripts across different samples, it is difficult to determine from these results if these findings could translate into differences in receptor activity levels across the brain regions sampled. The most notable finding that is clear is that there is no central expression of the sIL-18rap transcript. These results were also replicated with another semi-nested PCR strategy utilising different primers, which also failed to detect any expression across brain micropunch samples, despite strong positive control results in the spleen (data not shown). Thus, these results suggest that in the rat this splice variant is not expressed in the brain. The original study to report the existence of the sIL-18rap splice variant detected its expression in various brain regions by RT-PCR, and similarly another study in mice reports the presence of murine sIL-18rap in the brain using RT-PCR, and the equivalent human splice variant has been reported to be expressed in the brain, and the results here are therefore not in accordance with other reports in the literature across various species.

The expression of the IL-18rap transcript in the brain has been shown to be upregulated on exposure to LPS in two studies conducted in mice, also using RT-PCR. One of these also examined the expression of the murine sIL-18rap splice variant and found that this was also upregulated after LPS exposure, but that the ratio of expression of the sIL-18rap splice variant to the normal full length IL-18rap transcript was not changed. One possible explanation to reconcile the results here with other works showing sIL-18rap expression in the brain in rats and mice is the presence of inflammatory stimuli; expression levels of IL-18rap in the brain appear to be regulated by inflammatory stimuli, and the rats used in the present study were housed in SPF (Specific Pathogen Free) conditions where even food is autoclaved prior to consumption. Animals studied here may therefore exhibit a lack of sIL-18rap expression due to a lower level of inflammation than the animals used in other works by Andre et al. and Alboni et al. However, whether this could account for the lack of sIL-18rap expression seen here is unknown, as the normal IL-18rap transcript was readily detectable in brain samples, and even if sIL-18rap were expressed in relatively lower amounts than the normal IL-18rap transcript (which was observed to be the case in the two other reports by Andre et al. and Alboni et al.), the semi-nested PCR technique here would be more...
than capable of amplifying even minute levels of sIL-18rap expression. Thus, the reason for the discrepancy between the results of this experiment and other reports in the literature are not easily reconciled.

Regarding the results of in situ hybridisation studies shown here, at the outset of this thesis there were no published results of in situ hybridisation experiments conducted to detect the expression of the genes encoding the IL-18 receptor in the brain. The experiments shown above in Section 8.4 showed no signal for expression of IL-18 receptor subunits in the mouse brain, despite positive signals being detected in the spleen. During the course of conducting this thesis, the results of similar experiments have been published by others, which include: (i) the Allen Brain Atlas 807, which shows little or no central expression of IL18r1 (probe number 75831726) nor IL18rap (probe number 71529941) in the mouse brain; (ii) studies from Bruno Conti’s laboratory, which examine the central expression of IL18r1 804 and IL18rap 805 in the mouse using both in situ hybridisation and immunohistochemistry techniques, suggesting a relatively low-level but ubiquitous expression throughout the brain; and (iii) the Eurexpress developmental transcriptome which shows no IL-18r1 808 nor IL-18rap 809 in the developing mouse embryo by in situ hybridisation 810. Each of these other studies have utilized digoxigenin (DIG) probes, which would be expected to have a lower sensitivity than the radioactive probes utilized in this study. On the one hand, the utility of radioactive probes is best observed once slides incubated with radioactive probes are dipped in photographic emulsion and left to develop for a number of weeks. In this study, slides incubated with radioactive probes were exposed to autoradiographs, which have a lower sensitivity than emulsion-dipped slides, although in the case of in situ hybridisation experiments for the expression of IL-18r1 and IL-18rap these were left to develop for 16 and 25 days respectively, longer than autoradiographs would often be left to develop, in order to increase the possibility of detecting a low radioactive signal. Thus, the methods used here could arguably be more sensitive than the above studies utilizing DIG probes. However, there is obvious discrepancy amongst these works, with the experiments presented here, the Allen Brain Atlas, and the Eurexpress project showing no evidence of expression of the genes encoding the IL-18 receptor by in situ hybridisation, and the works from Alboni et al. showing a ubiquitous, low-level expression 804,805.

Other papers have used RT-PCR to detect expression of receptor subunits in the brain (e.g. 775,803), as has also been performed here. The various results from these studies are not necessarily in concordance with each other. The issue of whether there is a ubiquitous low-level expression vs. none in the brain could be viewed as a question of sensitivity or specificity, particularly where a lack
of sensitivity could result in a negative result (no expression). Even the results presented here in this thesis are not necessarily concordant across different techniques – whereas we have been unable to detect any reliable signal of receptor expression by *in situ* hybridisation using sensitive radioactive probes, multiple RT-PCR experiments have amplified IL-18 receptor subunit transcripts from various brain regions, albeit producing bands of weak intensity. One explanation for the discrepancy between methodologies found here and reported in the wider literature could be that RT-PCR experiments detect a much lower level of transcript than can be visualized with *in situ* hybridisation such that RT-PCR experiments produce positive signals which are not observed by *in situ* hybridisation; this would certainly be expected based on the ability of PCR experiments to amplify even very small levels of mRNA transcripts.

One potential confounding factor which could go some way to explaining the results presented across different studies is the presence of IL-18 receptor mRNA in blood-borne cells. The experiments presented here were performed on brain micropunches collected from tissue which were collected immediately after decapitation, and not perfused with paraformaldehyde or saline. This method of tissue collection is often used for examining mRNA expression as tissue can be collected and frozen prior to degradation of RNA, and it avoids the use of drugs for deeply anaesthetizing animals which may influence gene expression in unknown ways. However, a potential downside from this method is the presence of blood within tissue samples. Following the initial report by Alboni *et al.* describing the IL-18r1 type II transcript in the mouse, the presence of this transcript was investigated in the human by the author by RT-PCR, using RNA purified from whole blood (unpublished results from the author), in which both IL-18r1 type I and II were amplified from whole blood RNA. Thus, these transcripts are likely to also be present in cells within the bloodstream in rodent species and this could account for their detection in RT-PCR experiments. Other studies, such as Andre *et al.* and Wheeler *et al.* report the amplification of IL-18 receptor subunit transcripts from various brain regions in the Sprague-Dawley rat following saline perfusion prior to RNA extraction. This experimental approach would decrease, although not entirely eliminate, the possibility of false positive results through amplification of transcripts present in blood-borne cell types, and it is possible that the expression reported in those studies could also arise from amplification of IL-18 receptor expression in blood-borne cells remaining in the bloodstream.

Since RT-PCR was performed here and not qPCR, it cannot be known whether, for example, the weak intensity bands produced are reflective of a low level of transcript, or of a poor amplification efficiency of the primer pairs used.
small capillary vessels. The residual presence of such cells following perfusion could also explain the relatively homogenous expression across different brain regions reported by Alboni et al. by *in situ* hybridisation \textsuperscript{804,805}. However, contrary to this argument are reports of receptor expression in cell cultures of neurons, microglia and astrocytes \textsuperscript{803}, in which such contamination would not be present.

Regardless of the above inconsistencies, one finding which emerges is that the results from these experiments and other published studies is that none of these works suggest a focus of IL-18 receptor in the interpeduncular nucleus. Given the clear results obtained here (Chapter 7) and others (Sugama \textit{et al.} \textsuperscript{777} and Aizawa \textit{et al.} \textsuperscript{811}) of IL-18 being present in the dorsomedial subnucleus of the medial habenula, and of IL-18 containing fibres projecting down the fasciculus retroflexus and terminating in the interpeduncular nucleus (Chapter 7 and Sugama \textit{et al.} \textsuperscript{777}), a receptor for IL-18 would be expected to be present at increased levels in this nucleus. In light of the inconsistency, it seemed possible that habenula-produced IL-18 could act via another, separate, receptor system. This led to the examination of possible unknown mediators of IL-18 signalling in the brain detailed in the following chapter.
Chapter 9: Examining unknown mediators of IL-18 signalling in the CNS

9.1 Overview

IL-18 and its receptor belong to the interleukin-1 family of cytokines, and this family is ever-evolving with additional ligands and receptors being discovered. A number of unanswered questions remain about how members of the family interact; for example, it has recently been elucidated that the IL-18 receptor itself, IL-18r1, is also used by IL-37 and can combine with a different accessory protein, Single IgG IL-1 Related Receptor (SIGIRR), in order to exert anti-inflammatory actions. IL-18 forms a relatively weak bond with the IL-18r1 receptor subunit, and binding to IL-18rap is required before a high affinity bond is formed. Curiously, IL-18BP, which acts as a natural circulating antagonist, forms a high affinity bond with IL-18 and yet bears little resemblance to the IL-18 receptor subunits. For example, while IL-18r1 (and most other ligand-binding members of the IL-1 receptor family) contain three immunoglobin (Ig)-like domains, IL-18BP contains only one, and nucleotide and protein sequences share little similarity with the interleukin-18 receptor subunits. Experiments utilizing IL-18 and IL-18r1 knockout animals in the study of autoimmune processes demonstrate that the two exhibit divergent phenotypes. These have been interpreted as evidence of another ligand for the IL-18 receptor (a possible explanation for these findings is the recent discovery, noted above, of IL-37 exerting anti-inflammatory actions via IL-18r1), although these findings could also signify an alternative receptor for IL-18. To what extent these results could be explained in hindsight by other reports suggesting that aspects of the IL-18r1 knockout mouse phenotype arise from other co-inherited genetic material is unclear.

Thus, given (i) the acknowledged incomplete understanding of the IL-1 cytokine family, (ii) prior evidence within the IL-1 cytokine family of multiple ligands acting on different members of the IL-1 family of receptors, (iii) the fact that IL-18 is known to bind to both the IL-18 receptor and IL-18BP, two disparate and seemingly unrelated proteins, (iv) the apparent lack of central expression of the IL-18 receptor despite strong central expression, and (v) the published behavioural effects of IL-
18 on appetite, the notion that IL-18 could bind a separate centrally-expressed receptor seemed plausible.

This chapter details bioinformatics approaches and immunohistochemistry experiments undertaken to investigate this hypothesis. Given the exploratory nature of this line of investigation, initial results from bioinformatics approaches led to further hypotheses which were examined in subsequent experiments. In order to preserve the logical flow from one experiment to the other, each of these experiments is described in a separate section, with the background, methodological detail, results, and discussion of results and available literature contained within each section.
9.2 Examining a putative central receptor for IL-18: BLAST searches for proteins related to IL-18r1 and IL-18 BP

The initial line of enquiry began by examining the possibility of another putative central receptor for IL-18 using BLAST searches of reference human, mouse and rat protein sequences.

9.2.1 Bioinformatics methodology

BLAST searches:

To examine the hypothesis that IL-18 could act via a different receptor, the extracellular sequence of IL-18r1 (which forms the primary site of ligand binding during IL-18 signal transduction) and the sequence of IL-18 BP were entered into BLAST searches for related proteins for the respective protein sequences of rat, mouse and human species. Searches were conducting using the blastp interface to mine the database of reference sequence proteins (refseq_protein) with searches limited to each respective organism. Pairwise alignment between selected sequences was performed using the 'blastp' query interface set to align two sequences.

9.2.2 BLAST search results

The ten results with the lowest E-values from BLAST searches for proteins related to the extracellular portion of human, rat and mouse IL-18r1 are shown in Table 12, and those related to IL-18BP of each species in Table 13. Searches for proteins related to IL-18r1 consistently produced similarities for various members of the IL-1 receptor family across the three species, including the IL-1 receptor (interleukin-1 receptor type I and type II, interleukin-1 receptor accessory protein), IL-33 receptor (interleukin-1 receptor-like 1 \(^{812}\)), interleukin-1 receptor-like 2 (which binds IL-1 family members IL-1F6 and IL-1F8 \(^{813}\)), and interleukin-1 receptor associated protein-like 1 (IL1RAPL1, until recently an orphan receptor \(^{818,819}\)).

Searches for proteins similar to IL-18 BP produced much more varied lists across species (Table 13). Indeed, only one protein was consistently within the first ten results for all three species, interleukin-1 receptor accessory protein-like 2 (IL1RAPL2).
Table 12: BLAST search results using amino acids encoding the extracellular portion of IL-18r1 as a query against reference protein sequence databases for rat, mouse and human species. The first ten results are listed for each species using the amino acid queries as listed. Searches were limited to proteins from each respective species. Numerous members of the IL-1 receptor family showed similarity for each species. Shown shaded is IL1RAPL1, which was examined further in the following experiments.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>Total score</th>
<th>Query coverage</th>
<th>E value</th>
</tr>
</thead>
<tbody>
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<td><strong>Rat: proteins similar to amino acids 1 – 326 of rat IL-18r1 (NP_001100375)</strong></td>
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<tr>
<td>NP_001100375.1</td>
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<td>85%</td>
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<td>NP_037255.3</td>
<td>interleukin-1 receptor type 1</td>
<td>63.5</td>
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<tr>
<td>NP_001161312.1</td>
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<td>NP_037100.1</td>
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<td>NP_808796.1</td>
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<td>NP_598259.1</td>
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<td>XP_001055013.2</td>
<td>PREDICTED: leucine-rich repeats and immunoglobulin-like domains 3</td>
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<td>interleukin-1 receptor accessory protein isoform d precursor</td>
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**Human: proteins similar to amino acids 1 – 330 of human IL-18r1 (NP_003846.1)**

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<td>interleukin-1 receptor-like 2 precursor</td>
<td>54.3</td>
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Table 13: BLAST search results using IL-18BP protein sequence as a query against reference protein sequence databases for rat, mouse and human species. First ten results are listed for each species using the protein reference sequences as listed. Searches were limited to proteins from each respective species. Shown shaded is the only protein result common to the first ten results in all three species, IL1RAPL2.

<table>
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<tr>
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<th>Query coverage</th>
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<td>28.5</td>
<td>27%</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td>NP_001008846.1 RT1 class II, DO beta</td>
<td>28.1</td>
<td>52%</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>NP_062160.2 apolipoprotein B-100 precursor</td>
<td>28.5</td>
<td>31%</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>NP_958738.1 SH3 and multiple ankyrin repeat domains protein 2 isoform a</td>
<td>27.3</td>
<td>52%</td>
<td>0.068</td>
</tr>
<tr>
<td></td>
<td>NP_786939.1 immunoglobulin superfamily member 1 precursor</td>
<td>26.6</td>
<td>46%</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>NP_001102261.1 PHD finger protein 3</td>
<td>26.6</td>
<td>46%</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>NP_001100338.1 protein-cysteine N-palmitoyltransferase HHAT-like protein</td>
<td>26.6</td>
<td>37%</td>
<td>0.11</td>
</tr>
<tr>
<td><strong>Mouse: proteins similar to mouse IL-18 BP (NP_034661.1)</strong></td>
<td>NP_034661.1 interleukin-18-binding protein precursor</td>
<td>393</td>
<td>100%</td>
<td>1e-144</td>
</tr>
<tr>
<td></td>
<td>NP_034685.1 interleukin-1 receptor type 2 precursor</td>
<td>33.5</td>
<td>37%</td>
<td>3e-04</td>
</tr>
<tr>
<td></td>
<td>XP_923633.3 PREDICTED: tyrosine-protein phosphatase non-receptor type 1-like</td>
<td>33.1</td>
<td>39%</td>
<td>3e-04</td>
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<tr>
<td></td>
<td>NP_001002898.1 signal-regulatory protein beta 1</td>
<td>32.3</td>
<td>53%</td>
<td>7e-04</td>
</tr>
<tr>
<td></td>
<td>NP_001166931.1 signal-regulatory protein beta 1B</td>
<td>32.0</td>
<td>24%</td>
<td>8e-04</td>
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<tr>
<td>Gene Accession</td>
<td>Protein Description</td>
<td>Score</td>
<td>E-value</td>
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<td>NP_109613.1</td>
<td>X-linked interleukin-1 receptor accessory protein-like 2 precursor</td>
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<td>0.001</td>
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<td>NP_001166930.1</td>
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<td>NP_932138.1</td>
<td>SCY1-like protein 2</td>
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<td>0.035</td>
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<tr>
<td>NP_034519.2</td>
<td>histocompatibility 2, O region beta locus</td>
<td>26.9</td>
<td>0.038</td>
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<tr>
<td></td>
<td><strong>Human: proteins similar to human IL-18 BP (NP_766630.2)</strong></td>
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<td></td>
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<tr>
<td>NP_001034748.1</td>
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<td>341</td>
<td>2e-123</td>
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</tr>
<tr>
<td>NP_766632.2</td>
<td>interleukin-18-binding protein isoform d precursor</td>
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<td>4e-92</td>
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<td>interleukin-18-binding protein isoform b precursor</td>
<td>157</td>
<td>7e-52</td>
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<td>X-linked interleukin-1 receptor accessory protein-like 2</td>
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<td>XP_003119930.1</td>
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<td>PQ-loop repeat-containing protein 2 isoform 1</td>
<td>27.7</td>
<td>0.052</td>
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<td>NP_443137.2</td>
<td>formin-like protein 2</td>
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<td>0.066</td>
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<tr>
<td>NP_037421.2</td>
<td>killer cell immunoglobulin-like receptor 3DL1 precursor</td>
<td>27.3</td>
<td>0.077</td>
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<tr>
<td>NP_001077008.1</td>
<td>killer cell immunoglobulin-like receptor 3DS1 precursor</td>
<td>26.9</td>
<td>0.092</td>
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</tr>
</tbody>
</table>
Alignment of IL-18BP with IL1RAPL2 revealed a series of regions showing differing degrees of similarity spaced across different portions of the respective amino acid sequences (shown for rat sequences in Figure 18). Given the similarities between IL-18BP and IL1RAPL2 revealed by BLAST searches, IL-18r1 was aligned with the related protein IL1RAPL1, which also featured in lists of similar proteins from BLAST searches of the extracellular portion of IL-18r1. Alignment revealed a certain degree of similarity across almost the entire length of both proteins (shown in Figure 19 with rat sequences). Comparing rat sequences reveals 148 of 544 (27 %) of amino acids are identical, with a further 100 (18 %) exhibiting similar polarity (‘positives’). (Similar relationships are found with human and mouse species).

9.2.3 Discussion

The interleukin-1 family of ligands and receptors consists of a number of different members, all with varying degrees of similarity. The observed similarities between IL-18r1 and other IL-1 family members shown above was therefore expected, given the high degree of homology between family members, and the current understanding that these various family members appear to have arisen by gene duplication and deletion events. However, despite their similarity the IL-1 family members obviously exhibit a certain degree of specificity, such that IL-18, for example, does not activate the highly homologous IL-1 or IL-33 receptors. Therefore, similarity alone would not be sufficient to determine IL-18 binding to related family members. The similarity of IL-18 BP to IL1RAPL2, however, was not expected, and has not been reported previously.

IL1RAPL1 and IL1RAPL2 are highly homologous and are thought to have evolved by gene duplication. They are also interesting members of the IL-1 family by virtue of their chromosomal location; almost all members of the IL-1 receptor family reside within a cluster in the same autosomal region (chromosome 2 in humans, chromosome 1 in the mouse, and chromosome 9 in the rat; the interleukin-1 receptor accessory protein is the only exception, residing on a different autosomal chromosome in each species). The two genes for IL1RAPL1 and IL1RAPL2, however, both reside on the X chromosome in all three species. The mammalian X chromosome is enriched for genes involved in reproduction and brain function, and in accordance with this enrichment studies have identified IL1RAPL1 and IL1RAPL2 expression in the brain with little expression elsewhere. Given the bioinformatics searches conducted here were undertaken to identify a putative centrally expressed receptor for IL-18, the IL1RAPL proteins thus exhibit the required gross anatomical localisation necessary to fulfil this role. The IL1RAPL proteins were also regarded as orphan receptors until very recently, and at the time of conducting these investigations had no known ligands.
Figure 18: Alignment of rat IL-18BP (NP_445826.1) vs IL1RAPL2 (NP_001159814.1) proteins produces four regions of modest homology. Panels A - D: Schematic diagrams show the regions of similarity between the two proteins, followed by the aligned amino acid sequences for each of the four regions. The region of alignment for IL1RAPL2 shown in Panel C sits in the cytoplasmic domain, the remainder are extracellular.

A.

Segment 1: Identities = 26/90 (29%), Positives = 38/90 (42%), Gaps = 15/90 (17%)

IL-18BP 25 LARATSAPLTTATVLRSSKDCSSWSPAVPTKQYPTLDVIWPEKEVPLNGTLTLSCTACSRFP
     L + T+    TA +  + K  P  P +  P++  +V L  L + C A F
IL1RAPL2 222 LVRHTTELKVTALLTDKPPK--------PLFMENQPSV--------IDVQLGKPLNIPCKAFFGFS

IL-18BP 89 NFS--ILYLWGLNDSFIEHLPGRLREG 112
     S ++YW+    FIE L G +REG
IL1RAPL2 273 GESGPMIYYWMGEKFIEELAGHIREG 298

B.

Segment 2: Identities = 16/46 (35%), Positives = 21/46 (46%), Gaps = 6/46 (13%)

IL-18BP 17 LLYHVVLARATSAPLTTATVLRSSKDCSSWSPAVPTKQYPTL 62
     LL   VV  A +T+  ++  R+S D C WS  V K Y L
IL1RAPL2 5 LLLALVCSAVSTNLKMVS-----KRNSVDGCIDWS----VDLKYMAL 44

C.

Segment 3: Identities = 10/33 (30%), Positives = 15/33 (45%), Gaps = 1/33 (3%)

IL-18BP 52 PAVPTKQYPTLDVIWPEKEVPLNGTTLTLSCTAC 84
     P V K Y  + PE+++  +GT  T C
IL1RAPL2 432 PDVLEKHY-GYKLFIPERDLIPSGLTYIEDLTRC 463

D.

Segment 4: Identities = 4/12 (33%), Positives = 7/12 (58%), Gaps = 0/12 (0%)

IL-18BP 124 WLHRALVLEELS 135
     W+  +EEL+
IL1RAPL2 281 WMGEKFIEELA 292
**Figure 19**: Alignment of rat IL-18r1 (NP_001100375.1) vs IL1RAPL1 (NP_808796.1) proteins. Identities = 148/544 (27 %), Positives = 248/544 (46 %), Gaps = 58/544 (11 %).

<table>
<thead>
<tr>
<th>IL-18r1</th>
<th>23</th>
<th>IRRSQIHVVEGEFFYLKPCDSAPMHHNETA--------TMRFWKGANSHYRELNMRRRSSPFRAFHHALEFWPFVELEDKTGYFSQVGDND----RQNW 112</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I++ Q+ V GEF +K + N T ++ W+K + + E R++ ++ FF P L+D G Y + N ++</td>
</tr>
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<table>
<thead>
<tr>
<th>IL1RAPL1</th>
<th>38</th>
<th>IKKYQVLV--GEPVR1KCALFYGYIIRTNYTLAQASGLSLMWYKSSPGDFEEPIAFDSGSMKSEDSIWRPTLLQDGSGLYACVIRNSTYCMKVS 131</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>+L V + + C++ K + E + KS I+C + L YK C+ + P +++K+ + D G Y+C + + G +</td>
</tr>
</tbody>
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<table>
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<tr>
<th>IL-18r1</th>
<th>113</th>
<th>TLNVTKRKHSCFSEKLVTNRDVEVKSLWMITCENPSYGELINH---TLLYKNCIEISKTP----------MILKDAEFGDEGYSCFSDKHNGQQY 197</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>+L V + + C++ K + E + KS I+C + L YK C+ + P +++K+ + D G Y+C + + G +</td>
</tr>
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<table>
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<tr>
<th>IL1RAPL1</th>
<th>132</th>
<th>SLTVGENDTGLCYNSKMKYFKAELSKSKEISCRDEDFLIPQFTEPIWYKECRTKTRPSISFVRDTLLIKEVEKEDIGNYCTC--ELKYG---F 223</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>+L V + + C++ K + E + KS I+C + L YK C+ + P +++K+ + D G Y+C + + G +</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>IL-18r1</th>
<th>198</th>
<th>NITKVNITVGNSKIPAFGSKSAKVGE----LGEDVELCNASAVLNRLNDE-----LFYWSIRKE--DSLDNP-VHEDRNERTTWFEKGLHASKL 283</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+T +TV + P + +K++ LG L C A + L YW ++ + LD N V E G+ S L</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IL1RAPL1</th>
<th>224</th>
<th>VVRRTETLVTAPLTDKPKLPPMEKSTILQTETQGSSNLCTRAFFYGSDVPLIYWKKGAEFDLDENVWEDIRILEKHGQEVSISL 319</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+T +TV + P + +K++ LG L C A + L YW ++ + LD N V E G+ S L</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>IL-18r1</th>
<th>284</th>
<th>RIKVTEKYNLWVYNTVEANETATTDSKFILVRRKETQDHPGFMGTVMTLVSAVVCVILCIYKVDLVLFLYRVRSAQETLTDGKYDAFV 379</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+V E L Y+C V N S +L ++E + + E C V Y ++ YK+++L FYR +D K YDA++</td>
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<table>
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<th>IL1RAPL1</th>
<th>320</th>
<th>IVDSVEEGDLGNYSCYVENGNGRRHASVLLHKREL--------MITYVELAGGLGAILLLLVCSTIYWYKIEIMLFYRHNFGAEELDGDKNYDAYL 409</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+T +TV + P + +K++ LG L C A + L YW ++ + LD N V E G+ S L</td>
</tr>
</tbody>
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<table>
<thead>
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<th>IL-18r1</th>
<th>380</th>
<th>SYL-----ECOSHENGEYTFAVTTLPRLEQKQGKYLMCIFERDVFPFGAVGADVIEHSLIKESRLLIIVLSKSYMT--NGRRLELESGLHQAVERKI 465</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+ + + E GEE FA+E LP +LEK +GKY I +RD++F G ++++ +S+RLIIV++ +Y Y + ELE+ L LV +I</td>
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</tbody>
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<table>
<thead>
<tr>
<th>IL1RAPL1</th>
<th>410</th>
<th>SYTVKVDQFQWNQQTGETGEERFAELIPDMLEKHYKYLQFIRDLPITGTYEDVARCDQSKRLIYYTFNYVVRGSIFELETLRNMLVTGEI 505</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>+T +TV + P + +K++ LG L C A + L YW ++ + LD N V E G+ S L</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IL-18r1</th>
<th>466</th>
<th>KIILIEFTPSNAT-------FLPSLKLKSYSRVLWRKAKDPLSSKSFWRKNLLYLMMPAKVP 523</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>K+ILIE + I L ++KL V++W K S+FWK L Y MP K ++P</td>
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<table>
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<tr>
<th>IL1RAPL1</th>
<th>506</th>
<th>KVILIECSELKGGIMNYQGEVEALKHTIKLLT-----VIKWHGPKCNKLNSKFWKRLQYEMPFFKRIE 566</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+T +TV + P + +K++ LG L C A + L YW ++ + LD N V E G+ S L</td>
</tr>
</tbody>
</table>
9.3 Examining conservation of residues necessary for IL-18 binding in the IL-1 receptor family

The actions of a number of IL-1 family members are regulated by soluble versions of their respective receptors. IL-18, however, is unusual among cytokines in that its activity is regulated by a distinct circulating binding protein, which shows little resemblance to the IL-18 receptors other than the fact that they each contain immunoglobulin (Ig)-like domains. Given a role for IL-18 in the immune response and viral clearance, many forms of \textit{Poxviridae} have evolved a mechanism to increase their virulence by producing their own IL-18 binding proteins (including \textit{Variola virus}, the causative agent of smallpox 826), having possibly done so by horizontal gene transfer from their host species. Various studies have examined human or viral forms of IL-18 BP through mutation analysis and functional assays, and determined amino acid residues necessary for inhibition of IL-18 action 805,826–828. In order to examine whether IL1RAPL1 or IL1RAPL2 could form central receptors for IL-18, the similarity of each to various forms of IL-18BP was studied by multiple sequence alignment.

9.3.1 Bioinformatics methodology

Multiple protein sequence alignment: In order to examine the similarity of human and viral IL-18 BP protein sequences with those of the IL1RAPL1 and IL1RAPL2 proteins, multiple protein sequence alignment was undertaken using the MAFFT (Multiple Alignment using Fast Fourier Transform) alignment tool available online from the European Bioinformatics Institute 829. For comparison, other members of the IL-1 receptor family were included to assess the sequence similarity between other homologous proteins from the same receptor family which do not bind IL-18, in order to attempt to discriminate between residues common amongst IL-1 family members and residues common between proteins capable of binding IL-18 (human and viral IL-18BP, human IL-18r1). Results were fed into 'Mview', a multiple sequence alignment visualisation tool, also available from the European Bioinformatics Institute 830, in order to generate the included figures and assess amino acid consensus sequences. Consensus amino acid sequences generated by 'Mview' are based on those reported and used by Taylor 831 and Bork \textit{et al.} 832 and are shown for reference in Appendix 4, Table 1. Reference sequence and source codes for human and viral sequences are shown in Appendix 4, Tables 2a and 2b.
9.3.2 Results: IL1RAPL proteins share residues with IL-18BP not present in other IL-1 receptor family members

Comparison of IL-18 binding proteins in the multiple sequence alignment reveals that the studies by Kim et al. 605, Krumm et al. 827, Esteban et al. 833, and Xiang and Moss 828 identify many similar critical residues on human and viral IL-18 binding proteins (Figure 20, Panel A). Examining the similarity between sequences there are a number of conserved amino acids, and at the 70 % consensus level this group of proteins was observed to share either common individual residues or residues with similar physicochemical properties. IL1RAPL proteins can be seen to share a characteristic 'pFIEpL' sequence (where 'p' represents an amino acid with a positive charge) with a number of forms of IL-18BP. As shown underlined in the sequences of human and viral IL-18 binding proteins, residues in this region were identified by the above studies as forming one of the sites necessary for inhibition of IL-18.

Removing the other IL-1 receptor family members from the multiple sequence alignment (Figure 19, Panel B), it can be seen that the IL1RAPL proteins share a large number of residues at the 100 % consensus level which are either identical to, or possess similar physicochemical properties as, those present in human and viral forms of IL-18BP. Intriguingly, at the nucleotide level, the coding sequences of these proteins show that while they encode similar proteins, the IL1RAPL sequences share many nucleotides across the sequence encoding the 'pFIEpL' motif with the sequences of Ectromelia, Variola and Cowpox viruses, which form a conserved viral IL-18 binding protein domain cc, while the coding sequence for the MCV054L protein (forming a separate conserved domain dd) exhibits much more similarity with the coding sequence for human IL-18BP, such that these viruses appear to possess sequences forming two groups of 'human IL1RAPL-like' and 'human IL-18BP-like' nucleotide sequences across this 'pFIEpL' region (Figure 20, Panel C).

9.3.3 Discussion

The analyses conducted here show a number of conserved residues between human IL1RAPL proteins and both human and viral forms of IL-18 BP, which suggest that the former could act as receptors for IL-18. Importantly, the portion of the IL1RAPL proteins showing similarity to IL-18 binding proteins depicted in Figure 19 maps to the extracellular region of these receptors,


- 159 -
A nucleotide sequences added, highlighting the apparent existence of two nucleotide sequence groups.
specifically, the third Ig-like domain of both IL1RAPL1 and IL1RAPL2. Notably, the third Ig-like domain of IL-18r1 forms a key binding site for IL-18r1 binding of IL-18. The multiple sequence alignments above and by others suggest that both human and Poxviridae IL-18 binding proteins align exclusively to this third Ig-like domain.

If the hypothesis that IL1RAPL proteins form central receptors for IL-18 were correct, one would expect the following to also hold true: (i) that key residues necessary for IL-18 binding would be conserved throughout the process of evolution in the IL1RAPL proteins, as with IL-18 BP itself (provided that motifs in the ligand IL-18 were also conserved), and (ii) that IL1RAPL proteins would be found at sites of action of IL-18. Moreover, since it is already known that the IL1RAPL proteins are predominantly found in the brain, these should be located in the interpeduncular nucleus; the terminal field of IL-18 containing fibres projecting from the medial habenula.
9.4 Examining conservation of residues shared between IL1RAPL proteins and IL-18 BP

In order to assess the question (i) raised above, of conservation of key motifs throughout evolution, the protein sequences of IL-18BP, IL-18, IL1RAPL1, and IL1RAPL2 for a number of different species were aligned to assess the extent to which key residues identified as playing a role in IL-18BP or IL-18 action were conserved throughout mammalian evolution.

9.4.1 Bioinformatics methodology

Multiple protein sequence alignment:

Alignment of multiple protein sequences of IL1RAPL1, IL1RAPL2, IL-18 BP and IL-18 from various species was undertaken using the MAFFT and Mview alignment tools as described above. Selection of species was performed by examining the listings of *Homo sapiens* IL1RAPL1, IL-18 and IL-18BP in the Ensembl database to identify homologous genes in other species (listed under 'Gene tree' tab of Ensembl database), or searching the NCBI Genbank database for available protein sequences in non-human species. A total of 23 species were identified for which reliable protein sequences (i.e. not containing long ambiguous 'X' read segments) were available across the NCBI or Ensembl databases. Of these, only 20 had available sequences for IL1RAPL2. Reference sequence and source codes are shown in Appendix 4, Table 3.

9.4.2 Results

Residues shared between IL1RAPL proteins and IL-18 BP are highly conserved:

Multiple sequence alignment of IL1RAPL proteins from various species showed a high degree of conservation of almost the entire coding sequences of the IL1RAPL proteins (Appendix 4, Figure 1, Panels A and B). Interestingly, a number of the species sampled appear to have IL1RAPL proteins which contain only one extracellular Ig-like domain (as opposed to three in human, mouse, and rat species), and this one Ig-like domain corresponds to the third Ig-like domain found in humans which contains the conserved sequences shown in Figure 20 above. Sequences of both IL-18BP and IL-18 also showed a high degree of conservation across the species sampled (Appendix 4, Figure 1, Panels C and D, respectively).
Alignment of the 80 % consensus sequences across species for IL1RAPL1, IL1RAPL2, and IL-18BP is shown in Figure 21 for the region corresponding to the third Ig-like domain of human IL1RAPL proteins. It can be seen that a number of residues are shared between the consensus sequences, with two cysteine residues at either end of the third Ig-like domain which are believed to form a disulphide bridge and are necessary for IL-18 BP function \( ^{833} \), and various residues between these two, including the 'pFIEpL' motif identified earlier, an upstream 'YW' sequence, and downstream glutamic acid (E) which has been identified as a key residue for IL-18 binding or inhibition in *Ectromelia virus, Molluscum contagiosum virus* and human IL-18 binding proteins.

### 9.4.3 Discussion

It is now recognised that the three dimensional structure of folded proteins is arguably more important for evaluating protein function than amino acid residue sequences, as even proteins with low levels of amino acid similarity can show similar function and three-dimensional structure. In the case of the IL1RAPL proteins, however, consideration of the three-dimensional structure yields little further insight beyond that already known, as the portions of the proteins shown in Figures 20 and 21 are already recognised as forming Ig-like domains, a feature common to all members of the IL-1 receptor family, and also IL-18 BP. Thus, given IL1RAPL proteins are known to exhibit a similar three dimensional structure to other proteins capable of binding IL-18 (the IL-18 receptor subunits and IL-18BP), and that they share a number of key residues with IL-18BP, the anatomical location of the IL1RAPL proteins in the rat brain was investigated by immunohistochemistry in order to address question (ii) raised above, and examine if the IL1RAPL proteins are localised in the interpeduncular nucleus.
Figure 21: Manual alignment of multi-species consensus sequences for IL-18BP, IL1RAPL1 and IL1RAPL2. Below is shown key shared residues present across each protein in many species, and below the dividing line the sequences of *Ectromelia virus*, *Molluscum contagiosum virus* and human IL-18 binding proteins with key residues identified by Krumm *et al*. 827, Esteban *et al*. 833, Kim *et al*. 605 and Xiang and Moss 828, as being necessary for binding of IL-18.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Consensus</th>
<th>Shared Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1RAPL1</td>
<td>80%</td>
<td>N C A S Y W G F I E L E L N C</td>
</tr>
</tbody>
</table>
9.5 Immunohistochemistry for IL1RAPL proteins in the rat CNS

In order to assess whether the IL1RAPL proteins could form central receptors for IL-18, immunohistochemistry experiments were undertaken in the rat, as previous work identifying IL-18 projections from the habenula had been performed in this species.

9.5.1 Methodology: immunohistochemistry

Brains were collected from adult male Sprague-Dawley rats and processed in preparation for immunohistochemistry as described in the General Laboratory Methods chapter, Section 6.6. Given that fibres within the interpeduncular nucleus are largely oriented in a rostral-caudal direction, brains were sliced sagitally in 40 µm sections in order to cut through fibres running along the sagittal plane.

Immunohistochemistry for the detection of IL1RAPL1 and IL1RAPL2 was performed using goat polyclonal primary antibodies raised against human IL1RAPL proteins (IL1RAPL1: sc-161736, IL1RAPL2: sc-131569, Santa Cruz Biotechnology, USA), using the immunohistochemistry procedure described in General Laboratory Methods, Chapter 6, Section 6.6.

Immunohistochemistry for IL-18 was performed as described previously in Section 7.2.2. For IL1RAPL1 immunohistochemistry, sections were incubated overnight at 4 °C in a primary antibody solution containing either a 1:1000 or 1:2000 dilution of anti-IL1RAPL1 antibody in wash buffer with 5 % normal horse serum. The two antibody concentrations produced similar results and images from either concentration are included here. For IL1RAPL2 immunohistochemistry, sections were incubated overnight at 4 °C in a 1:2000 dilution of primary antibody. Secondary antibody (Santa Cruz horse anti-goat biotinylated secondary antibody, catalogue number BA-9500) was added at 1:2000 dilution and sections incubated at room temperature for one hour. Antigen pre-absorption of primary antibody was performed to test the specificity of staining, with sections processed in parallel with the addition of either primary antibody at 1:2000 dilution, or primary antibody which had been pre-absorbed by incubation in PBS with a 5-fold concentration of their respective blocking peptides (Santa Cruz Biotechnologies, USA, product numbers sc-161736P and sc-131569P) overnight at 4 °C. Sections were incubated in pre-absorbed primary antibody added to provide the same final concentration of primary antibody (1:2000) as used in positive control sections. Although the antibodies were raised against peptides from the human protein sequences, IL1RAPL1 and
IL1RAPL2 are highly homologous between human and rat species (as illustrated in Appendix 4, Figures 2 and 3), and given the high homology it was expected that both antibodies would therefore also be suitable for use on rat tissue. Although the precise antigen sequences are not given by the manufacturer, the antibodies were raised against peptides mapping within the C-terminal cytoplasmic domains of the respective proteins. The carboxy tail of the IL1RAPL1 and IL1RAPL2 receptors features a unique region of approximately 120 amino acids, which, although similar between these two proteins, shares little resemblance with any other known protein. Given that IL1RAPL1 and IL1RAPL2 are thought to have arisen via gene duplication and show a high degree of homology, it was feared that antibodies raised against one of these proteins could also produce positive staining for the other. However, despite their homology, the protein sequences of the carboxy tails of these two proteins are not greatly similar (Appendix 4, Figure 4), and the manufacturers claim no cross-reactivity of each antibody with the respective homologue proteins.

9.5.2 Results

Immunohistochemistry for IL1RAPL1 produced positive staining across a number of brain regions. In the interpeduncular nucleus, the region of interest in this study, staining corresponded well to the pattern of immunostaining of IL-18 terminal fibres entering the interpeduncular nucleus (Figure 22). At higher magnification, the positive immunostaining could be seen to label fibres and processes in the interpeduncular nucleus, with some staining of cell bodies (Figure 22, Panel C).

In other brain regions, positive immunostaining was also observed throughout the cerebral cortex and hippocampus (Figure 23), and in the molecular layer of the cerebellar cortex (Figure 24), where immunostaining was the most intense of the regions labelled, and was localised to Purkinje cell bodies and processes. Antigen pre-absorption of the primary antibody reduced, but did not entirely eliminate all of the observed immunostaining. No reliable staining could be seen in the forebrain or midbrain, however, some residual staining was observed in the cerebellar cortex (Figure 24), with Purkinje cell bodies and some processes visible.

Figure 22 (following page): Immunostaining for IL-18 (A), as shown in Figure 7, Panel C, and IL1RAPL1 (B and C) in sagittal sections through the interpeduncular nucleus. The boxed region in B is enlarged in C. Images are oriented similar to the schematic diagram depicted earlier in Figure 5 with the rostral portion of the brain to the right, and the caudal portion to the left of the image. Scale bars: A and B = 500 μm, C = 100 μm.
Figure 23: Immunostaining for IL1RAPL1 in the cortex (A and B) and hippocampus (C and D). Boxed regions in A and C are enlarged in B and D, respectively. Scale bars: A and C = 500 μm, B and D = 100 μm.
Figure 24: Immunostaining for IL1RAPL1 in the cerebellar cortex after incubation with primary antibody (A and B) or primary antibody following preabsorption with binding peptide (C and D). Boxed regions in A and C are enlarged in B and D, respectively. The intensity of staining in the cerebellum is reduced but not entirely eliminated by peptide preabsorption. Scale bars = 500 μm.
IL1RAPL2 immunohistochemistry produced no identifiable positive staining. Although some faint labelling of Purkinje cell bodies was observed in sections incubated with anti-IL1RAPL2 antibody, this was also present in sections incubated with pre-absorbed primary antibody (not shown).

9.5.3 Discussion

The immunohistochemistry results presented here suggest expression of IL1RAPL1 in the interpeduncular nucleus. Sections processed for immunostaining of IL-18 showed a marked correspondence of the terminal field of IL-18 fibres with IL1RAPL1 immunostaining. Although pre-absorption of primary antibody with its binding peptide did not entirely prevent immunostaining, a reduction in the intensity of immunostaining is generally viewed as indicative of a staining signal which reflects binding to the desired antigen sequence. Assessing whether immunostaining reflects a signal specific to the antigen of interest in the tissue is an area fraught with problems, and interpretation of immunostaining is best done by relying on a variety of methods \textsuperscript{838,839}. Ideally, a lack of immunostaining in tissue from a transgenic animal with a deletion of the gene encoding the protein of interest provides one of the best pieces of evidence in support of specific immunostaining. However, in neither the case of IL-18 immunohistochemistry (reported in Chapter 7), nor IL1RAPL1 immunohistochemistry reported here, did we have access to tissues from transgenic knockout rats devoid of the IL18 or IL1RAPL1 genes to test antibody specificity. Other methods, such as antigen preabsorption, can add to a body of evidence regarding antibody specificity but also have their shortcomings; non-specific staining is still possible with antigen preabsorption, as is specific staining when the binding of antibody to peptide used in preabsorption is reversible. Perhaps one of the best sources of evidence for antibody specificity is demonstrating mRNA expression in the same distribution as the observed immunostaining through \textit{in situ} hybridisation techniques. This, for example, was performed earlier in Chapter 7 and provides strong support for the specificity of IL-18 immunostaining reported in this thesis. In the case of IL1RAPL1, this has not been performed here due to time constraints. However, experiments have been reported by others examining IL1RAPL1 protein distribution by Western blot or mRNA expression by \textit{in situ} hybridisation, and these show a reasonable correspondence with the observed immunostaining (discussed further below in Section 9.7). Of particular importance is whether there is also IL1RAPL1 mRNA expression in the interpeduncular nucleus, and this is more difficult to discern from available studies. Thus, the immunostaining observed here could be specific to IL1RAPL1, but a firmer body of evidence regarding antibody characterization is clearly necessary to properly interpret the observed immunostaining.
Immunohistochemistry with the anti-IL1RAPL2 antibody used here failed to produce any positive staining. However, it is possible that further experimentation with incubation conditions or antigen retrieval steps could result in reliable staining from this antibody. There is a paucity of commercially available antibodies raised against human or rodent IL1RAPL2 proteins, making investigations into their localization difficult. At present, based on the results obtained here, the presence of IL1RAPL2 in the interpeduncular nucleus can neither be confirmed nor ruled out. The results of other studies examining IL1RAPL2 mRNA expression and protein presence in the brain utilizing different methodologies are discussed further below in Section 9.7.
9.6 Preliminary investigation of IL1RAPL gene expression across rat tissues

The findings above, that IL1RAPL proteins share a number of key residues with IL-18 BP, and that IL1RAPL1 receptor expression in the interpeduncular nucleus is in complete accordance with IL-18 projections, strongly suggest that IL1RAPL forms a central receptor for IL-18. Given IL-18 is constitutively present as a circulating cytokine, these results also raise the question as to whether IL-18 could also act on IL1RAPL receptors in the periphery. A pilot investigation was thus undertaken using tissue samples collected from the same animal in order to examine the expression of the IL1RAPL genes across a number of different peripheral tissues. RT-PCR was conducted using these samples to examine the expression of IL1RAPL1, IL1RAPL2, and by comparison, IL-18r1, gene expression across tissues.

9.6.1 Methodology

RT-PCR

A range of tissues were collected from an adult male Sprague-Dawley rat on dry ice following sacrifice by decapitation and stored at -80 °C until use. Hypothalamus and cortex samples were collected by sectioning the brain coronally at the anterior and posterior limits of the hypothalamus as viewed from the inferior surface of the brain. From the resulting coronal section, the hypothalamus was grossly dissected from the inferior surface and a sample of the cortex taken from superior surface of the same section. Skeletal muscle samples were collected from the quadriceps and heart samples from the wall of the left ventricle. Tissues were homogenized using the Qiagen TissueLyser II system (catalogue number 85300, Qiagen, USA) according to manufacturers’ instructions and total RNA extracted from the homogenate using the Qiagen RNeasy Plus Universal Mini Kit (catalogue number 73404, Qiagen, USA). Generation of cDNA was performed with Superscript III (product number 18080-044, Invitrogen, USA) as described in ‘General Laboratory Methods’ (Section 6.2) with a final addition of RNase H to remove remaining RNA strands. Amplification of desired transcripts was performed using the primer combinations and thermocycler conditions listed in Table 14 below. Approximately 150 ng of cDNA was added per 25 µl reaction for each sample.
Table 14: Primer combinations (A) and reaction conditions (B) for RT-PCR of IL-18r1, IL1RAPL1, IL1RAPL2 and Actb transcripts across various rat tissues.

A.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Forward primer</th>
<th>binds nucleotides on desired transcript</th>
<th>Reverse primer</th>
<th>binds nucleotides on desired transcript</th>
<th>Expected product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-18r1 (NM_001106905.2)</td>
<td>ATCCTAGCTATGGAGAGCTG</td>
<td>804 - 823</td>
<td>ACTGGTTCCCTCAATAACCGT</td>
<td>980 – 1000</td>
<td>196 bp</td>
</tr>
<tr>
<td>IL1RAPL1 (NM_177935.2)</td>
<td>TGGAGCTGAGGAACTGGACGGA</td>
<td>1186 - 1207</td>
<td>AGATGCTCCAGCCCCCTTCGGAC</td>
<td>1454 - 1475</td>
<td>290 bp</td>
</tr>
<tr>
<td>IL1RAPL2 (NM_001166342.1)</td>
<td>GCAAGC CAAAAATGTGGAGAAGCA</td>
<td>1472 - 1495</td>
<td>TGGCTTGGAGGCTTGTCTGT</td>
<td>1630-1650</td>
<td>179 bp</td>
</tr>
<tr>
<td>Actb (NM_031144.2)</td>
<td>TACAACCTTTCTTGCAGCTCCTCCG</td>
<td>28 - 51</td>
<td>TGTAGCCACGCTCGGTCAGG</td>
<td>657 - 676</td>
<td>649 bp</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Initialisation</th>
<th>Denaturing</th>
<th>Annealing</th>
<th>Extension</th>
<th>Cycles</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>94 °C for 3 minutes</td>
<td>94 °C for 30 seconds</td>
<td>57 °C for 30 seconds</td>
<td>72 °C for 45 seconds</td>
<td>35</td>
<td>72 °C for 5 minutes</td>
</tr>
</tbody>
</table>
9.6.2 Results

Images from gel electrophoresis of PCR products are shown in Figure 25. As seen previously, IL-18r1 expression was particularly prominent in the spleen, and also the lung, with lesser expression seen in the brain, liver and heart. The expression of both IL1RAPL genes was almost entirely restricted to the brain. However, some expression in peripheral tissues was present, with IL1RAPL2 expression also seen in the adrenal gland and lung, and IL1RAPL1 expression detected in the spleen sample. However, a very faint IL1RAPL1 band could also be seen in the RT- sample of the spleen, such that the positive signal seen in the spleen RT+ sample is best interpreted with some caution.
Figure 25: RT-PCR of IL1RAPL1, IL1RAPL2 and IL-18r1 expression across rat tissues. A: RT positive samples; B: RT negative control samples. Bands of the expected size for IL-18r1, IL1RAPL1 and IL1RAPL2 are shown, with control Actb (encoding β-actin) bands below. Samples were run against a 100 bp ladder (100 bp) in the left-most lane, with the remaining lanes showing: spl - spleen, adr - adrenal, kid - kidney, liv - liver, lun - lung, hea - heart, mus - muscle, hyp - hypothalamus, cor - cortex, pan - pancreas.

A.

![RT-PCR bands](image1)

B.

![RT-PCR bands](image2)
**9.7 Discussion**

The bioinformatics approaches used here have identified that amino acid residues which form part of the IL-18 binding domains of human and viral IL-18 BP are also present in the third Ig domain of the human IL1RAPL1 and IL1RAPL2 sequences, although are generally not shared amongst homologous IL-1 receptor family members. In addition, immunohistochemistry experiments in the rat brain showed the presence of IL1RAPL1 protein in the interpeduncular nucleus, providing anatomical evidence of co-expression of the putative receptor/ligand combination. Together these results are consistent with the hypothesis that IL1RAPL1 forms a centrally-expressed receptor for habenula-produced IL-18. IL1RAPL2 may also function in a similar manner, however, further experimentation with immunohistochemistry procedures or Western blots of interpeduncular nucleus specimens would be necessary to deduce whether this receptor is also present in the interpeduncular nucleus. Amino acid sequences of the IL1RAPL proteins were also observed to be highly conserved amongst a selection of mammalian species assessed here, suggesting that these putative receptor/ligand interactions may be very old in evolutionary terms, and may currently operate in a wide variety of animals.

Reviewing the literature related to IL1RAPL1 and IL1RAPL2 genes shows that both were first discovered through analyses in humans with X-linked mental retardation \(^{823,824}\), and are particularly highly expressed in the brain. Surprisingly, given their identified role in mental retardation, and studies examining cerebellar defects in IL1RAPL1 knockout mice \(^{840}\), there are no previous reports which have examined the overall distribution of the IL1RAPL proteins in the brain using immunohistochemistry, such that the results presented here are novel findings. A few papers have examined IL1RAPL1 expression by immunohistochemistry in cultured cortical \(^{819}\) or hippocampal \(^{818,841}\) neurons, showing the presence of IL1RAPL1 in both. However, these results are of limited value in determining the wider distribution throughout the brain *in vivo*. A number of other studies have examined IL1RAPL gene expression and protein distribution by other methods.

**IL1RAPL1/2 localisation within the brain:**

One of the first papers describing IL1RAPL1 and its association with mental retardation conducted *in situ* hybridisation in the mouse brain, describing the presence of IL1RAPL1 in the hippocampus, olfactory bulb and mamillary bodies (Carrié *et al.* \(^{820}\)). A closer inspection of the figures presented in that paper, however, suggests that the positive signal reported in the mamillary bodies may be at a more caudal level of sectioning. Figure 26 shows Figure 5d from that study, along with edited...
images of coronal sections from the Paxinos and Franklin mouse brain atlas at the level of the mammillary bodies and more caudally at the level of the interpeduncular nucleus. The level of sectioning shown in Figure 5d of Carrié et al. in fact appears to align better with the more caudal reference image. A common error which can occur in producing brain sections for experimental work is sectioning at an off-plane angle, such that different sides of the sections produced correspond to different reference images in a rodent brain atlas. As is shown in Figure 26, this does not seem to have happened in this case, as features of both sides of the ventral aspect of the section (e.g. the form of the hippocampus bilaterally) are similar to the reference atlas image at the level of the interpeduncular nucleus. Thus, although Carrié et al. report a positive signal for mRNA in the mammillary bodies, their results could be interpreted as showing a high density of the IL1RAPL1 gene transcript within the interpeduncular nucleus. The remainder of their results, showing IL1RAPL1 gene expression in the cerebellum, hippocampus and olfactory bulb are consistent with the immunohistochemistry results here (with the caveat that the olfactory bulb was not included in brain sections for immunohistochemistry experiments described here).

The Allen Brain Atlas presents in situ hybridisation for IL1RAPL1 in sagittal sections (image series 70278045), which is largely in concordance with the findings of Carrié et al., showing relatively wide expression across the brain, with particularly high expression in the hippocampus, olfactory bulb and cortex. It does, however, also show signals corresponding to the mammillary bodies (which is more convincing in the sagittal plane given the clear demarcation of the cerebral peduncular fossa) and hypothalamus. Only two sagittal sections are presented in the Allen Brain Atlas which include the interpeduncular nucleus, covering the lateral portion of the nucleus. A possible explanation to reconcile these in situ hybridisation results is that, while the coronal image presented by Carrié et al. appears to show the interpeduncular nucleus (shown in Figure 26), the authors may indeed have observed IL1RAPL1 expression in the mammillary bodies, as they report in the text, while the Allen Brain Atlas shows expression in the mammillary bodies but does not provide evidence to clearly refute the presence of IL1RAPL1 in the interpeduncular nucleus. Results of a preliminary RT-PCR experiment presented here (Section 9.6) and the Allen Brain Atlas suggest there is IL1RAPL1 mRNA present in the hypothalamus, although protein was not observed immunohistochemistry experiments.

The Eurexpress project, which has examined the expression of a wide number of genes in the developing mouse embryo, shows widespread IL1RAPL1 expression throughout the brain and spinal
Figure 26: In situ hybridisation in the mouse brain for IL1RAPL1 expression from Carrié et al., Nat Genet, 1999, 23: 25–31. Top panel shows image presented as Figure 5d of Carrié et al., with panels below showing reference atlas images at the level of the interpeduncular nucleus and mammillary bodies for comparison. Reference atlas images adapted from Paxinos and Watson showing coronal sections at Bregma -3.64 mm (interpeduncular nucleus) and Bregma -2.92 mm (mammillary bodies).

cord during development, which, however, does help discern the location of its expression at later stages of adult development. Other studies have utilised Western blot to show the presence of IL1RAPL1 protein in hippocampal neurons, the cerebellum, the olfactory bulb, and all three of these regions. However, there are no studies which provide Western blot data to confirm or refute the presence of IL1RAPL1 in the interpeduncular nucleus.

In sum, the results of these studies provide some support for the same distribution of IL1RAPL1 gene expression and translation into protein as the immunohistochemistry results presented here, with the presence of IL1RAPL1 in the cortex, hippocampus and cerebellum, which supports the validity of these results. Regions with discordant results are the interpeduncular nucleus (the area of interest for the current work), hypothalamus and mammillary bodies.

Regarding the localisation of IL1RAPL2 in the brain, mRNA expression has been detected in the human and mouse brain by Northern blot, and in situ hybridisation performed in the mouse brain by Ferrante et al. shows expression in the cortex, olfactory bulb, and cerebellum, similar to the IL1RAPL1 transcript. In contrast, the Allen Brain Atlas presents results for IL1RAPL2 from two different probes (image series 73994672 and 69735389) which show little expression of IL1RAPL2 across the mouse brain (with the exception of some weak signal in the pontine reticulotegmental nucleus), and Born et al. show only weak expression in the fetal brain. Likewise, the Eurexpress project shows no expression of IL1RAPL2 in the developing mouse embryo. Immunohistochemistry experiments conducted for this thesis in the adult rat brain failed to produce any specific staining. Thus, currently available studies present disparate results and cannot currently be easily reconciled to form a consensus picture as to the localisation of expression of IL1RAPL2 in the brain.

**IL1RAPL1/2 function and potential ligands:**

The IL1RAP proteins have been largely regarded as orphan receptors since their discovery around the turn of the millenium, although one paper has presented evidence that IL-1 can signal via IL1RAPL1 to induce phosphorylation of JNK.

Late in 2011 two papers simultaneously reported that IL1RAPL1 forms a trans-synaptic ligand-receptor complex with pre-synaptic protein tyrosine phosphatase receptor delta (PTPRD; Valnegri et al. and Yoshida et al.). These studies not only identify a ligand for the IL1RAPL receptors, but also greatly advance the understanding of how these receptors function.
the IL1RAPL receptors was initially assumed to follow that of the other IL-1 family members, these and other studies now provide sufficient insight to reveal that these function in an entirely separate manner, and in fact seem to regulate the formation and maintenance of excitatory (glutamatergic) synapses both via pre-synaptic and post-synaptic effects. The post-synaptic effects of IL1RAPL proteins can also be isolated to different portions of the cytoplasmic domain; the toll/interleukin-1 receptor (TIR) domain is necessary for inducing changes in dendrite architecture (although the signalling mechanisms involved are unknown), and the effect of IL1RAPL proteins on synaptic localisation occurs via the C-terminal domain, which induces phosphorylation of JNK and phosphorylation of post-synaptic density protein 95 (PSD-95). Importantly, studies in mice suggest that PTPRD is not expressed in the interpeduncular nucleus, and in fact the only overlap between PTPRD and IL1RAPL1 expression in the brain appears to be in the hippocampus. Given both the studies of Valnegri et al. and Yoshida et al. identified the interaction of IL1RAPL1 and PTPRD by utilizing whole brain homogenates it seems likely that their results arise from identifying an interaction which occurs in the hippocampus. Valnegri et al. mention approximately 200 proteins identified by their co-precipitation assays, although subsequent experiments only focused on PTPRD, and this suggests that a number of other IL1RAPL1-interacting proteins exist; phrased in a different manner, their results in no way exclude the existence of other IL1RAPL1 ligands. In addition, given that PTPRD and IL1RAPL1 could only interact in areas of co-expression, and furthermore, that the interaction only appears to occur with specific PTPRD splice variants, these studies raise the question of what potential ligands could interact with IL1RAPL1 in other areas of the brain with no evidence of PTPRD expression, such as in the cortex or cerebellum. The argument is made here that, based on the work described in this chapter, IL-18 is a prime candidate for forming an IL1RAPL1 ligand in the interpeduncular nucleus.

One study thus far has examined whether the IL1RAPL proteins could form receptors for IL-18 (Born et al. 848), utilizing two different experimental methods. In one, COS-7 cells were transfected with IL1RAPL proteins as well as an NF-κB luciferase reporter, finding no effect of IL-18 on luciferase reporter activity. Given most members of the IL-1 receptor family function by the combined action of ligand-binding and accessory protein chains (a.k.a. α and β chains), in another experiment the cytoplasmic domain of various members of the IL-1 receptor family, including the IL1RAPL proteins, were fused to the extracellular portion of the IL-1 receptor to generate chimeric receptors. Cells transfected with these receptors were stimulated with IL-1 using NF-κB luciferase activity as a marker of signal transduction. This experiment showed no effect of IL-1 stimulation on NF-κB activity when various members of the IL-1 receptor family were coupled with the
cytoplasmic domains of IL1RAPL proteins. These experimental procedures, however, are based on the assumption that the IL1RAPL receptors function in a similar manner to other members of the IL-1 receptor family and result in NF-κB activation, which now does not appear to be the case. At the time of conducting these experiments, it had been suggested that the longer C-terminal amino acid sequences of the IL1RAPL proteins could be inhibitory, and thus further experiments were conducted in which truncated versions of the IL1RAPL proteins, lacking the C-terminal domain, were utilised, again showing no activation of NF-κB. However, it is difficult to know from these experiments whether (i) this is simply because the toll-like receptor domain of the IL1RAPL proteins is unable to activate NF-κB even in the absence of the C-terminal domain, (ii) whether the IL1RAPL proteins simply do not function as ligand binding/accessory protein diads in the same manner as other members of the IL-1 receptor family, or (iii) whether they are in fact capable of activating NF-κB but simply require another accessory protein or ligand binding chain different from the known IL-1 receptor family members tested in this study. In support of a lack of possible NF-κB activation from IL1RAPL receptors are the findings of Khan et al., who have published evidence that IL1RAPL1, but not IL1RAPL2, can activate the JNK pathway, with no evidence that either activates the NF-κB, nor the p38 or ERK MAPK kinases. Despite its recent discovery, the TIR domain of IL1RAPL1 was the first member of the IL-1 family of receptors to have a crystal structure produced and published. The crystal structure shows a number of interesting insights, such as the apparent ability of IL1RAPL1, and presumably IL1RAPL2, to form homodimers, which is not seen with other TIR domains and may be involved in initiating the unique signalling pathways of these receptors.

The unique C-terminal cytoplasmic domain of IL1RAPL proteins has also been shown to interact with neuronal calcium sensor-1 (NCS-1) and can regulate exocytosis in endocrine PC12 cells via an effect on voltage-gated calcium channels. A recent analysis of gene sequence conservation across 29 mammals identified conserved regions within the final exon of IL1RAPL1 and IL1RAPL2, the portion identified as encoding the neuronal calcium sensor 1 (NCS-1) interacting domain, indicating that this interaction with NCS-1 probably forms a key, highly conserved, biological function of the IL1RAPL proteins.

Interactions of the homologous IL-1 cytokine with IL1RAPL1/2:

Of particular importance to the proposed interaction of IL-18 with IL1RAPL proteins is the recent findings of another study by Yoshida et al., which offer supportive evidence that the highly related cytokine IL-1 binds to a CNS-specific receptor which interacts with PTPRD, analogous to the
proposed action of IL-18 binding to IL1RAPL receptors (which are highly expressed within the brain and also interact with PTPRD).

A few key works prior to that study had discovered the existence of a centrally-expressed splice variant of the accessory receptor for IL-1 (known as IL-1RAcP)\(^{856-858}\). This receptor is formed by the use of a separate final exon, downstream of the IL1RAcP transcript, which is specifically transcribed in the CNS, and appears to mediate a number of the central effects of IL-1. The work of Yoshida \textit{et al.} takes these results further and shows that the extracellular portion of IL-1RAcP/IL-1RAcPb (the extracellular portion is common to both isoforms) forms a trans-synaptic interaction with PTPRD\(^{855}\), functioning in a manner very similar to that described for IL1RAPL1; the extracellular interaction with PTPRD is necessary for presynaptic differentiation of excitatory synapses, while the neuron-specific IL-1RAcPb isoform, with its longer intracellular domain, induces post-synaptic differentiation. They also report that no synaptogenic activity was observed for IL-18rap; an observation in accordance with the hypothesis presented here, i.e. that the neuronal effects of IL-18 are likely to be mediated via the IL1RAPL proteins and not the IL-18 receptor. Interestingly, the existence of a separate centrally-expressed receptor system was predicted for IL-1 prior to the recent discovery of IL-1RAcPb\(^{859}\), and similarly in the case of IL-18, a separate centrally-expressed receptor system would be expected based on the limited central expression of IL-18 receptor subunits and the mismatch this creates with abundant habenula IL-18 expression and projections into the interpeduncular nucleus.

\textbf{Conclusions:}

The goal at the outset of this thesis was to examine the neuroendocrine role of IL-18; experiments published in two high profile journals (Netae \textit{et al.} in Nature Medicine\(^{502}\) and Zorrilla \textit{et al.} in PNAS\(^{806}\)) had described a role for IL-18 in controlling food intake, mediated by effects in the brain. The founding hypotheses upon which the experiments in this thesis were undertaken revolved around the role of IL-18 as a pro-inflammatory cytokine and a large body of evidence linking changes in inflammatory signalling with alterations in metabolism; more specifically, that inflammation within the brain could alter hypothalamic function. As such, it was initially proposed that IL-18 could exert its effects via activating IL-18 receptors in the hypothalamus.

A number of experiments were then undertaken to examine the central distribution of the subunits of the IL-18 receptor. Although the results of those experiments are not entirely concordant across experimental techniques (as described earlier in Chapter 8), those experiments present results which
could be interpreted as showing either a lack of central IL-18 receptor expression, or a low-level, relatively even expression across different brain regions. Indeed, other published works appear to fall into either of these categories. These results are rather puzzling; on the one hand, a lack of focused expression in the interpeduncular nucleus is at odds with observed IL-18 projections to this brain region, on the other, there also appears to be no focus of IL-18 receptor expression in the arcuate nucleus, the most likely site of action for circulating IL-18 exerting a metabolic role through activation of hypothalamic inflammatory pathways (as hypothesized in this thesis).

In light of these findings, it seemed plausible that IL-18 could act through a distinct centrally-expressed receptor. The works in this Chapter have identified IL1RAPL receptors as potential central receptors for IL-18. Although this thesis does not provide evidence of IL-18 initiating signal transduction events downstream of the IL1RAPL receptors, nor provide evidence from binding studies, a number of pieces of evidence converge to suggest the IL1RAPL receptors may in fact form a centrally-expressed receptor for IL-18. These are: (i) the aforementioned mismatch between central IL-18 expression and a lack of central IL-18 receptor expression reported here and by other published studies; (ii) the overlapping presence in the interpeduncular nucleus of IL-18 containing projections and IL1RAPL1; (iii) conserved amino acid motifs shared between IL-18 BP and the IL1RAPL receptors; and (iv) that likewise, a CNS-specific receptor exists for the highly homologous IL-1 cytokine, which acts in a similar fashion to IL1RAPL1.

Clearly the proposed binding of IL-18 to IL1RAPL proteins will ultimately need to be experimentally tested, and in order to do so appropriate downstream signalling events as markers of IL-18 activation of IL1RAPL receptors will need to be identified. How, or if, these signalling events could translate downstream into effects on appetite and explain the hyperphagic phenotype of IL-18 knockout animals is currently unknown. One piece of evidence against this hypothesis is the phenotype of IL1RAPL1 knockout mice; the authors of the paper describing the generation of these mice report no gross abnormalities distinguishing them from wild-type mice. This could be interpreted as evidence that even if habenula-produced IL-18 does act on interpeduncular nucleus IL1RAPL proteins, this may be unrelated to the effects of IL-18 on appetite. However, IL-18 knockout animals appear to have been in existence for some time before their hyperphagia and overweight phenotype was published. In terms of phenotypes and associations with disease reported in humans, there are

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860 See Jackson Laboratories information page for IL-18 knockout mice, which shows numerous publications prior to the study of Netea et al. describing their overweight phenotype.
also no reports of IL1RAPL proteins being associated with obesity or alterations in body weight. This uncertainty aside, in the next Chapter a putative model of how IL-18 action on IL1RAPL receptors in the interpeduncular nucleus could mediate alterations in appetite is proposed and discussed, including future experiments which could test the proposed model. Although this model goes beyond the experimental evidence presented, building models such as these, and attempting to combine the proposed interaction between IL-18 and IL1RAPL receptors with other existing evidence regarding the anatomy and function of the habenula-interpeduncular nucleus connection, is a necessary key step in shaping future research questions and experimental approaches.

While the experiments described here were conducted with the principal focus on a neuroendocrine role of IL-18, they do raise other tangential questions relevant to related fields and other aspects of the biological role of IL-18. Of note, when conducting a mutation analysis of residues necessary for the Molluscum contagiosum virus IL-18 binding protein, MC054L, to bind human IL-18, Xiang and Moss identified that substituting the valine present in MC054L upstream of the ‘pFIEpL’ consensus sequence (‘VSLIYW’ shown in Figure 20, Panel A) for a phenylalanine, as is present in the other viral and human IL-18 BP sequences, caused the dissociation constant to decrease approximately 10-fold, to a value similar to human IL-18 BP 828. They concluded: "our finding that MC054L contained a nonoptimal valine in place of phenylalanine was puzzling, since we intuitively expected a virus to encode the most potent antagonist. There are several possibilities that might explain this apparent discrepancy. Although MCV replicates exclusively in humans, MC54L could have been acquired at an earlier stage of evolution from a host that had a valine instead of a phenylalanine in the IL-18 binding site. However, even the mouse IL-18BP has phenylalanine in this position, and there is no evidence for an ancestral IL-18BP with valine. Furthermore, the orthopoxvirus IL-18BP homologs have phenylalanine, not valine, in corresponding positions." (from pg 9950 of 828). Comparison of the respective amino acid sequences for each of these proteins shows that IL1RAPL1 also contains a valine in this position (‘VSPLIYW’ shown in Figure 20, Panel A). This could suggest, firstly, that the hypothesized binding of IL-18 to IL1RAPL exhibits a higher dissociation constant than that seen with IL-18BP hh. Secondly, an interesting research question which arises from the works above is the

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**Note** that two amino acids upstream of these residues, aligned by MAFFT in Figure 19 Panels A and B, are ‘YS’ (for IL1RAPL1) or ‘FS’ (for IL1RAPL2) residues which could also correspond to the respective VS or YS residues in viral IL-18BPs; these are shown aligned manually in Figure 21.

**At least for caspase-1 processed IL-18, which was tested in these studies. Discussed later is that IL-18 processed by other enzymes would be expected to exhibit different binding affinities.**
suggestion that *Poxviridae* may have obtained their own forms of IL-18BP not through horizontal transfer of the mammalian IL-18BP gene, but given similarities noted in the nucleotide coding sequences above, and the question raised by Xiang and Moss, possibly via incorporation of gene sequences encoding the third Ig-like domain of IL1RAPL proteins.

The position of the IL1RAPL proteins on the X chromosome also raises many interesting questions as regards their origins and function. The IL1RAPL1 gene in the human lies in the so-called 'short' arm of the X chromosome, also known as the 'X added region' (XAR) \(^{861}\), which is believed to have been transferred to the primordial X chromosome after the divergence of the sex chromosomes from autosomes. The IL1RAPL2 gene, in contrast, is located on the 'long arm' or 'X conserved region' (XCR) \(^{861}\) which is an evolutionarily older portion of the X chromosome. It has been hypothesized that as genes were transferred to the X chromosome their expression patterns became more restricted, tending towards brain-specific expression as opposed to wider expression in the body, (with the obvious exception of sexually-dimorphic expression of testis-specific genes being located on the X chromosome \(^{822}\)); in other words, that pre-existing genes transferred to the X chromosome evolved increasingly brain and reproduction-specific roles, and studies specifically investigating this hypothesis present data in support of this pattern \(^{821,862}\). In studies which have examined the expression of IL1RAPL proteins across various tissues, their expression appears to conform to this trend, with IL1RAPL2 (presumably having been present on the X chromosome for longer due to its location in the XCR) expression reported as restricted only to the brain in two studies \(^{823,825}\) or also in liver and placenta in one study \(^{848}\). In contrast, IL1RAPL1 (transferred to the X chromosome at a later stage based on its presence in the XAR) expression has been reported in the brain as well as peripheral organs such as the heart, kidney, skin, lung, thyroid, placenta, intestine, testis and ovarian tissues \(^{824,825,848}\). The pilot RT-PCR experiment conducted here in fact appears to show a more brain-specific expression of IL1RAPL1 than IL1RAPL2, i.e. in the direction opposite to the above hypothesis. Regardless of this difference, if the hypothesis presented here that IL-18 is able to act on the IL1RAPL receptors proves correct, given the association of IL-18 with a variety of diseases, including noncommunicable diseases such as cardiovascular disease \(^{464}\), and autoimmune diseases such as rheumatoid arthritis \(^{863}\), inflammatory bowel disease \(^{822}\) and asthma \(^{864,865}\), then IL-18 action on IL1RAPL1 and IL1RAPL2 receptors in the periphery may be of importance for the normal biological functions of IL-18, and their aberration in disease states.
Chapter 10: A model of habenula IL-18 function

Two papers published prior to, or early on in, the course of this thesis showed that IL-18 knockout mice exhibit hyperphagia, and that administration of IL-18 directly into the brain could reduce appetite $^{503,506}$. These studies established a metabolic role for IL-18, suggesting that this effect arises out of action within the brain. This gave rise to two hypotheses regarding IL-18 action: (i) that peripheral circulating IL-18 acts within the brain and thus functions in an endocrine manner, most likely exerting its influence via actions in the hypothalamus, and (ii) that IL-18 produced within the brain is responsible for its metabolic effects. Note that these two hypotheses are not necessarily mutually exclusive and cross-talk between peripheral and central IL-18 production could exist.

It is well established that IL-18 is constitutively expressed in the periphery, and can be readily detected in the circulation. The work in this thesis has shown that IL-18 is also constitutively expressed in brain, specifically within the habenula (at least in the Sprague-Dawley rat, the animal model used here). These findings are in agreement with those of Sugama et al. $^{777}$, and raise the possibility that the hyperphagia exhibited by IL-18 knockout mice derives from a lack of habenula IL-18 expression. When examining the available evidence of habenula function, discussed further below, a picture emerges suggesting the habenula could function as a metabolic sensor.

10.1 Pre-existing evidence for a role of the habenula in metabolism:

10.1.1 Circadian rhythm, seasonal change, and the habenula:

Indirect evidence for a potential role of the habenula in metabolism arises from considering the evolutionary role of the habenula across various species. Various fish, lizards, and amphibians show asymmetrical habenula nuclei, with either the right or left side larger than the contralateral nucleus $^{866}$. This asymmetry appears to be driven by an asymmetrical input from the parietal, or ‘third’, eye present in these species; the parietal eye forms a mid-line, translucent membrane, which contains
photosensitive cells which project to the habenula. Given the fact that the parietal eye is photosensitive, but appears not to encode visual information, it is theorised to play a role in encoding and entraining circadian rhythms, and thus the habenula, as one of the major efferent nuclei of fibres from the parietal eye, is likely to play a role in responding and adapting to diurnal rhythms or changes in day-night length. In accordance with this phenomenon, the habenula of some species of frog has been shown to undergo seasonal size variation. Habenular assymetry appears to a much lesser extent in chickens, and is absent in other mammals such as rats and humans.

Despite the obvious absence of a parietal eye in mammalian species, the possibility remains that the habenula in these species also receives non-visual light information, with studies in mice showing that melanopsin-containing neurons present in the retina project to a wide range of neuronal targets, including the habenula. In rats, the spontaneous firing rate of neurons in the medial and lateral habenula have been shown to be modulated by retinal illumination, as well as exhibiting diurnal variation in firing rate. The lateral habenula has been shown to be a target organ of the superchiasmatic nucleus in hamsters, and exhibits diurnal variations in stress-induced fos immunoreactivity. Interactions between the habenula and pineal gland also exist, with evidence for projections from the habenula to the pineal gland. Intracerebroventricular IL-18 administration in rats and rabbits has also been shown to modulate aspects of sleep, such as length of non-REM sleep, which raises the possibility that habenula-derived IL-18 may act in a similar fashion.

Thus, there is some evidence from a range of species that the habenula may function as a sensor of circadian rhythm. This may not necessarily implicate the habenula in the regulation of metabolism, particularly as seasonal changes in habenula size or function observed in some species could be related to the seasonality of reproduction rather than seasonal metabolic adaptations.

10.1.2 The habenular ependymal layer:

The distribution of IL-18 producing cells observed here (Chapter 7), and previously by Sugama et al., were seen not only to be present in the medial habenula, which lies either side of the dorsal third ventricle, but moreover were specifically clustered around the most medial surface of the medial habenula, directly adjacent to the ventricle, corresponding to the superior medial habenula subnucleus (MHbS) described by Andres et al. Such a constrained distribution so close to the ventricle raises the question as to whether these IL-18 producing cells in fact participate in sensing the composition of, or secreting factors into, the cerebrospinal fluid. The ventricular ependymal layer of the medial habenula of the rat has been investigated by a one laboratory in detail. Their...
findings show that the ventricular surface of the habenulae exhibit microvilli and cilia\(^ {877,878}\), whose exact function is unknown, but appear similar to microvilli present on the choroid plexus\(^ {879}\). In addition, the ventricular surface is host to a number of tanycytes\(^ {880}\), similar to other circumventricular organs known to exhibit endocrine roles such as the area postrema and median eminence. The ependymal surface of the medial habenula also shows properties of being 'leaky', with for example, entrance of horseradish peroxidase into the habenula from the cerebrospinal fluid\(^ {881}\). Thus, a number of features of the habenular ventricular wall suggest that the habenula could act as a sensor of cerebrospinal fluid contents, which influence brain function at a wide level\(^ {882}\). Notably, the position of the medial habenula either side of the dorsal third ventricle is also immediately adjacent to the choroid plexus of the third ventricle, placing these IL-18 producing cells in a prime position to monitor, or secrete into, the neighbouring cerebrospinal fluid.

10.1.3 The habenula as mediator of reward and drug addiction:

Animals with electrodes placed in the habenula show self-stimulatory behaviour\(^ {883}\), a classic example of the involvement of a brain region in reward. A number of studies have also implicated the habenula or connected interpeduncular nucleus in the intake of drugs of abuse, including morphine\(^ {884,885}\), heroin\(^ {886,887}\), cocaine\(^ {888-892}\) and nicotine\(^ {893-897}\), as well as a possible role in alcohol intake and addiction\(^ {898-900}\). The habenula is the site of the heaviest density of nicotinic receptors in the brain\(^ {901,902}\), and manipulation of habenular nicotinic receptors in rodents alters self-administration of nicotine\(^ {897,903}\). Under chronic nicotine administration the habenula and its projections to the interpeduncular nucleus undergo degeneration\(^ {896,904,905}\). Similar degeneration is also observed upon exposure to other drugs of abuse, which has led some authors to describe the habenula-interpeduncular nucleus pathway as a ‘weak link’\(^ {896,906}\) which may potentially underlie addiction to many drugs of abuse.

A number of studies have shown interactions between the habenula and ventral tegmental area (VTA)\(^ {907-910}\), and median raphe\(^ {883}\). Habenula projections can modulate dopamine activity in the VTA\(^ {907}\) and other brain regions including the nucleus accumbens and striatum\(^ {911}\). Efferent projections from the habenula to the midbrain also appear to be reciprocal, with evidence for VTA projections along the fasciculus retroflexus to the habenula\(^ {912-914}\), and from the median raphe to the habenula\(^ {912,915}\). Interestingly, the distribution of IL-18 producing cells in the habenula occupy the exact same distribution as the terminal fields of dopamine projections from the VTA\(^ {913}\) (and in addition habenula substance P expression\(^ {916,917}\)). Such connections thus provide an anatomical basis for an interaction of habenula-derived IL-18 with brain reward systems. Both the median raphe\(^ {918}\)
and the VTA are known to influence feeding behaviour, and thus interactions with IL-18 producing cells could form a mechanism by which IL-18 influences appetite. In addition, lesions of the habenula (both medial and lateral nuclei) produce a sustained activation of mesocortical dopamine neurons. Although the above evidence of interactions with reward pathways does not necessarily implicate the habenula in the control of food intake, there is currently a large amount of scientific interest in evidence showing cross-over of the reward of eating behaviour with reward pathways involved in addictive behaviour. Also of note is that alterations in VTA dopaminergic activity is an established effect of other metabolic hormones such as leptin and insulin.

10.1.4 Metabolic signalling, food intake, and the habenula:

More specifically with regard to a role in metabolism, the habenula has been observed to exhibit expression of receptors for leptin, insulin, NPY, GLP-1, melanocortins (MC3R) ii and sweet taste (Tas1r2, Tas1r3) receptors, as well as the peptide adropin. The habenula has also been shown to respond to metabolic stimuli, with studies showing changes in habenula leptin receptor expression with fasting, habenula opioid receptor expression in obesity or fasting, and changes in habenula expression of c-fos with intake of a palatable meal. Clearly, showing that the habenula responds to metabolic signals may not necessarily imply that it influences eating behaviour, as metabolic signals could be integrated in this nucleus but any subsequent output utilised for other means, such as effects on reproduction and fertility, growth, or the immune system, which need to be tied to the metabolic health and functioning of an organism. More direct evidence for a role for the habenula in food intake comes from a study showing lesions of the habenula or interpeduncular nucleus influence the intake of water laced with the bitter substance quinine. Electrical stimulation of the lateral habenula has also been observed to reduce sucrose intake.

Furthermore, with regard to the effects of IL-18 on appetite, Zorrilla et al. report that IL-18 knockout mice overeat on a normal chow, or low-fat diet, but not on a high fat diet, suggesting that some form of taste or food preference may underlie their hyperphagia, rather than a simple drive to overeat per se.

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ii Unpublished in situ hybridisation experiments in the rat, H Phillipps, 'Grattan laboratory', Dept of Anatomy, University of Otago.
10.2 A theoretical model for a neuroendocrine role of IL-18

10.2.1 Projections and transport from the habenula:

The primary output from the habenula is the fasciculus retroflexus, with fibres from the medial habenula travelling down the core of the fasciculus and lateral habenula fibres forming the surrounding outer sheath \(^{938}\). Once they reach the interpeduncular nucleus, fibres can be seen to undergo a somewhat unusual pathway of crossing to the contralateral side and then returning ipsilaterally \(^{835}\). Whether these fibres ultimately terminate ipsilaterally or also form *en passant* axonal connections on the contralateral side is not clear. It has been shown here, and by Sugama *et al.* \(^{777}\), that IL-18 containing fibres can be observed within the fasciculus retroflexus, terminating in the IPN.

10.2.2 Processing of IL-18 in the habenula:

IL-18 has typically been investigated with a focus on its role following caspase-1 processing. Indeed the IL-18 precursor is often referred to as 'pro-IL-18' which is cleaved by caspase-1 to 'active' or 'mature' IL-18. However, there is also clear evidence that the IL-18 precursor can be processed by mast cell chymase-1 \(^{939}\) (as can IL-1 \(^{940}\)), proteinase-3 \(^{941}\), granzyme-B (at the same site as caspase-1) \(^{942}\) and caspase-3 \(^{943}\). Given the forms of IL-18 produced by processing from these enzymes form shorter amino acid sequences (with the exception of granzyme-B), it would be expected that these forms of IL-18 would show differing affinities to the IL-18 receptor and IL-18 BP. There appears to be little available literature regarding the expression of these other IL-18-processing enzymes in the brain, and while the Allen Brain Atlas shows no experiments for detecting caspase-1 or proteinase-3 as yet, they have performed experiments for the detection of mast cell chymase-1 (encoded by the Cma1 gene) showing a high density of expression in the habenula \(^{944}\), and caspase-3 \(^{945}\), showing light expression in the habenula. It is proposed that differential processing of the percursor IL-18 protein has evolved as a mechanism which allows, on the one hand, for the deactivation of 'mature' IL-18 (as has been shown for caspase-3 processing of IL-18 \(^{943}\)), but also for the IL-18 precursor to be directed toward greater or lesser affinity to differing receptors or isoforms of the IL-18 binding protein. Given the high expression levels of mast cell chymase-1 in the habenula, it is proposed that chymase-processed IL-18 shows a greater affinity for the IL1RAPL receptors than caspase-1-processed 'mature' IL-18. Indeed, mast cell chymase-1-processed IL-18 has be shown to exhibit a reduced ability to elicit IFN-γ production in cell culture (approximately 20 % of that observed for caspase-1-processed IL-18) \(^{939}\), suggesting a reduced affinity or ability to activate downstream signalling for the IL-18 receptor.
10.2.3 Proposed actions of IL-18 on interpeduncular IL1RAPL receptors

Of particular relevance to proposed effects of IL-18 on IL1RAPL receptors are findings from Yoshida et al. who describe the interaction of another member of the IL-1 receptor family, IL-1RAcP(b), with PTPRD. They present a model of the functioning of this receptor as shown in Figure 27. The binding of the extracellular portion of IL-1RAcP or IL-1RAcPb (since both share the same extracellular domain) with PTPRD forms a trans-synaptic adhesion resulting in differentiation of the pre-synaptic terminal, specifically in excitatory synapses. Binding of IL-1, however, is theorized to disrupt this trans-synaptic interaction and result in the typical pro-inflammatory effects of IL-1 (Figure 27, Panel B). However, missing from the proposed model is that, while the interaction of the extracellular portion of IL1-RAcP(b) with PTPRD is sufficient to induce pre-synaptic differentiation, only the longer neuron-specific IL-1RAcPb isoform is capable of inducing post-synaptic differentiation of excitatory synapses, as shown in Figure 28, Panel A. Importantly, IL-1RAcPb also appears to lack the ability to induce pro-inflammatory signalling (Figure 28, Panel B), and thus, based on current knowledge it appears that the binding of IL-1 may not be important in terms of inducing novel downstream signalling, but through disrupting the trans-synaptic interaction with PTPRD.

In terms of the actions of the IL1RAPL receptors, the majority of research has focused on IL1RAPL1. The trans-synaptic interaction of this receptor with PTPRD has been shown to induce both pre- and post-synaptic differentiation of excitatory synapses (Figure 29, Panel A), in a similar fashion to IL-1RAcPb. Effects on a number of downstream signalling molecules have been shown across various studies, including phosphorylation of JNK, which leads to phosphorylation of PSD-95, as well as recruitment of Rho-GAP2 to excitatory synapses and interaction with NCS-1. Trans-synaptic interaction of PTPRD with IL1RAPL2 also induces pre-synaptic differentiation of excitatory synapses, although based on the works of Valnegri et al. this appears to be of a lesser extent than that seen for IL1RAPL1. In addition, while they show that IL1RAPL2 is capable of inducing post-synaptic effects, such as an increase in dendritic spine number, it is unclear how these effects are mediated, as they found that IL1RAPL2 does not activate Rho-GAP2, unlike IL1RAPL1, and in fact appeared to reduce IL1RAPL1-induced Rho-GAP2 activation, presumably by competitive binding with pre-synaptic PTPRD. Thus, while the signalling effects of IL1RAPL2 are less clear, it is known that this receptor also exerts effects which result in the strengthening of excitatory synapses (Figure 28, Panel B). As detailed above, it is hypothesized here that IL-18 binds to the third Ig domain of both IL1RAPL receptors. Currently, it is unknown what the stoichiometry of this interaction is; given evidence from the crystal structure of IL1RAPL1 it appears that the TIR domains of these receptors...
receptors are capable of forming homodimers, and thus IL-18 could putatively bind either single IL1RAPL receptors, or form dimers with either IL1RAPL1, IL1RAPL2, or both (Figure 29, Panel C). What the downstream signalling effects of these interactions could be cannot be easily hypothesized. However, the effects of IL-18 binding may not lie in any specific signalling mechanisms induced, but in the resulting lack of interaction with pre-synaptic PTPRD, which would be expected to reduce the strength of excitatory synapses; in this manner, IL-18 binding to the IL1RAPL proteins may be similar to IL-1 binding of the neuron-specific IL-1RAcPb isoform. IL-18 binding to IL1RAPL receptors could therefore form a mechanism by which alterations in habenula IL-18 expression (such as those observed by Sugama et al. following an acute stress) could dynamically regulate the strength of interpeduncular nucleus excitatory synapses.

The cytokines IL-1 and IL-18 are known to share a number of similarities in their processing, protein sequences, mechanisms of receptor activation and downstream signalling, and the model proposed here would therefore also extend their similarities to include the manner in which they regulate the strength of excitatory synapses via neuron-specific receptors. A key difference between the case of IL-1 and the proposed relationship here of IL-18 with the IL1RAPL receptors, is that in the case of IL-1 the centrally-expressed IL-1RAcPb receptor is formed by alternative splicing of a gene previously known to mediate IL-1 actions in the periphery, whereas the case is made here that IL-18 functions in the CNS via separate receptor(s), which show(s) similarity to IL-18 BP. Clearly, the IL-1 signalling system does not possess a similar circulating binding protein, such that a completely analogous process is impossible. Nevertheless, the existence in the case of IL-1 of a distinct receptor isoform specifically expressed in the CNS which mediates excitatory synaptic strength via PTPRD gives strong support to the possibility that IL-18 acts in a similar manner via interactions with the IL1RAPL proteins.

10.2.4 A model of habenula IL-18 function governing food intake:

From the literature summarized above and other published works, a model of habenula IL-18 function is proposed here, which is described below and depicted in Figure 30.

Many works linking habenula function to reward focus on the lateral habenula, although interestingly the medial habenula possesses projections to the lateral habenula, yet the reverse does not appear to be the case (the lateral habenula does not project to the medial habenula). Therefore it is proposed that the medial habenula possesses the ability to influence lateral habenula activity, but not vice versa. Exactly what the nature of these fibres are is unknown, and therefore whether...
Figure 27: A model of IL-1RαC function as proposed by Yoshida et al. Panel A - IL-1RαC interaction with pre-synaptic PTPRD induces the pre-synaptic differentiation of excitatory synapses. Panel B - binding of IL-1 results in the formation of an IL-1r1 and IL-1RαC complex inducing pro-inflammatory signalling.
Figure 28: The model of Yoshida et al. altered to account for the specific actions of IL-1RaCpb. Panel A - Only IL-1RaCpb is capable of also inducing post-synaptic differentiation of excitatory synapses. Panel B - Recruitment of IL-1RaCpb to the IL-1/IL-1r1 receptor complex does not induce pro-inflammatory signalling.
Figure 29: A similar model of IL-18 and IL1RAPL function. Panel A - The trans-synaptic interaction of IL1RAPL1 with PTPRD has been shown to induce both pre- and post-synaptic differentiation of excitatory synapses, with a range of post-synaptic signalling events implicated. Panel B - The trans-synaptic interaction of IL1RAPL2 with PTPRD appears to exert effects on pre-synaptic, but not post-synaptic, differentiation. Panel C - Binding of IL-18 to the third Ig domain of the IL1RAPL proteins is theorized to disrupt the interaction with PTPRD, but whether this results in any unique post-synaptic signalling effects is unknown. Also unknown is whether IL-18 binds to just one of the IL1RAPL proteins, or can form homo- or hetero-dimers.
they are excitatory or inhibitory of lateral habenula neurons is depicted in Figure 30 with a question mark. Fasciculus retroflexus projections from the lateral habenula are principally glutamatergic\textsuperscript{908,909}, while the medial habenula projections contain glutamate \textsuperscript{953}, substance P \textsuperscript{916}, acetylcholine \textsuperscript{916}, and IL-18. Fibres from both the lateral and medial habenula exhibit distinct topography both within the fasciculus retroflexus and in the location of their terminations in the interpeduncular nucleus\textsuperscript{906,910,916}, suggesting that any direct effect of IL-18 (and indeed, IL1RAPL presence) is likely to be limited to a distinct sub-region of the interpeduncular nucleus. Within this system, the specific role of IL-18 release in the interpeduncular nucleus is proposed to act on IL1RAPL1, and possibly IL1RAPL2 receptors, as described above, resulting in a reduction in the strength of excitatory synapses, and thus forms a mechanism by which the strength of excitatory glutamatergic inputs from the medial habenula are regulated. In this manner, IL-18 may function in many respects as a type of co-transmitter, whose function in-and-of-itself is simply to modulate the strength of co-localized and co-existent glutamatergic synapses. Interpeduncular nucleus efferents project to the ventral tegmental area and median raphe \textsuperscript{799,954}, and thus decreased habenular glutamatergic input is proposed to influence in turn ventral tegmental area dopamine neurons, alterations in forebrain dopaminergic activity, and ultimately effects on appetite. Other projections from the lateral habenula to the ventral tegmental area and median raphe \textsuperscript{907,915,938} may also play an indirect role through projections from the medial to lateral habenula. Importantly, blocking fasciculus retroflexus transmission has been shown to increase forebrain dopaminergic activity \textsuperscript{911}, providing experimental support for an effect of fasciculus retroflexus fibres in modulating forebrain dopamine release in the manner proposed here. In addition, effects in the ventral tegmental area, and downstream changes in forebrain dopamine content and feeding behaviour have been previously observed as a mechanism of leptin action \textsuperscript{926,927}.

Dopamine neurons project from the ventral tegmental area to the same portion of the medial habenula which expresses IL-18 \textsuperscript{913}, and serotonergic fibres from the median raphe form intraventricular axons lining the ependymal layer of the medial habenula \textsuperscript{955}, and thus these may form a feedback system which regulates the overall activity of the combined signalling circuit formed by these nuclei.

This hypothesis could be tested by the use of viral-mediated RNA knockdown to reduce habenula IL-18 expression over a medium term period (days to weeks depending on the longevity of the virus employed). This would allow sufficient time for changes in appetite, and possibly also body weight, to manifest, and would be predicted to recapitulate the phenotype of increased appetite observed by
Netea et al. 503 and Zorrilla et al. 506 in global IL-18 knockout animals. The nature of IL-18 effects in the interpeduncular nucleus and proposed actions on midbrain dopaminergic systems could also be analysed by such experiments, which would avoid potential confounding by possible developmental effects of a lack of IL-18 across the lifespan in global knockout animals. A similar approach could be used to reduce IL1RAPL expression in the interpeduncular nucleus, which would be expected to bring about similar changes in behaviour.

Within this proposed system, it is also important to take note of additional results presented by Netea et al. 503, as IL-18r1 knockout animals and transgenic animals overexpressing IL-18 BP showed similar phenotypes to IL-18 knockout animals. Given results from Sugama et al., showing an increase in IL-18 in the medial habenula 777, and also an increase in peripheral IL-18 levels 626 following an acute stress, it is proposed that there is a neuroendocrine mechanism which couples habenula IL-18 expression with circulating IL-18. Other metabolic hormones such as cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1) have been shown to exert central effects via activation of receptors on vagal afferents 956,957. A similar mechanism could exist whereby circulating IL-18 activates neural signals which feed back to regulate habenula IL-18 levels. In other words, habenula IL-18 expression may be driven by, and dependent on, peripheral IL-18 signals. If such a signalling mechanism were to occur via activation of the IL-18 receptor (IL-18r1), this would form a coherent explanation for the model proposed here where habenular mast cell chymase processed IL-18 regulates appetite via effects on IL1RAPL1 receptors, and also explain the reported phenotypes of IL-18r1 and IL-18 BP genetically modified animals. This hypothesis could be tested by administration of IL-18 (to raise circulating IL-18 levels), or by the use of monoclonal antibodies or IL-18 BP (to reduce circulating IL-18 levels), and examining the expression of IL-18 in the habenula. If an effect were found whereby peripheral IL-18 levels modulate central IL-18 production, repetition of similar experiments in IL-18r1 knockout animals could help to elucidate whether this effect is mediated by the IL-18 receptor, and potentially explain the reported phenotype of IL-18r1 knockout animals 817.

While there are many unknowns with regard to the role and action of brain-derived IL-18, it is clear that it is constitutively produced in a specific group of cells in the dorsomedial habenula and

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ii Although both also act directly on receptors in the brain.

kk It is difficult to know to what extent the phenotype of IL-18r1 KO mice could be explained by later evidence suggesting that there are additional genetic alterations in this strain beyond the deletion of IL-18r1 817.
Figure 30: Schematic diagrams depicting a model of habenula function governing food intake. **A** – line diagram showing a coronal view of the medial (MHB) and lateral (LHB) habenula. Black dots represent the location of IL-18 expressing cells in the dorsomedial habenula. Glutamatergic projections travelling in the fasciculus retroflexus (fr) are shown terminating in the interpeduncular nucleus (IPN). Fibres from the medial habenula form the ‘core’ of the fasciculus retroflexus, with lateral habenula fibres surrounding these. **B** – outline of the rat brain in sagittal section. The approximate positions of the habenula (Hab), interpeduncular nucleus (IPN), ventral tegmental area (VTA), and median raphe (Raphe) are all shown, although these nuclei in fact exist in different planes of sagittal section. The fasciculus retroflexus is shown as a solid arrow, with additional arrows representing fibres seen to project to the VTA and median raphe from the lateral habenula. The hypothesized final downstream effect of IL-18 action is predicted to be an increase in dopamine (DA) release in forebrain targets, which is shown by dotted lines. See text for a full description and explanation of the model with supporting references.
transported along the length of the fasciculus retroflexus. In this regard, centrally-produced IL-18 appears to be functioning in a fashion typically reserved for neurotransmitters or neuropeptides; although the downstream effects are unknown, these qualities grant it a unique role in brain function, as no other cytokine is known to exhibit such characteristics.
Section 4:

Discussion
Chapter 11: Advancing theory and practice in the treatment and prevention of cardiometabolic diseases

11.1 The inflammatory hypothesis

The Introduction to this thesis focused on the history of research into the treatment of diabetes and its complications and the theory of their causation. During the 20th century, the prevailing theory of diabetes pathogenesis revolved around the central role of blood glucose as a cause of diabetes complications, and therefore unsurprisingly this was also the central focus of the major diabetes-related treatment trials of this time period. In the wake of these studies, questions have been raised regarding the underlying assumptions inherent in the focus on blood glucose, and evident in the medical literature is an increasing interest in examining other possible pathological mechanisms of diabetes.

Claude Bernard noted in 1877: "The cause of diabetes is more profound that the causes of (hyper)glycaemia… the real etiological element of this affliction is that cause, as yet unknown, which causes the weakening of the primitive organs. It is this cause which should be addressed and not the symptoms of (hyper)glycaemia and glycosuria" (pg 414 of ).

Over the course of the last 140 years since this statement an alternative interpretation of the pathogenesis of diabetes is evident in the medical literature; an interpretation in which inflammation is posited as a cause of insulin resistance and diabetes. As discussed in the Introduction (Chapter 1), a number of investigations have been conducted over the course of the 20th century into the potential benefits of anti-inflammatory medications on insulin sensitivity. However, these works have been largely overlooked. There has been a recent resurgence in earnest into the potential use of anti-inflammatory agents in the treatment of diabetes, which appears to have arisen from a

ii Translated from the original French by the author.
‘discovery science’ route; with an increasing focus on, and understanding of, the intracellular pathways of inflammatory and insulin signalling, and observations of increased inflammation in obesity and diabetes, the idea was arrived at that anti-inflammatory medications could form a potential therapy for diabetes. Researchers involved in some of the modern clinical trials of anti-inflammatory agents in diabetes themselves state that they arrived at such investigations via this ‘discovery science’ route, unaware of previous clinical research. This alternative interpretation has therefore certainly not been at the forefront of the diabetes literature, and given the scarce attention paid to this theory it is not surprising that there has been little real progress in the development or application of this theory; as is evident in the sobering lack of progress seen in the pages of the following publication excerpts, where the same links between rheumatism, diabetes and salicylates are re-stated over the course of 128 years:

1881: “Dr Latham had been led to try salicylic acid in cases of diabetes, from theoretical considerations, arising out of a hypothesis as to the curative action of the same drug in acute rheumatism”. G.E. Paget, British Medical Journal, January 1881.


2009: "Salicylates are commonly used to treat rheumatic pain and have an established safety profile. As chronic, subclinical inflammation is often associated with obesity and increases the risks of obesity-related insulin resistance and type 2 diabetes mellitus, researchers suggest that salicylates could prevent or ameliorate obesity-related diabetes mellitus.” J Vig, Nature Reviews Endocrinology, April 2009.

With a recently renewed interest in pursuing the theory of inflammation as a cause of insulin resistance, a number of clinical trials are now currently registered which aim to examine the potential of anti-inflammatory agents to treat diabetes and related metabolic conditions, whether these will result in new treatment indications for these pre-existing agents is yet to be seen.

Given the failure then of recent trials to show reductions in cardiovascular mortality in diabetes, the withdrawal of diabetes medications, and a continued inability to design safe drugs for the treatment of obesity, a major diabetes risk factor, one of the key questions which arises is...
whether there may be better methods and ways of identifying potential drug targets in type 2 diabetes. Specifically, moving away from a focus on glycaemic control, with the plethora of biochemical alterations associated with diabetes how could we identify biochemical parameters which could form good treatment targets, and therefore could hold the best hope of treatment success, and warrant increased focus and investigation?

11.2 Theoretical considerations for future research into, and pharmacological approaches to, the treatment of type 2 diabetes

Although few researchers or drug manufacturers explicitly state that by designing drugs for use in diabetes they are directly attempting to mimic the beneficial effects of lifestyle modification, in effect this is by default the unstated aim of any medical therapy for a disease in which the principal risk factors are lifestyle-related; drug treatment aims to either reverse the metabolic abnormalities induced by a ‘poor lifestyle’ or at least manage the pathogenic changes sufficiently to prolong life. This is indeed the aim of prescribing hypoglycaemic agents to patients with diabetes – to reverse the biochemical abnormality on which the diagnosis of diabetes is made. In the case of management of diabetes by diet or lifestyle modifications only, this approach makes sense. Dietary and lifestyle factors are among the principal risk factors for the development of diabetes, and various studies in individuals with ‘pre-diabetes’ have shown that alterations in diet and increasing physical activity can avoid or delay progression to overt diabetes 972 mm. Notably, the effectiveness of lifestyle intervention in improving glycaemia in people with diabetes was first demonstrated in 1939 974, around the same time in which diabetes was being recognized as including insulin sensitive and insulin resistant phenotypes.

One of the most important, yet scarcely mentioned, logical progressions arising from measuring the effectiveness of diet and lifestyle modification by using measures of glycaemia is that this creates a scenario whereby the same risk factors which are associated with worsening glycaemic control are those being modified to improve glycaemic control. As noted earlier, type 2 diabetes is associated with changes in a range of biochemical and physiological parameters and, indeed, the effectiveness

\[\text{mm} \] That these studies have been undertaken in participants with ‘pre-diabetes’ as opposed to frank diabetes does not undermine the argument here, as it is well known that the diagnosis of diabetes itself is based on ‘drawing a line in the sand’ and establishing a cut-off for a continuous risk factor, a cut-off which itself traces back to rates of development of the diabetes complication retinopathy 973.
of lifestyle modification could also theoretically be assessed by measuring changes any of these variables, alone or in concert. In contrast, in the case of utilising pharmacological therapy as a treatment for type 2 diabetes, the dynamics of the above scenario are changed, such that the self-same risk factors which underlie the pathogenesis of the disease are no longer the factors being modified as part of the treatment of the disease; instead an exogenous pharmacological agent is introduced, and its effectiveness is monitored on the basis of changes in one of the biochemical abnormalities associated with type 2 diabetes. However, as Archie Cochrane noted, “it may well be the wrong parameter that is being altered” 975, and importantly, the above logical progression no longer holds. Of particular concern to the current day management of type 2 diabetes is that new drug agents for the treatment of type 2 diabetes have been licensed and approved on the basis of their ability to modify this one biochemical parameter

The question which arises then is how to best identify other metabolic variables that could serve as potential drug targets. If one accepts the premise that diabetes medication, by definition, must attempt to reverse or alleviate the metabolic derangements brought about by the disease, then clearly the examination of these derangements and how they relate to the incidence of complications will highlight potential drug targets. This is obviously in many ways the method by which elevated blood glucose came to be investigated as a potential cardiovascular risk factor, and serves as a reminder that this method of investigation may throw up 'false postives' – factors which are associated with the incidence of diabetes complications but whose manipulation will not appreciably influence rates of disease. Chronic sub-clinical inflammation is another diabetes-related metabolic derangement which forms part of this thesis and the potential for anti-inflammatory agents to improve diabetes outcomes is discussed in the Introduction. Likewise, studies examining the role of endoplasmic reticulum stress suggest that its alleviation may show promise in diabetes 100,976–988. There remain a variety of other known alterations associated with diabetes, and indeed we may soon discover additional metabolic derangements. Whether the manipulation of these factors will alter

This is not necessarily to say that these drugs are completely ineffective, however; although the logic underpinning their approval and how their effectiveness is monitored may be erroneous, they may still exert favourable metabolic changes which are clinically relevant. The problem arises as to how to assess this on the basis of the currently completed trials, as their designs reflect the underlying hypothesis, which was not to examine whether ‘medication x’ is useful for reducing diabetes complications, but whether a common effect of various drug agents (the reduction of blood glucose) alters diabetes-related end points.
rates of diabetes complications is, of course, a completely separate question, and it is here that an improved understanding of the ‘basic science’ of the pathogenesis of type 2 diabetes will be necessary.

11.2.1 Combining hypothesis-free and hypothesis-driven research to identify molecular targets in type 2 diabetes

Recently, in many areas of medicine and research, more ‘broad brush’ approaches to examining factors influencing disease onset have been carried out. Most notably, and perhaps most well-known, has been in the field of genetics, which has moved from a candidate gene approach based on the hypothesized role of a gene of interest, to genome-wide association studies (GWAS).

In the case of type 2 diabetes, this approach has yielded a number of novel insights, including establishing the role of the Wnt signalling pathway in glucose control with the TCF7L2 gene emerging as the strongest genetic risk marker, an unexpected finding. Similarly, GWAS in cardiovascular diseases has highlighted a section of DNA on chromosome 9q21 as showing a strong influence on risk, which was of completely unknown function (studies conducted since implicating a role in IFN-γ signalling).

A similar change in experimental approach has also occurred in examining the association of environmental risk factors with type 2 diabetes; whereas environmental exposures are typically derived from validated questionnaires to assess exposure to specific chemicals, a recent study performed an ‘environment wide association study’ examining the association of type 2 diabetes with a number of chemical entities directly measured in bodily tissue samples. This approach identified the association of diabetes with a number of previously-recognized environmental exposures, such as pesticides and polychlorinated biphenyls, thus confirming the validity of this approach, as well as novel associations such as γ-tocopherol (a component of vitamin E).

Another example of a broader mass-screening approach to investigate the acute metabolic effects of insulin was also recently undertaken in individuals with type 1 diabetes using proton magnetic

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Interestingly, this same locus has been identified in GWAS of type 2 diabetes.
resonance spectroscopy and liquid chromatography tandem mass spectrometry, and similar studies have been done in type 2 diabetes to identify changes in common metabolites associated with the disease, as well as metabolic predictors of diabetes incidence. Of note, these studies have identified alterations in amino acid and lipid metabolite profiles, reaffirming the scientific consensus of the early 20th century that diabetes involves not just alterations in glucose metabolism, but also disorders of protein and fat metabolism. These approaches highlight not only the technological advances of recent high-throughput analytical techniques, and the validity of such approaches – in that many have reliably identified previously known associations – but, more importantly, the promise of such techniques to identify many novel, unexpected associations, and the promise of such ‘hypothesis-free’ research to uncover unknown associations and mechanisms.

This form of hypothesis-free investigation is more in line with the method of scientific investigation known as ‘inductive reasoning’ supported by Francis Bacon, who believed it offered a number of advantages over hypothesis-driven research. In terms of the application to type 2 diabetes, such approaches have already delivered, and in the future could offer more, new insights into the pathogenesis of diabetes.

Clearly, however, it is one thing to be able to identify metabolic, genetic, or biochemical differences between states of health and disease, and another to understand how those changes ‘work’, both in terms of the biological pathways involved and how they influence the incidence and progression of complications arising from the disease. It is these aspects which will require a substantial investment of ‘discovery science’ research into the basic functions of metabolic pathways, and will arguably require the greater length of time and amount of research funding before observed differences in biochemical or metabolic parameters can be converted into viable therapies. A research approach of this nature will also require a great deal of collaboration and cross-pollination between epidemiological and clinical research methods and discovery science laboratory-based investigations.

The results of this study are, in itself, testimony to the fact that insulin has a diverse range of metabolic effects which extend far beyond a reduction in blood glucose levels.

An important logical extension of acknowledging that diabetes is a disease of protein, lipid and carbohydrate metabolism, is that HbA1c and glucose, which form the basis of the diagnosis and monitoring of diabetes, could just as validly be replaced by other biomarkers related to other aspects of metabolism, which could be used independently, or in combination with, markers of glucose metabolism.
This thesis represents in many ways an application of the investigative approach outlined above, utilizing both a clinical research approach to examine changes in inflammatory markers brought about by lifestyle modification in people with type 2 diabetes, in concert with a discovery science approach to attempt to further understand the biological role of one of the inflammatory markers modified by dietary intervention. This research has identified that a dietary intervention in people with type 2 diabetes, who are concurrently treated according to national guidelines, produces reductions in IL-18 and neopterin. These reductions, however, do not appear to be related to changes in adiposity or glycaemia, which raises many interesting questions for further research. The 'discovery science' aspect of this thesis has investigated the neuroendocrine role of IL-18, coming to the conclusion that IL-18 is unlikely to act through its currently recognized receptor (composed of the IL-18r1 and IL-18rap subunits) but may instead act through related receptor(s) (IL1RAPL1 and possibly IL1RAPL2) which are almost exclusively expressed in the brain and show similar amino acid motifs as seen in IL-18 BP. Specifically, the action of habenula-derived IL-18 on these receptors is hypothesized to modulate the strength of glutamatergic synapses within the interpeduncular nucleus. Exactly how this may relate to previously reported effects of IL-18 on appetite is unknown, although it is interesting to note that the habenular-interpeduncular nucleus pathway interacts with dopaminergic reward pathways, and modulation of these pathways is known to alter eating behaviour. Indeed, altering dopaminergic transmission has been shown to be one of the central effects of other metabolic hormones such as leptin and insulin.

11.2.2 The need for joint clinical and discovery science initiatives

While the above blueprint represents a logical template and way forward with regard to the medical management of type 2 diabetes, investigations of this nature will clearly necessitate a substantial investment of research personnel, participants, funding, and time before any discoveries find their way into routine clinical use. However, the need for such approaches is highlighted by the recent example of the case of rosiglitazone, which was licensed for use in diabetes and released on the basis of its ability to lower blood glucose levels. In documentation concerning the initial licensing of rosiglitazone, the pharmacology review states: “Rosiglitazone is a thiazolidinedione derivative that has antihyperglycemic action which might be a useful antidiabetic agent. The mechanism of action has not been clearly established, although its stimulatory effect on peroxisomal proliferator activated

However, in this case a more traditional hypothesis-driven approach has been used by studying a selection of cytokines based on prior evidence suggesting potential roles for these cytokines in metabolic disorders, and examining the neuroendocrine role of one of these cytokines in greater detail.
receptor gamma (PPARγ) is well known. The primary molecular target for the action of rosiglitazone might be the nuclear receptors, but how its activation of the receptors improves glycemic control remains to be elucidated” (pgs 2-3 of Pharmacology Review Part 1 from 1997). The medication was subject to much controversy 1998, and is now largely withdrawn, and yet, further research has highlighted the lack of original understanding as to this drug’s effects and the foolhardiness of embracing the use of medications whose mechanisms of action are poorly understood. ‘Discovery science’ research published after the use of rosiglitazone began to fall out of favour suggest that it’s profile of activity was far broader than necessary 1999-2001, with it’s glucose-lowering effect correlating specifically with Cdk5 (cyclin-dependent kinase 5)-mediated phosphorylation of PPAR-γ in adipose tissue 2001, and that effects on appetite, body weight, and to some extent glycaemic control, arise via actions in the hypothalamus 2002,2003. Scenarios such as these highlight the need for a greater understanding of the molecular mechanisms and systemic alterations in metabolism present in type 2 diabetes prior to attempting to modulate them for clinical benefit.

A disturbing consequence of the failure of trials such as the ACCORD, ADVANCE and VADT to reduce clinical diabetes-related endpoints is that it is subsequently clear that the current pharmacological management of type 2 diabetes is not founded on firm evidence; the majority of the present day medications indicated for use in diabetes have not been shown to lower micro- or macrovascular endpoints. Furthermore, given the progressive nature of diabetes, many patients are given ‘add on’ therapies with the aim of maintaining glycaemic control and the safety and efficacy of different drug combinations has received even less attention. Given, in addition, a current estimated worldwide adult prevalence of diabetes of approximately 7 % and projected increases in the number of people living with diabetes 2004, we are faced then with a large population of individuals with diabetes receiving treatment which is not evidence-based 2005, and will not become so without a substantial research investment to investigate the utility of these medications in reducing the incidence of diabetes complications.

And yet, research shows us that substantial improvements could be made in the treatment and prevention of type 2 diabetes (and associated non-communicable diseases) through greater, more widespread use and uptake of lifestyle modifications, which could translate into improvements in public health were it not for want of uptake and execution. Indeed, in the current absence of sufficient action to reduce the mean population risk of development of diabetes, and in turn its complications, the weight of diabetes prevention and treatment falls therefore by default on the shoulders of discovery science, when clearly the greatest gains in health could be achieved if both
discovery science and population health measures worked together in concert – and the full array of available resources and strategies were utilized to reduce the burden of cardiometabolic diseases.
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- 256 -


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Section 5:

Appendices
Appendix 1:

Chapter 3, LOADD study supplementary information

<table>
<thead>
<tr>
<th></th>
<th>Intervention</th>
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<td>48</td>
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<td>58.4 (8.8)</td>
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<td>4 (9)</td>
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<td>42 (88)</td>
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<td>2 (4)</td>
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<tr>
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<td>4 (8)</td>
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<tr>
<td>Medication:</td>
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<tr>
<td>Oral hypoglycaemic agents</td>
<td>31 (69)</td>
<td>34 (71)</td>
</tr>
<tr>
<td>Insulin</td>
<td>1 (2)</td>
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</tr>
<tr>
<td>Insulin and oral hypoglycaemic agents</td>
<td>13 (29)</td>
<td>14 (29)</td>
</tr>
<tr>
<td>Lipid modifying drugs</td>
<td>26 (58)</td>
<td>31 (65)</td>
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<tr>
<td>ACE inhibitors, A2 receptor blockers</td>
<td>24 (53)</td>
<td>30 (63)</td>
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<tr>
<td>Other antihypertensive agents</td>
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<td>Aspirin</td>
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Appendix 1, Table 2: Dietary intake across intervention and control groups. Difference (95 % CI) and p values adjusted for age, sex and baseline measures. As previously published in Coppell KJ, Kataoka M, Williams SM, et al. Nutritional intervention in patients with type 2 diabetes who are hyperglycaemic despite optimised drug treatment - Lifestyle Over and Above Drugs in Diabetes (LOADD) study: randomised controlled trial. BMJ, 341 (7766): c3337. (2010).

<table>
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<th>Dietary component</th>
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<td>Baseline</td>
<td>6 months</td>
<td>Baseline</td>
<td>6 months</td>
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<td></td>
<td>(n = 39)</td>
<td>(n = 39)</td>
<td>(n = 39)</td>
<td>(n = 39)</td>
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<tr>
<td>Energy (kJ)</td>
<td>8020 (1899)</td>
<td>6855 (1770)</td>
<td>7845 (2085)</td>
<td>7171 (2087)</td>
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<td>Protein (% TE)</td>
<td>19.7 (3.7)</td>
<td>22.1 (3.9)</td>
<td>19.2 (3.5)</td>
<td>20.4 (4.1)</td>
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<td>Total fat (% TE)</td>
<td>30.9 (6.1)</td>
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<td>29.8 (6.1)</td>
<td>29.9 (6.6)</td>
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<td>Saturated fatty acids (% TE)</td>
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<td>26.3 (7.3)</td>
<td>26.4 (5.6)</td>
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**Appendix 1, Table 3:** Pairwise correlation coefficients of the change in BMI, HbA1c, IL-18 and neopterin for participants from both control and intervention groups.

|        | Δ BMI | | | | Δ HbA1c | | | | Δ IL-18 | | | |
|--------|-------|---|---|---|-------|---|---|---|---|-------|---|---|---|
|        | r     | p  | n |   | r     | p  | n |   | r     | p  | n |   |
| Δ HbA1c| 0.302 | 0.003| 92|   | 1    | -  | - |   | -     | -  | - |   |
| Δ IL-18| -0.038| 0.724| 90|   | 0.137| 0.199| 90|   | 1     | -  | - |   |
| Δ neopterin| -0.112| 0.336| 76|   | -0.024| 0.835| 76|   | 0.299| 0.009| 76|   |
Appendix 1, Figure 1: Scatter graphs showing the correlation of change in neopterin levels with various dietary components, as follows: A – dietary fat; B – polyunsaturated fat (PUFA) intake; C – monounsaturated fat (MUFA) intake; D – saturated fat (SFA) intake; E – SFA intake for participants in control group only; F – SFA intake for participants in intervention group only; G – protein intake; H – carbohydrate (CHO) intake; I – dietary fibre intake. Note that the two outlying Δ neopterin values shown in Figure 2, Chapter 3 have been excluded.
G

change in neopterin levels (nmol/L)

change in protein intake (%)

\( r = 0.08, p = 0.53, n = 64 \)

H

change in neopterin levels (nmol/L)

change in CHO intake (%)

\( r = 0.14, p = 0.26, n = 64 \)

I

change in neopterin levels (nmol/L)

change in fibre intake (g)

\( r = 0.14, p = 0.28, n = 64 \)
Appendix 1, Figure 2: Scatter graphs showing the correlation of change in IL-18 levels with various dietary components, as follows: A – dietary fat; B – polyunsaturated fat (PUFA) intake; C – monounsaturated fat (MUFA) intake; D – saturated fat (SFA) intake; E – carbohydrate (CHO) intake; F – protein intake; G – dietary fibre intake.

A

B

C

D

E

F

\[ r = 0.03, \ p = 0.79, \ n = 76 \]

\[ r = 0.06, \ p = 0.60, \ n = 76 \]

\[ r = 0.02, \ p = 0.89, \ n = 76 \]

\[ r = 0.00, \ p = 0.97, \ n = 76 \]

\[ r = 0.08, \ p = 0.94, \ n = 76 \]

\[ r = 0.15, \ p = 0.21, \ n = 76 \]
$r = 0.03, p = 0.79, n = 76$
Appendix 1, Figure 3: Distribution of baseline measures of adipokines and inflammatory markers in the LOADD study, used for the generation of Pearson pairwise correlation coefficients shown in Chapter 3, Table 6. Outliers described in Chapter 2, Sections 2.4 & 2.5 have been excluded.
Appendix 1. Figure 3 cont:
Appendix 1. Table 4. Panels A - G: Pearson correlation coefficients, p-values, and number of observations for correlations shown in Chapter 3, Table 6. \( r \) = Pearson pairwise correlation coefficient; \( p \) = p-value; \( n \) = number of observations. Units for measurements are as shown in Table 6, Chapter 3.

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Appendix 1, Figure 4: Scatter plots of statistically significant pairwise correlations between inflammatory markers & adipokines (y-axes) and age & sex (x axes) identified in Table 6. For each panel (e.g. A, B) a linear regression line plotted with GraphPad Prism (red dashed line) and the corresponding r, p, and n values are shown below the graph. Panels marked A’, B’, etc. show the same scatter as the corresponding upper panel but with outliers suspected of influencing the correlation (marked in red) removed.

- Panel A: IL-1β vs. age
  - r = 0.25, p = 0.05, n = 63

- Panel B: Neopterin vs. age
  - r = 0.32, p < 0.01, n = 79

- Panel C: TNFα vs. age
  - r = 0.28, p < 0.01, n = 90

- Panel A’: IL-1β vs. age
  - r = 0.22, p = 0.08, n = 62

- Panel B’: Neopterin vs. age
  - r = 0.35, p < 0.01, n = 78

- Panel C’: TNFα vs. age
  - r = 0.22, p = 0.03, n = 89
**Diagram D:**

- sTNF-R1
- Age: 30 to 80
- Correlation: $r = 0.22$, $p = 0.04$, $n = 90$

**Diagram E:**

- sTNF-R2
- Age: 30 to 80
- Correlation: $r = 0.24$, $p = 0.02$, $n = 90$

**Diagram F:**

- Leptin
- Sex
- Correlation: $r = 0.38$, $p < 0.01$, $n = 83$
Appendix 1, Figure 5: Scatter plots of statistically significant pairwise correlations between inflammatory markers & adipokines and smoking identified in Table 6. For each panel (e.g. A, B) a linear regression line plotted with GraphPad Prism (red dashed line) and the corresponding r, p, and n values are shown below the graph. Panels marked A’, B’, etc. show the same scatter as the corresponding upper panel but with outliers suspected of influencing the correlation (marked in red) removed.
Appendix 1, Figure 6: Scatter plots of statistically significant pairwise correlations between inflammatory markers & adipokines and anthropometric measures identified in Table 6. For each panel (e.g. A, B) a linear regression line plotted with GraphPad Prism (red dashed line) and the corresponding r, p, and n values are shown below the graph.

A

IL-18 vs. waist circumference

r = 0.31, p < 0.01, n = 91

B

Leptin vs. waist circumference

r = 0.30, p < 0.01, n = 83

C

sTNF-R1 vs. waist circumference

r = 0.22, p = 0.04, n = 90

D

IL-18 vs. weight

r = 0.28, p < 0.01, n = 91

E

Leptin vs. weight

r = 0.27, p = 0.01, n = 83
F

IL-18

BMI

r = 0.26, p = 0.01, n = 91

G

leptin

BMI

r = 0.48, p < 0.01, n = 83
Appendix 1, Figure 7: Scatter plots of statistically significant pairwise correlations between inflammatory markers & adipokines and markers of cardiovascular risk identified in Table 6. For each panel (e.g. A, B) a linear regression line plotted with GraphPad Prism (red dashed line) and the corresponding r, p, and n values are shown below the graph. Panels marked A’, B’, etc. show the same scatter as the corresponding upper panel but with outliers suspected of influencing the correlation (marked in red) removed.
E:

\[ r = 0.29, p < 0.01, n = 83 \]

F:

\[ r = 0.26, p = 0.02, n = 83 \]

G:

\[ r = 0.23, p = 0.05, n = 76 \]
Appendix 1, Figure 8: Scatter plots of statistically significant pairwise correlations between inflammatory markers & adipokines and markers of glycaemia identified in Table 6.
For each panel (e.g. A, B) a linear regression line plotted with GraphPad Prism (red dashed line) and the corresponding r, p, and n values are shown below the graph. Panels marked A’, B’, etc. show the same scatter as the corresponding upper panel but with outliers suspected of influencing the correlation (marked in red) removed.
Appendix 1, Figure 9: Scatter plots of statistically significant pairwise correlations between inflammatory markers and adipokines identified in Table 6. For each panel (e.g. A, B) a linear regression line plotted with GraphPad Prism (red dashed line) and the corresponding r, p, and n values are shown below the graph. In order to examine whether associations were robust or unduly influenced by possible outliers influencing the strength of association, suspected outliers (marked in upper graphs with red circles) were excluded in corresponding panels below (e.g. A’, B’), which show the same analysis with circled points removed.

**Panel A:**
- Scatter plot of IL-1ra vs. neopterin
- $r = 0.499$, $p = 0.004$, $n = 31$

**Panel B:**
- Scatter plot of sTNF-R1 vs. neopterin
- $r = 0.218$, $p = 0.053$, $n = 79$

**Panel C:**
- Scatter plot of sTNF-R2 vs. neopterin
- $r = 0.247$, $p = 0.029$, $n = 79$

**Panel A’:**
- Scatter plot of IL-1ra vs. neopterin with suspected outliers removed
- $r = 0.170$, $p = 0.396$, $n = 27$

**Panel B’:**
- Scatter plot of sTNF-R1 vs. neopterin with suspected outliers removed
- $r = 0.251$, $p = 0.028$, $n = 77$

**Panel C’:**
- Scatter plot of sTNF-R2 vs. neopterin with suspected outliers removed
- $r = 0.446$, $p < 0.001$, $n = 78$
Appendix 2:

Section 3 supplemental information, detecting IL-18 system splice variants by RT-PCR
Appendix 2.1: Establishing methodology to examine expression of an IL-18 splice variant

Appendix 2.1.1 Designing primers to amplify an IL-18 splice variant by RT-PCR:

Primers were designed to bind to either the 57 bp segment present only in the full-length IL-18 transcript, or to span the nucleotides either side of this segment to amplify the IL-18s transcript, as shown in Appendix 3, Figure 1 and Appendix 3, Table 1. A trial PCR was set up to test these primers, using RT products extracted from male Sprague-Dawley spleen and adrenal tissue. PCR tubes were prepared as stated in the General Laboratory Methods section, Chapter 6, Section 6.2, using PCR settings of 94 °C for 3 min as an initial denaturing step, followed by 35 cycles of 94 °C for 30 seconds for denaturing, 57 °C for 30 seconds for annealing, and 72 °C for 45 seconds for extension steps. A final extension step of 72 °C for 5 minutes concluded the PCR. Gel electrophoresis of reaction products is shown in Appendix 3, Figure 2.

Appendix 2.1.2 Performance and testing of IL-18 splice variant primers:

While the primer combination F and the ‘common reverse’ primer should theoretically amplify both transcripts, this is difficult to visualise; a lighter band corresponding to the IL18s transcript could be present in the adrenal RT+ lane (3rd left from ladder) although the intensity of the full-length IL18 band is such that it is difficult to know if the lighter IL18s band is truly present. The primer combination F and ‘IL-18s’ reverse primer, designed to amplify the IL-18s transcript, produced a clear band the expected size in the spleen, and in the adrenal RT+ product apparently shows some ability to amplify the full-length IL-18 transcript as well, with a heavier band corresponding to the expected size of the full-length IL-18 transcript visible (expected size shown in Appendix 2, Table 1). This could potentially arise due to the first 13 nucleotides of the ‘IL-18s’ reverse primer providing sufficient adhesion to the full-length IL-18 transcript to allow some amplification, despite the 3’ instability introduced by the remaining nucleotides of the primer not matching the reference sequence. The primer combination F and ‘IL-18 full only’ reverse produced clear single bands in both spleen and adrenal samples showing amplification of the full-length IL-18 transcript.
Appendix 2, Table 1: Primer details for establishing methodology for amplifying an IL-18 splice variant and examining its expression in the habenula (Chapter 7, Section 7.5).

<table>
<thead>
<tr>
<th>Transcript(s)</th>
<th>Primer pair</th>
<th>Primer sequences</th>
<th>Primers designed to bind</th>
<th>Expected product</th>
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<tr>
<td>IL18: both transcripts</td>
<td>Forward (F)</td>
<td>CTGTGTTCGAGGACATGCCTG</td>
<td>ENSRNOT00000013093, 191-211; IL18 reference sequence within exon 3</td>
<td>full IL18: 353 bp</td>
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<tr>
<td></td>
<td>Common reverse</td>
<td>ATCCCCATTTTTCATCTCC</td>
<td></td>
<td>IL18s: 296 bp</td>
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<tr>
<td>IL18s *</td>
<td>Forward (F)</td>
<td>CTGTGTTCGAGGACATGCCTG</td>
<td>ENSRNOT00000013093, 191-211; IL18 reference sequence within exon 3</td>
<td>IL18s: 183 bp</td>
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<tr>
<td></td>
<td>IL18s reverse</td>
<td>CTGGCACACGT TT-CTCAAAG</td>
<td>ENSRNOT00000013093 354-360/418-430; IL18 reference sequence within exons 4 - 5</td>
<td>(full IL18: 240 bp)</td>
</tr>
<tr>
<td>IL18 full transcript only †</td>
<td>Forward (F)</td>
<td>CTGTGTTCGAGGACATGCCTG</td>
<td>ENSRNOT00000013093, 191-211; IL18 reference sequence within exon 3</td>
<td>full IL18: 232 bp</td>
</tr>
<tr>
<td></td>
<td>IL18 full reverse</td>
<td>CGTTTCTGAAAGAATATGAGATCAC</td>
<td>ENSRNOT00000013093, binding 398-422; IL18 reference sequence within exon 5</td>
<td></td>
</tr>
</tbody>
</table>

* Reverse primer was designed to cross the exon sequences either side of the 57 bp segment which is missing in the IL18s transcript. The point of division between exons 4 and 5 is shown as a dash in the primer sequence.

† Underlined bases show those binding with the 57bp segment missing in the IL18s transcript.
Appendix 2, Figure 1: Schematic diagram showing location of primers designed to amplify rat IL-18 splice variants, shown against reference sequence NM_019165.1/ENSROT00000013093. One common forward primer ‘F’ was combined with three different reverse primers spanning the 57 bp segment which is present only in the full-length IL-18 transcript. ‘Common reverse primer’, binding nucleotides 524-543 of the reference sequence, should theoretically amplify both transcripts; ‘IL-18 full only’ primer, binding nucleotides 398-422, is almost entirely within the 57 bp segment and thus designed to amplify only the full-length IL-18 transcript; ‘IL-18s’ reverse primer corresponds to the nucleotides either side of the 57 bp segment, nucleotides 418-430 and 354-360, and theoretically should only amplify the the IL-18s transcript.
Appendix 2, Figure 2: RT-PCR testing the amplification of IL-18 transcripts. Primer combinations are shown above. Samples from left-to-right are: 100 bp ladder, spl = spleen, adr = adrenal, H20 = control lane with water only. Positive and negative symbols indicate RT+ and RT- lanes. Expected product sizes are shown in Chapter 6, Table 7. The darker band towards the middle of the DNA ladder is 600 bp. Expected product sizes - IL-18: 352 bp, IL-18s: 182 bp (239 bp for amplification of full-length IL-18), full IL-18 only: 231 bp.
Appendix 2.2: Establishing methodology to examine expression of an IL-18r1 splice variant

A splice variant of IL-18r1 has been reported by Alboni et al. \textsuperscript{1}, which contains an inserted intron sequence downstream of exon 9. The inserted nucleotides form a stop codon shortly into the previously intronic sequence (Appendix 2, Figure 3), such that the receptor is thought to form a truncated version of the IL-18r1 reference sequence, encoding the transmembrane region but lacking much of the intracellular amino acid sequence. The corresponding intron in the rat was compared to that in the mouse (Appendix 2, Figure 3), showing that a similar sequence could be encoded by the equivalent intron in the rat. A reverse primer was therefore designed to bind the equivalent nucleotides in the rat as those targeted by Alboni et al. in the mouse. A PCR reaction was carried out using cDNA reverse transcribed from rat spleen total RNA, using primers and reaction conditions as described in Chapter 7, Tables 9 and 10. Gel electrophoresis showed a band of the expected size, and samples were purified by column purification (as described in the General Laboratory Methods section, Chapter 5) and sequenced by the Genetics Analysis Services, Department of Anatomy, University of Otago. Resulting reaction sequences are shown aligned to the predicted rat IL-18r1 type II sequence in Appendix 2, Figure 4. Sequences showed the amplification of the desired transcript.
Appendix 2, Figure 3. Alignment of rat intronic sequence with the corresponding mouse intronic sequence transcribed in the IL18r1 type II splice variant described in Alboni et al. (nucleotides 25812–26100 of ENSMUSG00000026070) and rat sequence (nucleotides 19354–19634 of ENSRNOG00000015027; transcript codes refer to the Ensembl database entries). The initial codons in the sequence are shown by alternating grey highlighting; the final nucleotide in exon 9, upstream of the shown intron, forms the first base of the first codon in both species and is shown underlined. Underlined and bold nucleotides show common stop codon. Nucleotides in bold and double-underlined show position of reverse primer used to amplify the type II transcript by Alboni et al. in the mouse and the equivalent bases in the rat sequence used as the basis of the reverse primer used here, as shown in Chapter 7, Table 9. Vertical lines indicate matching nucleotides across the species.
Appendix 2, Figure 4: Sequencing results for rat IL-18r1 type II. Shown is the manual alignment of reaction product from ‘Forward C’ and ‘IL-18r1 type II reverse’ primers as shown in Chapter 7, Table 9 (‘Product’) from sequencing reactions with expected sequence (above, ‘Ref’) derived from reference sequence ENSRNOG00000015027 from the Ensembl database. The previously intronic region which is inserted in IL-18r1 type II downstream of exon 9 is shown in grey highlighting in the reference sequence. A: product from sequencing reaction using the forward primer ‘Forward C’. B: product from sequencing reaction using the reverse primer ‘IL-18r1 type II reverse’.

A.

Ref:    CCAACGAAGAAGCCACAGACACCAAAAGCTCTCATCTTGTAGAAAAAGAAACACCTGACATCCAAGGCCATCTTTATGAGAGGAATAACCATGGTTGTTCTCACCTCTGTGGCA
            ||| |   |   |  ||  |||||  |||| |||| ||||||||||| ||||||| ||  || | ||||| |||||||||||
Product: CTTTGCTGKMWAAGAAMCTTACG-TCCAGGGCCCTGTCTTTATGAGAAATGCGYMGGGTTCCTCMCTTCTGTGGCA

Ref:    GCAGTGTGCGTTGATTTTGTGCAATTTTATAAGTTTAGTATTGTTTATTCTATAGGTACGGTAGCAGAAAGAGAGCATCAGACTGTTATCTTGCGCAGTCCAGGACG
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Product: GCAGTGTGCGTTGATTTTGTGCAATTTTATAAGTTTAGTATTGTTTATTCTATAGGTACGGTAGCAGAAAGAGAGCATCAGACTGTTATCTTGCGCAGTCCAGGACG

Ref:    TCCTAGCTCATTTTTGCACTGCCCATCAGAAATGCAAGAGGACGCATCTTGCTCTTTTATTGTAGCTAACATCTGACGTGTCCACGGCTTGAGTACAC
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Product: TCCTAGCTCATTTTTGCACTGCCCATCAGAAATGCAAGAGGACGCATCTTGCTCTTTTATTGTAGCTAACATCTGACGTGTCCACGGCTTGAGTACAC

Ref:    TGTGTGGCTTACACTGAAGCCGGTCATATGAGCAGGCTTGTGTAGCCTACTGAGGTGTTTCTCTTCTGCTGCTCTCTAGTGCTCTCTGCTACATGCTCCGTGCT
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Product: TGTGTGGCTTACACTGAAGCCGGTCATATGAGCAGGCTTGTGTAGCCTACTGAGGTGTTTCTCTTCTGCTGCTCTCTAGTGCTCTCTGCTACATGCTCCGTGCT
B.

Ref: CCAACGAAGAAGCCACAGACACCAAAAGCTTCATCTTGGTGAGAAAAGAAACACCTGACATCCAAGGCCATGTCTTTATGAGAGGAATAACCATGGTTGTTCTCACCTCTCTG GCC

Product: TCCAACGAAGAAGCCACAGACACCAAAAGCTTCATCTTGGTGAGAAAAGAAACACCTGACATCCAAGGCCATGTCTTTATGAGAGGAATAACCATGGTTGTTCTCACCTCTCTG GCC

Ref: AGCAGTGTGCGTGGTGATTTTGTGCA

Product: AGCAGTGTGCGTGGTGATTTTGTGCA

Ref: GTCCTAGCTCATTTTTCATGCCTGCACCAAGTAAAGGACGCACTGGTCTTCCTTTTATTTGGATGACGCTGCTAACAATCTGAGACTGCTTCCCAGCCTGGAGTCAGGAGTTACA

Product: GTCCTAGCTCATTTTTCATGCCTGCACCAAGTAAAGGACGCACTGGTCTTCCTTTTATTTGGATGACGCTGCTAACAATCTGAGACTGCTTCCCAGCCTGGAGTCAGGAGTTACA

Ref: CTGTGCTTACACTGACCGGGCGAGCTATGTGCAAGTCTGTGCTTCTGTGTAGTGACTACTCA--GCTGTGTTTCTTCTGGT--GCTTCAATGTCTGTGAACCTGTCTTCCACATGTTCCGGT

Product: CTGTGCTTACACTGACCGGGCGAGCTATGTGCAAGTCTGTGCTTCTGTGTAGTGACTACTCA--GCTGTGTTTCTTCTGGT--GCTTCAATGTCTGTGAACCTGTCTTCCACATGTTCCGGT

Ref: GCT
Appendix 2.3: Establishing methodology to examine expression of sIL-18rap

An IL-18rap splice variant had been reported in the rat \(^3\) and human \(^4\) at the time of undertaking the experiments described in this thesis. Subsequently the equivalent splice variant has been described in the mouse \(^5\). This has been referred to as sIL-18Rβ by Andre \(et\ al\). in the rat \(^3\), as IL-18RAPshort by Fiszer \(et\ al\). in the human \(^4\), and as short IL-18Rβ by Alboni \(et\ al\). in the mouse \(^5\). In this thesis, this splice variant will be referred to as sIL-18rap. Primers were designed to amplify this transcript in the rat, as described in Chapter 7, Tables 9 and 10, using a semi-nested PCR protocol. These primers were tested on cDNA obtained from reverse transcription of rat spleen samples, using the PCR settings described in Chapter 7, Section 7.2. Gel electrophoresis showed a band of the expected size, and the remaining PCR product was purified by column purification, as described in the General Laboratory Methods chapter, Chapter 5, and sequenced by the Genetics Analysis Service, Department of Anatomy, University of Otago. Sequencing results are shown below in Appendix 2, Figure 5 manually aligned against the expected reference sequence. Results demonstrated that the desired transcript had been amplified.
Appendix 2. Figure 5: Sequencing results for rat sIL-18rap. Shown is the manual alignment of reaction product (386 bp; ‘Product’) from sequencing reactions with expected sequence (above, ‘Ref’) derived from reference sequence ENSRNOT00000020194 from Ensembl database. The previously intronic region which is inserted in sIL-18rap is shown in grey highlighting in the reference sequence. A: product from sequencing reaction using the forward primer, B: product from sequencing reaction using the reverse primer.

A.

Ref: CAAATCATCCACAGTCTGAAATTATTCTCAATTTCTTTCTAGACAAAGCTGCTGACAGAACTTTCTCTGTCTCTCAGGAGGCCAGGATACAGCTTGTTGTGTGAAGACTGTC

Product: CYMKGATCTTTTT-WYAGAC-AGCGCTGACAGAACTTTCTCTGCTCTGAGAGGCCCAGGATACAGCTTTGTTGTGGAAGACTGTC

Ref: TTGGAAGTTAAGCCTCAGGAAATAATATATCTGTGGGAGCTCAGCAGAAAGCTGCTGACAGAACTTTCTCTGTCTCTCAGGAGGCCAGGATACAGCTTGTTGTGTGAAGACTGTC

Product: TTGGAAGTTAAGCCTCAGGAAATAATATATCTGTGGGAGCTCAGCAGAAAGCTGCTGACAGAACTTTCTCTGTCTCTCAGGAGGCCAGGATACAGCTTGTTGTGTGAAGACTGTC

Ref: TGTACGGAGTCCAGAGATGACCTGGTACAAGGATGGAAGACTGCTGCCCGAGTATAAGAAAAATCCAATTGAGATGGAGGATATTTATGATTTTCACCAAGGCTTGTACGTGCG

Product: TGTACGGAGTCCAGAGATGACCTGGTACAAGGATGGAAGACTGCTGCCCGAGTATAAGAAAAATCCAATTGAGATGGAGGATATTTATGATTTTCACCAAGGCTTGTACGTGCG

Ref: ATTACACTCAGTCAATATGTGTGAGTCTCTGG--ACCGTC

Product: ATTACACTCAGTCAATATGTGTGAGTCTCTGG--ACCGTC

---
B. 

Ref: CAATCTCCAGCTGAAAAATATTCTCAATATGTCTTTTATCTAGACAAGCGCTGACAGAACTTTCTCTGTCTCTCAGGAGGCCCGAGGATACAGCTTGTTGTGTGTGAAGACTGTC

Product: TCAAATCATCCCAGTCTGAAAATATTCTCAAATGTTCTTTTATCTAGACAAGCGCTGACAGAACTTTCTCTGTCTCTCAGGAGGCCCGAGGATACAGCTTGTTGTGTGTGAAGACTGTC

Ref: TTGGAAGTTAAGCCTCAGAGAAATATATCTCTGTGAGGAGCTCAACACAAGATGAGCAAGTCCTACTACTTTGGCAGCACTGGCTCCATTCACTGTCCCAGCCTGAGCCAAAAGTGTGAT

Product: TTGGAAGTTAAGCCTCAGAGAAATATATCTCTGTGAGGAGCTCAACACAAGATGAGCAAGTCCTACTACTTTGGCAGCACTGGCTCCATTCACTGTCCCAGCCTGAGCCAAAAGTGTGAT

Ref: GTACGGAGTCCAGAGATGACCCTGCTGACAGATGAGCAGCTGCCAGGAGTATAAGAAAATCCAATTGAGATGGAGGATATTTATGATTTTACCAATTGAGGAGCTATTTATGATTCTACCCAGCTTGACGTGTCGAT

Product: GTACGGAGTCCAGAGATGACCCTGCTGACAGATGAGCAGCTGCCAGGAGTATAAGAAAATCCAATTGAGGAGCTATTTATGATTCTACCCAGCTTGACGTGTCGAT

Ref: TACACTCAGCTCAGATAATGTGAGTCCCTGGACCAGTC

Product: ATTGTA
References, Appendix 2:


Appendix 3:

Section 3 supplementary information, images of micropunch procedure
Appendix 3, Figure 1: Micropunches of rat brain habenula and arcuate nuclei. A - frozen slide-mounted section; B - the same section after micropunching of the habenula and arcuate nuclei; C - approximate position shown in Nissl-stained coronal section, adapted from http://brainmaps.org, showing the position of the habenula and arcuate nucleus of the hypothalamus.
Appendix 3, Figure 2: Micropunches of rat brain habenula and ventromedial hypothalamic nuclei. A - frozen slide-mounted section; B - the same section after micropunching of the habenula and ventromedial hypothalamic nuclei; C - approximate position shown in Nissl-stained coronal section, in the rat brain, adapted from http://brainmaps.org, showing the position of the habenula and ventromedial hypothalamic nucleus (VMH).
Appendix 3, Figure 3: Micropunches of rat brain paraventricular hypothalamic nuclei. A - frozen slide-mounted section; B - the same section after micropunching of the paraventricular hypothalamic nuclei; C - approximate position in Nissl-stained coronal brain section, adapted from http://brainmaps.org, indicating the position of the paraventricular hypothalamic nucleus (PVN).
Appendix 3. Figure 4: Micropunches of rat brain interpeduncular nuclei and ventral tegmental area. A - frozen slide-mounted section; B - the same section after micropunching of the interpeduncular nuclei and ventral tegmental area; C - approximate position shown in Nissl-stained coronal section, adapted from http://brainmaps.org, indicating the position of the interpeduncular nucleus (IPN) and ventral tegmental area (VTA).
Appendix 4:

Chapter 9 supplementary information: IL1RAPL1 and IL1RAPL2 bioinformatics comparisons
Appendix 4, Table 1: Consensus amino acid residue codes

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Appendix 4, Tables 2a and 2b: Reference protein and nucleotide sequences used for multiple sequence alignment in Figures 19 and 20 (Chapter 9).

**Appendix 4, Table 2a:** Sequences aligned at protein level only

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<td>NP_001128525.1</td>
<td>single Ig IL-1-related receptor</td>
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**Appendix 4, Table 2b:** Sequences aligned at protein and nucleotide level

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<td>CPXV024</td>
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### Appendix 4, Table 3: Sources of amino acid sequences for 23 species used in multiple sequence alignment of IL1RAPL, IL-18BP and IL-18 proteins. Protein codes refer to either entries in the NCBI or Ensembl databases. For the 23 species, 3 were lacking available sequences for IL1RAPL2.

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<th>Binomial name</th>
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Appendix 4, Figure 1: (Following pages) Multiple species alignment of IL-18BP and the third IgG domain of the IL1RAPL proteins in 23 species (for IL-18BP and IL1RAPL1), and 20 of these species for which IL1RAPL2 sequences are available. **A:** alignment of third Ig domain of IL1RAPL1. **B:** third Ig domain of IL1RAPL2. **C:** IL-18BP. **D:** Alignment of IL-18 from the same species. Underlined residues on the human IL-18 sequence are those identified across the studies of Kato et al. \(^1\), Meng et al. \(^2\), and Kim et al. \(^3\),\(^4\) as necessary for binding IL-18 BP or IL-18r1.
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**Consensus/100%**

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**Consensus/90%**

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**Consensus/80%**

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Consensus/100%: PPKPLF MENQPSVIDVQLGKNI PKCAFFGS GESGMPIYW MKGEKF IEELAGHIREIR LKELLKGEKE VELALIFDSV VEAD LANYTCHVENRNGRKHASVLLR

Consensus/90%: PPKPLF MENQPSVIDVQLGKNI PKCAFFGS GESGMPIYW MKGEKF IEELAGHIREIR LKELLKGEKE VELALIFDSV VEAD LANYTCHVENRNGRKHASVLLR

Consensus/80%: PPKPLF MENQPSVIDVQLGKNI PKCAFFGS GESGMPIYW MKGEKF IEELAGHIREIR LKELLKGEKE VELALIFDSV VEAD LANYTCHVENRNGRKHASVLLR
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<th>Protein Identity</th>
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</tr>
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<td>70.7%</td>
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<tr>
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<tr>
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<td>FPAAKCPALEVTPEVEPLNLGLACVSRPNFSIYLYWLGNGSFIEHLPG</td>
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<td>FPAAKCPALEVTPEVEPLNLGLACVSRPNFSIYLYWLGNGSFIEHLPG</td>
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<td>61.5%</td>
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<td>Hedgehog</td>
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<tr>
<td>Pika</td>
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<tr>
<td>Mouse Lemur</td>
<td>59.8%</td>
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<td>Rat</td>
<td>58.6%</td>
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<td>Tasmanian Devil</td>
<td>54.1%</td>
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<tr>
<td>Platypus</td>
<td>30.1%</td>
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Consensus/100% consensus/90% consensus/80%

Additional notes:
- s.h.tsL...pLo...ps.hp..............
- s.shthhhpt.ths.s.sLaLChCS+...sFLyWLGNSFIEHLPoFECgLPS.L.Euphph... .tttT.L+sLVEpLoPsLpsSFSCL13.Da..sh.sh.hhh...
- s.sppCuLpVpWpC-.hPhNGTLoLScsACS+Fs.FS1LYWLGNSFIE+LPoGhEgpTphp.tstT.L+sLVEpLoPsL+sTNFCIIhhDpsspshH11LA
1 Marmoset 100.0% MAEE----------PVEDNCINFVFEMKFTGDALYFAEEDDENLTDHFAKL-KE-MA-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRGLAV
2 Rhesus monkey 79.8% MAEE----------PAEDNCINFVFAMKFIDSTLYFAEEDDENLEDSYFGLK-ES-LSLAIKMFDDYFLDQFRDQGLQH-HPFEDMTSDCERNDAPRTIFIYKYKDSFRQGMAV
3 Human 80.8% MAEE----------PVEDNCINFVFAMKFIDTLYFAEEDDENLEDSYFGLK-ES-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRGLAV
4 Chimpanzee 78.2% MAEE----------PVEDNCINFVFAMKFIDTLYFVAEEDDENLEDSYFGLK-ES-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRQGMAV
5 Orangutan 78.2% MAEE----------PVEDNCINFVFAMKFIDTLYFVAEEDDENLEDSYFGLK-ES-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRQGMAV
6 Gorilla 78.2% MAEE----------PVEDNCINFVFAMKFIDTLYFVAEEDDENLEDSYFGLK-ES-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRQGMAV
7 Gibbon 77.7% MAEE----------PVEDCNVFAMKFIDTLYFVAEEDDENLEDSYFGLK-ES-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRQGMAV
8 Pig 73.1% MAEE----------P-EDNCISVFEMKFTGNTLYFAEEDDENLEDSYFGLK-EP-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRGLAV
9 Horse 73.1% MAAG----------PVEDNCINFVFAMKFIDTLYFVAEEDDENLEDSYFGLK-EP-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRGLAV
10 Panda 71.5% MAAN----------T-EDNCISVFEMKFTGNTLYFAEEDDENLEDSYFGLK-EP-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRGLAV
11 Cow 73.1% MAAE----------Q-EDNCISVFEMKFTGNTLYFVAEEDDENLEDSYFGLK-EP-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRGLAV
12 Dog 70.5% MAAN----------TIEDNCISVFEMKFTGNTLYFVAEEDDENLEDSYFGLK-EP-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRGLAV
13 Alpaca 68.9% MAVQ----------PVEDCNSMFEMKFTGNTLYFVAEEDDENLEDSYFGLK-EP-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRGLAV
14 Mouse Lemur 68.9% --------------------------NLESDYFGLK-EP-FLSIRLNNQYFVFDKMD--G-ERIFEDMFKDKDQNLAQTRFIYKYKDSFRGLAV
15 Elephant 63.6% MAEE----------LVEDSCINFVFAMFINTLYFVAEEDDENLEDSYFGLK-ERP-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRGLAV
16 Hyrax 45.3% MAEE----------P-EDNSINFVMKFTGNTLYFVAEEDDENLEDSYFGLK-EP-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRGLAV
17 Mouse 58.5% MAAM----------SED-SCNFKEMKFINTLYFVAEEDDENLEDSYFGLK-EP-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRGLAV
18 Rat 60.5% MAAM----------SEEGSVNFKEMKFINTLYFVAEEDDENLEDSYFGLK-EP-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRGLAV
19 Fika 61.7% MAEE----------RF-TEEDCFVAMKFTGNTLYFVAEEDDENLEDSYFGLK-EP-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRGLAV
20 Microbat 61.3% MAAM----------TLEDYFGR1-EP-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRGLAV
21 Hedgehog 42.8% MAEE----------FPVEDFIDVDMEFINTLYFVAEEDDENLEDSYFGLK-EP-LSLAIKMFDDYFLDQFQVHLQGAGH-HPFEDMTDSCEANASRTIFIYKYKDSFRGLAV
22 Platypus 32.2% --------------------------VRLWSTPCCQLQENINGSFESDAMKHFNP-KFQVFQSSTKVLVMQDQSFRAMESQEIKNDAPQTIFIYKYKDSFRQGMAV
23 Tasmanian Devil 28.7% MAAKDQCQVACSPSEENCK-MLNFNVDEMEPFEREDCECLNLCAILKYYSVP-MCILKNSNKEISIFNQQEVEVIIFEEMNNEKRENAWPQFMLQRYCSGPRSL-V consensus/100% ----------------------------------------------------------------------------------------------------------------------------------------
consensus/90% .... ...........hhshsh...h.p...sLEsDhFh+h ps httli+s.sspVLFhtptp .slFE-Ms--pntNtpThhI1.hY+sS.sShsV consensus/80% MAs. ..Et.hhshhtMpFhssTLYyh.pt.tsLdSdA+Fu+l ps +hulIRNiNsQVLFLa+pt .slFEDMsDpDppsNuspThF1I.hYKDS.sKghAV
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<th>Identity (%)</th>
<th>Amino Acid Sequence</th>
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<td>100.0%</td>
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</tr>
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<td>2</td>
<td>Rhesus</td>
<td>79.8%</td>
<td>AIsvkC-eKistIscn-RiIsFkEmnPpDnItdkIstlIfFqrrsvpghDnkQfEssyeyFyFlaceKerEd=Lyk=LlkKrKoELGd-</td>
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<tr>
<td>3</td>
<td>Human</td>
<td>80.8%</td>
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<td>4</td>
<td>Chimpanzee</td>
<td>78.2%</td>
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<tr>
<td>5</td>
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<td>78.2%</td>
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<td>Gorilla</td>
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<td>77.7%</td>
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<td>73.1%</td>
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<td>73.6%</td>
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<td>71.5%</td>
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<tr>
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<td>70.5%</td>
<td>TlsvkC-kmIstIscn-KtIlsFkQmsSpIIndegIstlIfFqrrsvpghDkIqfEssyLkgYFllacKeEnd=Lfk=LlkKrKoELGd-</td>
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<td>13</td>
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<td>64.8%</td>
<td>TlsvrC-kktyIstIscn-KiVsFkEmSpFpIIndegIstlIfFqrrsvpghDkIqfEssyLkgYFllacKeEnd=FFk=LlkKrKoELGd-</td>
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<tr>
<td>14</td>
<td>Mouse</td>
<td>68.9%</td>
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<td>63.6%</td>
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<td>45.3%</td>
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<tr>
<td>17</td>
<td>Mouse</td>
<td>58.5%</td>
<td>TlsVkd-SkmstIscn-KiFsFeEmPpFnIIdIqIstlIfFkrvPgh-NkMfEssyLkgYFllacKeDed=Afk=LlkKrKoELGd-</td>
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<td>60.5%</td>
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<tr>
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<td>Pika</td>
<td>61.7%</td>
<td>TlsVkn-KkIstIscn-KIIsFk-</td>
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<tr>
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<td>Microbat</td>
<td>63.1%</td>
<td>TlsVkc-PemPtlsCnn-Kn-</td>
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<td>21</td>
<td>Hedgehog</td>
<td>42.8%</td>
<td>Sfsvys-KkMytsCkrd-KiIsFkEmnPpDnInednkIstlIfFqklvGhNmkMfEssyLkgCfLackRegd=RFk=LvlKdPNEelpIsvMFIL-</td>
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<td>22</td>
<td>Platypus</td>
<td>32.2%</td>
<td>TlsvksGdrtFhlScg-NIhFkGevQqKEdqIstlIfFkqGvFkDvQqFqQsCpgYlAcKqGqNqYst-MklkvksGdN-dstHmTl-</td>
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<td>23</td>
<td>Tasmanian Devil</td>
<td>28.7%</td>
<td>AfsVktEnsNyIsCnnHQLHfkgDfYfEyEedkOEdIIFQskVIGy-EKVFESFLYPEHYLANEIQKD-KfEmvlkEkdGv-</td>
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<td>consensus/80%</td>
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Appendix 4, Figure 2: Alignment of rat (NP_808796.1) vs human (NP_055086.1) IL1RAPL1 amino acid sequences. Note only four amino acids differ between species across the entire protein. Identities = 688/696 (99%), Positives = 692/696 (99%), Gaps = 0/696 (0%).

Rat 1  MKAPIPHLILLYATFTQSLKVVRLGFAKCALFYIRTNYLAQSAGLSLMWYKSSGPGDFEEFIAFDGSRMSKEEISWFPRPLLQDSGL  115
Human 1  MKAPIPHLILLYATFTQSLKVVRLGFAKCALFYIRTNYLAQSAGLSLMWYKSSGPGDFEEFIAFDGSRMSKEEISWFPRPLLQDSGL  115

Rat 116  YACVIRNSTMKVSISTLVGDYLGYSLCALFEGRTNYLAQSAGLSLMWYKSSGPGDFEEFIAFDGSRM  115
Human 116  YACVIRNSTMKVSISTLVGDYLGYSLCALFEGRTNYSLAQSAGLSLMWYKSSGPGDFEEFIAFDGSRM  115

Rat 231  LTVTAPLTDKPKLYPMESKLIQETQFLGSSANLTCRAFFGSGVSLIYWMKGEKFIEDLDENRVWEDDIRLKEHGGQEVSISLVDSVEEGLNGYSCYVENGNRRA  345
Human 231  LTVTAPLTDKPKLYPMESKLIQETQFLGSSANLTCRAFFGSGVSLIYWMKGEKFIEDLDENRVWEDDIRLKEHGGQEVSISLVDSVEEGLNGYSCYVENGNRRA  345

Rat 346  SVLLHKREMYTVELLAGGLALLLVCSTVYKCYIEIMLYRHFGAELDGKDNYDAYLSTYKVDpqwNqETEGERFEALEILPMLEKHYGKLFPDRLPITGYI  460
Human 346  SVLLHKREMYTVELLAGGLALLLVCSTVYKCYIEIMLYRHFGAELDGKDNYDAYLSTYKVDpqwNqETEGERFEALEILPMLEKHYGKLFPDRLPITGYI  460

Rat 461  EDVARCDQSKRLLIMYTFMNYYRGSWFELETRLNNMVTGEIKVILIECSELGMINYQEVAKHLKTIITLTVKWHGPKCNKLNSKFWKRLQYEMPFFKRIEIPITHQALDV  575
Human 461  EDVARCDQSKRLLIMYTFMNYYRGSWFELETRLNNMVTGEIKVILIECSELGMINYQEVAKHLKTIITLTVKWHGPKCNKLNSKFWKRLQYEMPFFKRIEIPITHQALDV  575

Rat 576  SEQGFEGLQTVSASIAMMTAATAHDFLRTSFHNTYSHQMRKHYYRSEYDVPPTGLTLPSTIGNQHTymiPMTLINGRPTQKSREPNNPDATHNSSLPLMRETS  690
Human 576  SEQGFEGLQTVSASIAMMTAATAHDFLRTSFHNTYSHQMRKHYYRSEYDVPPTGLTLPSTIGNQHTymiPMTLINGRPTQKSREPNNPDATHNSSLPLMRETS  690

Rat 691  ISSVIW  696
Human 691  ISSVIW  696
Appendix 4, Figure 3: Alignment of rat (NP_001159814.1) vs human (NP_059112.1) IL1RAPL2 amino acid sequences. Note that 37 amino acids differ between species across the entire protein. Identities = 649/686 (95%), Positives = 664/686 (97%), Gaps = 0/686 (0%).

Query 1  MKLPLLLALVCSAVSTNKLKVSRNSVGDIDSVLKDVTYMALAPGEFVRVKCALFYSYIRTNSMAQTSTGRLMWRNKGDLEEIPIFSEVRMSKEEANIWFHSAEQDGSFY
MK P LLALVCVS VSTNKLKVSRNSVGDIDSVLKDVTYMALAPGEFVRVKCALFYSYIRTNSAQTSTGRLMWRNKGDLEEIPIFSEVRMSKEEANIWFHSAEQDGSFY 115

Sbjct 1  MKPFFLLALVCSVSTNKLKVSRNSVGDIDSVLKDVTYMALAPGEFVRVKCALFYSYIRTNSAQTSTGRLMWRNKGDLEEIPIFSEVRMSKEEANIWFHSAEQDGSFY 115

Query 2  CVMRNCVIALGGLNLLVLLLLYVETYCNIEMLFLYRFGQDETFDDDNEKYDAYLSYTKVDQDTLDNTEEEQFAELIPDLVLEKYKLFPERDLIPSGTYIEDL
CVMRNCVIALGGLNLLVLLLLYVETYCNIEMLFLYRFGQDETFDDDNEKYDAYLSYTKVDQDTLDNTEEEQFAELIPDLVLEKYKLFPERDLIPSGTYIEDL 230

Sbjct 2  CVMRNCVIALGGLNLLVLLLLYVETYCNIEMLFLYRFGQDETFDDDNEKYDAYLSYTKVDQDTLDNTEEEQFAELIPDLVLEKYKLFPERDLIPSGTYIEDL 230

Query 3  VSSALTDKPPKLFPENQPSVIDVLKPLNPCKAFFGFSGEGPMIVWKMKEFIEEELAGHIREGIEILLLKEHLEGEKEVLTLIFDVSVEADLANYCHVENRNGRKHASVL
VSSALTDKPPKLFPENQPSVIDVLKPLNPCKAFFGFSGEGPMIVWKMKEFIEEELAGHIREGIEILLLKEHLEGEKEVLTLIFDVSVEADLANYCHVENRNGRKHASVL 345

Sbjct 3  VSSALTDKPPKLFPENQPSVIDVLKPLNPCKAFFGFSGEGPMIVWKMKEFIEEELAGHIREGIEILLLKEHLEGEKEVLTLIFDVSVEADLANYCHVENRNGRKHASVL 345

Query 4  LSRRMDFQPSITQRLVLLLEFLSSVRSLNLSVEIKVILIECTLGKVCNPVEELSHKIKLKLILKWGPKSSLKNKWFKHLVLYEMPITKKEMLSHCVLDSAEQ
LSRRMDFQPSITQRLVLLLEFLSSVRSLNLSVEIKVILIECTLGKVCNPVEELSHKIKLKLILKWGPKSSLKNKWFKHLVLYEMPITKKEMLSHCVLDSAEQ 575

Sbjct 4  LSRRMDFQPSITQRLVLLLEFLSSVRSLNLSVEIKVILIECTLGKVCNPVEELSHKIKLKLILKWGPKSSLKNKWFKHLVLYEMPITKKEMLSHCVLDSAEQ 575

Query 5  GLGELQIPSIAMTSATMATMVSQALDPFHSMDQMRRHCCRYKHEMPANTLSVFSLGHNHYTNCNLPTLLNGQLPLNLSKEETEESRNNPLLPSKELSFTSDIW
GLGELQIPSIAMTSAT+V SQADLPEFHN DSMQ+RHCCRYKHE+PA TL VFSLGNHYTNCNLPTLLNGQLPLNN+LK+T+E RSN+LPLPSKELSFTSDIW 686

Sbjct 5  GLGELQIPSIAMTSATMATMVSQALDPFHSMDQMRRHCCRYKHEMPANTLSVFSLGHNHYTNCNLPTLLNGQLPLNNTLKDQFHRNSSSLPSKELSFTSDIW 686
Appendix 4, Figure 4: Rat IL1RAPL1 (NP_808796.1) aligned with rat IL1RAPL2 (NP_001159814.1). Identities = 421/696 (60%), Positives = 541/696 (78%), Gaps = 10/696 (1%).

IL1RAPL1 1 MKAPIPHLILLYATFTQLVTKRGASDGCTDWSDVIKKYQVLGPEPVIKCALFYGYIRTNYLQAQSLM1WYKSSGPGDFEEPIADGSRMKSEEISIFRPTLLQD 112
MK P++ + + + + + +K+KR S DG DWSV+K Y L GEPVR+KCALFY IRTNY2+AQSL GL LMW++ G D EPI F +RSKEED+IWF QD
IL1RAPL2 1 MKLPLLALVCSAVSTNNKMVSQKSNVDGCDWSDKTMYALGEPVRVVKCALFSYIRTNSQASTGLRLM1WYRNKG=+DLEPIIFSEVRSKEDAIWFHSAEQD 110

IL1RAPL1 113 SGLYACVIRNSTDLYMKSNSMKVFEKARLSKSEIKISCRDIEDFLPLTTREPIELWYKCEKRTKWRPS1VFDRDRTIL1KEHLEQGEQYSISLIVDVEEGLGNQCY 224
SG Y CV+RNSTDLYMKS+N+LSKSEKISCRDIE++ D+++ E+++WYKE+ K W+ I+ K + +E+LV+EV+E+D GNYTCELGY+ G +
IL1RAPL2 111 SGFYTCVLRNSTDLYMKSMSTVSAENSLGYSRNLSRIILEKSEVTKKEISCPMDDFKSDKQDFVWYKECKPKMWRI1QQGGLALQLQEVECGNYTCELGYEKL 222

IL1RAPL1 225 VRRTELTVTAPLTDKPKLLPYMESKTLTIQETQLGGSANLTCRARGGGYGDVPLIYWMKGEKFIIDLDENVWESDIR1LEKHELGEQVEVSISLIVDVEEGLGNQCY 336
VRRTE+ VTA LTDKPPK L+ME+++ ++ + QLG N+ C+AFFG+SG+ P+IYWMKGEKFIE+L + E+IR+LKEHLE+EV+LI DSV E DL NY+C+V
IL1RAPL2 223 VRRTEKVTALLTDKPKLFLPMENQPSVIDIQLGKPLINPMKGFESNGPMY1M1WYGKFXFIEL=+A+R+GE+IRLL1KEHLEVEETL1FSVEEADLANITC 333

IL1RAPL1 337 ENGRKRHASVLLHKLKMYTVELLAGGLGALVLLLCSVTIYKCYKEIMLFYR+RNFAGAELGGNDKDYDAYLSYTKDQPDQWQETGE+EE+R+L+LP+DLEKHYGYKLFI 448
EN NGR+HASVLL K++LY +ELAGGL+L1 + +YKCY IE+MLFYR FG +E DNK Y+YLDYKTVKD D ++ EEE+FA+LP+DEKHYGYKLFI 445
IL1RAPL2 334 ENRGRKHASVLLHKLKMYTVELLAGGLGALVLLLCSVTIYKCYKEIMLFYR+RNFAGAELGGNDKDYDAYLSYTKDQPDQWQETGE+EE+R+L+LP+DLEKHYGYKLFI 445

IL1RAPL1 449 PDRDLPFGTYEIDVARCVDQQLRIL1VMYNPVYVRGRWSIFELETILRLMLTVGIEKVI1ECSELRLGINMYQEVAELKHTIK1LTV1XKGPKCNKSNKFWMRLQYEMP 560
P+RDLPPGTYEID+ RCVQ+SRL11V+T+Y++RRGWSIF+ +RL NMLV+GEIKVIELC+EL+G +N E+LVK IK+11+IKP +K+LNSKFW L YEMP
IL1RAPL2 446 PERDLPSGYETCVESQRR1L1IVLTPDYL1VRGSE1LSRLHNLMLVSEIKVIELCSTELJKGNCPEVESLKHNIKL1L1IKWKPKS+KLNSKFWHLYVEMP 557

IL1RAPL1 561 FKR+PETHEQALDVOGFGFELQTVSAISAATSTALATAPHLDRSF1ILYQHSMRQKHYRSVEYDVFP1GTLPLISG11QGHT1QPNG1TPQGCTGSSREP+NP 672
K E ++H LD +EQG FGELQ + +I+ M +TS + + DL FH++ QMR H R Y++++ P TL + S+GNHTYCNP+P+TL+NGQ P S +E
IL1RAPL2 558 IKKKEMLSHCVLDSLAEQ1GLGFEL1QPIF1SIMAT-TSTSTAMVSPOADL-PEF1H1DSMQMR=+HCCRGYKHEM-PANTLVSPLGNHHTYCNLPLTLNQLPLNLSLKE=+T 662

IL1RAPL1 673 DEIGHTNSAILLLPLARETSISSVIT 696
+E N+ +LPL +E S +S IW
IL1RAPL2 663 EELMRNPLLPLSSKELSFTSDIW 686
References to Appendix 4:


